Aus dem Institut für Mikrobiologie und Tierseuchen
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

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Inaugural-Dissertation
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vorgelegt von
Constanze Hoffmann
Tierärztin aus Chur, Schweiz

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All figures were made by Constanze Hoffmann and not have been published elsewhere.

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List of Abbreviations

aDNA	Ancient DNA
AdV	Adenovirus
AFLP	Amplified fragment length polymorphisms
B. anthracis	*Bacillus anthracis*
Bcbva	*Bacillus cereus* biovar *anthracis*
B. cereus	*Bacillus cereus*
BC	Before christ
BLAST	Basic local alignment search tool
bp	Base pair
C	Celsius
cAMP	Cyclic adenosine monophosphate
CA	Cameroon
CAR	Central African Republic
CDC	Centers for Disease Control and Prevention
CI	Côte d'Ivoire
COI	Cytochrome oxidase I
DFTD	Devil facial tumour disease
DJR	Dja Reserve
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
DSPA	Dzanga-Sangha Natural Protected Areas
EBHS	European brown hare syndrome
eDNA	Environmental DNA
EF	Edema factor
e.g.	Exempli gratia (for example)
EID	Emerging infectious disease
ELISA	Enzyme linked immunosorbent assay
ENP	Etosha National Park
et al.	Et alia (and others)
FTA	Technology for analysis of nucleic acids
GMYC	Generalized mixed Yule-coalescent model
GNF	Grebo National Forest
HAdV	Human adenovirus
HIV	Human immunodeficiency virus
iDNA	Invertebrate DNA

i.e.	It est (that is)
kbp	Kilo base pair
kD	Kilo Dalton
KNP	Kruger National Park
LF	Lethal factor
MAPKK	Mitogen-activated protein kinase kinase
MERS-CoV	Middle east respiratory syndrome coronavirus
MLVA	Multiple-locus VNTR analysis
mtDNA	Mitochondrial DNA
NCBI	National Center for Biotechnology Information
NHP	Non-human primate
NUMT	Nuclear mitochondrial DNA segment
OIE	Office International d`Épizooties (World Organization for Animal Health)
PA	Protective antigen
PCR	Polymerase chain reaction
PHRANA	Progressive hierarchical resolving assay using nucleic acid
PPE	Personal protective equipment
rDNA	Ribosomal DNA
rt PCR	Real-time polymerase chain reaction
SAGIR	National network for the surveillance of the health status of wildlife)
SARS	Severe acute respiratory syndrome
SIV	Simian immunodeficiency virus
SNP	Single nucleotide polymorphism
SGNP	Serengeti National Park
TB	Tuberculosis
tMRCA	Time to most recent common ancestor
TNP	Taï National Park
UNESCO	United Nations Educational, Scientific and Cultural Organization
US	United States of America
VNTR	Variable number tandem repeat
WHO	World Health Organization
WWF	World Wide Fund For Nature or World Wildlife Fund

1. Introduction

Emerging infectious diseases (EIDs) pose a global threat to public health and national economies [1,2]. The Ebola virus disease outbreak in Western Africa in 2014 and 2015 was the latest impressive evidence for how EIDs can spread in today's globalized world [3,4]. Most EIDs are zoonoses transmitted from animals to the human population [2,5]. Of these emerging zoonoses, 72% originate in wildlife [2]. Identifying wildlife diseases with the potential for zoonotic transmission before entering the human population would contribute significantly to minimizing the risk of infectious disease emergence. This demonstrates the strong interrelationship of human and animal health and is a good example of the need for research initiatives in the One Health framework [6].

However, monitoring wildlife diseases is challenging. Wild animals are elusive and shy, and obtaining samples suitable for pathogen analysis is laborious and cost-intensive. Additionally, the regions at highest risk for disease emergence, and thus of special interest with regard to circulating wildlife diseases, often have the fewest resources (i.e. tropical Africa, Asia and South America) [2]. Sustainable monitoring tools are required to facilitate the surveillance of wildlife diseases in those regions where it is most needed.

Recently, Grubaugh et al. introduced the new term *xenosurveillance* [7]. They suggested that blood meal analyses of mosquitoes (*Anopheles gambiae*) could provide insights into pathogens circulating in human populations in resource-poor areas. However, mosquitoes generally exhibit strong feeding preferences towards certain species, reducing the applicability for wildlife disease surveillance. There is also evidence that non-biting flies belonging to the dipteran families Calliphoridae and Muscidae take up pathogens during their lifecycles [8–10]. To this point, there have been no studies regarding whether non-biting flies may serve as a practical and cost-effective monitoring tool in resource-poor areas.

The aim of this study is to investigate the potential of flies for wildlife disease surveillance in rural areas. Flies have advantages that make them promising candidates: they are ubiquitous, visiting animal material for oviposition and as food source is a fundamental part of their lifecycle and they are easy to trap and store. We hypothesize that fly-based wildlife disease surveillance can cover different scales (small and large), resolutions (first screening and in-depth analysis) and dimensions (space and time). Two pathogens with zoonotic potential known to affect wildlife in the main study area were chosen as sample targets: *Bacillus cereus* biovar *anthracis* (*Bcbva*) and adenoviruses (AdV). The main study site is the Taï National Park (TNP), a remote tropical rainforest in Côte d'Ivoire (CI).

The objective of this thesis is to assess the potential for fly-based wildlife disease surveillance based on:

i) a small-scale fly-based in-depth analysis of *Bcbva* in TNP,
ii) a large-scale fly-based screening of *Bcbva* in sub-Saharan Africa,
iii) a small-scale fly-based screening of AdV in TNP.

To achieve this objective, more than 1,500 flies were collected in TNP, and around 1,000 flies at ten different sites throughout sub-Saharan Africa. For pathogen detection in flies, molecular tools were applied (polymerase chain reaction). Subsequent phylogenetic analyses based on whole genomes or genetic markers were used to assess genetic diversity. Identification of the mammalian source of the latest fly meal complemented the overall results. Objectives i) and ii)

were addressed in publication I (Persistent anthrax as a major driver of wildlife mortality in a tropical rainforest, Nature, 2017), and objective iii) was described in publication II (Assessing the feasibility of fly based surveillance of wildlife infectious diseases, Scientific Reports, 2016)

2. Background

To provide the reader with the background information for a deeper understanding of the two studies included in this thesis, an overview of the relevant literature is presented here. I start by elaborating on emerging infectious diseases, their implications and sources to justify the need for wildlife disease surveillance in general (section 2.1). Section 2.2 demonstrates different concepts of wildlife disease surveillance and major impediments, outlining the need for new innovative monitoring tools. Section 2.3 provides all information needed to comprehend the role that flies could play for wildlife disease surveillance. Subsequently, the main study site, Taï National Park in Côte d'Ivoire, is briefly presented to underline the exceptional setting in which the two studies were carried out (section 2.4). The final two sections, 2.5 and 2.6, present the targeted pathogens and the diseases they cause. Section 2.5 describes the disease anthrax and its two causative pathogens, *B. anthracis* and *B. cereus* biovar *anthracis*. Section 2.6 is dedicated to adenoviruses, the second targeted pathogen.

2.1 Emerging infectious diseases

EIDs are defined as "infectious diseases that have newly appeared in a population or that existed before but are rapidly increasing in incidence or geographic range" [11–16]. Newly appearing pathogens causing outbreaks of global significance could either be recently evolved strains, such as multi-drug-resistant tuberculosis [17], or pathogens that have entered the population for the first time, e.g. the human immunodeficiency virus (HIV) [18]. Diseases already known to occur in the human population may, due to environmental changes, for example, rapidly increase in incidence and geographic range and thus act as emerging diseases. For instance, in the late 1990s borreliosis (Lyme Disease) incidence increased dramatically in the United States of America (US) after reforestation led to an expansion of the deer population that carries the tick vector of this bacterial disease [19].

The global incidence of EIDs has significantly increased in recent decades, posing a growing threat to global health and the economy [2,20]. According to official numbers from the World Health Organization (WHO; status August 2017), 916 people died during the Severe acute respiratory syndrome (SARS) outbreak that started in 2002 in China; 18,449 people died from the pandemic influenza H1N1, starting in 2009; 710 people have died due to the Middle east respiratory syndrome coronavirus (MERS-CoV) since 2012; and most recently, 11,310 people died during the Ebola virus outbreak in 2014/2015. Beyond the loss of thousands of human lives, disease outbreaks of this magnitude cause severe short- and long-term effects on the societies and economies involved. For example, in the case of the Ebola virus outbreak in 2014/2015, mental health problems caused by stigmatization and traumatization continue to have a significant impact on the affected societies even today [21,22]. Furthermore, food insecurity in the short term due to quarantine-imposed travel restrictions, and in the long run due to harvest losses caused by farm labor shortages, have resulted in social deprivation [23]. According to the World Bank, economic growth in Liberia shrank by approximately 50% due to the Ebola crisis in 2014/2015 [24,25].

The majority (54%) of EIDs are caused by bacterial (including many rickettsial) pathogens, mainly drug-resistant bacterial strains, 25% by viral or prion pathogens, 10% by protozoa and the rest by fungi and helminths [2,5]. Most interestingly, more than 60% of EIDs are zoonotic diseases, meaning that most EIDs originate in animals. More specifically, 70% of animal-source

EIDs are caused by wildlife pathogens, whereas only 30% come from pathogens circulating in domestic animals [2]. The importance of this finding is further supported by the fact that there is an increasing trend in the number of EID events of wildlife origin [2]. This highlights the critical need to consider wildlife disease surveillance as a fundamental part of EID research. To combat EIDs, major efforts in various disciplines must be undertaken to identify risk factors and sources of disease emergence as a basis on which to develop effective countermeasures.

Various risk factors that explain the acceleration in the emergence of infectious diseases have been identified [2,11,12,14,26]. Nearly all of these are anthropogenic. The most striking factor is human population growth, which could also be seen as a proxy for many other risk factors. In the past 30 years the world population has increased by nearly 150%, from 3.0 billion in 1960 to 7.55 billion in 2017. This is predicted to continue growing in the future (to 9.8 billion in 2050 and 11.2 billion in 2100) [27]. Urbanization is accompanying this rapid development, often resulting in overcrowding, poor housing, and inadequate sanitation and solid waste removal, thus providing ideal conditions for the transmission of infectious diseases such as cholera and dengue fever [28,29]. In the case of dengue fever, for example, inadequate management of water and waste has led to an increase of urban breeding sites for the disease's arthropod vector, *Aedes aegypti*g contributing significantly to a major expansion in urban dengue [30,31]. Along with this expanding urbanization, the growing human population is also encroaching on remote areas and wildlife habitats. Increased contact of humans and wildlife can lead to the emergence of diseases from wildlife reservoirs. This was the case, for example, with HIV, which originated in the non-human primate (NHP) population of Central Africa [18,32]. Certainly, many more factors, such as international travel, contributed to the successful spread of the disease. Related to population growth is the change in land use and agricultural intensification. For instance, in Malaysia in the late 1990s agricultural intensification caused an extensive growth in mango plantations located near intensively managed pig populations. This combination led to the spill-over of Nipah virus from fruit bats to pig populations, resulting in a subsequent outbreak in humans [33–35]. Pandemic influenza may also have an agricultural origin. In China integrated pig-duck farming may have provided the fatal exchange platform of the major reservoir of influenza (waterfowl) and pigs, which can serve as "mixing vessels" for new highly pathogenic strains [36]. As indicated earlier, in most cases several factors interact, forming complex ecological interaction networks. To explain these complex interplays of factors precipitating the emergence of infectious diseases, further research must be undertaken, including the identification of potential sources of EIDs.

The risk of disease emergence from wild animal populations is not equal across the globe. Predictive models showed that lower-latitude developing countries in tropical Africa, Latin America and Asia, are what is often called EID "hotspots" [2]. This means that zoonotic EIDs caused by wildlife pathogens are much more likely to occur in these areas than elsewhere. Certain risk factors in particular foster the transmission of wildlife pathogens to humans in these regions, such as increased human-wildlife contact due to land-use changes and human encroachment [2,26]. Also high species diversity improves the probability of interspecies disease transmission [2,37–39]. Additionally, the poor infrastructure and health care systems also present in remote regions of sub-Saharan Africa further support disease dissemination [11]. Focusing on these EID hotspots would be beneficial for trying to identify wildlife diseases with zoonotic potential.

The criteria that define human EIDs can also be applied to wildlife, resulting in a range of relevant wildlife EIDs [26]. The implications of wildlife EIDs are threefold. First, as discussed above, they can act as predecessors for human EIDs. Second, they pose a severe threat to biodiversity and conservation [14,26,40]. For example, amphibian chytridiomycosis, a fungal infection, causes mass mortalities, population declines and possibly species extinctions in amphibians worldwide [41,42]. Another example is the Devil facial tumor disease (DFTD), an aggressive non-viral transmissible parasitic cancer affecting Tasmanian devils that has caused population declines estimated to be up to 50% [42,43]. Third, wildlife EIDs serve as maintenance

hosts for relevant domestic animal diseases. For instance, in the US brucellosis circulating in elk and bison poses a potential threat to sympatric domesticated cattle [44]. The underlying factors driving disease emergence in wildlife are, as for humans, largely consequences of human-induced environmental change. Of particular importance is the translocation and introduction of animals to new geographic regions in the course of global travel and commerce. This include international livestock trade-induced EIDs such as rinderpest in Africa and bovine spongiform encephalopathy in Europe [14]. Translocation of wildlife, whether due to conservation efforts or interest in exotic animal species as non-traditional pets, can also cause disease outbreaks in animals and humans. One example is an outbreak of monkeypox in humans in the US that could be traced back to the import of Gambian rats for the pet trade [45]. In another case, in 2015 three fatal cases of encephalitis in squirrel breeders were associated with variegated squirrel (*Sciurus variegatoides*) 1 bornavirus [46].

2.2 Wildlife disease surveillance

This section describes various concepts related to wildlife disease surveillance, as well as major impediments concerning study design, sample collection and data analysis, in order to show the need for new practical tools such as fly-based disease surveillance. The primary focus is wildlife diseases caused by infectious agents.

There are various definitions of wildlife disease surveillance and monitoring [47–49]. In this context wildlife disease surveillance or monitoring is the detection of the presence or absence of wildlife diseases and identification of spatial and temporal trends with a view toward the prevention of disease emergence. Ideally, it should further include the recording of population data such as host abundance and distribution to provide a good basis for epidemiological inference.

Unfortunately, the surveillance of wildlife disease faces a number of serious challenges concerning the collection of samples and field data, as well as the analysis and interpretation of data obtained [48–51]: Wildlife is not confined by boundaries and can extend over large distances. In particular, cryptic species, species of small body sizes and those living in remote areas (deserts, polar regions, rainforests) are extremely difficult to detect. Additionally, ethical aspects must be taken into account when sampling wildlife to strictly adhere to conservation guidelines. Furthermore, obtaining samples is generally non-random and opportunistic. Diagnostic tests originally designed for domestic animals lack validation and interpretation may be limited due to the lack of denominator data. In addition to these science-related and ethics-related difficulties, there are also political problems that often hinder wildlife disease surveillance. In order to overcome this political problems major interdisciplinary effort must be undertaken, including veterinarians, public health professionals, authorities, wildlife biologists, behavioral ecologists and others, in order to establish effective wildlife surveillance practices that are often lacking [49,52].

2.2.1 Wildlife disease surveillance – from the beginnings to today

The first wildlife disease surveillance programs were established in the early 1930s in Scandinavia [50]. These programs were based on analysis of non-randomly submitted dead animals to national veterinary laboratories throughout the country. Later, they were extended with the targeted surveillance of rabies [53] and trichinellosis [54]. The first scientific meetings on wildlife diseases were held in the early 1990s in France (Symposium on the health and management of free-ranging mammals held in Nancy, France, in 1991; First conference of the European section of the Wildlife Disease Association EWDA, in Paris, France, in 1994). In 1994 a wildlife disease working group of the World Organisation for Animal Health (OIE) was established. The occurrence of classical swine fever in Eurasian wild boar (*Sus scrofa*) in the late 1990s [55] and pandemic influenza in 2003 [56] contributed significantly to an increased interest in wildlife diseases of relevance for livestock and zoonotic diseases [57]. A number of official wildlife disease surveillance organizations throughout Europe now exist [58]. For example, the SAGIR network (Réseau national de surveillance de l'état sanitaire de la faune sauvage: National network for the surveillance of the health status of wildlife) in France collects data on wildlife necropsies performed throughout the country. It is widely recognized that countries maintaining wildlife disease surveillance networks are more likely to detect zoonotic diseases and are therefore better prepared to protect human, animal and wildlife health [50]. However, it can be said that wildlife disease monitoring is still in its infancy [47], and establishing continuous and accurate wildlife disease surveillance programs locally, national and globally should be a high priority for health authorities.

2.2.2 Forms of wildlife disease surveillance

Wildlife disease surveillance may be divided into two different approaches: general surveillance (also called passive or scanning surveillance) and targeted (or active) surveillance (Table 1, page 13) [47,49,52,59].

General surveillance

General surveillance is the opportunistic collection of data about wildlife health [47,49,52,59]. In practice, this means examination of all animals found dead and submitted to national animal health laboratories to determine their cause of death. Submitted carcasses and tissues are mainly road-kill animals, randomly found carcasses or routinely collected hunter-killed samples [52]. In France, for example, more than 100 different causes of death were identified for 4,000 dead roe deer (*Capreolus capreolus*) analyzed under their national general surveillance program (SAGIR) [60]. Theoretically, general surveillance also includes the non-invasive recording of observations of clinically diseased animals. However, the observation of clinical signs in wildlife is rare. Clinical expression in wildlife is usually brief and survival behaviors often mask clinical signs.

The success of general surveillance depends largely on the awareness of the public and people in the field, such as hunters, landowners and wildlife guards, and their willingness to report cases to national authorities [52]. Furthermore, the non-random and opportunistic nature of general surveillance, as well as the varying quality of samples, limits the analytical approaches that can be applied and affects the conclusions that can be drawn from the data. Still, general surveillance of wildlife pathogens constitutes the foundation for national wildlife health programs [59]. It is the only way to find out which pathogens are circulating in wildlife populations and detect new pathogens. For example, in the early 1980s when Scandinavian wildlife surveillance programs were initiated, they were the first to observe the European brown hare syndrome (EBHS) in European brown hares (*Lepus europaeus*) [61].

Targeted surveillance

Targeted surveillance is the proactive collection of wild animal health data for investigating a specific pathogen in a particular wildlife population [47,49,52,59]. The wildlife populations investigated may be of apparent good health condition. Usually, targeted surveillance is implemented to facilitate disease management actions or to confirm that a certain region is free of a particular pathogen. This is especially relevant for pathogens that either have zoonotic potential or pose a threat to livestock and the economy. For example, Berke et al. investigated the spatiotemporal distribution of *Echinococcus multilocularis* in red foxes (*Vulpes vulpes*) in northern Germany based on data collected from 1991-2005 [61]. They showed that *Echinococcus multilocularis* is steadily emerging in red foxes, necessitating disease management to avoid further cases in humans.

Compared to general surveillance, targeted surveillance requires greater effort, human resources and expense. Designing targeted surveillance requires high expertise in wildlife disease epidemiology (see 2.2.3). Furthermore, targeted surveillance is often linked to invasive sampling methods such as the capture or killing of animals with traps, net guns, pharmacological immobilization and other methods. These methods require profound expertise and are related to certain risks and difficulties, as described in section 2.2.4. However, non-invasive sampling methods, such as fecal sampling, can also be used for targeted surveillance (again, see 2.2.4). The advantage over passive surveillance is certainly that, if the circumstances allow, samples can be gathered according to a statistical sampling plan, which enables standard statistical estimates and analyses. With proactive sampling, the sample quality is often higher, yielding more reliable and interpretable results.

2.2.3 Study design of targeted wildlife disease surveillance

When designing a targeted wildlife disease study it is important to take several factors into consideration: first, the targeted host; second, the targeted pathogen; and third, further ecological factors that must be taken into account.

The selection of the targeted host species can greatly influence the outcome of a study. Consideration must be given not only to disease susceptibility, distribution, abundance and degree of protection of the targeted host species, but also to practicability in terms of sample collection and available diagnostic tests [48]. For example, in Spain Eurasian wild boar was selected as major tuberculosis (TB) surveillance target although red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) were similarly affected [62]. However, wild boar had a wider distribution, higher abundance, high availability as a game species and an anatomical lesion distribution that facilitated sample collection [48,63]. If a study aims to detect and monitor wildlife diseases with zoonotic potential, some species, so called "high potential" or "sentinel species", with certain demographic, ecological and behavioral characteristics are particularly promising to target [64,65]. High potential or sentinel species may be high-density animals that exhibit a broad niche overlap with humans, such as rodents and bats [66]. Another criterion identifying animals as sentinel species is close relatedness to humans, as is the case for non-human primates [67–70]. Focusing on such sentinel species may greatly enhance the probability to detect and understand zoonotic pathogens before they enter the human population.

In addition to the targeted host, the targeted pathogen(s) and the according sampling and diagnostic methods must also be chosen carefully. As most tests were originally designed for domestic animals, it is advisable to use those parameters and detection tools whose effectiveness has been evaluated in previous wildlife disease studies [48]. Further, sample size and sampling strategy should be specified in a way that allows epidemiological inference but is still feasible in the field and does not exceed budget limits [48]. For an expected disease prevalence of 0.1% and an assumed population size >10,000, Boadella et al. suggest a sample size of approximately 3,000; for an expected prevalence of 10%, 29 samples are recommended (estimates based on Win Episcope 2.0) [48]. Simultaneous collection of population data such as abundance is critical to allow for reasonable epidemiological inference. If the targeted disease occurs also in humans and livestock in the same area, the monitoring must be extended accordingly in order to detect any trend of mutual inference.

Further ecological factors must be taken into consideration while designing and conducting wildlife disease studies. The same pathogen may behave very differently in different host species for various reasons, e.g. different social behavior (gregarious versus solitary) or feeding habits. Disease transmission can also depend significantly on seasonal or climatic conditions and related mating seasons, vector abundance, migratory behavior and food availability. Environmental factors such as altitude, vegetation, soil composition and rainfall can play a major role in disease dynamics. Taking all these factors into consideration and focusing on areas with a high probability of disease occurrence and potentially serious impact on local human, animal and wildlife health can greatly help to save costs and improve the outcome [49]. Finally, particular attention should be paid to mass mortality events involving wildlife. Events involving a great number of deceased wild animals often are valuable indicators of new diseases with a potential impact on human and animal health [50].

2.2.4 Sample collection

Sampling for wildlife disease surveillance can be performed either invasively or non-invasively. In general, invasive sampling is accompanied by higher sample quality, but also higher risks for both the humans and animals involved in the process of sampling. In contrast, non-invasive sampling is less precarious, obtrusive and expensive, but often results in reduced/impaired sample quality.

General wildlife disease surveillance usually includes invasive sampling (post-mortem examination of animal carcasses) and only rarely the non-invasive observation of clinical signs (see also 2.2.2). Targeted wildlife disease surveillance was traditionally based on invasive sampling *in vivo* (capture of animals) and post-mortem (pathological examination). However, non-invasive sampling techniques, such as fecal sampling, are on the rise.

