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Disease occurrence in free-ranging raccoons (*Procyon lotor*) from rural and urban populations in North-eastern Germany

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To my mother and sisters…

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2. Rentería-Solís Z, Michler F-U, Michler BA, Riehn K, Grobbel M, Wittstatt U, König M, Wibbelt G: **Disease occurrence in raccoons (*Procyon lotor*) from urban and rural populations in Germany – preliminary results.** Gesellschaft für Wild- und Jagdforschung, International Symposium, 11th – 14th of April 2013, Halberstadt, Germany, oral presentation.

3. Rentería-Solís Z, Michler F-U, Michler BA, Riehn K, Grobbel M, Wittstatt U, König M, Wibbelt G: **Diseases in invasive species: Investigations in free-ranging raccoons (Procyon lotor) in Germany**. 62nd International Conference of the Wildlife Disease Association, 27th of July – 2nd of August 2013, Knoxville, USA, oral presentation.

4. Rentería-Solís Z, Michler F-U, Michler BA, Riehn K, Grobbel M, Wittstatt U, König M, Wibbelt G: **Diseases in invasive species: Investigations in free-ranging raccoons (*Procyon lotor*) in Germany**. VIII International Symposium on Wild Fauna of the WAVES-Sociedad Euromediterránea para la Vigilancia y Conservación de la Fauna Salvaje, 31st of October – 3rd of November 2013, Leon, Spain, oral presentation.

5. Rentería-Solís Z, Michler F-U, Michler BA, Wittstatt U, Grobbel M, Riehn K, König M, Rossi L, Wibbelt G: **Diseases in invasive species: investigation in urban raccoons in Berlin, Germany**. 11th conference of the European Wildlife Disease Association, 25th – 29th of August 2014, Edinburgh, Great Britain, oral presentation.

6. Rentería-Solís Z, Wittstatt U, Grobbel M, Mayer-Scholl A, König M, Rossi L, Wibbelt G**: Invasive species in urban settlements: diseases in free-ranging North American Raccoons (*Procyon lotor*) from Berlin, Germany**. International Urban Wildlife Conference, 17th – 20th of May 2015, Chicago, Illinois, USA, oral presentation.

CONTENT

LIST OF ABBREVIATIONS

A. alata	*Alaria alata*
B. procyonis	*Baylisascaris procyonis*
C. perfringens	*Clostrifium perfringens*
CAV-1	Canine Adenovirus-1
CDV	Canine Distemper Virus
CNS	Central Nervous System
ha	Hectare
MEV	Mink enteritis virus
MNP	Müritz National Park
PV	Parvovirus
RT-PCR	Reverse transcription polymerase chain reaction
S. canis	*Streptococcus canis*
S. enterica	*Salmonella enterica*
S. scabiei	*Sarcoptes scabiei*
SuHV-1	Suis Herpesvirus-1
USA	United States of America
Y. enterolitica	*Yersinia enterolitica*

1. INTRODUCTION AND OBJECTIVES

Today, an established raccoon population is widespread throughout Germany and groups of free-ranging animals are found in several European countries. Information on raccoon disease occurrence in Germany is sparse and is limited to few parasitological surveys focussed on the nematode *B. procyonis* (Lux & Priemer 1995, Gey 1998, Winter 2005, Anheyer-Behmenburg 2013). These studies have helped to elucidate the distribution of *B. procyonis* in Germany. For raccoons in central Germany