Invasive sampling

Invasive sampling of wildlife can be performed post-mortem or *in vivo*. Post-mortem sampling includes the examination and sampling of wildlife that was found dead (roadkill or unknown cause of death), that was actively killed for other purposes (hunted game, bush meat, slaughtered farmed wildlife) or that was euthanized for wildlife disease studies [49,52,66,69]. Killing wildlife purely for surveillance purposes must be performed in strict adherence to local conservation guidelines and should be used as a last resort. Ideally, the examination of wildlife carcasses comprises a complete necropsy with pathological examination and subsequent sampling of all internal organs for microbiological, genetic and histopathological analyses. Only experienced staff such as veterinarians and specially trained biologists should perform necropsies. If no trained expert is available, there are simplified protocols based on swab taking. Wildlife necropsies can be carried out either in animal health laboratories or directly in the field. In regions where highly pathogenic microorganisms are known to occur, e.g. Ebola virus or *Bacillus anthracis* in tropical Africa [71,72], necropsies should only be performed with full personal protective equipment (PPE) and strictly following a standardized protocol including de-contamination. The detection and sampling of wildlife carcasses can be significantly impeded by scavenging, predation and decomposition [51,52]. For example, only 6% of marked duck carcasses were detected by search teams in a study in Texas [73] and only 27% of deer carcasses during a survey in Montana [74]. Furthermore, small animals are particularly difficult to detect, resulting in a detection bias towards large mammals. Utilizing dogs trained to detect diseased or dead animals can significantly increase the detection rate compared to humans. For example, dogs detected 92% of passerine carcasses whereas humans detected only 45% [75–77]. Drones can also be useful for the enhanced detection of carcasses, as indicated by Koh et al. [78].

For *in vivo* sampling, wildlife must be captured with methods such as traps or pharmacologically immobilized using tranquillizing guns or blowpipes [52]. *In vivo* sampling facilitates the collection of blood samples from diseased or healthy individuals, which can provide extremely valuable information about the health status of the sampled population. However, trapping of wildlife is a challenging task, requiring high expertise, putting humans and animals at risk, incurring costs and time and often resulting in low sample sizes. Anesthesia of wildlife brings along various risks, such as over-dosage, arboreal species falling from trees (e.g. primates), injuries at the injection site and disorientation in the awakening phase increasing the risk for predation [69,79]. Furthermore, *in vivo* sampling requires permission from national authorities, which they are particularly reluctant to grant for endangered species, including various non-human primates. Trapping and immobilization entail eventual habituation programs that create important knowledge about the social behavior and interactions. Occasionally, invasive sampling can be performed in the course of translocation programs or when animals are admitted to sanctuaries.

Invasive sampling has the great advantage of high-quality samples, such as blood and tissue samples, which may even facilitate the complete reconstruction of the pathogen's genetic information (as shown in publication I). Furthermore, the sample obtained is 100% attributable to a certain individual and enables the eventual re-sampling of the same individual. However, these advantages both come at a significant cost. Both post-mortem and *in vivo* sampling require highly experienced staff, are labor- and cost-intensive and put animals and humans at risk (see above). Therefore, invasive sampling should be subjected to careful scrutiny before implementation, and non-invasive sampling methods should be given consideration.

Non-invasive sampling

Non-invasive sampling of wildlife for pathogen detection mainly involves the collection of feces, urine and food remains (saliva samples) [80,81]. The concept of non-invasive sampling is borrowed from the fields of endocrinology and genetics [82], where it has been applied much longer. In the field of pathogen detection this approach is a rather recent development that has gained increasing prevalence in wildlife disease research. There are several cases of successful implementation. For example, it was shown that non-invasive fecal samples can be used for the detection and monitoring of malaria and respiratory pathogens in wild great apes in CI [83–85]. The same is true for the surveillance of TB in badgers in the United Kingdom [86] and chlamydia and retroviruses in koalas (*Phascolarctos cinereus*) in Australia [87]. All these studies rely on the detection of the pathogen's genetic material by polymerase chain reaction (PCR) and real-time polymerase chain reaction (rt PCR). Antibodies can also be detected in fecal samples, as demonstrated in a study of simian immunodeficiency virus (SIV) prevalence in gorillas (*Gorilla gorilla gorilla*) in Cameroon [88]. Schaumburg et al. showed that *Staphylococcus aureus* carriers can be identified based on bacterial culture from fecal samples and saliva samples (food wadges) [89]. Further, it was shown that viruses shed in the oral cavity can be detected by PCR from material that animals have chewed on (e.g. ropes) [90,91]. Urine samples have provided insights into retrovirus prevalence in chimpanzees in CI using a specially established western blot to detect antibodies in urine [92].

Compared to invasive sampling, the collection of feces, urine and food remains requires less experience, observation and training effort. Thus, non-invasive sampling has high practicability in the field as well as time- and cost-effectiveness, enabling the monitoring of larger populations, including healthy animals, without disturbing their natural behavior or incurring the risks of chemical immobilization. This is of particular relevance for species that are subject to legal conservation guidelines but are promising candidates for the detection of novel zoonotic agents such as NHPs. If a system based on non-invasive sampling aims to assess infectious disease prevalence, the sensitivity of the approach should be estimated by analyzing matched blood and feces or urine samples from individuals with known infections (e.g. animals in captivity) [69]. In this way, it is certain that the presence of a pathogen in feces or the presence of antibodies in urine indicate the presence of disease. Unfortunately, non-invasive samples are often of low quality and the nucleic acids they contain are often degraded. For fecal samples PCR inhibition can play a major role in the analysis [86,87]. Appropriate deoxyribonucleic acid (DNA) extraction kits can minimize this issue.

Another possibility for non-invasive gathering of health data is the use of imaging devices (e.g. remote camera traps, infrared imaging). This was done, for example, for the diagnosis of sarcoptic mange in Spanish ibex (*Capra pyrenaica*) and Iberian wolf (*Canis lupus*), for rabies in racoons and foot-and-mouth-disease in ungulates [49,93–96].

Logistics

Determining the appropriate sampling material must take into account how feasible sample collection is under the given circumstances (situation in the field and availability of trained personnel), as well as the cooling and storage facilities needed, the transport and shipping requirements and related costs [49]. In particular, invasive samples (e.g. blood and tissue samples) of endangered animal species are subject to strict export restrictions that must be considered when planning a study. In order to obtain optimal results, all sample materials (i.e. blood, other bodily fluids, tissue samples, swabs, fecal samples, urine samples and food remains) must be as fresh as possible and be frozen as soon as possible after collection. However, immediate access to cooling facilities is often problematic. This is especially true in remote regions where electricity is absent or unreliable, and refrigeration facilities are rarely available. Liquid nitrogen containers can help meet this need but require certain logistical solutions and have limited storage capacity.

Different sample preservation methods can be used when immediate freezing is not possible. Depending on the sampling material and test used for diagnostics, sample quality may then still be sufficient [69]. For example, the preservation of small slices of tissue or feces in "RNAlater" (Qiagen, Hilden, Germany) allows shipping and storage at room temperature for up to two weeks or longer, with reliable results when tested by PCR or serological assays [97–99]. For bacterial culture, tissue and fecal samples can be preserved in 10% glycerol or other specific bacterial conservation buffers enabling transitional non-frozen storage [69]; however, feasibility depends on the biology of the targeted pathogen. Preservation of tissue and feces in 10% buffered formalin for histological and parasitological examination enables long-term storage at room temperature [69]. Whole blood, serum and urine can be stored dried using Guthrie filter cards or equivalent filter papers making them also storable long-term at room temperature. The detection of antibodies seems to remain possible and the detection of nucleic acids has also been described [69,92]. An alternative to Guthrie filter cards is the fast technology for analysis of nucleic acids (FTA), a paper-based system fixing and storing nucleic acids directly from fresh tissue [100]. Blood smears can be kept without cooling and, in addition to classical microscopic examination, are usable for DNA extraction [101]. For genetic analysis, it has been shown that feces stored dried on silica at room temperature is still suitable for genetic analysis [82].

To ensure usable results, the infrastructure in the sampling environment must be taken into consideration when designing a wildlife disease study. If refrigeration facilities are not available, alternative storage techniques must be used. It has to be kept in mind that alternative storage solutions always come with reduced sample quality. However, depending on the research question and diagnostic tests used, valuable results can still be obtained. Regardless, the mode of storage should always be included in the discussion of results.

2.2.5 Data analysis and interpretation

Prior knowledge (previous clinical, pathological and histological findings) determines whether a specific or general screening for pathogens is the preferred approach. If samples originate from regions with the potential occurrence of highly pathogenic microorganisms such as Ebola virus or *Bacillus anthracis*, these "candidates" should be tested first, before any other analysis is performed [69]. This should be irrespective of the type of downstream analysis (e.g. PCR, (meta)genomic analysis, culture) to ensure adherence to biosafety guidelines.

Depending on sample material and quality, a diagnosis can be made based on classical microbiological diagnostic tests and on clinical or pathological examination. Microbiological methods include detection of pathogens that may be direct (microscopy, bacterial culture, cell culture, PCR) or indirect (serological assays such as ELISA and western blot). In the case of freshly preserved samples, direct isolation of bacteria and viruses can be successful, facilitating biochemical or morphological identification. However, culture and isolation are often limited due to reduced sample quality and the specific properties of the pathogens. On a molecular basis, broad screening for bacteria can be relatively easily performed using PCR. Generally, the targeted region is the highly conserved 16S rDNA region found in all bacterial species [102]. For viruses, no such universal conserved regions exist, an there are only "generic" PCR systems that can detect different members of a certain virus family [103]. As these systems are relatively specific, related but divergent viruses may be missed. PCR systems for the detection of specific known pathogens are generally well described in the literature. Metagenomic approaches based on shotgun sequencing extend the opportunities for molecular diagnostics and will most certainly play a major role in the near future [7]. Indirect tests based on the serological detection of antibodies can provide insight into past or chronic infection. In acute cases, however, antibodies may not have been produced until the time of sampling. This means that the absence of specific antibodies does not mean the absence of disease. Exposure to pathogens indicated by antibody presence is also not equivalent to the expression of disease. Some individuals/species may only

be carriers of the disease without showing clinical symptoms [49].

Diagnostics face other limitations in addition to sample quality. As indicated above, most diagnostic tests were originally designed for domestic animals. When used for wildlife, the level of sensitivity and specificity is often reduced [49]. This applies particularly to serological assays involving commercial species-specific antibodies that often do not exist for wild animal species. Consequently, the most closely related domestic animal species antibodies are taken instead, sometimes yielding unreliable results. Cross-reacting antibodies can also affect the reliability of serological tests. Thorough validation with experimental trials could solve this problem, but is often complicated to perform. PCR-based approaches are also not always reliable. Sequence similarities of closely related pathogens or contaminant sequences can produce false positives. To increase confidence in PCR results, additional measures have to be taken, e.g. physical separation of pre- and post-PCR work. Furthermore, positive and negative controls are often rarely available. Establishing and validating diagnostic tests for wildlife is challenging, as controlled conditions imitating the natural environment are difficult to create [49]. Sanctuaries and other wildlife in captivity may be an option.

Wild animal health data must be interpreted with caution. To ascertain that a microorganism is really causing disease and death, monitoring of healthy animals is of great importance. If the microorganism occurs in both healthy and diseased or dead animals, making a direct link between the presence of a microorganism and disease or death appears questionable [69]. Pathological and histopathological findings, as well as the properties of known pathogens, may show evidence for the detected pathogen as the cause of death. It should be kept in mind that manifestation of disease often depends on co-infections, emphasizing the relevance of broader screenings (e.g. helminth parasites and anthrax in zebra in Etosha National Park) [69,104].

Additionally, the type of sample material, sample origin and sampling conditions must be taken into account [69]. For example, not being able to detect a pathogen in a carcass at an advanced stage of decomposition does not necessarily mean that it is not there or was not there previously. Similarly, finding respiratory pathogens in a muscle sample should raise concern. Environmental contamination with, e.g., soil-borne bacteria should be considered as a possibility when interpreting results obtained from wildlife disease monitoring [69]. A substantial impediment to interpretation of wildlife health data is the frequent absence of relevant population and biological data (regarding both hosts and infectious agents), such as population size, density, age structure, home range sizes, food habits, microscopic appearance of normal tissue, susceptibility, transmission potential and others. Such data is extremely labor-intensive to collect and thus is often not available, limiting the extent to which conclusions can be drawn [49,51,52].

Statistical analysis of data obtained from wildlife disease surveillance often requires non-parametric testing because the data is rarely distributed normally [105]. Generalized linear mixed models are applied to take into account data generated at different scales (individual, population, region) by including random variables to control for pseudo-replication [106]. In recent years, molecular epidemiological approaches based on inference from phylogenetic data based on genetic information obtained (whole genomes or suitable genetic regions) have contributed significantly to understanding zoonotic disease dynamics [107,108]. However, conclusions derived solely from molecular findings can be misleading. Instead, results from molecular analysis and field data should be combined.

	General surveillance	**Targeted surveillance**
Synonyms	Passive surveillance Scanning surveillance	Active surveillance
Definition	Opportunistic collection of wild animal health data by examining all animals found dead and submitted to national animal health laboratories "Cases as they occur"	Proactive collection of wild animal health data for investigating a particular pathogen in a particular wildlife population "Targeting individuals to detect the disease"
Aim	Detect pathogens circulating in wildlife population Establish basis for national wildlife health programs	Measure the presence of a certain pathogen in a population Facilitate disease management actions confirm that a certain region is free of a certain pathogen
Sampling methods	Mainly invasive sampling (post-mortem) Rarely non-invasive observation of clinical signs	Invasive sampling post-mortem and *in vivo* Non-invasive sampling (collection of feces, urine, food wadges)
Targeted pathogens	No a priori targeted pathogen, but rather determination of cause of death	Pathogens that have zoonotic potential or pose a threat to livestock or biodiversity and require management action
Data analysis	No epidemiological estimates can be inferred (non-random and opportunistic data collection)	Sometimes epidemiological estimates can be inferred if the sampling can be performed according to a statistical sampling plan
Advantages	Good cost-benefit ratio Easy to implement at large scale	Sampling according to a statistical sampling plan possible Ultimately higher sample quality
Disadvantages	Dependent on the awareness of people in the field and their willingness to report cases Limited analytical approaches (non-random opportunistic data)	Requires expertise in wildlife disease epidemiology Cost- and labor-intensive Risky due to invasive *in vivo* sampling
Representative studies	Lamarque et al. 1991 Gavier-Widén & Mörner 1991	Berke et al. 2008

Table 1: Forms of wildlife disease surveillance. The table provides an overview of the characteristics of the two main forms of wildlife disease surveillance: general and targeted surveillance. The table indicates the definitions, advantages and disadvantages and suitable sampling methods, among other characteristics, to illustrate the differences between the two forms.

2.3 Flies in wildlife disease surveillance

Section 2.3 provides the background information needed to comprehend the potential role of flies for wildlife disease surveillance. It is important to understand the ecology of flies to assess their possible use for disease surveillance. Unfortunately, and especially with respect to tropical flies, the current data is sparse, making interpretation of results a challenging task. Because the species of flies might be of importance for the outcome of the approach, the identification of fly species is described. Further, this section also describes the ways in which flies are already used for biodiversity assessment and which pathogens have been detected in flies previously.

2.3.1 Fly ecology

In the context of fly-based wildlife disease surveillance, the term "flies" refers to the two non-bloodsucking dipteran families Calliphoridae (blow flies) and Sarcophagidae (flesh flies). Both fly families contribute significantly to the process of carcass decomposition and are therefore also called carrion flies [109,110]. Carrion flies are a cosmopolitan group of insects found worldwide, from the tropics to the arctic during summer [111]. Around 1,000 Calliphoridae and 2,000 Sarcophagidae species have been described [112]. Equally broad is the range of lifecycles the different species exhibit, which differ depending on geographic location, making them a challenging group to study [109]. In particular, the African community of carrion flies is dramatically understudied. The few investigations that exist focus mainly on arid ecosystems and rarely on tropical regions [113,114]. According to Villet et al., Africa has around two dozen indigenous species and some introduced species of Calliphoridae, and around 200 species of Sarcophagidae [109]. However, it can be assumed to be much more. Most research is generally undertaken in the field of forensic entomology, as the colonization of carcasses with larvae of carrion flies, among other insects, facilitates the estimation of a minimum post-mortem interval [115]. Thus, most studies in this field focus on the ecology of larvae, especially on the developmental time, whereas studies concerning nutritional ecology and the flight and mating behavior of adult flies are rare. The lack of baseline data about the ecology of flies in general, and tropical flies in particular, requires cautious interpretation of results, and conclusions are often based on assumptions.

Carrion flies are holometabolous insects that undergo four life cycle stages: egg, larva, pupa and adult [116]. The duration of each stage depends largely on environmental factors, especially ambient temperature, and varies across fly species and ecosystems [116]. Generally, the duration of the three developmental stages ranges within days, and the survival of adults within weeks. Adult females lay their eggs (up to 200 per deposition) in decaying animal matter that serve as a nutritive source for their offspring. The vast majority of Calliphoridae and Sarcophagidae species oviposit on carcasses and are therefore called carrion flies. A few species also exploit feces and wounds or multiple sources [116,117]. Sarcophagid species are also viviparous in some cases and deposit larvae instead of eggs [116,118].

The decomposition process of carcasses is largely driven by the succession of different insects (flies, beetles, ants), in addition to non-living factors such as humidity and temperature [119]. Carrion flies are among the first insects that colonize fresh corpses, so-called early colonizers, because their larvae develop best in fresh tissue [120]. The efficacy of carrion flies in detecting decaying animal material is outstanding: they can arrive at carcass sites within minutes after death [121,122]. They are highly responsive to carcass volatiles and even capable of detecting differences in carcass age through the volatile profile as it changes throughout the process of decomposition [123,124]. Males and females are both attracted to carrion odor. Johansen et al. even reported that both sexes are equally attracted [124]. While females attend fresh carrion for oviposition and the intake of protein required for the maturation of their ovaries, males obtain food and increase the probability of successful mating [125,126]. To facilitate coexistence, carrion

fly species have developed niches based on season and succession (time of arrival at the carcass and thus stage of decomposition), and perhaps carcass type, to partition the carrion resource [127]. Single reports exist suggesting that some species are more attracted to large carcasses while others are more prone to seek out smaller carcasses [128,129].

There is little data on the nutritional ecology of adult flies. It is generally assumed that adult carrion flies feed on nectar, carcasses and/or feces [116,130]. Finally, the food source also depends on availability. As mentioned earlier, female adult flies require additional protein for the development of their reproductive systems. Thus they exploit carcasses not only as breeding sites but also as a nutritive source for the maturation of their ovaries [110,126]. It is often described in the literature that flies exhibit a special feeding behavior: after feeding they rest in the vicinity of the food source, regurgitate to facilitate digestion and defecate [131,132]. This behavior is relevant in the dissemination of diseases.

Studies of flight behavior are also rare. Two experiments on the mobility of flies showed that flies can spread up to 60 km per generation [132,133]. However, there are various studies showing that the abundance of flies varies with season and habitat type [114,134–136].

The flies analyzed in publications I and II were attracted using either meat or an artificial bait containing animal proteins designed to imitate the odor profile of fresh carcasses. Entomologists working in the field clearly identified them as carrion flies belonging to the family Calliphoridae. The species, however, have yet to be identified. This is mainly due to the fact that many tropical fly species are cryptic and have never been described.

2.3.2 Fly species identification

Species identification of flies is traditionally performed morphologically based on taxonomic keys. DNA-based methods are also now widely used. The cytochrome oxidase I (COI) gene located on the mitochondrial DNA (mtDNA) has been shown to be useful for the molecular identification of insects [137–139]. The mutation rate of the COI gene is fast enough to allow for the identification of closely related species, which often still show more than 2% divergence at this locus [138]. When using molecular identification it is important to take into account the intra- and interspecies sequence differences, which can vary from species to species. Interspecies divergence can vary from 3-5% and intraspecific variation from 0.2–0.8% [137,139,140]. For cryptic species, which are to be expected in under-researched areas, molecular identification as such, based on the reliable information in databases, may be not fruitful. Africa most likely has a large number of cryptic species. Phylogenetic approaches not requiring any a priori information to define species and instead relying solely on phylogenetic information might be a better option [141,142].

2.3.3 Using fly-derived DNA for mammalian biodiversity assessment

It has been shown that DNA extracted from carrion flies can be used to assess local mammalian biodiversity [143,144]. The rationale behind this approach is that carrion flies, due to their lifecycle, act as natural samplers of mammalian DNA. Uptake of DNA can occur during the larval stage, when animal carcasses represent the major nutritive source, and during the adult stage, when females visit carcasses or dung to acquire the proteins needed for the development of their reproductive system or for oviposition and larvaposition. It can be assumed that flies, unlike humans, are unbiased samplers of DNA [143]. For example, flies are able to detect very small rodents that can easily be overlooked by conventional methods based on observations made by humans. In addition to having little host preference, additional benefits over other mammalian biodiversity assessment methods include high abundance across an extremely wide spectrum of ecosystems and high practicability in the field [143,144].

Methodologically, fly-based mammalian biodiversity assessment is based on metabarcoding. This

technique is borrowed from the field of environmental DNA (eDNA), which identifies biodiversity based on environmental samples such as soil and water [145–147]. These samples are characterized by a complex mix of DNA that usually exhibits a certain stage of degradation [146]. Metabarcoding helps decipher this complex mixture based on high-throughput sequencing and classical barcoding. For species assignment, barcoding uses relatively short genetic markers that are usually located on mtDNA [138]. DNA extracted from carrion flies, also referred to as invertebrate DNA (iDNA), displays qualities corresponding well to environmental samples [148]. Thus, using metabarcoding in the context of iDNA is a logical solution. The first iDNA studies were based mostly on Sanger sequencing [143]. However, the application of Sanger sequencing was limited in cases of multiple meals significantly reducing the final output with regard to number of detectable species. High-throughput sequencing can easily handle a mix of meals, resulting in an increased number of detectable species. Further high-throughput sequencing enables the pooling of samples, leading to a decreased workload. Using specific assays targeting a species of interest also increases detection probability compared to non-targeted approaches [144]. To this point, fly-based biodiversity assessment has been carried out only in the tropical ecosystem of TNP [143,144]. However, in one unpublished study and in one recently published study we were able to show that this approach applies to a variety of African ecosystems, including arid ecosystems and the urban environment in Berlin, Germany [149].

2.3.4 Pathogen detection in flies

It appears likely that while visiting animal material flies take up the genetic material not only of the host but also of microorganisms present. Indeed, various studies have provided evidence for "hitchhiking" microorganisms and also for the involvement of flies in disease transmission [150,151]. Pathogens can hitchhike mechanically via mouthparts and the body surface or via ingestion and subsequent fecal deposition or regurgitation [151]. The capacity of flies to act as vectors has also been demonstrated under laboratory conditions by the successful transfer of microorganisms from flies to agar plates [151,152]. Most obvious is the involvement of flies in gastrointestinal disease. Many enteric pathogens of humans and animals have been detected in flies, including primarily bacteria (*Salmonella* spp., *Shigella* spp. *Campylobacter* spp., pathogenic strains *E. coli*) but also viruses (rotavirus, poliovirus; [153–159]). It also has been shown that flies can indicate the status of nosocomial infections in hospitals, including any antibiotic resistance present (*Klebsiella* spp., *Pseudomonas* spp. [160,161]. Other non-primary enteropathogenic agents have been detected in flies for which their role in the epidemiology is not entirely understood. For example, Newcastle disease virus (NDV) RNA was detected in flies collected in the vicinity of infected chickens (isolation of virions from flies was also possible), and H5N1 RNA was found in flies collected near a poultry farm with infected birds [10,162]. Anthrax may also be cultured from flies caught near anthrax victims [9]. However, in most of these studies flies were caught near pathogen hotspots, such as latrines or carcasses or in high-density animal populations.

2.4 Taï National Park

Taï National Park (TNP) is the main study site of the two studies presented in this thesis. TNP is what was defined above as an EID hotspot: this remote tropical rainforest harbors high biodiversity, and land-use changes are altering the human-wildlife interface, increasing the probability of cross-species transmission events.

Figure 1: Geographic location of Taï National Park. The main study area for both publications, Taï National Park (TNP), displayed in dark green, is in Côte d'Ivoire at the border with Liberia and is one of the largest remaining evergreen rainforests in Africa. It harbors exceptionally rich flora and fauna, including one of the largest remaining chimpanzee populations of Western Africa.

TNP is located in the western part of CI close to the Liberian border and is one of the largest remaining tropical rainforests in Africa. The area, spanning 5,300 km^2 (including a 2,000 km^2 buffer zone), was once part of the Upper Guinea forest belt (Figure 1). This forest belt at one point stretched from Ghana to Sierra Leone but has been largely displaced in recent decades due to major land use changes in the region. The local climate is sub-equatorial with a mean temperature of approximately 25 °C, humidity of approximately 85% and an annual rainfall of 1,800 mm. There are two annual rainy seasons: the minor rainy season from March to June and the major one from August to October [163]. Due to its exceptional flora and fauna, TNP was recognized as a national park in 1972 and granted UNESCO Natural World Heritage Site status in 1982. The unique fauna of the park comprises rare and endangered species, including the pygmy hippopotamus (*Hexaprotodon liberiensis*), seven species of duikers (*Cephalopus jentinki*, *C. silvicultor*, *C. ogilbyi*, *C. dorsalis*, *C. zebra*, *Philantomba monticola, P. maxwelli*), giant pangolin (*Manis gigantea*), leopard (*Panthera pardus*) and a unique community of non-human primates. In addition to one of the largest remaining Western chimpanzee communities (*Pan troglodytes verus*), known for their unique nut cracking behavior, ten species of monkey are known to occur: three colobus *(P. badius, C. polykomos, and C. verus),* four cercopithecoids *(Cercopithecus diana, C. petaurista, C. campbelli and C. nictitans),* the sooty mangabey *(Cercocebus atys),* the dwarf galago *(Galago dernidovii),* and the Bosman's potto *(Perodicticus potto*; [163,164]. Unfortunately, this remarkable biodiversity has dramatically decreased recently as a direct consequence to population growth and subsequent increasing human pressure on the park [165,166]. In 20 years (1971-1991) the number of inhabitants per km^2 in the region around the park increased 15-fold. This has been accompanied by agricultural intensification (rubber and cacao plantations), triggering deforestation and hence human encroachment in wildlife habitat [167,168]. These factors (high biodiversity, population growth, land use change and human encroachment in wildlife habitat) make the Taï region a potential EID hotspot and an area of special interest for One Health research.

In 1979 Christophe Boesch, later head of the Department of Primatology at the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany, initiated a long-term research project focusing primarily on the behavioral ecology of chimpanzees. In 2001 the project was complemented by a veterinary program under the lead of Fabian Leendertz, Robert Koch-Institute in Berlin, Germany. Since then a veterinary unit is constantly at site performing necropsies on every wild mammal found dead in the forest. This program and previous short-term studies were able to reveal a broad range of pathogens circulating in the ecosystem. Ebola virus killed chimpanzees in 1994 [71]; in 2001 a new anthrax-like bacterium, *Bacillus cereus* biovar *anthracis* (*Bcbva*), was discovered [72]; and in 2012 monkey pox virus was isolated from a deceased sooty mangabey (*Cercocebus atys*) [169]. Aside from these highly pathogenic microorganisms, other pathogens of relevance for the ecosystem have been intensively studied: retroviruses in monkeys, malaria in chimpanzees and adenoviruses in chimpanzees [83,170–173]. Unfortunately, devastating respiratory disease outbreaks in chimpanzees occurred as a consequence of anthropozoonotic disease transmission [174]. Since then, strict hygiene measures have been undertaken to prevent transmission of respiratory viruses from researchers working in the forest to habituated chimpanzees.

2.5 Anthrax

Anthrax is one of the two diseases targeted in this thesis for investigating the potential of fly-based wildlife disease surveillance. Although anthrax has been known for millennia and has a relevant impact on human and animal health, the disease is relatively understudied. Many aspects remain to be investigated, particularly in tropical ecosystems. This section provides a detailed overview of the anthrax literature to provide a sound basis for the discussion of my results.

Anthrax is a zoonotic disease whose causative agent is traditionally the spore-forming bacterium *Bacillus anthracis* (*B. anthracis*). The disease affects mainly herbivores but also an exceptionally broad spectrum of other animals, including humans. It can cause high fatality rates in susceptible animals and humans and occurs on all continents except Antarctica. Its clinical manifestation is caused by three exotoxins (protective antigen, edema factor, lethal factor) that are encoded extrachromosomally on a plasmid (pXO1). This plasmid, together with a second plasmid (pXO2) encoding a capsule essential for infection, are seen as typical of *B. anthracis* [175–177].

Relatively recently, in 2001, a hitherto unknown pathogen causing a disease equivalent to anthrax was discovered [72,178]. It also contained the two plasmids pXO1 and pXO2, but was designated *Bacillus cereus* biovar *anthracis* due to the fact that the chromosomal background was suggestive of *Bacillus cereus* [99,179,180].

The disease anthrax is known since early history and was already mentioned 5,000 BC [181]. The causative agent of anthrax was first described by Robert Koch in 1876 [182]. The disease was a serious problem in livestock until the early 20th century. For instance, in 1914 in Russia more than 20,000 animal deaths were recorded [183]. The development of an effective livestock vaccine by Sterne in the 1930s [184], the availability of an effective treatment (penicillin) [185] and the enforcement of quarantine rules reduced the burden of disease in many countries [186]. Nevertheless, in many parts of the world anthrax has remained or reemerged as a non-negligible problem (see section 2.5.1 Epidemiology). The relevance of anthrax is also seen in its role in biological warfare. Anthrax-infected carcasses were used as primitive biological weapons as early as the Middle Ages and thrown into fortifications to weaken the enemy inhabitants. In World Wars I and II *B. anthracis* was tested for its potential as biological warfare agent but never put into action [187]. However, 64 people died in 1979 of inhalational anthrax after an accidental release of spores from a biological warfare institution in Sverdlovsk (former Soviet Union) [188,189]. Most recently, in 2001 the potential of *B. anthracis* for bioterrorism was imposingly demonstrated when letters containing spores were sent by post to the White House. Twenty-two people were infected by inhalation of spores and five of them died [190,191]. Due to its applicability for biological warfare and bioterrorism *B. anthracis* is listed as a Category A agent by the Centers for Disease Control and Prevention (CDC). Category A comprises agents that constitute a major threat to the public because they can be easily disseminated, cause high mortality rates and might incite panic among the public.

2.5.1 *Bacillus anthracis*

General characteristics

B. anthracis belongs to the *Bacillus cereus* group (*Bacillus cereus sensu lato*), which is comprised of Gram-positive, rod-shaped, endospore-forming bacteria. Its members, *B. anthracis*, *B. cereus*, *B. mycoides*, *B. thuringensis*, *B. pseudomycoides* and *B. weihenstephanensis*, exhibit different phenotypes and pathogenicity. Genetically they are extremely closely related and share a highly conserved chromosome [192]. Apart from the microbiological group characteristics, *B. anthracis* is a non-motile, aerobic and facultative anaerobic. Its genome consists of a circular chromosome of approximately 5,230,000 base pairs (bp) and the two virulence plasmids pXO1 and pXO2 of approximately 180,000 bp and 95,000 bp, respectively [193]. Until the discovery of *Bcbva*, *B.*

anthracis was the only known member of the group to possess the two plasmids pXO1 and pXO2.

Epidemiology

In general, anthrax is prone towards more temperate regions as sporulation, a substantial part of the *B. anthracis* lifecycle, is hindered by temperatures below 9-12 °C [176]. The occurrence of anthrax in cooler regions can in most cases be traced back to the import of contaminated animal products [186]. Unless effective prevention measures are available anthrax remains relevant for livestock, wildlife and humans. Anthrax is endemic in certain regions of the world: southern and Sub-Saharan Africa, Central Asia, countries of the former Soviet Union, western China, India, Central and South America and also small pockets of the USA and Canada. In Europe, sporadic outbreaks and cases occur predominantly in the south but also in other more temperate areas [177,186]. For example, the most recent cases in Germany were reported in July 2012 in cattle in Saxony-Anhalt (not imported cases) [194]. In general, the anthrax situation in livestock determines the incidence of natural disease in humans [177]. Non-natural outbreaks in humans increased recently in 2001 as part of a bioterrorist attack (contaminated letters in the US) and in 2009-2010 when contaminated heroin was circulating in northern Europe [190,195]. Unfortunately, worldwide anthrax surveillance faces deficiencies. Cases and outbreaks are often underreported due to a lack of experience, interest and training among veterinary personnel. This is the case in developing countries as well as in the Western world, where anthrax has tended to fall into obscurity as effective control measures successfully reduced incidence of the disease [186]. Therefore, the worldwide distribution of anthrax can only be approximated.

The ability of *B. anthracis* to form spores significantly shapes the dynamics of the disease. *B. anthracis* endospores are highly resistant to various environmental conditions and can survive in contaminated soils for decades before entering a new cycle of infection [196,197]. If a dormant spore enters a suitable host, the conditions for germination are met: vegetative bacilli are produced, multiply and express potent toxins, causing fatal septicemia. During the process of death and decomposition, bacilli are shed, sporulate on contact with oxygen, contaminate the surroundings and remain in a dormant phase till they enter the next infection cycle [176,177]. In contrast to spores, vegetative bacilli are poor survivors and die within hours when released into the environment [198]. Whether a full lifecycle can occur outside a mammalian host is intensively debated within the scientific community [176,199,200]. The *B. anthracis* cycle of sporulation and germination is sensitive to seasonal variations. Climate conditions influence the availability of spores in the environment and the constitution of the potential hosts [177]. The infection cycle described here is a typical point-source infection. Due to its non-invasive character, transmission of *B. anthracis* from animal to animal and human to human is very unlikely. However, there have been reported cases that challenge the assumption of sole point-source infection, suggesting an infection cycle involving arthropods, as described in detail below [9,132,201].

Under natural conditions, humans contract the disease directly or indirectly from infected animals or contaminated animal products via skin lesions, ingestion or inhalation. Anthropogenic spread of aerosolized spores in bioterrorist attacks or biological warfare constitutes another potential source of infection [177].

How exactly animals contract the disease, as Turnbull (2008) stated, "remains in the realm of theory." It is assumed that animals generally acquire anthrax through the ingestion of spores and that lesions must be present to initiate infection. The traditional scenario is that during the dry season spores are taken up while grazing near the ground. Arid and thorny vegetation causes orogastrointestinal lesions that facilitate infection. However, epidemiological observations have shown that there is no consistent correlation of hot and dry climate conditions with anthrax incidence. Furthermore, it is not known to what extent the inhalation of spores while grazing on dusty contaminated soil contributes to infection [177]. Contaminated fodder such as hay, bone

meal and meat also constitutes a major source of infection, especially for non-grazing livestock and animals in captivity [177].

It has been repeatedly described that biting and non-biting flies may play a non-negligible role in the transmission of anthrax. For example, biting flies of the dipteran family Tabanidae (horseflies) were most likely involved in a fatal anthrax outbreak in white-tailed deer (*Odocoileus virginianus*) in Arkansas, USA [202]. The same was suspected for a large anthrax epidemic in Zimbabwe in 1978-1979 [203] and for anthrax outbreaks in bison (*Bison bison*) in Northern Canada [204]. At present, the transmission of anthrax through biting flies remains a hypothesis. However, the transmission of anthrax through biting flies was shown experimentally already in the early 1900s [205–207]. Non-biting flies have also been suspected to be involved in the dissemination of anthrax. Hypothetically, they feed on the bodily fluids of anthrax victims and subsequently contaminate surrounding vegetation via regurgitation and defecation, putting susceptible browsers such as kudu or deer at risk [9,132]. This hypothesis was first proposed by Braack and de Vos to explain anthrax outbreaks affecting browsing kudus in KNP [132]. Blackburn et al. later called this the "case multiplier hypothesis" in the context of enzootic outbreaks of anthrax in white-tailed deer (*Odocoileus virginianus*) in West Texas, USA [208]. In the early 1990s, Braack and de Vos proved environmental contamination in an experimental setting using radioactively labelled bacteria that could be detected with a Geiger counter [132]. Blackburn et al. were able to prove part of the hypothesis under natural conditions and succeeded in isolating *B. anthracis* from flies collected in the field [9,208]. *B. anthracis* in surrounding vegetation was detectable only by PCR [208]. Under laboratory conditions, it has been shown that *B. anthracis* can be isolated from emesis and feces of flies that fed on *B. anthracis*-contaminated matter [201]. It is debatable whether replication of bacteria takes place in the gut of arthropods. Fasanella et al. showed evidence that this might be the case, but experiments by von Terzi et al. contradicted these findings [201,209].

Vultures and other carrion eaters have also been implicated in disseminating *B. anthracis* in the large National Parks of South Africa. The extent to which this contributes to the overall local dynamics of anthrax remains questionable, as the number of disseminated bacteria usually remains low [197,198].

Animal-to-animal transmission seems unlikely given the non-invasive character of *B. anthracis*. Scavenging constitutes an exception. In general, scavengers are less prone to infection, but there are cases of anthrax in scavengers, for example lions [210]. A remarkable report of a massive anthrax outbreak in hippopotamus (*Hippopotamus amphibius*) in Uganda was also explained by either scavenging or cannibalism [211].

The range of *B. anthracis* hosts is exceptionally broad, but susceptibility varies significantly across species. Herbivores are regarded as most susceptible to the disease, whereas omnivores and carnivores show varying levels of natural immunity [212]. Interestingly, susceptibility can differ within species across ecosystems. For example, in Etosha National Park (ENP), Namibia, zebras are most commonly affected by anthrax, whereas kudus are only occasionally affected [198]. Conversely, in Kruger National Park (KNP), South Africa, kudus have significantly higher incidence than zebras [197]. This can probably be explained by ecological and behavioral differences putting species with comparable susceptibility at unequal risk. In KNP, for example, it has been hypothesized that flies contaminate the vegetation at 1-3 m height, which augments the risk for browsers, such as kudus, whereas grazers, such as zebras, are less at risk [132,210]. Ecological and behavioral differences may also account for the fact that in a large proportion of outbreaks primarily one species is affected although equally susceptible species live in the same habitat but may use a different ecological niche [104].

Pathogenesis

It is the common perception that *B. anthracis* is non-invasive and enters the host organism via

abrasions, inhalation, ingestion or injection. Regardless of the mode of infection, *B. anthracis* spores must overcome the respiratory, cutaneous or digestive epithelial barrier in order to initiate infection [213]. To cross the epithelial barrier, two models of dissemination have been described depending on the intactness of the barrier: the "Trojan horse" model and the "jailbreak" model. The well-established "Trojan horse" model posits a cell-mediated transport of spores (alveolar macrophages or dendritic cells) across the intact epithelial barrier to the local lymph node [214]. Germination of spores and initial multiplication take place while in transit to or after reaching the lymph node. Whenever the number of invading macrophages or dendritic cells exceeds the capacity of the lymph node, cytotoxic effects facilitate deliberation of vegetative forms into the blood system [175]. The "Trojan horse" model applies only to the inhalation route of infection, which has long been the primary focus of research given its relevance in biological warfare and bioterrorism. The more recently described "jailbreak" model assumes a damaged epithelial barrier and applies to all routes of infection [215]. Germination of spores takes place in situ and subsequent multiplication of vegetative bacilli and expression of virulence factors enable local colonization of the host and migration via the lymphatic system to the bloodstream. Evidence for the "jailbreak" model of dissemination has been shown experimentally for all major routes of infection [216,217].

The *B. anthracis* toxin complex and *B. anthracis* poly-γ-D-glutamic capsule constitute the principal virulence factors. Regardless of the mode of crossing the epithelial barrier, vegetative bacilli that have entered the bloodstream express the potent *B. anthracis* toxin complex that determines the fatal progress of the disease. The toxin complex consists of three proteins, protective antigen (PA, 83 kD), lethal factor (LF, 90 kD) and edema factor (EF, 89 kD), which form a so-called AB toxin. PA functions as a cell-binding B component that can bind to LF and/or EF (enzymatic active A components) to form the operative lethal toxin (PA+LF) and edema toxin (PA+EF), respectively, and that can also bind to highly abundant cell surface receptors to mediate entrance of the toxins into the cells [175,176]. The edema toxin functions as an adenylate cyclase that increases the intracellular cyclic adenosine monophosphate (cAMP) level, leading to increased permeability of ionic channels. This results in the loss of intracellular liquid and subsequent edema in surrounding tissues, as seen in cutaneous anthrax. The lethal toxin is a zinc-dependent protease that cleaves the amino termini of mitogen-activated protein kinase kinases (MAPKKs) and thereby disturbs various regulatory processes of the cell, including cell growth, maturation and cellular stress responses. It stimulates the release of proinflammatory cytokines, including tumor necrosis factor α and interleukin 1β, promoting a hyperinflammatory condition that results in shock and subsequent death [175,176]. The second integral cause of *B. anthracis* virulence, the poly-γ-D-glutamic capsule, reduces the host's immune response in favor of bacterial survival via an antiphagocytic effect [218] and a monotonous linear structure that is only weakly immunogenic [219]. Both the capsule and the toxin components are encoded on the two virulence plasmids, pXO1 and pXO2. The genes encoding the toxin components, *pagA*, *lef* and *cya,* and their regulator genes are located on the lager plasmid pXO1, and the capsule gene and its regulator genes, the *capBCADE* operon, are on pXO2 [218,220]. The loss of either pXO1 and/or pXO2 leads to a dramatic reduction of virulence, providing the basis for attenuated live vaccines [184].

Clinical manifestation

Depending on the route of infection, four different clinical manifestations in humans have been described: cutaneous anthrax, ingestion anthrax, inhalational anthrax and, a rather recent manifestation, injectional anthrax [175–177]. Generally, two clinical phases can be distinguished: an initial phase with non-specific symptoms and localized infection, and a later acute phase caused by systemic infection, septicemia and toxemia. A rare but severe complication of all forms is anthrax meningitis (hemorrhagic meningitis), which is almost always fatal [175,177]. The outcome of the disease depends on the route of infection, the risk of systemic infection, possible

complications and the onset of treatment.

Cutaneous anthrax is the most common form of human anthrax infection, representing 95% of all cases worldwide [177]. *B. anthracis* non-invasive endospores enter the organism through small cuts or abrasions of the skin, usually in areas with the highest risk of exposure, e.g. hands, extremities and the neck; a few cases of transmission by insect bites have also been suspected [221,222]. After an incubation period of usually 2 to 6 days (although up to three weeks have been described), characteristic black lesions arise, surrounded by marked edema (eschars) [177]. Most cutaneous infections stay localized and are self-limiting. Antibiotic treatment is still recommended to prevent further damage and complications such as shock and anthrax meningitis [175,223]. Although antibiotic therapy is normally successful, lesions can take weeks to fully resolve due to substantial tissue damage by anthrax toxins [175]. Only a small proportion of cases become systemic and end up being fatal. It has been reported that 10-40% of untreated cutaneous anthrax cases and < 1% of treated cases result in death [177].

The ingestion of *B. anthracis* with contaminated food products and beverages can result in two clinical manifestations: gastrointestinal and oropharyngeal anthrax. After an incubation period of 3 to 7 days, the course of both forms can range from asymptomatic to severe, including sepsis, septic shock and death. Gastrointestinal anthrax lesions can develop in all parts of the gastrointestinal tract, most commonly in the ileum and cecum. The usually multiple, superficial and ulcerative lesions, surrounded by edema, can lead to hemorrhage, obstruction, perforation and ascites. Initial clinical symptoms of gastrointestinal anthrax may be unspecific. As the disease progresses symptoms may become more severe, including abdominal pain, hematemesis, bloody diarrhea and massive ascites. Oropharyngeal anthrax occurs only rarely, mostly in regions where gastrointestinal anthrax is not uncommon. Lesions are generally localized in the oral cavity and covered with a grey pseudomembrane surrounded by extensive edema. At a later stage of the disease, extensive swelling of the neck region and the anterior chest wall can lead to acute respiratory distress syndrome. Both manifestations can result in sepsis, toxemia and subsequent shock and death. The outcome of the disease depends largely on the time of recognition and onset of treatment. Non-treated cases are usually fatal [177].

Inhalational anthrax occurs only rarely and has been described in people working with animal hides (tanneries, drum builders) and via bioterrorist attacks. The incubation period ranges from 4 to 6 days, followed by fever, sweating, fatigue and other non-specific symptoms. Along with bacteremia, patients develop dyspnea, cyanosis, disorientation with coma and death. Generally, the number of inhalational anthrax cases in history is lower than perceived given its involvement in bioterrorist attacks; it most often results in a fatal outcome [177].

Injectional anthrax emerged relatively recently in the early 2000s among heroin users, most likely caused by injection of contaminated heroin [224]. Severe soft tissue infections around the injection sites were observed, but no eschars as are typical for cutaneous anthrax. Patients also showed non-specific gastrointestinal symptoms such as nausea, vomiting and abdominal pain. Due to the high proportion of systemic manifestations of the disease, fatality rates are significantly higher than for cutaneous anthrax [223].

The clinical manifestation of anthrax in animals depends largely on their susceptibility. The clear distinction of three clinical manifestations of anthrax as described for humans (cutaneous, intestinal and inhalational anthrax) is not applicable to animals. Rapid onset of disease, on the one hand, and mild unrecognized symptoms, on the other hand, result in a lack of clinical data. The incubation period for naturally infected cattle is up to two weeks. Very often, the only recognized symptom is sudden death in apparently healthy animals with a short period of distress and disorientation before falling. Bloody discharges from bodily orifices are frequently observed post-mortem, along with swelling in the area of the head, incomplete rigor mortis and absence of blood clotting. Depending on the progress of disease, treatment can still be successful after

symptoms area apparent [177]. Cutaneous lesions, as seen in humans, are rarely seen in animals, but reported cases exist [221].

Bacillus anthracis in African wildlife

B. anthracis is known to affect wildlife in several national parks throughout southern and central Africa, all of which represent savannah ecosystems [212]. The general setting is an enzootic anthrax situation interspersed with periodic epidemics. These usually affect primarily one species (generally herbivores) and mortalities sometimes occur in a wave-like pattern, with high incidence at the start followed by decreasing cases afterward [177,212]. They are normally self-limiting and can in some cases be seen as part of the local ecosystem, not substantially threatening wildlife populations [197]. The chronology of outbreaks varies greatly between locations and most likely depends on environmental factors such as climate and soil properties, among other unknown factors. The most frequently affected species also differs from park to park, and even within a park from outbreak to outbreak [197,198]. This may be explained by behavioral (e.g. feeding habits) and constitutional differences (e.g. impaired immune status due to parasite infection) that put different species at unequal levels of risk.

Most research has been undertaken in Kruger National Park (KNP), South Africa, in Etosha National Park (ENP), Namibia, and Serengeti National Park (SGNP), Tanzania. The results indicate a broad variety of disease dynamics that remains poorly understood.

In KNP anthrax cases have been recorded since the early 1950s, but archaeological findings suggest that the disease may have been present in the area for more than 200 years [197]. Anthrax endemicity is restricted to the northernmost part of the park, where outbreaks peak in the driest months of the year (July-August) [197,225]. Kudus (*Tragelaphus strepsiceros*) represent the main host, followed by impalas (*Aepyceros melampus*). Epidemics sweeping from the northern region through the whole park occur with a periodicity of ten years. They coincide with rainfall cycles in the northern part of the park and fall into dry spells that follow years with exceptionally heavy rainfall [197]. The onset of rainfalls usually terminates outbreaks abruptly. For the anthrax transmission cycle in KNP, it is assumed that *B. anthracis* spores concentrated in water holes during the dry season are the major source of infection. Incipient rainfalls have a dilution effect that interrupts the transmission cycle [197]. Further, blow flies are thought to contribute significantly to the dissemination of the disease [132,197]. The role of vultures in the carryover of spores into water holes has also been discussed [197,210]. It has been shown that anthrax outbreaks are density-dependent and thus self-limiting; the epidemic ratio for kudu is 1:500, meaning that 500 susceptible kudus are required to replace one case with exactly one new case [197]. In KNP anthrax may be seen as an integral part of the ecosystem, but its unnatural fenced-in setting puts endangered species such as the roan antelope (*Hippotragus equinus)* at risk of extermination [197].

In ENP the first anthrax cases were reported in 1964 [226]. Distinct anthrax endemic and non-endemic areas can be differentiated. In endemic areas, sporadic cases occur year-round, interspersed with small-scale outbreaks. Larger epidemics are rarely seen and have been reported only three times since the recording of anthrax mortality started in the 1970s [198]. Elephant (*Loxodonta africana*), Burchell's zebra (*Equus quagga*), blue wildebeest (*Connochaetes taurinus*) and springbok (*Antidorcas marsupialis*) account for 97% of all anthrax cases, and Burchell's zebra alone for 52%. Of the three major outbreaks, two only affected elephants, whereas the third affected Burchell's zebra and blue wildebeest among other ungulates [198]. In contrast to KNP, outbreaks in ungulates peak in the rainy season. Outbreaks in elephants, however, occur at the end of the dry season. The source of infection, as well as the reason for seasonal variation of anthrax dynamics in ENP, remains unidentified [198]. It has been suspected that the host immune status, influenced by the parasite burden, and behavioral characteristics play important roles [104]. Anthrax contributes significantly to overall wildlife mortality in ENP and is responsible for 13%

of animals found dead since 1975 (n=6,000) and suspected in another 11% [227]. Elaborate vaccination programs (dart-gun inoculation from a helicopter) are only implemented for rare and endangered species such as black rhinoceros (*Diceros bicornis*) and roan antelope (*Hippotragus equinus*) [227].

In the Serengeti sporadic outbreaks occur in relatively localized endemic foci [228]. Since veterinary records started in 1996, outbreaks have been found every year, affecting a few focal species, mainly impala, zebras, blue wildebeest and buffalo (*Syncerus caffer*). It has been observed that outbreaks involving grazers occur in the dry season, whereas those involving browsers followed heavy rains [229]. Further, soil alkalinity was identified as a spatial and temporal predictor of infection risk. Four larger outbreaks were observed that varied with regard to the main host between the four species mentioned above. These outbreaks were associated with extreme weather conditions such as heavy rains or droughts [229].

Anthrax outbreaks in wildlife have also been recorded in other national parks. Examples include a mass outbreak in hippopotamus (*Hippopotamus amphibius*) in the Luangwa River Valley, Zambia and the Queen Elizabeth National Park, Uganda in 2004-2005. The fact that hippopotamus do scavenge may have played an important role in the transmission dynamic of this exceptional outbreak [211,230,231]. Further outbreaks were reported in Zimbabwe in 2004 and Kenya in 2005, affecting various ungulates [232,233]. It can be taken for granted that many more anthrax outbreaks occur that are not published because they are not noticed or investigated.

Genotyping and evolution

B. anthracis is one of the most genetically homogeneous pathogens described. Its low genetic diversity may lead to the conclusion that *B. anthracis* emerged recently, as mutations lead to genetic heterogeneity over evolutionary time. However, evolutionary processes are greatly reduced during the dormant phase of *B. anthracis* and only take place during the infection-death-infection cycle. This may contribute to the extreme homogeneity of *B. anthracis* [234].

The nearly monomorphic molecular nature of *B. anthracis* long made it almost impossible to differentiate strains (serotypes or genotypes). Traditional typing methods, such as serotyping, ribotyping and amplified fragment length polymorphisms (AFLP) showed little or no discriminatory power [234,235]. In 1996 Andersen et al. identified a region of genetic variability that allowed for the discrimination of distinct *B. anthracis* strains [235]. This variable number tandem repeat sequence, called *vrrA*, contains a 12-bp repeat that can be present in different copy numbers, creating a DNA polymorphism. For variable number tandem repeat (VNTR) analysis, fluorescently labelled PCR primers are used to produce amplicons of the variable locus. Their size is then determined using a fluorescent image of an electrophoresis gel. Strains can now be differentiated by the length of the PCR fragment generated. In the following years, further VNTR regions were identified, increasing discriminatory power. In 2000 Keim et al. developed a multiple-locus VNTR analysis (MLVA) that uses eight markers (including *vrrA*), of which two marker loci are also localized on the two plasmids [236]. This MLVA-8 system made it possible to identify two major clonal lineages (A and B) and 89 distinct genotypes out of 426 *B. anthracis* isolates [236]. Four years later Keim et al. suggested a MLVA-15 system nested within a hierarchal approach called progressive hierarchical resolving assay using nucleic acid (PHRANA) [237]. In addition to MLVA analysis, this technique employs canonical single nucleotide polymorphism (canSNP) and single nucleotide repeat (SNR) analysis. Canonical SNPs are nucleotide substitutions that define *B. anthracis* strains in different major clades at a lower resolution. SNRs are a class of VNTRs that exhibit extreme mutability and can be used for high-resolution molecular typing of *B. anthracis*. The three methods exhibit different resolution and stability (the higher the resolution the less stable). The hierarchical application of the three methods has more resolving power and accuracy than any of the three assays alone. PHRANA identified 476 unique genotypes among 1067 *B. anthracis* genotypes (canSNPs alone 8, MLVA-15

209 and SNR 418) [237]. In the same year, whole genome sequencing of five diverse strains and subsequent SNP analysis revealed a third rare clonal anthrax lineage C, suggesting that there are more diverse anthrax genotypes [238]. In 2007 van Ert et al. used 12 canSNP and MLVA-15 to identify the global genetic structure of *B. anthracis*. A diverse global collection of 1,000 isolates was tested [239]. CanSNP analysis subdivided the isolates in the three previously recognized lineages (A, B, C) and 12 further sub-groups. The more rapidly evolving MLVA-15 marker identified a further 221 unique genotypes. Regarding the evolutionary history of *B. anthracis*, it is assumed that it emerged from *B. cereus* through the acquisition of the two virulence plasmids, pXO1 and pXO2, as well as several chromosomal mutations. Subsequent evolution is reflected by differences in the global distribution and abundance of *B. anthracis* isolates belonging to the major clonal lineages A, B and C. The majority of isolates are in the A lineage, whereas the B and C lineages comprise rarer genotypes and fewer isolates. A strains are present worldwide, whereas B and C strains occur in narrower ranges. For example, B strains are almost entirely restricted to South Africa. Molecular clock models show that within the A clade two major radiation events occurred that may relate directly to events involving increased human activity. Assuming the substitution rate was properly estimated, the primary radiation event was in the mid-Holocene, i.e. the era of animal domestication and domesticated population expansion. The secondary radiation coincided with the colonial era and importation of *B. anthracis* into the new world. This is clear evidence for how human activity dramatically influenced the distribution and abundance of *B. anthracis* [239].

B. anthracis genotyping in KNP using MLVA-8 on 98 isolates revealed two very distinct strains, one each to the A and the B clade. This may indicate two separate introduction events [225]. In ENP all strains were identified with PHRANA using a 31 MLVA marker system clustered within the *B. anthracis* A branch. All 24 MLVA genotypes differed by only one or two MLVA markers, representing a closely related clonal group [240]. Co-infection with different genotypes has been shown to occur [241].

2.5.2 *Bacillus cereus* biovar *anthracis*

Bacillus cereus biovar *anthracis* (*Bcbva*) was first described in 2001 in chimpanzees in CI [72]. Over the course of eight months, eight chimpanzees (*Pan troglodytes verus*) of apparently good health died with no onset of symptoms. Six of them were sampled and tested negative for pathogens that could have potentially caused sudden death, such as Ebola, Marburg and Lassa. Pathological and histological examination was performed in two cases and revealed small hemorrhages (ecchymoses) in nearly all inner organs and edema and emphysema of the lungs. Microscopic examination showed Gram-positive rod-shaped bacteria intra- and extravascularly in all tissues examined (spleen, liver, lung, lymph nodes and intestine). Analysis of the bacterial 16S gene revealed identity with both *B. cereus* and *B. anthracis*, which cannot be discriminated by the 16S sequence. Subsequent *B. anthracis*- specific rt PCR targeting genes encoded on the *B. anthracis* virulence plasmids was positive for all individuals sampled (6/8). These were the first reported cases of anthrax in chimpanzees [72]. In 2004 *B. anthracis* was also confirmed in bone and muscle tissue from three wild chimpanzees and one gorilla (*Gorilla gorilla gorilla*) found dead in Dja Reserve (DJR), Cameroon (CA), about 2,000 km from TNP. This was the first reported case of anthrax in a gorilla [99,178].

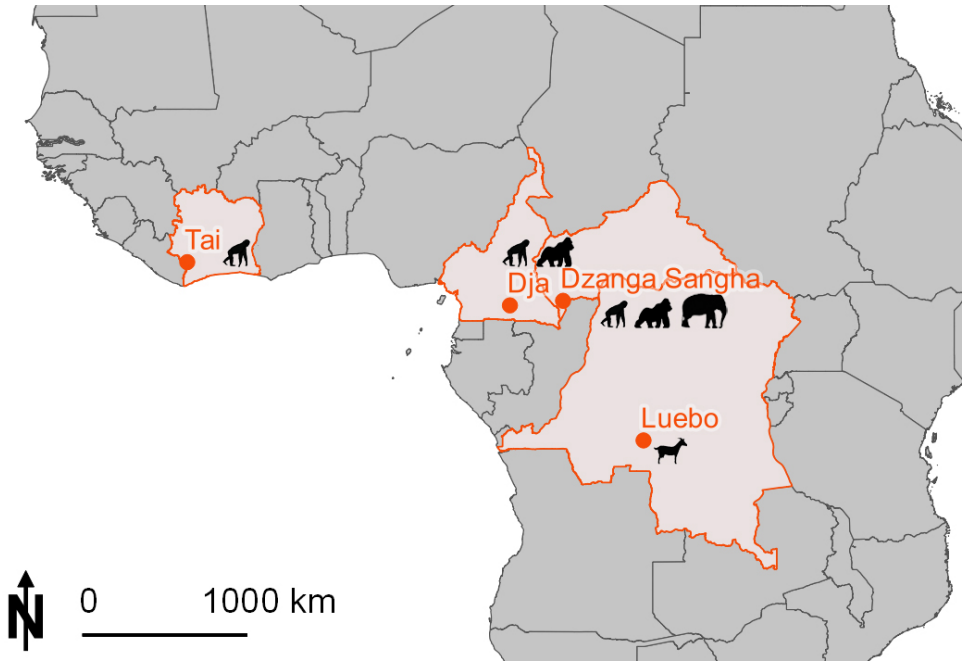

Figure 2: Sites in sub-Saharan Africa where *Bcbva* has been described. *Bcbva* was first described in 2001 in six chimpanzees in Taï National Park, Côte d'Ivoire. In 2004 *Bcbva* was confirmed as the cause of death of three chimpanzees and one gorilla in Dja Reserve, Cameroon. Next, *Bcbva* cases occurred in 2012 in 2013 in Dzanga-Sangha Natural Protected Areas, Central African Republic in a chimpanzee, a gorilla and an elephant and in a domestic goat in Luebo.

In 2006 it was shown that the pathogen causing these fatalities in wild living primates, and that was assumed to be *B. anthracis*, exhibited some major discrepancies. On a microbiological and molecular basis, it became clear that the pathogen under investigation was an anthrax-causing microorganism other than *B. anthracis* [99,179].

B. anthracis has typical microbiological features used for diagnostic differentiation of other members of the *B. cereus* group: *B. anthracis* is non-motile, lacks beta-hemolytic activity and is sensitive to penicillin G and to lysis by the gamma phage (diagnostic tool). The isolates from CI and CA, however, differed significantly from *B. anthracis*. They were motile and resistant to the gamma phage, and the isolates from CA were also resistant to penicillin G. A capsule was expressed not only under CO_2 conditions but also under normal growth conditions. As *B. anthracis* protective antigen was expressed and secreted in the medium under CO_2 conditions. Traditional microbiological diagnostics would probably miss the pathogenic CI and CA strains [179].

The molecular findings also pointed towards an atypical form of *B. anthracis*. MLVA analysis based on six chromosomal markers and two markers localized on the plasmids was performed on all DNA isolates (n=10) from CI (n=6) and CA (n=4) [99]. No genetic difference was found among the isolates from CI and CA. Surprisingly, however, the strain from Côte d'Ivoire (CI strain) and the strain from Cameroon (CA strain) formed a group clearly distinct from *B. anthracis* groups A and B. This new group was called F (for "forest anthrax cluster") [99].

These findings were supported by multilocus sequence typing (MLST), a molecular tool that displays relatedness based on housekeeping genes' allelic profiles. MLST also showed that the two "forest anthrax" strains were identical and did not cluster with *B. anthracis* strains, but were more closely related to a pathogenic *B. thuringiensis* serovar konkukian 97-27 strain and a *B. cereus* strain (*B. cereus* strain E33L) [179]. Also differing from *B. anthracis*, the new "forest anthrax" strains lack four recently identified *B. anthracis*-specific prophage regions (genomic insertion) A,

B, C and D, which are present only in *B. anthracis* and not in *B. cereus* [99,242].

In 2010 the fully sequenced genome of an isolate from CI was published. It consists of four replicons: a chromosome of 5,488,8191 bp, two larger plasmids of 181,907 bp and 94,469 bp and a smaller plasmid of 14,219 bp. Comparative sequence analysis revealed high similarity of the chromosome with the two strains already identified by MLST (*B. thuringensis* serovar konkukian 97-27, *B. cereus* strain E33L). The two larger plasmids showed 99-100% identity with the two virulence plasmids of *B. anthracis*, pXO1 and pXO2. The smaller plasmid, pCI-14, fits into a group of smaller cryptic plasmids of *B. cereus* with unknown functions and was later only found in some isolates from CI. Based on these and previous findings, the isolate was designated *Bacillus cereus* biovar *anthracis* (*Bcbva*) [180].

Further *Bcbva* cases were recorded in 2012 and 2013 in Luebo, Democratic Republic of Congo (DRC) in a domestic goat (*Capra aegagrus hircus*) and in Dzanga-Sangha Natural Protected Area (DSPA), Central African Republic (CAR), in an elephant (*Loxodonta cyclotis*), a chimpanzee and a gorilla (Figure 2, page 27) [243]. Recently, phylogenetic analysis of five *Bcbva* genomes, one each from CI, CA and DRC and two from CAR, showed that all *Bcbva* strains form a distinct chromosomal clade not closely associated with *B. anthracis*. The closest relative was *B. cereus* ISP 3191, an environmental strain of *B. cereus* isolated from a food source in Belgium. Approximately 39,000 SNPs separated the strains within the *Bcbva* clade from *B. anthracis*, whereas an average of only 12,000 SNPs separated them from their closest relative. Interestingly, other *B. cereus* strains were more closely related to *B. anthracis* than *Bcbva* (all findings from a maximum likelihood tree based on core chromosomal SNP data) [243].

Bcbva is the first anthrax-causing bacterium described that possesses both anthrax virulence plasmids but does not belong the monophyletic *B. anthracis* group. It differs from *B. anthracis* in important diagnostic features, increasing the probability of misdiagnosed cases. Nothing is known about *Bcbva*'s transmission cycle, but it has been shown in animal models that its virulence is comparable with *B. anthracis* [244]. Furthermore, in addition to the *B. anthracis* polyglutamate capsule, *Bcbva* expresses a hyaluronic acid capsule, also encoded on pXO1, that retains full virulence even if pXO2 is cured [244]. The occurrence of *Bcbva* in four different areas spanning a maximum distance of 2,000 km suggests a broad distribution throughout Western and Central Africa. The origin of *Bcbva* remains only a matter of speculation. It is most likely that *Bcbva* emerged recently when an unknown *B. cereus* acquired the *B. anthracis* plasmids.

2.6 Adenoviruses

Adenovirus (AdV) is the second pathogen we targeted to assess fly-based wildlife disease surveillance. In contrast to *Bcbva*, AdVs are primarily shed with feces and are generally low-pathogenic. As we conducted only a first screening for AdV in TNP, in contrast to an in-depth study of *Bcbva,* I will provide a much shorter overview of the AdV literature that still allows for an adequate understanding of the results obtained.

2.6.1 General characteristics

Adenoviruses are double-stranded DNA viruses that can be detected in all vertebrate classes [245]. Their name originates from the Greek term for "gland," the tissue AdVs were first isolated from [246]. AdVs are facultative pathogens and most AdV infections proceed with mild or no clinical symptoms.

The taxonomy of AdVs relies on the assumption that they are strictly host-specific and have coevolved with their vertebrate hosts. This assumption led to five AdV genera according to the presumed hosts: Mastadenoviruses (mammals), Aviadenovirus (birds), Siadenovirus (amphibians), Ichtadenoviruses (fish) and Atadenovirus (scaled reptiles) [247,248]. The concept of strict co-divergence has now been challenged and AdVs have been isolated from vertebrates that do not match "their" AdV genus; the taxonomic structure, however, was maintained [249,250]. AdV genera can be further divided into species (e.g. HAdV A) and subtypes. There exist different criteria for defining species based on serological, biological and genetic features. For example, distinct species should show at least 5-10% sequence difference of the hexon and DNA polymerase gene [245]. Differentiation of serotypes relies mainly on serology, but here molecular information can also be protracted [245].

AdVs are non-enveloped viruses and consist of an icosahedral capsid and an inner nucleoprotein core. The icosahedral capsid is comprised of 252 capsomeres that are mainly represented by the hexon protein and complemented at the vertices with penton proteins forming protruding fibers [251]. Like most non-enveloped viruses, AdVs display a relatively high tenacity, being resistant to temperature and pH alterations, as well as disinfectants and lipid solvents, facilitating waterborne infection.

The AdV genome is on average 36 kbp long and has an average G+C content of 51% [245]. The central part of the genome contains genes that encode structural and replication proteins and that are well conserved within genera. The genome ends, however, vary in length and the layout of their genes [248]. Homologous recombination, the horizontal exchange of related gene sequences between viruses, has been commonly described for AdVs [252,253]. It has been stated that for HAdV D recombination might play a greater role in generating genomic diversity than base substitutions [254].

Pathogenicity of AdV is based on a stimulating effect on cell proliferation to facilitate virus replication, as well as the induction of cell lysis by apoptosis. Further, virus proteins hinder the adaptive immune response by preventing the transport of mature major histocompatibility complex I to the cell surface, which normally displays pathogen particles for recognition by cells of the acquired immune system [255].

2.6.2 Epidemiology and clinical manifestation

AdVs occur worldwide, and for humans it has been stated that nearly all adults show serologic evidence of past infection with AdV [256]. For animals only few data on prevalence is available. However, the high diversity of affected species and the broad spectrum of involved ecosystems (e.g. tropics, Antarctica) suggest that AdV infection in animals also occurs around the globe [257,258]. In TNP AdVs have been described only in NHPs, but it can also be assumed that non-

simian AdVs are circulating in the region [108,173]

AdVs are mainly transmitted horizontally, directly or indirectly via the fecal-oral route and later via the eyes and throat, and urine. The relatively high tenacity of AdVs facilitates the waterborne spread of the virus. In several situations, ingestion of contaminated water can be considered the major route of infection. In dense populations, transmission via aerosols can be facilitated. Vertical transmission of the virus has also been described, e.g. in cattle [259], and this route plays a significant role in virus spread in poultry [260]. In general, the incubation period lasts from 1-7 days, depending on the dose of infection. AdVs can be shed up to several weeks after primary symptomatic infection or stay latent in lymphatic tissues (e.g. tonsils or the intestinal tract) [261–263]. In immune-compromised hosts, shedding may take even longer [264].

Most AdV infections occur during early stages of life and are usually self-limiting, with only mild or even no clinical symptoms. Nevertheless, severe outbreaks with fatal outcomes do occur [256]. Most at risk for severe infections are individuals that impaired for other reasons. With regards to humans, this includes the so-called YOPIs (young, old, pregnant or immunocompromised, e.g. due to HIV). Among animals, poor hygiene conditions and crowding constitute predisposing factors for severe disease outcomes [255]. Similarly, crowding in military camps led to the outbreak of severe respiratory disease in humans [265]. Generally, clinical symptoms, or their absence, depend on the genus and species of the virus. AdVs infecting humans (HAdVs) all belong to the genus Mastadenovirus. Depending on the species (HAdV A-G), clinical symptoms, if shown, are mainly gastrointestinal and respiratory. It is noteworthy that HAdV F is one of the most common viruses involved in acute gastroenteritis in children [266,267]. AdVs infecting animals are largely represented by Mastadenovirus with respect to mammals, but also by Atadenoviruses. Avians are infected mainly with Aviadenoviruses but also with Atadenoviruses and Siadenoviruses. In animals as well, gastrointestinal and respiratory symptoms are predominant. In addition to most asymptomatic or subclinical infections, some clinical manifestations are non-negligible. For example, egg drop syndrome in chicken and the hemorrhagic enteritis virus in turkeys can have severe consequences for the poultry industry [268,269]. In small animal medicine infectious canine hepatitis and the involvement of AdV in infectious tracheobronchitis are relevant (ITB or kennel cough).

No specific treatment for AdV infection is available. However, preventive measures such as adhering to hygiene standards can contribute significantly to preventing outbreaks of disease. Live and inactivated vaccines are available. For humans, an oral vaccine is used to prevent respiratory outbreaks in military camps [265]. For poultry and dogs, vaccination against AdV is common [270,271].

2.6.3 Cross-species transmission and zoonotic potential

It is generally assumed that AdVs have often codiverged with their respective vertebrate hosts, meaning that the divergence of AdV lineages mirrors the divergence of vertebrate classes [247,248]. This hypothesis is broadly applicable to many AdV lineages. However, there is evidence for cross-species transmission, and thus against strict co-evolution. In particular, viruses belonging to the genus Atadenovirus and Siadenovirus cannot be assigned to one specific vertebrate class. This could be explained by multiple host switches from reptiles to birds, marsupial mammals and ruminants, resulting in an exceptionally wide host spectrum across vertebrate classes [272]. Within the genus Mastadenovirus, principally following the concept of co-evolution of virus and host, evidence for cross-species transmission has also been found. A remarkably close genetic relationship between canine AdV and bat AdV indicates an interspecies transmission event and points to a bat origin of canine AdV. This assumption is further substantiated by a relatively high pathogenicity and broad host range of canine AdV [273,274]. A comparable close genetic relationship has been shown for equine AdV-1 and bat AdV, suggesting a common ancestor for bat, canine and equine AdV [275].

Cross-species transmission of a zoonotic nature has also been reported. Phylogenetic analyses of human and simian AdVs showed that the human AdV species B, E and G might have evolved from simian AdVs and originate in AdVs of NHPs: HAdV B most likely originated in gorilla and HAdV E in chimpanzee [108,173,276,277]. Further, the genome of HAdV 4, the only member of HAdV E obtained from a human, is a recombinant of human and simian AdV sequences [278]. The only member of HAdV G, HAdV-52, is closely related to SAdV-1 and SAdV-7 and was isolated from a patient with gastroenteritis [279]. The fact that zoonotic transmission occurred during AdV evolution is also suggested by the fact that NHPs shed AdVs belonging to every HAdV species [173,280].

Cases of zoonotic AdV transmission from NHPs to humans have also been directly observed. In a Primate Research Center in California, a novel titi monkey AdV was obtained from a *Callicebus cupreus* specimen and from a diseased human in close contact with the New World monkey [281]. This was also the case for a simian AdV C retrieved from a captive baboon (*Papio hamadryas anubis*) as well as a human with respiratory and flu-like symptoms [281,282].

It can be expected that the reverse transmission of AdV from humans to animals also occurs. For example, anti-HAdV antibodies were detected in chimpanzee.

3. Publications

3.1 Publication I

Persistent anthrax as a major driver of wildlife mortality in a tropical rainforest

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Received: 25.10.2016

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Authorship shared with Fee Zimmermann.

Own contributions to this publication:

Sample collection:

- Collection of flies in TNP, CI ("fly snapshot") together with Fee Zimmermann.
- Coordination of fly sampling in sub-Saharan Africa.

Lab work:

- DNA extraction from flies under BSL 2 and 3 conditions.
- Implementation of PCRs for the detection of anthrax in fly samples.
- Implementation of PCRs for the detection of mammalian DNA in flies.
- Metabarcoding for species assignment of fly meals containing mammalian DNA.
- Barcoding for species assignment of flies.

Data analysis:

- Phylogenetic and statistical analyses for fly species assignment (GMYC analysis).
- Bioinformatic pipeline for the analysis of metabarcoding data for fly meal analysis.
- Statistical analyses (impact of season on anthrax positivity of carcass and fly samples, impact of amount of mammalian DNA in a fly on anthrax positivity, impact of genus of fly meal on anthrax positivity, geographic distribution of anthrax positive flies in TNP).
- Analysis of GPS data and creation of distribution maps

DOI: https://doi.org/10.1038/nature23309

3.2 Publication II

Assessing the feasibility of fly based surveillance of wildlife infectious diseases

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SCIENTIFIC REPORTS

Assessing the feasibility of fly based surveillance of wildlife infectious diseases

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Monitoring wildlife infectious agents requires acquiring samples suitable for analyses, which is often logistically demanding. A possible alternative to invasive or non-invasive sampling of wild-living vertebrates is the use of vertebrate material contained in invertebrates feeding on them, their feces, or their remains. Carrion flies have been shown to contain vertebrate DNA; here we investigate whether they might also be suitable for wildlife pathogen detection. We collected 498 flies in Taï National Park, Côte d'Ivoire, a tropical rainforest and examined them for adenoviruses (family *Adenoviridae*), whose DNA is frequently shed in feces of local mammals. Adenoviral DNA was detected in 6/142 mammal-positive flies. Phylogenetic analyses revealed that five of these sequences were closely related to sequences obtained from local non-human primates, while the sixth sequence was closely related to a murine adenovirus. Next-generation sequencing-based DNA-profiling of the meals of the respective flies identified putative hosts that were a good fit to those suggested by adenoviral sequence affinities. We conclude that, while characterizing the genetic diversity of wildlife infectious agents through fly-based monitoring may not be cost-efficient, this method could probably be used to detect the genetic material of wildlife infectious agents causing wildlife mass mortality in pristine areas.

The recent epidemic of Ebola virus in West Africa has again shown the relevance of emerging infectious diseases (EID) for global public health and economies[1,2]. Like the Ebola virus, most EIDs are of zoonotic origin and involve wildlife reservoir hosts[3]. Emergence is likely facilitated by increased contact of humans and domestic animals with wildlife combined with insufficient access to health care[4]. Therefore, it is most intense in resource-poor tropical regions, such as those in Central and West Africa where the Ebola virus repeatedly emerged[3,5]. To prevent disease emergence, monitoring of wildlife infectious agents in these hotspot regions is essential. Some prior knowledge on these microorganisms' genetic diversity may accelerate the identification of early-stage spillover events and help mitigate their outcome.

Surveillance of wildlife infectious agents faces many hurdles. Poor infrastructure and limited resources in remote areas hinder sample collection, storage and transport in such a way that setting up the logistic framework necessary for acquiring wildlife samples is often challenging. This is even more complicated when endangered species are to be monitored. For instance, non-human primate species, which are promising candidates for detecting potential zoonotic agents[6], are generally strictly protected.

This means that only non-invasive sampling methods can be used, e.g. fecal sampling[7]. Fortunately, nucleic acids of vertebrate-infecting microorganisms exhibiting a variety of tissue tropisms have already been recovered from fecal samples[8–10]. These methods come with some disadvantages: they incur additional organizational costs, e.g. extra staff often needs to be recruited and specifically trained, they are labor-intensive and they exclude candidate reservoir species for which fecal sampling is impractical or impossible, e.g. wild rodents. This highlights the need for the development of complementary and cost-efficient monitoring techniques.

Blood-sucking invertebrates might represent interesting alternative sources of vertebrate-pathogen nucleic acids. Blood meals of many hematophagous arthropods have been demonstrated to contain DNA from their vertebrate hosts and the pathogens for which they act as vectors, i.e. malaria in birds[11]. Besides vector-borne pathogens, it is also possible that non-vector-borne pathogens could be contained in blood meals. Interesting examples include the detection of RNA from H5N1 avian influenza A viruses in a mosquitoes' blood meal[12–14].

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Grubaugh and colleagues went a step further and proposed to use blood meal analysis as a tool to survey human pathogens in remote tropical locales, which they refer to as *xenosurveillance*[15]. Blood-sucking arthropods however often exhibit strong host preferences, which may be suboptimal when the objective is to survey infectious agent diversity in complex ecosystems with high biodiversity.

Non blood-sucking invertebrates feeding on vertebrate fecal matter and/or carrion, such as blow and flesh flies (here referred to simply as flies), might also be suitable for the surveillance of wildlife infectious agents. Flies are abundant and ubiquitous, have little host preference and are easy to trap[16,17]. We also recently showed that flies often contain DNA fragments of their mammalian hosts[16,17]. Finally, the genetic material of a number of hitchhiked microorganisms was already detected in flies, including food borne bacteria, e.g. *Salmonella* spp., and enteric viruses[18–21]. For example, Newcastle disease virus (NDV) RNA was detected in, and even virions were isolated from, flies collected in the vicinity of infected chickens. Similarly, H5N1 RNA was found in flies collected in the surroundings of a poultry farm with infected birds[22,23].

These studies however focused on flies caught near high-density vertebrate populations, which raises questions about the broad applicability of this method. In this study, we investigate whether flies are suitable for vertebrate-infecting microorganism surveillance in complex ecosystems with high species richness. We analyzed flies collected in a remote tropical rainforest, Taï National Park (TNP), Côte d'Ivoire, and focused on an *a priori* favorable target: adenoviruses (AdV; family *Adenoviridae*). AdV are shed massively in feces, are usually host-specific and have already been detected in many vertebrates in TNP[24,25].

Results

Out of 498 flies, 156 (31%) contained mammalian 16 S mt DNA. We considered all mammal positive flies as suitable for AdV screening, but due to shortage of material we could only test 142 of these flies. From eight flies AdV DNA could be amplified and sequenced once and six AdV sequences could be confirmed a second time. A BLAST search revealed that four of the six sequences were $\geq$98% identical to a simian AdV sequence determined from a mona monkey (*Cercopithecus campbelli*, KP274048) in Côte d'Ivoire (Fly 92, Fly 101, Fly 740, Fly 1355)[26]. The remaining two sequences (Fly 381, Fly 1375) showed 100% identity with simian AdV sequences obtained from captive chimpanzees in the US (FJ025905, FJ025926) and from a wild chimpanzee in TNP (JN163974) and 98% identity with a murine AdV 2 sequence (NC014899), respectively (Table 1).

We also performed phylogenetic analyses in both maximum likelihood and Bayesian frameworks to better determine the position of fly-derived AdV sequences within the AdV family tree (Fig. 1). In line with the BLAST search, sequences from Fly 92, Fly 101, Fly 740 and Fly 1355 formed a well-supported clade with the mona monkey AdV sequence (aLRT 0.99, pp 1; Fig. 2C). The sequence of Fly 381 clustered with AdV sequences from captive and wild chimpanzees (FJ025905, FJ025926, JN163974, FJ025906, FJ025904, FJ0295899; aLRT 0.98, pp 1; Fig. 2B)[24,27]. These sequences nested within the clade corresponding to species *Human mastadenovirus C*, albeit with a much lower statistical support (HAdV-C; aLRT 0.93, pp 0.85). The sequence of Fly 1375 was most closely related to the murine AdV B (Murine AdV 2) sequence (aLRT 0.99, pp 1; Fig. 2A).

Meal analyses based on Sanger sequencing identified plausible hosts in 3 of the 6 AdV positive flies (Fly 92, Fly 101, Fly 1355; Table 1). To further investigate potential hosts, we performed an in-depth fly meal analysis of all six AdV positive flies using a metabarcoding approach. After quality trimming, 65,552 reads–8,683 to 12,278 reads per fly - were used for taxonomic assignment. Overall, we identified hosts from 9 mammal families and 10 genera/species. Five flies contained DNA from multiple hosts (Fly 92, Fly 101, Fly 381, Fly 1355, Fly 1375; Table 1). The 3 plausible hosts identified by Sanger sequencing were confirmed by this approach. Fly 740, which harbored one of the simian AdV sequences, only contained rodent DNA fragments. For the last two flies, the metabarcoding approach revealed the presence of DNA fragments belonging to plausible hosts, i.e. rodents in Fly 1375 and a hominid in Fly 381. As the hominid family contains the two closely related genera *Homo* and *Pan,* we manually checked the according sequences and were able to refine the assignment to *Pan troglodytes*.

Discussion

We investigated the feasibility of using DNA derived from flies for the surveillance of wildlife infectious diseases. We were able to detect short AdV sequences in 6 flies, that is 4.2% of all mammal positive flies. We used these sequences for phylogenetic analyses and found that most represented AdVs known to infect monkeys and great apes in the region[24,25]. The close relationship of four sequences with an AdV sequence obtained from a single mona monkey supports the notion that this AdV may be relatively abundant in the region[26]. The fifth fly-based simian AdV sequence clustered with HAdV-C sequences and clearly belonged to the chimpanzee clade. HAdV-C viruses are very host-specific and seem to have co-diverged with their hominid hosts[25]. We also detected what is likely a new rodent AdV, thereby underlining the potential of flies to also monitor small-bodied species. Finally, our high-throughput fly meal analyses identified multiple hosts, including plausible ones, in 5 of 6 AdV positive flies. These results demonstrate that fly-based analyses allow for the simultaneous characterization of microorganism genetic diversity and their distribution in local mammalian hosts.

In comparison with detection rates in fecal samples (11 to 58%), the AdV detection rate in flies appears low[25,28]. This might result from the extreme dilution of vertebrate-infecting microorganisms in carrion flies, which itself results from the interplay of meal quantity, quality, frequency and the speed of digestive processes[29]. Given this low detection rate, systematic screenings would probably only make sense where fly collections established for other purposes, e.g. mammal diversity assessment, are available. Sample pooling combined with deep sequencing of PCR products may help decrease the workload and costs of such a screening approach.

Further investigations are needed to determine the extent to which the approach described here is applicable to other microorganisms. The low detection rate of AdV sequences in flies suggests that surveillance of non-enteric microorganisms might be complicated. However, in the case of outbreaks with massive production of microorganisms, e.g. Ebola virus outbreaks[30], there might be a good chance that pathogen nucleic acids

AdV Fly ID	closest AdV BLAST hit (identity/host/accession)	Mammal 16 S - fly meal analysis			
		Sanger		NGS MiSeq	
		closest BLAST hit	species assignment (assigned raw reads)	genus assignment (assigned raw reads)	family assignment (assigned raw reads)
92	Simian AdV (98%/*Cercopithecus campbelli*/KP274048)	*Cercopithecus nictitans* 96%	*Cercopithecus nictitans* (9289)	*Cercopithecus* (9859)	Cercopithecidae (11194)
			Colobus guereza (1335)	*Colobus* (1335)	Suidae (123)
			Cercopithecus campbelli (559)	*Sus* (123)	not assigned (38)
			Sus scrofa (123)	not assigned (38)	
			not assigned (49)		
101	Simian AdV (99%/*Cercopithecus campbelli*/KP274048)	*Cercopithecus diana* 90%	*Cercopithecus diana* (9327)	*Cercopithecus* (10266)	Cercopithecidae (10266)
			Cercopithecus campbelli (825)	*Sus* (395)	Suidae (395)
			Sus scrofa (352)	not assigned (24)	not assigned (24)
			not assigned (181)		
381	Simian AdV (100%/*Pan troglodytes*/JN163974, FJ025905, FJ025926)	dirty sequence	not assigned (10526)	*Felis* (6657)	Felidae (6657)
			Felis catus (178)	not assigned (4047)	Hominidae[2] (4047)
740	Simian AdV (98%/*Cercopithecus campbelli*/KP274048)	unknown rodent 100%[1]	*Cricetomys* sp. 1 PG-2014 (6767)	*Cricetomys* (7050)	Nesomyidae (7050)
			not assigned (5511)	not assigned (5228)	not assigned (5228)
1355	Simian AdV (100%/*Cercopithecus campbelli*/KP274048)	*Cercopithecus campbelli* 99%	*Cercopithecus campbelli* (7529)	*Cercopithecus* (7571)	Cercopithecidae (11805)
			not assigned (4318)	not assigned (4259)	not assigned (42)
				Cercocebus (17)	
1375	Murine AdV 2 (98%/*Mus musculus*/NC014899)	Cephalophini 97%	not assigned (5252)	not assigned (3196)	Cercopithecidae (3139)
			Civettictis civetta (2598)	*Civettictis* (2598)	Viverridae (2598)
			Rattus rattus (620)	*Cephalophus* (1916)	Bovidae (1916)
			Neoromicia somalicus (213)	*Rattus* (760)	Muridae (760)
				Neoromicia (213)	Vespertilionidae (213)
					not assigned (57)

Table 1. Results of AdV screening and in depth fly meal analysis. [1]Assignment based on a local database. [2]Manual BLAST search revealed 100% identity with *Pan troglodytes*.

are detectable in flies. Of course, the detection probability will depend on the biology of the microorganism of interest. Here also, high throughput sequencing approaches (including shotgun sequencing) could open up new perspectives, as recently shown with mosquitoes[13,14]. It was recently demonstrated that portable sequencing devices such as the MinION (Oxford Nanopore Technologies, Oxford, United Kingdom) can be used for on-site sequencing in outbreak situations[31,32]. These technologies only require a basic molecular laboratory in the field. Such laboratories currently allow users to perform sequencing, though the high error rate and relatively low throughput of MinIONs currently limit them to amplicon sequencing based approaches. The limited needs in the present study suggest it could be feasible to conduct fly/amplicon-based wildlife surveillance during major outbreaks, including in resource-poor countries.

Invertebrates other than carrion flies and blood sucking arthropods might also constitute a valuable source of information on vertebrate-infecting microorganisms. For example, leeches can ingest several times their weight host blood in a single meal and could therefore be seen as long-term blood tanks. Most recently, a number of viruses (with DNA or RNA genomes) were shown to persist up to four months in experimentally fed aquatic leeches, with bovine parvovirus being detectable for up to six months[33]. Terrestrial leeches, whose lifestyle might be more compatible with broad, undirected wildlife molecular epidemiology, were also recently shown to allow retrieval of their host's DNA[34]. Both aquatic and terrestrial leeches warrant a careful examination of their potential as tools for wildlife microorganism sampling.

Finally, an alternative to microorganism nucleic acid detection might be the detection of antibodies reactive to these microorganisms. If this is feasible, it would open the potential to examine wildlife exposure to microorganisms. Detection of trypanosome-reactive antibodies from a number of haematophagous dipterans was reported as early as 1962 [35]. This potential tool then fell into a long-lasting oblivion until its recent rediscovery by Barbazan and colleagues, who showed that blood-fed mosquitoes contain detectable levels of various virus-reactive antibodies[36]. Determining whether invertebrate-based serological surveys can be conducted in the wild promises to be an exciting area of future research.

Material and Methods

Sample collection. Sample collection was performed with the permission of the Ivorian national parks authorities (OIPR) and the ministry of research of Côte d'Ivoire. Flies used in this study were captured in Taï National Park, Côte d'Ivoire, a tropical rainforest with remarkable mammal biodiversity. Overall, 498 flies were captured using customized fly traps consisting of a pyramidal mosquito net over a plastic bowl containing a

Figure 1. Maximum clade credibility (MCC) tree of mastadenoviruses. This MCC tree is based on the Bayesian analysis of a 370 bp long alignment of hexon gene sequences. Posterior probabilities are plotted above branches. The tree was built under a clock model and thus is rooted. Enlarged section A, B and C are shown in Fig. 2.

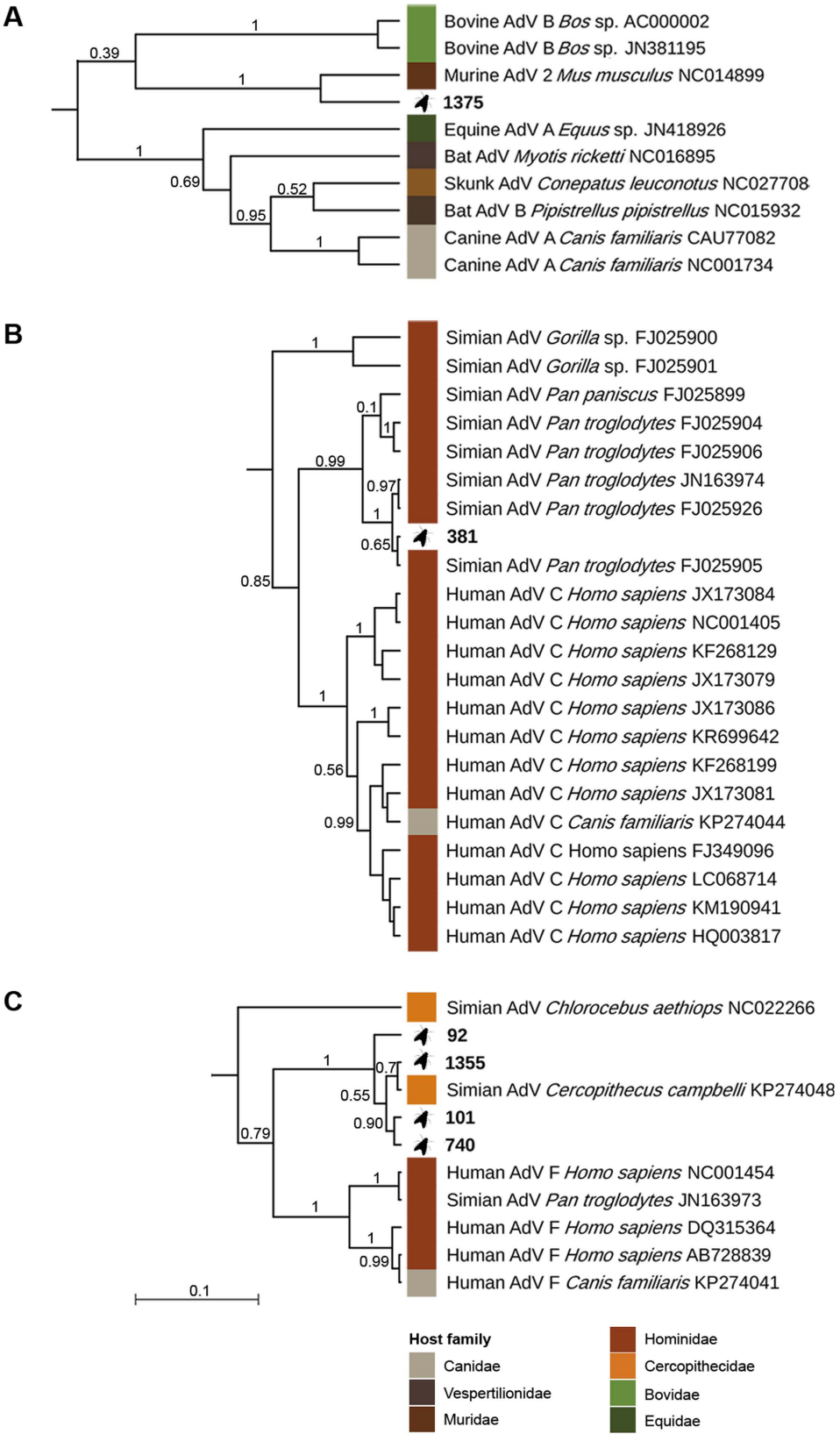

Figure 2. Enlarged sections of the maximum clade credibility of mastadenoviruses. The original MCC tree (Fig. 1) was based on the Bayesian analysis of a 370 bp long alignment of hexon gene sequences. Posterior probabilities are plotted above branches. Study sequences are in bold, reference sequences are represented by host name and accession number.

commercial bait (Unkonventionelle Produkte Feldner, Waldsee, Germany) or a piece of meat[16]. After collection, flies were either placed in Cryotubes (Thermofischer, Waltham, MA, USA), and stored in liquid nitrogen tanks, or in 50 ml Falcon tubes (Carl Roth, Karlsruhe, Germany) containing silica and stored either at ambient temperature or 4 °C.

Nucleic acid extraction. Single flies were cut with sterile scissors and crushed in 250 μL phosphate buffer saline using the FastPrep®-24 Instrument (MP Biomedicals, Illkirch, France). One hundred μL of the resulting mix were then used to extract carrion fly DNA using the EURx GeneMatrix Stool DNA Purification Kit (Roboklon, Berlin, Germany).

Identification of flies suitable for adenovirus screening. We considered that flies containing mammal DNA would be suitable for adenovirus screening. To show the presence of mammal DNA, we first ran a real time PCR targeting a short 130 bp fragment of mitochondrial 16 S rRNA (16Smam1 5′-CggTTggggTgACCTCggA-3′ and 16Smam2 5′-gCTgTTATCCCTAgggTAACT-3′)[37]. Reaction volume was 12.5 μL and contained 6.25 μL GoTaq® qPCR Master Mix (Promega, Fitchburg, WI, USA), 0.2 μM of each primer, 1 μM of blocking primer (human blocking primer 16Smam_blkhum3 5′-CggTTggggCgACCTCggAgCAgAACCC—spacerC3[38]), 1 μL DNA extract or 1 μL of a diluted African palm civet (*Nandinia binotata*) PCR product (10^1–10^5 molecules per μL) for standard reactions. Cycling conditions were: 95 °C 5 min, 40 cycles [95 °C 15 sec, 64 °C 1 min], and 95 °C 1 min, 55 °C 30 sec, 95 °C 30 sec. For all positive flies, we attempted to generate PCR products for sequencing using the same amplification primer pair as for the real-time PCR[16,37]. PCRs were carried out in a total volume of 25 μL and seeded with 200 ng of DNA (DNA concentration > 40 ng/μL) or 5 μL DNA (DNA concentration <40 ng/μL). Reactions contained 0.2 mM dNTP (with dUTP replacing dTTP), 4 mM $MgCl_2$, 0.2 μM of each primer, 1 μM of two different blocking primer (human blocking primer 16Smam_blkhum3 5′-CggTTggggCgACCTCggAgCAgAACCC—spacerC3[38], pig blocking primer 16Smam_blksus1 5′-CggTTggggTgACCTCggAgTACAAAAAAC—spacerC3[16]), 0.3 U Amperase® uracil N-glycolsylase (Invitrogen), 1.25U Platinum® Taq Polymerase (Invitrogen) and 2.5 μL 10x PCR Buffer (Invitrogen). Cycling condition were: 45 °C 7 min, 95 °C 15 min, 42 cycles [95 °C 30 sec, 64 °C 30 sec, 72 °C 1 min], and 72 °C 10 min. PCR products were cleaned up with ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA) and sequenced in both directions according to Sanger's method using the BigDye Terminator kit v3.1 (Thermofischer). All chromatograms were evaluated using the software Geneious Pro v9.1.3 (Biomatters Ltd., Auckland, New Zealand)[39]. Sequences were assigned to species or higher taxa using BLAST[40] and following the rationale depicted in Calvignac-Spencer and colleague's study[16]. Most of these assignments were made in course of the study of Schubert & Stockhausen *et al.* 2014. Flies that produced a band of the expected size but did not yield interpretable sequences were also used for AdV screening.

Adenovirus screening. We implemented various countermeasures to minimize contamination. To avoid cross-contamination with native AdV DNA, DNA extraction was never performed simultaneously with other sample types (fecal and tissue samples). To minimize contamination with PCR products, PCR setup and post-PCR analysis steps were performed in separate, dedicated rooms. In addition, a glovebox exclusively dedicated to fly analysis was used to set up all PCR performed for this study. We also used dUTP instead of dTTP and cleaned our confirmatory reactions with uracil n-glycosylase (UNG) to further reduce the likelihood of carry over contamination with PCR products (see below). It should also be noted that before this study, AdV sequences had never been amplified in the laboratory where AdV screening was performed.

A semi-nested PCR system described by Pauly *et al.*[26] was used for detection of adenoviruses[26]. Primers had been designed for the generic detection of mastadenoviruses and targeted a short 160 bp fragment of the hexon gene (6500 s 5′CgCAgTggKCNTWCATgCACAT-3′, 6500 s 5′-ACCCACgAYgTSACNACNgA-3′, 6500as 5′-gTgCCggTgTANggYTTRAA-3′). All PCRs were carried out in a 25 μL mix containing 0.2 mM dNTP (with dUTPs replacing dTTPs), 4 mM $MgCl_2$, 0.2 μM of each primer, 1.25U Platinum® Taq Polymerase (Invitrogen) and 2.5 μL 10x PCR Buffer (Invitrogen). Reactions of the first round were seeded with 200 ng DNA extract or 5 μL if DNA concentration was below 40 ng/μL and the second round with 1.5 μL of 1:40 diluted PCR product of the first round. Cycling conditions were: 95 °C 5 min, 40 cycles [95 °C 30 sec, 56 °C 30 sec, 72 °C 60 sec], and 72 °C 10 min. PCR products were Sanger sequenced as described above. Flies apparently containing amplifiable AdV nucleic acids were confirmed using the same assay but including 0.3 U Amperase® UNG (Invitrogen) in the first round reaction so as to minimize the risk of contamination with PCR products. Again, PCR products that yielded a band were sequenced using the Sanger method. All chromatograms were evaluated using Geneious Pro v9.1.3[39] and the respective sequences were confirmed to be adenoviral by a BLAST search[40].

Phylogenetic analysis. The dataset used for phylogenetic analysis comprised AdV sequences generated in this study (n = 6) and hexon gene sequences extracted from all available complete genomes of the genera *Mastadenovirus* and *Atadenovirus* (n = 506). This set of sequences was reduced to only contain unique sequences using FaBox v1.41[41]. All remaining 363 sequences were aligned at the nucleotide level in SeaView v4[42], using the MUSCLE algorithm[43]. Conserved blocks were selected using Gblocks[44] (as implemented in SeaView v4) resulting in an alignment of 370 positions. After block selection, sequences were de-replicated again using FaBox v1.41 (218 unique sequences). The best-fit model of nucleotide substitution was selected using JModelTest v2.14 and the Bayesian information criterion[45] (SYM + I + G). Maximum likelihood (ML) as well as Bayesian frameworks were used for tree reconstruciton. The ML tree was reconstructed using PhyML v3.0[46]. Branch support was estimated using SH-like approximate likelihood-ratio tests. The Bayesian phylogeny was estimated using BEAST v1.8.2[47] under the assumption of a relaxed clock (lognormal) and a Yule tree prior. Multiple Markov chain Monte

Carlo analyses were run; convergence and effective sample sizes were checked in Tracer v1.6 (combined effective sampling size was >200). Tree files of all runs were combined using LogCombiner v1.8.2 and the maximum clade credibility tree was extracted using TreeAnnotator v1.8.2.

Identification of fly meals. We performed in depth meal analysis of the 6 AdV-positive flies using a metabarcoding approach. Primary 16 S amplicons were generated with the same primers and under the same conditions as mentioned above[16,37]. Preparation of the generated amplicons for the Illumina MiSeq (San Diego, CA, US) sequencing platform included a first PCR in which Illumina specific overhang adapters are added to the fragment and a second PCR in which sequencing adapters and sample specific indexes were added. The first PCR reaction contained 25 μL of 1:50 diluted PCR-product, 0.2 mM dNTP, 4 mM $MgCl_2$, 0.2 μM of each fusion primer (16Smam primer plus Illumina specific adapter sequence), 1 μM of each blocking primer, 1.25 U Platinum® Taq Polymerase (Invitrogen) and 10x PCR Buffer (Invitrogen) in a total volume of 50 μL. Cycling conditions were: 95 °C 5 min, 5 cycles [95 °C 30 sec, 64 °C 30 sec, 72 °C 1 min], and 72 °C 10 min. The second PCR mix contained 5 μL of cleaned PCR product from the first PCR, 25 μL KAPA HiFiTM Hot Start ReadyMix (PeqLab, Erlangen, Germany), 10 μM of each Nextera Index Primer (Illumina) in a total volume of 50 μL. Cycling conditions were 95 °C 3 min, 15 cycles of 95 °C 30 sec, 55 °C 30 sec, 72 °C 30 sec followed by elongation at 72 °C for 5 min. Both the first and second PCR products were cleaned using the Agencourt® AMPure® XP PCR1 Purification system (Beckman Coulter, CA, USA). The final dual-indexed amplicon libraries were quantified using Quant-iT™ dsDNA Broad-Range Assay Kit (Invitrogen) and pooled equimolarly. The pool was sequenced on an Illumina MiSeq platform using the MiSeq Reagent Kit v2 (2 × 150 bp; Illumina). Raw reads were analyzed using a custom bioinformatic pipeline: paired-end reads were first merged with the program illuminapairedend of the software package OBITools v1.1.18 setting the minimum alignment score to 40. Primer sequences were then removed using the program Cutadapt v1.2.1[48,49] before quality trimming was conducted with the program Trimmomatic v0.35 [50] setting the quality score to 30 over a sliding window of four bases. We then de-replicated identical sequences and filtered out those that occurred less than 10 times using the obiuniq and obigrep commands of OBITools. For taxonomic assignment a reference database was built by performing an *in silico* PCR on all mammalian and vertebrate sequences available at Genbank (http://www.ncbi.nlm.nih.gov/genbank/) using the program ecoPCR v0.2 [51,52]. This not only contained the reference sequences themselves, but also a unique *taxid* that links each sequence to a taxonomy database where the taxonomic information was stored. For the assignment itself we used the ecotag command of OBITools. Ecotag uses the global alignment algorithm Needleman-Wunsch to find the most similar sequence to the query sequence in the reference database with a minimum identity level of 0.95 (primary reference sequence). The query sequence is then assigned to the most common recent ancestor of the primary reference sequence and the most similar reference sequence to the primary reference (secondary reference sequence).

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Author Contributions

S.C., M.S., C.H. and F.L. designed the study. K.M., M.S. and C.H. performed the experiments. C.H. and S.C. analyzed the data and C.H., M. S. and S.C. wrote the manuscript. F.L. contributed to the interpretation of the data and critically read the manuscript.

Additional Information

Accession codes: AdV sequences and raw reads of fly meal analysis are available at the European Nucleotide Archive under the accession numbers (Fly 92: LT617635; Fly 101: LT617636; Fly 381: LT617637; Fly 740: LT617638; Fly 1355: LT617639; Fly 1375: LT617640) and study accession number PRJEB14554 (sample accession numbers ERS1272921- ERS1272926).

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4. Discussion

Wildlife diseases constitute a potential source for emerging infectious diseases affecting humans and livestock and pose a threat to species of conservation concern. Consequently, wildlife disease surveillance has immediate public health and veterinary relevance. However, the tools available for wildlife disease surveillance require improvement, especially with regard to application in resource-poor regions. This thesis has investigated the feasibility of using flies for wildlife disease surveillance using two pathogens: *Bacillus cereus* biovar *anthracis* (*Bcbva)*, and Adenovirus (AdV). Both pathogens are known to cause infection in wildlife in Taï National Park (TNP), the main study site. *Bcbva* and AdV differ greatly with regard to their microbiological features and the severity of diseases they cause. *Bcbva* is a highly pathogenic spore-forming bacterium and AdV is a facultative pathogenic virus.

We proposed that fly-based surveillance of wildlife diseases could cover multiple dimensions (space and time), scales (small-scale and large-scale) and resolutions (initial screening and in-depth analysis). Once a pathogen has been found to be detectable in flies, the extent to which fly-based surveillance can be applied must be investigated. This depends largely on the pathogen's features (e.g. tenacity, route of transmission), but also on the habitat studied (e.g. prevalence). Based on the two pathogens studied, *Bcbva* and AdV, we demonstrated different levels of fly-based disease surveillance. The first, more comprehensive, study comprised an in-depth fly-based analysis of *Bcbva* on a small scale in TNP over space and time. This included the isolation of bacteria from flies and subsequent phylogenomic analysis. Furthermore, on a large scale, throughout sub-Saharan Africa, a first screening of *Bcbva* using flies was performed. The second study represents a first fly-based screening of AdV on a small scale in TNP including the amplification and sequencing of viral genetic material allowing phylogenetic analysis. Both studies were complemented with fly meal analysis, adding a valuable layer of information.

4.1 *Bcbva* in TNP: an in-depth analysis on small scale

The first publication of this thesis presents a unique wildlife health data set gathered since the first outbreak of *Bcbva* in TNP in 2001 and beyond. It comprises 204 carcass samples, 75 bones and 1634 flies collected in TNP, as well as 136 bones and 1089 flies collected at 16 other sub-Saharan sites (see 4.2). This chapter focuses on the results deduced from fly analyses. However, I first briefly present the overall results of the study to put the fly-based results in the appropriate context. Carcass monitoring in TNP revealed that 40% of the observed wildlife mortality is attributable to *Bcbva*. Further, it was possible to isolate bacteria from all three sample types (i.e. carcass samples, bones and flies). 178 bacterial genomes were fully sequenced and used for phylogenomic analysis. This analysis revealed a remarkably high genetic diversity when compared to classical *Bacillus anthracis* (*B. anthracis*) strains. The detection of *Bcbva* in bones provides evidence that *Bcbva* was already present in the area in the early 1990s. Genetic analysis even suggested that the time to the most common recent ancestor of all isolates was 146 years [284]. The overall data was suggestive of an unusual disease dynamic of *Bcbva* in TNP characterized by an on-going outbreak and an exceptionally broad host-range. For more details, see the doctoral thesis of Fee Zimmermann "Epidemiology and Ecology of *Bacillus cereus* biovar *anthracis* in Taï National Park, Côte d'Ivoire" [284].

Based on the analyses of flies, insights into the spatial and temporal distribution of *Bcbva* in TNP were obtained. Of a total of 1634 analyzed flies, 726 were randomly collected within the research area of TNP over a period of four years (2008, 2009, 2012, 2013; referred to as "random flies"; Table 2, page 114; Figure 3A, page 113). Another 908 flies were collected based on a grid system inside and outside the research area over a period of 19 days (May/June 2014, referred to as "snapshot flies"; Table 2, page 114; Figure 3B, page 113). In 5% of all flies (80/1634) *Bcbva* genetic material was detected by real-time polymerase chain reaction (rt PCR) and isolation of bacteria was possible in most cases for the subset of PCR-positive flies (43/50).

Considering that the flies were collected independently of carcass sightings, the detection of *Bcbva* genetic material in every 20th fly supports the overall finding that *Bcbva* is exceptionally active in TNP. Even of the "random flies" that were regularly collected for four years and exclusively within the research area, 8.5% (62/726) were positive for *Bcbva*, whereas for the "snapshot" dataset 2% of the flies (18/908) were *Bcbva* positive. This difference could be explained by a temporary lull of anthrax incidence within the limited time frame of 19 days of the "fly snapshot". Furthermore, the area covered during the "fly snapshot" is substantially larger and comprises traps that were located outside the research area (Figure 3B). Campbell et al. showed that research activity in TNP has a protective effect on wild mammal communities and increases mammal density [166]. Differences in mammal density might influence anthrax dynamics and prevalence and thus also *Bcbva* prevalence in flies (see also 4.1.1).

Depending on how long flies carry Bcbva and on their flight range, Bcbva prevalence in flies gives a more or less simultaneous and local picture of the anthrax situation. According to experimental data, B. anthracis can remain detectable in flies and on the surface of flies for several days [131,201,209]. Thus, the contamination with spores led to longer detectability than with vegetative bacilli. Based on these findings, it can be argued that the detection of Bcbva in flies gives a relatively simultaneous picture of the anthrax situation in TNP. Little is known about the flight range of flies in tropical habitats. Capture-recapture experiments in savannah regions indicated that single flies can travel up to 40 km in 17 days [133]. It is doubtful that this is also true for tropical habitats in which dense vegetation and heavy rainfalls hinder fly dispersal. More likely, flies move in a constrained space and thus depict the anthrax situation of a smaller area. However, accidental anthropogenic transport could facilitate movement over large distances in a limited time frame [285]. In this case, the picture of the local anthrax situation reflected by flies would be skewed.

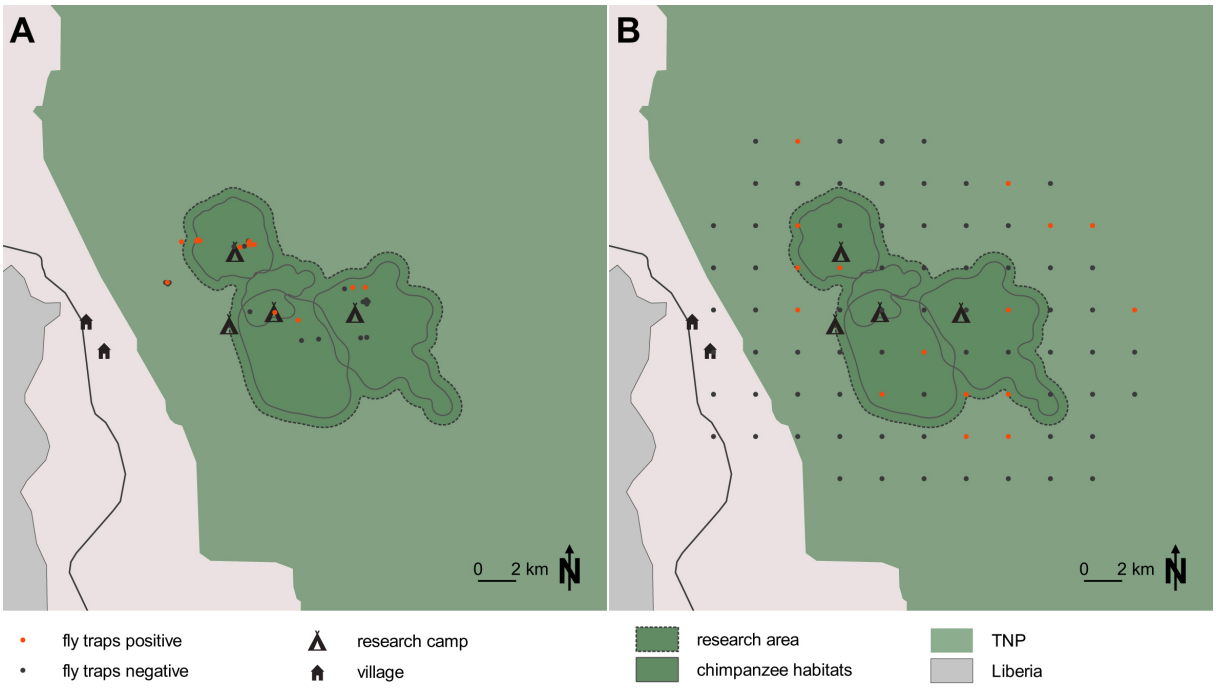

Figure 3: Fly sampling on a small scale in Taï National Park for publication I. A. For the "random flies" data set, flies were caught at random locations within the research area over four years at different times of the year. B. The "snapshot flies' were caught within a 2×2 km grid system within 19 days in May 2014.

For classical anthrax, various experimental studies have shown the recovery of *B. anthracis* from flies and fly feces and vomit after exposure to contaminated material [131,152,201,205,209]. However, few studies have been published that report the successful detection and isolation of *B. anthracis* from flies under field conditions. Braack and de Vos, who intensively studied anthrax ecology in Kruger National Park (KNP) in the 1990s, mentioned unpublished data according to which isolation of *B. anthracis* spores was possible from fly feces and vomit from vegetation surrounding anthrax carcasses [132]. Steele and Helvig very briefly noted that they found *B. anthracis* in flies collected from cattle carcasses during an anthrax outbreak in the United States of America (US) in the 1950s [286]. Later, Blackburn et al. presented two comprehensive datasets showing the successful recovery of viable *B. anthracis* in maggot and fly pools collected during three anthrax outbreaks in white-tailed deer (*Odocoileus virginianus*) and cattle (*Bos taurus*) in West Texas, US [9,208]. However, in all studies, flies or maggots were sampled from or near anthrax carcasses. Thus, to the best of our knowledge no data exist on the prevalence of *B. anthracis* in flies that were randomly collected. The extent to which these findings can be transferred to *Bcbva* is not entirely clear. Unfortunately, there is no comparative data concerning sporulation, tenacity and germination of *Bcbva* and *B. anthracis*.

It is most likely that flies take up vegetative bacilli and/or spores while laying eggs or imbibing the bodily fluids of anthrax victims (whether as a general food source or a protein source for their reproductive systems). How exactly flies take up *Bcbva* can only be speculated. It is conceivable that flies may either be externally contaminated with spores or vegetative bacilli or that they ingest them. It has been repeatedly shown that flies externally contaminated with pathogens mechanically transmit disease via their mouthparts and body surface, especially their hairy and sticky legs (see also 2.3.4) [131,151,156,287,288]. Förster et al., for example, showed that flies collected in the vicinity of urban settlements carried up to 20 different bacteria on their body surface [151]. For *B. anthracis*, there is also evidence for the external contamination of flies with spores. Buchanan et al. transferred flies that had contact with *B. anthracis*-infected material to

agar plates and allowed them to walk for a short time on the surface. After incubation, growth of *B. anthracis* was clearly detectable [152].

	Sampling Approach	# analyzed	PCR positive	Isolates	Year
In-depth analysis of *Bcbva*					
	Random flies	726	62	24 of 32	2008/2009/ 2012/2013
	Snapshot flies	908	18	17 of 18	2014
First screening of AdV					
	Random flies	498 (156)*	6	-	2008/2009/ 2012/2013

* Out of 498 flies 156 flies contained mammalian DNA were regarded as suitable for analysis.

Table 2: Samples and results for fly-based surveillance of *Bcbva* and AdV on a small scale in Taï National Park (TNP), Côte d'Ivoire. This table shows the number of flies collected and analyzed for the small-scale datasets in TNP (in-depth analysis of *Bcbva* and first screening of AdV), as well as PCR results and whether the isolation was successful.

There is also evidence for flies ingesting pathogens and subsequently disseminating them via their droppings [150,289]. For *B. anthracis*, von Terzi et al. and Fasanella et al. showed that flies ingest *B. anthracis* spores and that they remain detectable in their digestive tract and in their droppings for up to several days [201,209]. They obtained contradictory results as to whether *B. anthracis* can multiply within the fly or not. Fasanella et al. suggested that spores germinate in the gut of flies and eventually multiply, whereas von Terzi et al. obtained opposing results. If bacteria do not replicate in the gut of flies, it appears most appropriate to assume a *contamination* of flies with *B. anthracis*, *Bcbva* respectively. If this multiplication does occur, it remains debatable whether an *infection* or a *colonization* with *B. anthracis* or *Bcbva* is present. For other *Bacilli* it has been shown that arthropods are capable hosts. *Bacillus thuringiensis*, for example, infects a broad spectrum of insects and is also used as biological pesticide [290]. For *Paenibacillus larvae larvae*, the causative agent of American Foulbroot, a deadly epizootic of honey bee broods, insights were obtained concerning how exactly the infection in the arthropod gut is facilitated [291]. S-layer proteins, structures expressed on the cell surface, were identified as a determining factor for enabling adhesion to host cells. However, it is also broadly assumed that *Bacilli* represent parts of the microbiome and colonize the intestinal tract of flies [292,293].

Flies may also take up the pathogen while feeding on feces. For classical anthrax, it has been described that carnivores and other scavengers generally believed to be largely immune to the disease shed spores with feces after feeding on anthrax victims [226,294–296]. The authors of the aforementioned studies assumed that the concentration of spores in feces is not high enough to infect suspicious animals without having performed experiments to substantiate this assumption. Still, it appears unlikely that the concentration of spores in feces is sufficient to contaminate flies. Dragon et al. and Lindeque and Turnbull reported spore concentrations in scavenger feces that ranged from 34-6740 cfu/g [198,294]. Based on these numbers one could assume that 250 g of

scavenger feces contains a maximum of 10^6 spores. Infective material used in the experiments by Fasanella et al., who showed that *B. anthracis* is detectable up to 24 hours in fly spots (vomit and feces), contained about 800 times the amount of spores that is expected to be in feces) [201]. Based on this rough calculation it appears unlikely that flies are significantly contaminated through the contact with feces.

More research must be conducted to understand the interplay of *Bcbva* and flies. From the current perspective, it appears most likely that flies are both externally and internally contaminated when feeding on carcasses. Still, it is not known how exactly flies in TNP come into contact with *Bcbva* (carcass, feces or unknown source) and whether there is a *Bcbva* lifecycle in flies outside the mammalian host and, if so, whether or not this affects the fly.

Our findings rely on the detection of pathogen genetic material by rt PCR. Various countermeasures were taken to minimize the risk of false positive samples. To avoid cross-contamination with native *Bcbva* genetic material, desoxyribonucleic acid (DNA) extraction was never performed with other sample types, such as necropsy samples. Further, the setup of PCR reactions for flies was physically separated from those for high-quality samples that potentially contain high loads of bacterial DNA, such as necropsy samples. To minimize contamination with PCR products, PCR setup and post-PCR analysis steps were performed in separate, dedicated rooms. All flies were tested in duplicate and only those positive in both reactions were considered positive for *Bcbva*. As cultivation of *Bcbva* is time- and cost-intensive (BSL 3 pathogen), only a subset of 50 PCR-positive flies was underwent culture attempts to confirm the PCR result. In 43 of the 50 attempts (86%), the culture of living bacteria was successful. This number appears reasonable, as genetic material may be present even if isolation of bacteria is not possible [297,298].

Flies were not washed before testing, nor was the intestinal tract dissected. Thus a differentiation of internal and external infection with *Bcbva* was not possible. It has been shown experimentally that flies do ingest *B. anthracis* bacilli that subsequently pass through the gastrointestinal tract [131,201,209]. It has also been experimentally shown that flies can become externally contaminated with *B. anthracis* [131,152]. However, for estimating the prevalence of *Bcbva*, it is irrelevant whether the flies are externally or internally infected. Cross-contamination during the storing process (20 flies per silica tube) was not regarded as problematic, as unpublished data by Fee Zimmermann showed that bacterial culture from the silica itself was not successful.

4.1.1 *Bcbva* across space

Comparison of *Bcbva* prevalence within the research area and outside the research area revealed a higher prevalence of *Bcbva* in the zone of research activity. These findings are based on a statistical test (Fisher's exact test) that showed a positive association between *Bcbva* presence and location inside the research area.

Campbell et al. demonstrated that research activity in TNP reduces poaching pressure on the local mammal community, resulting in a higher mammal density within the research area [166]. It can be hypothesized that a higher mammal density favors constant *Bcbva* transmission chains, leading to higher *Bcbva* prevalence in the research area. For *B. anthracis* in KNP, it has been shown that outbreaks in this area are density-dependent: if the number of susceptible animals falls below a threshold, the transmission chain is interrupted [197]. It would have been desirable to have mammal density data for TNP in order to build a logit model that predicts *Bcbva* positivity based on mammal density. However, the mammal density data in the study by Campbell et al. did not entirely cover the area in which flies were collected and thus could not be used [166]. Furthermore, the rather small sample size would not have supported more complex modelling, such as generalized linear mixed models. However, the application of the Fisher's exact test, which is well suited for small sample sizes, generated data that provide a good hint. It would be

of interest to repeat the "fly snapshot" sampling to increase sample size and generate mammal density data as a basis for more precise and complex predictions. Regular fly-based surveillance covering the entire park could provide useful information regarding *Bcbva*'s temporal and spatial variation throughout the area and shed light on the interplay of mammal density, fly abundance and *Bcbva* prevalence.

Phylogenomic analysis performed by Fee Zimmermann on bacterial isolates retrieved from flies caught during the "fly snapshot" (n=17) revealed that within the period of 19 days, 13 different strains (differing by more than two single nucleotide polymorphisms (SNPs)) were co-circulating (thesis by Fee Zimmermann, "Epidemiology and Ecology of *Bacillus cereus* biovar *anthracis* in Taï National Park, Côte d'Ivoire") [284]. These 13 strains differed by up to 48 SNPs (median: 25), supporting the notion that *Bcbva* exhibits extremely high activity in TNP (the maximum distance described for two *B. anthracis* isolates during an outbreak in the French Alps was 20 SNPs) [299]. There are no comparable data sets based on whole genome sequencing for African ecosystems. Only studies applying progressive hierarchical resolving assay using nucleic acid (PHRANA) and multiple-locus variable number tandem repeats analysis (MLVA) have been published regarding Etosha National Park (ENP) and KNP [240,300]. It is likely that each identified strain represents a different *Bcbva*-infected carcass, as co-infection with more than one strain was not observed, for example, in the ENP study [241]. It is noteworthy that during the "fly snapshot" only three *Bcbva*-positive carcasses were found, all of which were infected with strains that corresponded well to those found in flies. This demonstrates that flies are far more efficient than humans in detecting carcasses and may thus allow us to investigate disease dynamics in greater depth than is possible based on carcasses alone. It seems that the mortality observed by carcass monitoring greatly underestimates the real situation.

4.1.2 *Bcbva* across time

In addition to spatial patterns, the analysis of flies also shows temporal variations. *Bcbva* in flies was detected year-round but peaked from December to March. This time of the year falls between the two rainy seasons and is the only distinct dry period in the TNP ecosystem. The occurrence of *Bcbva* carcasses, however, showed no seasonality.

Although seasonality in *Bcbva* carcass prevalence was not detected in TNP, the fly-based results suggest that climatic conditions also play a role in *Bcbva* ecology in TNP. How exactly climatic conditions influence the disease remains only a matter of speculation: the availability of infective spores could depend on rainfall, and the immune status of potential hosts could vary with season or the abundance of potential vectors. For *B. anthracis* the role of climate in disease ecology has been intensively described and discussed. Although it has been proven that anthrax incidence is often climate dependent, the patterns described are not consistent across different habitats. For some regions anthrax incidence peaked at the end of dry periods, and in others this occurred with the onset of rainfalls or after heavy rains [197,198,228]. It is clear that seasonal and climatic influences constitute only one of many factors that shape the dynamics of the disease.

A remarkable finding is that *Bcbva* is detectable in flies at every time of the year. This underlines the overall finding that *Bcbva* is a permanent cause of wildlife mortality in TNP and contrasts with what is observed with savannah anthrax, which is generally perceived as sporadic with major outbreaks occurring on a somewhat regular basis.

It would have been preferable to include climate data, such as rainfall data and temperature, in the statistical model, but at the time of publication this data was not available.

4.2 *Bcbva* in sub-Saharan Africa: a first large-scale screening

On a large scale throughout sub-Saharan Africa, 1089 flies were collected at ten different sites. These included sites where *Bcbva* had been detected in necropsy samples before (n=2) and sites where *Bcbva* had not been detected but also never looked for (n=8; Table 3, page 120). All ten sites represent chimpanzee habitats but different ecosystems, ranging from moist evergreen rainforests to savannah regions. The two most distant sites were Kayan in Senegal and Budongo Forest in Uganda, about 5,000 km apart (Figure 4). In addition to flies, bones were also collected at all sites with unknown *Bcbva* occurrence.

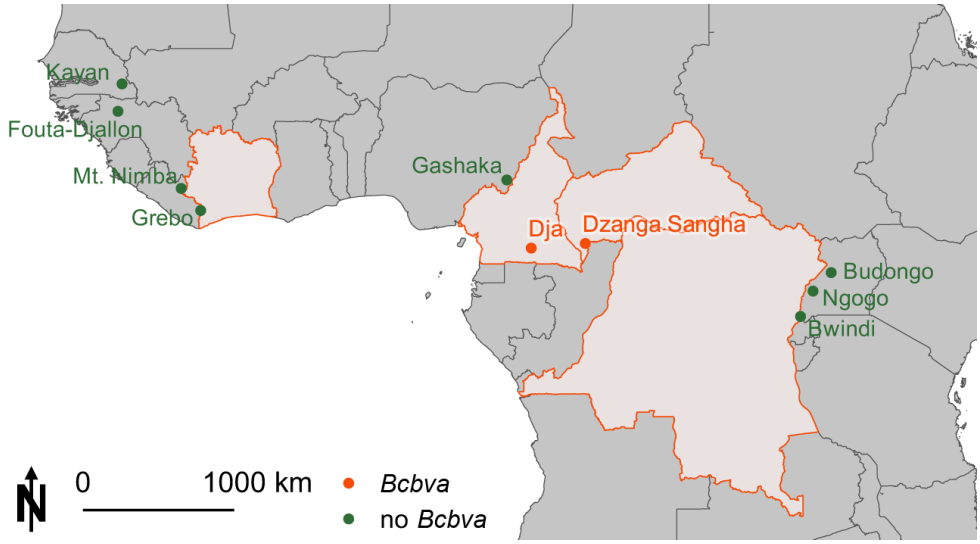

Figure 4: Fly sampling on a large scale in sub-Saharan Africa for publication I. Flies (105 per site) were collected at eight sites with no reported *Bcbva* cases **(**dark green) and at two sites with known *Bcbva* occurrence (orange).

4.2.1 Sites with unknown *Bcbva* occurrence

Sites with unknown *Bcbva* occurrence were represented by two lowland rain forests (Grebo National Forest and Mount Nimba Nature Reserve), three highland rain forests (Ngogo in Kibale National Park, Budongo Forest and Bwindi Impenetrable National Park) and three dry savannah regions (Fouta Djallon, Gashaka-Gumti National Park and Kayan). At each site, 105 flies from 15 different traps, generally at least 1 km apart, were analyzed (in total 784 flies; for Mount Nimba Nature Reserve only 49 suitable flies were available). Two flies from one site, Grebo National Forest (GNF), a lowland rainforest in Liberia approximately 40 km from TNP, tested positive for *Bcbva* by rt PCR and were confirmed by culture (Figure 5, page 118). A bone from GNF also tested positive for *Bcbva*, but culture was not possible. Flies and bones from all other sites were negative for *Bcbva*.

This is the first reported detection of *Bcbva* in Liberia and supports the presumption that *Bcbva* is spread throughout sub-Saharan Africa [243]. A 2% *Bcbva* prevalence in flies (2/105) from GNF matches the *Bcbva* prevalence in flies collected during the "fly snapshot" in TNP (18/908). Flies in GNF were also collected within a limited time frame of 12 days. However, it is speculative to infer that the *Bcbva* situation in GNF is comparable to TNP.

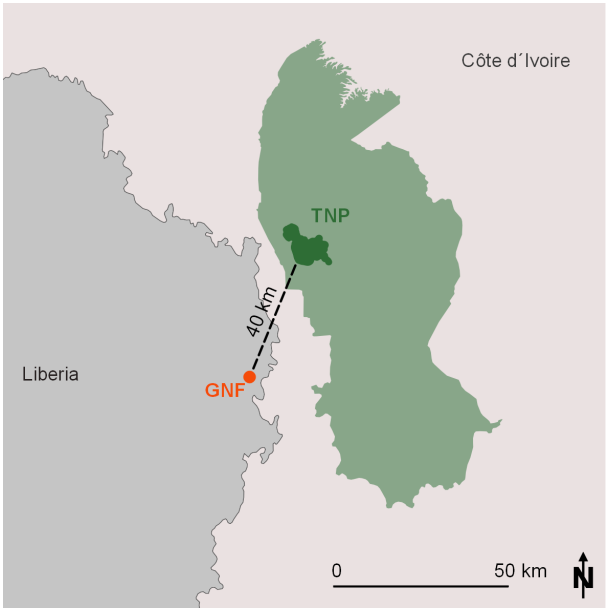

Figure 5: Geographic location of Grebo National Forest (GNF). Grebo National Forest is located in Liberia only 40 km from Taï National Park (TNP) across the Ivorian-Liberian border. Of eight sites with unknown *Bcbva* occurrence, GNF was the only site where flies tested positive for *Bcbva* (2/205). The geographic proximity to TNP and the close genetic relatedness with strains from TNP suggest a possible epidemiological link between GNF and TNP.

Phylogenomic analysis described in the thesis "Epidemiology and Ecology of *Bacillus cereus* biovar *anthracis* in Taï National Park, Côte d'Ivoire" by Fee Zimmermann includes six chromosomal sequences of *Bcbva* isolates from Côte d'Ivoire (CI), Cameroon (CA), Central African Republic (CAR) and Liberia. This work showed that the two isolates from GNF cluster with those from TNP [284] and were more closely related to isolates from TNP than to those from CA and CAR. The analysis further implied that *Bcbva* has been present in sub-Saharan Africa for millennia (time to most recent common ancestor (tMRCA) 2157 years). Consequently, it appears likely that strains from TNP and GNF diverged more recently from one another than this date and that there may be an epidemiological link. Insects or birds that can cover a distance of 40 km could constitute such an epidemiological link. Based on the existing data we do not know whether flies, which are obviously contaminated with *Bcbva*, can fly 40 km through dense tropical rainforest (see 2.3.1). Birds, however, are known to be capable of transporting pathogens over thousands of kilometers [301]. However, birds are comparatively resistant to anthrax and the only outbreaks described occurred in ostriches [186,302]. No data exists about the external contamination of birds with anthrax apart from studies showing that anthrax spores could be retrieved from vulture feces [198,226,295].

All other sites except GNF were negative for *Bcbva*. There may be various reasons for this. First, it could simply be the case that *Bcbva* does not occur in the region. Another possibility is that *Bcbva* does occur in the region, but at a much lower frequency than in TNP or with strong seasonal variations such that the sample size was too small to detect it. Third, it may be that TNP occurs in the region, but due to ecological differences flies might not take up the pathogens. Also the species of flies collected could influence the outcome of the studies as discussed in section 4.2.2. Further fly sampling paired with carcass monitoring could clarify the findings of this first fly-based screening.

More research is needed in the GNF, including carcass monitoring, to evaluate the impact of *Bcbva* in the area. Further studies are also desirable at the other sites to rule out the presence of *Bcbva*.

4.2.2 Sites with known *Bcbva* occurrence

Flies collected at two sites in CA (n=200) and CAR (n=105) where *Bcbva* had been detected before in necropsy samples of chimpanzees, gorillas and an elephant (Dzanga-Sangha Natural

Protected Areas (DSPA), Dja Reserve (DJR)) were all tested negative for *Bcbva* [99,243]. This finding was surprising, especially for DSPA, where during the time of fly sampling a confirmed *Bcbva* case was observed in a duiker (unpublished data). We may assume that the overall *Bcbva* prevalence might be lower in these regions and that sample sizes were too low. This assumption might apply to DJR, but the DSPA dataset was comprehensive and a high mortality in wildlife was reported by the World Wildlife Fund (WWF), which has a permanent project at this site (unpublished data).

To determine whether the species of the flies collected plays a role in the detectability of *Bcbva*, we investigated whether the composition of fly species differed between TNP, DSPA and DJR. As little taxonomic and genetic data is available on tropical flies, we opted for an approach that relies solely on phylogenetic information (generalized mixed Yule coalescent model (GMYC)) in order to avoid taxonomic misidentification caused by cryptic species, which are to be expected [141,142]. Forty-five maximum-likelihood entities (species; 95% confidence interval: 42-56) were identified. These 45 species were then taxonomically assigned at family level when a clear barcoding gap was present, resulting in the identification of six different dipteran families (Calliphoridae, Glossinidae, Sarcophagidae, Staphylinidae, Syrphidae and Tachinidae) represented by one to eleven GMYC species. In nearly half of the cases, no clear barcoding gap could be identified. This may be due to nuclear pseudogenes, also called nuclear mitochondrial DNA segments (NUMTs), which are known to inflate the number of species identified, or due to erroneous and lacking data on cryptic species in the database [303,304]. At all three sites, flies of the family Calliphoridae predominated. However, in TNP one Calliphoridae species (GMYC entity 15) represented 67% of all flies analyzed. This same species represented only 7% of the flies collected in DJR and 22% of the flies collected in DSPA. These results raise the question of whether this Calliphoridae species plays a crucial role in the dynamics of *Bcbva* that may be exceptional for TNP. Entomological investigation should be performed to clearly identify fly species involved in *Bcbva* dynamics and to reveal associations between *Bcbva* presence and abundance of certain fly species.

***Bcbva* presence**	**Site**	**Country**	**# analyzed**	**PCR positive**	**Isolates**	**Year**
Known	Dja Faunal Reserve	Cameroon	105	0	0	2014
Known	Dzanga Sangha Protected Area	Central African Republic	200	0	0	2013
Unknown	Fouta Djallon	Guinea	105	0	0	2013
Unknown	Grebo National Forest	Liberia	105	2	2	2013
Unknown	Mount Nimba Nature Reserve	Liberia	49*	0	0	2013/2014
Unknown	Gashaka-Gumti National Park	Nigeria	105	0	0	2013
Unknown	Kayan	Senegal	105	0	0	2013
Unknown	Budongo Forest	Uganda	105	0	0	2012/2013
Unknown	Bwindi Impenetrable National Park	Uganda	105	0	0	2012/2013
Unknown	Ngogo in Kibale National Park	Uganda	105	0	0	2013
		Σ	1089	2	2	

* no more flies available

Table 3: Samples and results for fly-based disease surveillance of *Bcbva* on a large scale in sub-Saharan Africa. This table shows the number of flies collected and analyzed for the large-scale datasets in sub-Saharan Africa. It also includes the PCR results and the number of retrieved isolates. The ten sites are grouped according to known/unknown *Bcbva* presence.

4.3 Adenoviruses in TNP: a first small-scale screening

To investigate whether fly-based wildlife disease surveillance might also apply to viruses, we chose AdV as our target virus. AdV appears to be a promising viral pathogen to target, because these viruses display a relatively high tenacity and are intensively shed with feces. Furthermore, AdV prevalence in TNP is known to be high. To enhance detection probability, from a dataset comprising 498 flies we tested only those collected in TNP that had been shown to contain mammalian DNA. We reasoned that detectable mammalian DNA indicates a recent meal and that this increases the probability of detecting AdVs that were taken up while feeding [305]. Of the 498 flies, 156 (31%) had recently fed on mammals, but due to material shortage, only 142 flies were tested for AdV by PCR. AdV genetic material from eight flies was amplified and sequenced (Sanger sequencing), and six were confirmed in a second round, resulting in an AdV prevalence of 4.2% in flies with mammal DNA.

AdVs usually cause inapparent infections but are shed in large quantities in feces. Therefore, it is suggested that flies take up the pathogen while exploiting fecal matter and not while exploiting carcasses, as is assumed for *Bcbva*. As discussed for *Bcbva*, external and internal contamination of the fly with AdV was not differentiated and not regarded as meaningful for the overall question.

Although AdVs have been described in many wildlife species [258,306], few studies regarding prevalence in feces have been published. Three studies investigating AdV prevalence in non-human primates (NHP; chimpanzees, gorillas and bonobos), including one study conducted in TNP, revealed that AdV prevalence in NHP feces is surprisingly high, ranging from 38% to 58% [108,173,276]. Another study reported that 11% of rodent fecal samples collected in China contained amplifiable AdV [307]. Serological studies in Scandinavia and Australia showed a seroprevalence of up to 60% in wild ranging canids [254,308]. Although there is no data about overall AdV prevalence in wildlife feces, the above-mentioned results indicate that an AdV prevalence of 4.2% in all mammal positive flies appears rather low and underestimates the overall AdV prevalence in TNP. It is possible that the expected AdV prevalence in wildlife feces based on the data retrieved from the literature is overestimated, and that the true prevalence is instead within the range of the fly-based results. However, it appears more likely that a fly-based approach does not constitute the most efficient tool for detecting AdV prevalence in wildlife. For example, it could be the case that the flies sampled in TNP do not have sufficient contact with AdV-contaminated feces. It is not known to what extent flies in TNP prefer feces relative to carcasses. Furthermore, we do not know the mechanisms of pathogen-fly interaction and factors that could hinder detectability of AdV in flies (e.g. the extent of degradation in the fly intestinal tract). From a technical point of view, the PCR system used in this study may lack sensitivity. The PCR system used is a semi-nested approach described by Pauly et al. [309]. In her thesis "Adenoviruses in Côte d`Ivoire: Investigation of Diversity and Interspecies Transmission," she tested different PCR systems for the detection of Mastadenoviruses in animals and identified the semi-nested approach mentioned above as the most sensitive and specific assay [310]. However, she did not specifically evaluate the sensitivity of the semi-nested PCR system. In general, nested PCR systems are considered to be relatively sensitive because the two-step process minimizes the amplification of non-specific products (due to incorrect primer binding).

The genetic data retrieved from flies was suitable for phylogenetic analyses. These analyses included the confirmed fly-based AdV sequences and hexon gene sequences extracted from all available complete genomes of the genera Mastadenovirus and Atadenovirus. The positions of the AdV sequences in the trees obtained (maximum likelihood and Bayesian framework) were in line with the results of a basic local alignment tool (BLAST) search against the non-redundant National Center for Biotechnology Information (NCBI) database [311]. Four fly-derived sequences (Fly 92, Fly 101, Fly 740, Fly 1355) formed a well-supported clade with a simian AdV sequence (KP274048) that was obtained from a mona monkey (*Cercopithecus campbelli*) living in a

habituated group in a village near the border of TNP [309]. The authors, Pauly et al., suggested that this simian AdV might represent a new species, as pairwise observed genetic distance to all known human and simian AdVs was greater than 10% (Harrach et al. posited a 5-15% amino acid difference as a basis to designate new species) [245,309]. Our findings show that this simian AdV may be relatively abundant in the region. Another fly-based AdV sequence (Fly 381) belonged to a chimpanzee clade that clustered with HAdV-C sequences. HAdV-C are generally assumed to be strictly host-specific and seem to have co-diverged with their hominid host [108]. The AdV sequence retrieved from Fly 381 was most closely related to a simian AdV obtained from a captive chimpanzee in the US (FJ025905) [276]. However, this chimpanzee clade also comprised a simian AdV sequence obtained from a fecal sample from a chimpanzee in TNP (JN163974) among other simian AdVs retrieved from captive NHPs [173]. The sixth fly-based AdV sequence (Fly 1375) is most closely related to a murine AdV-2 sequence (NC014899). Murine AdVs are the oldest representatives of the genus Mastadenovirus on the basis of phylogenetic analysis [312]. The finding of a murine AdV sequence in flies demonstrates the potential of flies also to "sample" AdVs of small mammals, such as rodents. For humans, sampling rodent feces in an environment with extremely dense vegetation, like TNP, is a challenge. The rodent AdV study mentioned at the beginning of this section was not based on fecal samples collected in the field [307]. Instead animals were caught and feces was subsequently sampled or rectal swabs taken.

Isolation of AdV from PCR-positive flies was not attempted because flies in this study were mainly preserved dried on sililca at ambient temperature for weeks, and even months, before analysis. Resulting quality of the samples with regard to virus isolation was assessed as not being promising. Additionally, the success rate for isolation of AdV from wildlife fecal samples was described to be very low [276].

When fecal sampling is applicable, the collection of flies for detecting AdV in wildlife might not be the best first choice. However, the results show that the detection of viruses and the amplification of DNA sequences suitable for phylogenetic analysis can be successful. For the detection of AdVs in mammals of small body size, fly-based approaches might be beneficial.

4.4 Fly meal analysis

The analysis of fly meals has been shown to be extremely beneficial for the assessment of mammalian biodiversity in remote tropical habitats [143,144]. Publications I and II demonstrated that fly meal analyses also add a valuable layer of information to studies primarily focusing on disease.

4.4.1 Fly meal analysis and *Bcbva* in TNP

In the *Bcbva* study in TNP (publication I) the meals of 57 flies were analyzed. Because it is known that flies are attracted to carcasses as part of their reproduction cycle and as a food source, mammalian DNA found in flies may to some extent reflect wildlife fatalities (see also 2.3.1). According to a study published in 2015 mammalian DNA remains detectable in flies for four days after ingestion, meaning that flies provide a more or less simultaneous picture of wildlife mortality [305]. A total of 20 genera belonging to the six mammalian orders Artiodactyla, Carnivora, Chiroptera, Hyracoidea, Primates and Rodentia were found. Ten of the genera detected in flies were also detected by carcass monitoring, whereas flies were lacking three genera that humans found (Table 4, page 124). It is noteworthy that 61% of the flies (35/57) contained rodent DNA, whereas in two decades of carcass monitoring only 4% of all carcasses found were rodents (9/204). This finding points to the potential of flies for detecting wildlife mortality with less bias towards large mammals.

To investigate *Bcbva*'s host-range, half of the fly meal analyses performed originated from *Bcbva*-positive flies and the other half from *Bcbva*-negative flies. Statistical analysis showed no substantial difference between the two groups. This supports the assumption that *Bcbva* has a broad host range without one species taking precedence. Genera found in *Bcbva*-positive flies corresponded well to those identified from *Bcbva*-positive carcasses. Based on the flies, eleven additional potential host genera were identified, suggesting that *Bcbva* may exploit a much broader host range than assumed based on carcass monitoring. In other ecosystems as well, anthrax affects a broad range of species. For example in Africa's largest national parks, ENP, KNP and SGNP, anthrax cases have been described in various species of different orders (e.g. kudu, zebra, wildebeest, giraffe, warthog, hippopotamus, elephant, lion, cheetah and ostrich) [210,227,228]. However, these species are usually not affected evenly during a single outbreak. In most cases, one or two species account for the vast majority of total anthrax cases, which often vary from outbreak to outbreak. For example, at least three-quarters of all recorded anthrax cases are accounted for in ENP by Burchell's zebras, elephants and blue wildebeests, in KNP by kudus and in SGNP by impala, wildebeest, zebra and buffalo. Based on the data presented here, no clear shift towards a certain species in TNP was observable, suggesting that anthrax in tropical ecosystems may exhibit different dynamics.

It is striking that, while fatalities in chimpanzees have been frequently recorded in TNP, chimpanzee DNA in flies was not detectable. This is most likely due to a technical reason: to prevent human DNA from masking the signal from wildlife DNA, blocking primers inhibiting the amplification of human DNA were applied. These blocking primers presumably also hinder the amplification of chimpanzee DNA, as the sequence differences between the two closely related hominid genera are minor. The taxonomic assignment based on a short 16S barcode (130 bp) was performed at the genus level [313]. Insufficient information in available databases (cryptic species are not uncommon in remote tropical rainforests) and possible sequencing errors impeded assignment at the species level.

The biggest obstacle to the use flies as "wildlife mortality investigators" is the lack of knowledge about fly ecology. It is not entirely clear in what ratio flies exploit carcasses vs. feces. Consequently, we cannot tell whether mammalian DNA detected in flies stems from wildlife fatalities or from feces. Field observations suggest that the fly species predominantly caught in

TNP is primarily associated with carcasses. It would be of great interest to identify markers (e.g. specific bacteria or compounds) that enable the determination of the food source.

Order	Genus	Number of mammal-positive and *Bcbva*-positive flies (n=28)	Number of mammal-positive and *Bcbva*-negative flies (n=29)	Number of *Bcbva*-positive necropsies (n=81)	Number of *Bcbva*-negative necropsies (n=123)[1)]
Artiodactyla	*Cephalophus*	28	26	26	14
Artiodactyla	*Hexaprotodon*	0	1	0	1
Artiodactyla	*Potamochoerus*	2	2	0	3
Carnivora	*Civettictis*	1	0	0	0
Carnivora	*Crossarchus*	2	0	0	0
Carnivora	*Genetta*	2	0	0	1
Carnivora	*Herpestes*	2	0	2	0
Carnivora	*Nandinia*	0	1	0	2
Chiroptera	*Hypsignathus*	2	3	0	0
Chiroptera	*Myonycteris*	4	2	0	0
Hyracoidea	*Dendrohyrax*	1	0	0	0
Primates	*Cercocebus*	3	5	11	12
Primates	*Cercopithecus*	12	17	5	15
Primates	*Colobus*	6	4	2	4
Primates	*Pan*	0[2)]	0[2)]	31	24
Primates	*Perodicticus*	0	0	0	3
Primates	*Procolobus*	7	5	3	29
Rodentia	*Atherurus*	4	1	0	0
Rodentia	*Cricetomys*	2	1	0	0
Rodentia	*Hylomyscus*	10	11	0	0
Rodentia	*Hystrix*	0	0	1	0
Rodentia	*Praomys*	0	2	0	0
Rodentia	*Protoxerus*	1	3	0	0

1) Probably due to use of human blocking primer.
2) Seven carcasses were only identifiable at family level (Anomaluridae (n=2), Manidae (n=3), Soricidae (n=2)) and eight at order level (Rodentia).

Table 4: Results of fly meal analyses performed within the fly-based in-depth analysis of *Bcbva* in Taï National Park, Côte d'Ivoire. This table shows all mammalian genera (grouped according to orders) that were detected by fly meal analyses and carcass monitoring and the number of detections in *Bcbva*-positive and *Bcbva*-negative flies and carcasses.

4.4.2 Fly meal analysis and AdV in TNP

For all flies from which a confirmed AdV sequences could be retrieved (n=6), fly meal analyses were performed using Sanger sequencing and high-throughput sequencing (publication II). Sanger sequencing identified a plausible host in three cases (Fly 92, Fly 101, Fly 1355). High-throughput sequencing confirmed these results and showed the host species matching the AdV sequence in two other cases (Fly 381, Fly 1375). In the case of Fly 381 (containing a simian AdV sequence most likely of chimpanzee origin), the most plausible host only became visible through assignment at family level: metabarcoding results revealed a high proportion of non-assignable reads at the species and genus levels. At the family level, these reads were assigned to the family Hominidae, which contains only the two genera *Homo* and *Pan*. The Needleman-Wunsch algorithm implemented in the bioinformatic pipeline for the analysis of fly meals functions as follows. First, it finds the sequence most similar to the query sequence in the reference database (the primary reference sequence). This query sequence is then assigned to the most common recent ancestor of the identified primary reference sequence and the reference sequence most similar to the primary reference sequence (the secondary reference sequence) [314]. In the case of the two closely related and only genera of the family Hominidae, *Homo* and *Pan*, this resulted in an assignment at family level. A manual BLAST search of one of the representative metabarcoding sequences revealed 100% similarity with a *Pan troglodytes* sequence. For one fly (Fly 740) the plausible host DNA could not be detected. The AdV sequence of Fly 740 suggested that *Cercopithecus campbelli* was present, but instead only the genus *Cricetomys* (giant pouched rat) was detected. It can only be assumed that the more recent *Cricetomys* meal diluted the *Cercopithecus campbelli* DNA. Further sequencing errors or amplification bias during library preparation could explain the absence of *Cercopithecus campbelli* genetic material.

This dataset presents a good comparison of barcoding based on Sanger sequencing and metabarcoding based on high-throughput sequencing. Sanger sequencing indicated two genera belonging to two different families (Cercopithecidae, Cephalophidae) and one order (Rodentia) that did not comprise any of the two aforementioned families. High-throughput sequencing revealed ten genera belonging to nine mammalian families (Bovidae, Cercopithecidae, Felidae, Hominidae, Muridae, Nesomyidae, Suidae, Viverridae and Vespertilionidae). The genera/order detected by Sanger sequencing were reproduced using high-throughput sequencing. One retrieved Sanger sequence (Fly 381) displayed an inconclusive superposing chromatogram that made assignment impossible. This can occur when a sample contains mammalian DNA from more than one species (multiple meals). Metabarcoding results confirmed this assumption, showing a mix of Hominidae and Felidae sequences. Four further flies contained DNA from multiple hosts (Fly 92, Fly 101, Fly 1355, Fly 1375). Whereas with the Sanger method multiple meals are not detectable, metabarcoding can easily address this issue, resulting in higher numbers of detectable genera.

4.5 Feasibility of using flies for wildlife disease surveillance

We assessed the feasibility of using flies for wildlife disease surveillance based on two pathogens: the highly pathogenic spore-forming bacterium *Bcbva* and the low-pathogenic virus AdV. Both pathogens were detectable in flies. Isolation of the pathogen was possible for *Bcbva*. Phylogenetic analyses were performed: for *Bcbva* based on whole genomes and for AdV based on a 160-bp region of the hexon gene, displaying the pathogen's genetic diversity. For *Bcbva* information about its distribution across time and space was obtained. Fly meal analyses reflected the pathogen's distribution in local mammalian hosts. These results show that flies may be a useful tool for wildlife disease surveillance.

Whether a pathogen is suitable for fly-based surveillance depends on three main factors: the nature of the pathogen, the ecosystem studied and other available monitoring tools. First and foremost, the nature of the pathogen determines whether it is detectable in and retrievable from flies. Of relevance are the pathogen's tenacity and how it became accessible for flies (i.e. via feces, carcasses, wounds or other secretions). For example, due to its spore-forming character *Bcbva* is a promising candidate with regard to the isolation of viable bacteria from flies. AdVs, in contrast, although they exhibit rather high tenacity, have not been shown to be easily retrievable from non-invasive samples [276]. Obviously, the preservation of flies after sampling also influences detectability. In most cases, it can only be guessed how flies come in contact with the two pathogens in question. However, it appears plausible that *Bcbva* becomes easily accessible post-mortem, and AdVs through the fecal shedding of the virus. It has also been reported that flies take up pathogens (e.g. treponemes and *Chlamydia trachomatis*) while sitting and feeding on wounds and other secretions [8,315].

Another significant impact on the feasibility of fly-based wildlife disease surveillance is the ecosystem studied. This particularly concerns the prevalence of the pathogen of interest in the mammal community and the abundance and composition of fly species. The minimum prevalence necessary to allow for the fly-based detection of a pathogen in a specific ecosystem most likely varies across pathogens and ecosystems. For mass mortality events, e.g. due to anthrax and Ebola, with a steep increase in the amount of accessible pathogen material, fly-based surveillance would certainly be of interest. It is also self-evident that the abundance of flies influences the outcome of fly-based disease surveillance. Unfortunately, the abundance of arthropods in tropical Africa has not been studied adequately and most likely depends on climate factors. As suggested for *Bcbva*, the composition of fly species collected also has an impact on the outcome of fly-based wildlife disease studies (see 4.2.2). It is necessary to investigate which species show what degree of preference for carcasses, feces or other secretions to determine the species of relevance for the research questions of a particular study. The fly species attracted with a commercial bait containing animal protein throughout the two studies performed in TNP (according to preliminary entomological assignment *Calliphora chlorophyga*) seem to be attracted to both types of animal matter. According to observations in the field this species is usually seen on carcasses, whereas the majority of flies attracted with fecal bait belonged to the dipteran family Muscidae (unpublished data from a pilot study). It would be desirable to identify protein markers that would allow for identifying the source of the fly meal as carcass or feces.

Finally, it must be taken into account whether alternative methods for monitoring the targeted pathogen are available. For example, for AdV it has been demonstrated that surveys based on fecal sampling provide good data regarding prevalence as well as the genetic diversity of AdVs in wild living NHPs [173,276]. As with fly sampling, fecal sampling is easy to implement, including in resource-poor countries. However, it becomes less feasible when monitoring pathogens of small mammals. With regard to *Bcbva*, the only available alternative is carcass monitoring, which

is extremely cost- and time-intensive and of high risk for the person responsible for sampling. Furthermore, flies might even be more efficient in detecting carcasses, as shown in our "fly snapshot" data gathering. In this case, flies are highly beneficial.

Apart from the question of feasibility, fly-based disease surveillance has certain general advantages and disadvantages. Its easy applicability in resource-poor areas is a clear benefit. This applies to both field work and lab work. Collection of flies requires no special training, flies can be stored at ambient temperature, and only a basic molecular lab is required for groundwork analysis. Synergistic effects can be achieved when the technique is combined with biodiversity studies. Due to its high practicability in the field, the inclusion of fly sampling in citizen science projects could be considered. On the downside, individual DNA extraction from flies, as performed in the two studies presented here, requires significant labor. Technical advances such as high-throughput sequencing could facilitate pooling of samples to decrease the workload. Shotgun or, more likely, targeted resequencing could open up new possibilities, as shown for the blood meal analysis of mosquitoes [7].

In addition to biting and non-biting arthropods, other invertebrates may have potential for disease monitoring. It has been shown that aquatic and terrestrial leeches take up mammalian DNA as well as vertebrate-infecting microorganisms while feeding on warm-blooded vertebrates [316,317]. Although it has been reported that leeches exhibit no host preference, they are abundant only in certain areas of the world. To further develop the conceptual foundation of arthropod-based monitoring, it should also be investigated whether antibodies reactive to wildlife-infecting microorganisms are detectable. This would allow for the examination of wildlife exposure to certain pathogens. A few studies have shown that antibodies reactive to mammal-infecting microorganisms are detectable in hematophagous dipterans [318,319].

5. Conclusion and Outlook

Flies constitute an effective and practical tool for monitoring and investigating *Bacillus cereus* biovar *anthracis* (*Bcbva)* in Taï National Park (TNP). *Bcbva*'s spore-forming nature and its high virulence, which complicate post-mortem sampling, favor the use of fly-based *Bcbva* surveillance. Based on flies, valuable information about the dynamics of *Bcbva* in TNP was obtained, including seasonal variation, distinct spatial patterns and a broad host range. Genetic data deduced from flies showed that several *Bcbva* strains were co-circulating at a time in the park. Extending the collection of flies to the whole park and adjacent villages would be of interest to reveal further distribution patterns and dynamics in the area.

Whether flies are also suitable for *Bcbva* surveillance on large scale throughout sub-Saharan Africa is not entirely clear. A new potential *Bcbva* hotspot was identified in Grebo National Forest, Liberia. However, a preliminary attempt to use flies for *Bcbva* surveillance at other *Bcbva* sites failed, most likely due to ecological differences.

For Adenovirus (AdV), the feasibility of using flies to monitor the pathogen appears limited. Although these studies are the first to describe the detection of AdV in flies find a murine AdV in TNP, the prevalence in flies was relatively low when compared to the prevalence described in feces. Thus, feces-based approaches appear to be more efficient with comparable labor, cost and applicability in resource-poor countries. However, they might be biased toward large animals, which is not the case for flies, as shown by the detection of rodent AdV.

In principle fly-based surveillance of wildlife diseases is possible. However, the feasibility depends on the characteristics of the pathogen (i.e. tenacity, mode of transmission), environmental factors (i.e. prevalence of the pathogen in the habitat studied, the abundance of hosts and flies) and the availability of other monitoring tools (e.g. fecal sampling). Further studies should be conducted regarding the extent to which the approach is applicable to pathogens other than the two studied here. High-throughput sequencing, including shotgun and targeted resequencing, could provide new possibilities for the applicability of flies for disease surveillance. Further, the availability of alternative monitoring tools must always be taken into account to minimize effort and expens.

6. Summary

The surveillance of wildlife diseases is relevant for three main reasons. First, wildlife diseases constitute a source of human emerging infectious diseases (EIDs); second, wildlife diseases pose a potential threat to livestock; and third, they are of conservation concern. This applies especially to resource-poor areas with high biodiversity (e.g. tropical Africa and Asia). As most wildlife disease surveillance tools available are cost-, time- and resource-intensive, we investigated the feasibility of a fly-based approach for wildlife disease surveillance in resource-poor countries. Two sample pathogens were targeted: *Bacillus cereus* biovar *anthracis* (*Bcbva*), a spore-forming highly pathogenic bacterium causing anthrax, and adenovirus (AdV), a low-pathogenic virus that is generally shed with feces. Studies were performed at different spatial scales with different resolutions and dimensions. On a small scale, an in-depth study of *Bcbva* over time and space and an initial AdV screening were performed in a remote tropical rainforest (Taï National Park, Côte d'Ivoire). On a large scale, *Bcbva* screening was implemented at ten sites throughout sub-Saharan Africa.

Both pathogens were detectable in flies using molecular methods. Based on the genetic information obtained, phylogenetic analysis revealing the pathogens' diversity was performed. In the small-scale study, *Bcbva* was present in 5% of flies (detection by polymerase chain reaction (PCR)) and isolation of viable bacilli was successful. It was shown that *Bcbva* prevalence varies over time and space: prevalence in flies peaked from December to March and was higher within the research area. Fly meal analysis supported the overall finding that anthrax in the tropics exploits hosts that differ from those in savannah regions. Phylogenetic analysis based on whole fly genomes, as well as carcass and bone samples, showed exceptionally high genetic diversity. It was also shown that flies were more effective than carcass monitoring in detecting anthrax victims. Using flies, three different AdVs (two simian and one murine) circulating in the region were identified. However, prevalence in flies was far below prevalence reported for feces, calling into question the applicability of fly-based surveillance of AdVs.

In the large-scale study, a *Bcbva* screening identified one potential new *Bcbva* site in Grebo National Forest, Liberia. Surprisingly, flies from two sites where *Bcbva* had been detected previously all tested negative. Most likely, differences in the fly species collected accounted for the phenomenon.

In can be concluded that the feasibility of fly-based wildlife disease surveillance depends on three factors: the nature of the pathogen of interest, the ecosystem studied and the possible availability of alternative surveillance techniques. In the case of *Bcbva*, the use of flies was highly beneficial. For AdV, other surveillance tools such as fecal sampling might be more efficient. The extent to which this new approach is applicable to other wildlife pathogens remains to be investigated.

7. Zusammenfassung

Einschätzung der Anwendbarkeit von Fliegen für die Überwachung von Wildtierkrankheiten anhand zweier exemplarischer Mikroorganismen: *Bacillus cereus* biovar *anthracis* und Adenoviren

Wildtierkrankheiten sind aufgrund dreier Aspekte von hoher Relevanz: Erstens können Wildtierkrankheiten ein Reservoir für neu auftretende Infektionskrankheiten des Menschen sein, zweitens sind sie von potentieller Gefahr für die Gesundheit von Nutztieren und drittens bedrohen sie solche Tiere, die vom Aussterben bedroht sind. Besonders in ressourcenarmen Gegenden, deren Fauna eine hohe Biodiversität aufweist, spielen diese drei Aspekte eine übergeordnete Rolle (tropisches Afrika, Asien, Südamerika). Da die derzeit verfügbaren Überwachungsmethoden von Wildtierkrankheiten sehr zeit- und kostenintensiv sind, haben wir die Anwendbarkeit Fliegen-basierter Krankheitsüberwachung als neue Methode, vor allem für ressourcenarme Länder, näher untersucht. Dies erfolgte anhand zweier exemplarischer Erreger: *Bacillus cereus* biovar *anthracis* (*Bcbva*) ein sporenbildendes hochvirulent (Pathogenität ist die qualitative Eigenschaft, Virulenz die quantitative) Bakterium, das Anthrax verursacht, und Adenovirus (AdV), ein niedrigvirulentes Virus, das vor allem über den Kot ausgeschieden wird. Die durchgeführten Studien waren von unterschiedlichem Umfang hinsichtlich geographischer Ausdehnung und Probenanzahl und deckten unterschiedliche Dimensionen (Zeit und Raum) ab. Fokussiert auf den Taï National Park (TNP) in der Elfenbeinküste, einen entlegenen tropischen Regenwald, wurde eine umfangreiche Studie zu *Bcbva* über Zeit und Raum sowie ein erstes Screening von AdV realisiert, von größerem geographischen Maßstab ein *Bcbva* Screening, das zehn verschiedene Regionen in West- und Zentralafrika umfasste.

Beide Erreger konnten mittels molekularer Methoden (Polymerase-Kettenreaktion (PCR)) in Fliegen detektiert werden. Basierend auf den erhaltenen genetischen Informationen wurden phylogenetische Analysen durchgeführt und somit die Diversität der Erreger ermittelt. Fünf Prozent der Fliegen in TNP waren *Bcbva*-positiv und die Isolierung von Bakterien aus Fliegen war möglich. Es konnte gezeigt werden, dass die Prävalenz von *Bcbva* in Fliegen über Zeit und Raum variiert: sie war am höchsten während der Trockenzeit von Dezember bis März und höher innerhalb des Forschungsareals. Ergebnisse von Fliegenmahlzeitanalysen unterstützen die Annahme, dass Anthrax in tropischen Regionen anders unter seinen Wirten agiert als *Bacillus anthracis* in Savannen. Phylogenetische Analysen basierend auf Vollgenomen, neben von Fliegen stammenden Isolaten auch solche von Kadavern und Knochen, zeigten, dass *Bcbva* in TNP eine, verglichen mit *B. anthracis*, unerwartet hohe genetische Variabilität aufweist. Zudem waren Fliegen über einen festgelegten Zeitraum erfolgreicher an Anthrax verstorbene Tiere aufzuspüren, als ein gleichzeitig implementiertes Kadavermonitoring. Auf von Fliegen erhaltene genetische Informationen basierende phylogenetische Analysen identifizierten drei verschiedene Adenoviren (zwei simiane und ein murines AdV), die in der Region zu zirkulieren scheinen. Die detektierte AdV-Prävalenz in Fliegen lag jedoch unter der für Kot beschriebenen Prävalenz, was die Anwendbarkeit von Fliegen zur Überwachung von AdV in Frage stellt.

Das supranationale Screening von *Bcbva* in West- und Zentralafrika brachte Hinweise auf eine neue potentielle *Bcbva*-Region in Grebo, Liberia. Überraschenderweise konnte bei Fliegen aus zwei Regionen, von denen bekannt ist, dass *Bcbva* vorkommt (Dja Reserve in Kamerun und Dzanga-Sangha Natural Protected Area in der Zentralafrikanischen Republik), kein Bcbva nachgewiesen werden. Unterschiede in der Zusammensetzung der analysierten Fliegenspezies

könnten eine Ursache sein.

Zusammenfassend kann gesagt werden, dass die Anwendbarkeit von Fliegen-basiertem Überwachung von Wildtierkrankheiten maßgeblich von drei Faktoren abhängt: die Eigenschaften des zu untersuchenden Erregers (vor allem die Tenazität des Erregers sowie die Art und Weise wie er übertragen wird und somit in Kontakt mit Fliegen kommen kann), das untersuchte Ökosystem (z.B. die Prävalenz des Erregers in der zu untersuchenden Wildtierpopulation und die Abundanz von Wirt und Fliegen) und vorhandene alternative Überwachungsmethoden (z.B. Für die Überwachung von *Bcbva* waren Fliegen von großem Wert. Für AdV hingegen erscheinen andere Überwachungsmethoden, wie das Sammeln von Kot, effektiver bei vergleichbarem Aufwand. In welchem Maß Fliegen auch zur Überwachung anderer Erreger beitragen können, muss weiter erforscht werden.

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9. List of publications

Research articles

Hoffmann C, Merkel K, Sachse A, Rodríguez P, Leendertz FH, Calvignac-Spencer S (2018) Blow flies as urban wildlife sensors. *Molecular Ecology Resources*, 00, 1–9, doi:10.1111/1755-0998.12754

Hoffmann C & Zimmermann F et al. (2017) Persistent anthrax as a major driver of wildlife mortality in a tropical rainforest. *Nature*, 548, 82-86, doi: 10.1038/nature23309

Hoffmann C, Stockhausen M, Merkel K, Calvignac-Spencer S & Leendertz FH (2016) Assessing the feasibility of fly-based surveillance of wildlife infectious diseases. *Scientific Reports*, 6, doi: 10.1038/srep37952

Hoffmann C, Schubert G & Calvignac-Spencer S (2016) Assessing aquatic biodiversity for the lazy. *Molecular Ecology,* 25, 846-848, doi: 10.1111/mec.13535

Schubert G & Stockhausen M, Hoffmann C, Merkel K, Vigilant L, Leendertz FH & Calvignac-Spencer S (2014) Targeted detection of mammalian species using carrion fly-derived DNA. *Molecular Ecology Resources*, 15, 285-294, doi: 10.1111/1755-0998.12306

Oral presentations

Hoffmann C, Zimmermann F, Nowak K, Grützmacher K, Dupke S, Merkel K, Kühl H, Grunow R, Klee S, Calvignac-Spencer S, Leendertz FH. The wide spread distribution of *Bacillus cereus* biovar *anthracis* requires practical and cost-effective monitoring tools. 15th Medical Biodefense Conference, Munich (2016)

Hoffmann C, Zimmermann F, Calvignac-Spencer S, Leendertz FH. Blow-flies as a tool to obtain insights in the epidemiology of *Bacillus cereus* biovar *anthracis.* Robert Koch-Symposium Functional Molecular Infection Epidemiology, Berlin (2016)

Hoffmann C, Zimmermann F, Nowak K, Grützmacher K, Dupke S, Merkel K, Kühl H, Grunow R, Klee S, Calvignac-Spencer S, Leendertz FH. Monitoring *Bacillus cereus* biovar *anthracis* in sub-Saharan Africa. 7th International Conference on *Bacillus anthracis*, *B. cereus* and *B.thuringensis*, Delhi, India (2015)

Hoffmann C, Merkel C, Rodriguez P, Leendertz FH, Calvignac-Spencer S. Blow-flies as urban wildlife sensors 27th International Congress for Conservation Biology - 4th European Congress for Conservation Biology, Montpellier, France (2015)

Poster presentations

Hoffmann C, Zimmermann F, Merkel K, Kühl H, Klee S, Calvignac-Spencer S, Leendertz FH. Invertebrates as neglected zoonosis research assistants. National Symposium on Zoonoses Research, Berlin (2015)

Hoffmann C, Léguillon F, Dupke S, Klee S, Calvingac-Spencer S, Kühl H, Leendertz FH. Blow-flies as a tool to monitor the distribution of highly pathogenic *Bacillus cereus* biovar *anthracis*. National Symposium on Zoonoses Research, Berlin (2013)

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11. Selbstständigkeitserklärung

Ich bestätige, dass die vorliegende Dissertation in allen Teilen von mir selbstständig angefertigt wurde und die benutzten Hilfsmittel vollständig angegeben worden sind. Veröffentlichungen von Teilen der vorliegenden Dissertation sind angegeben. Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionsverfahren beantragt habe.

Berlin, den 25.06.2018

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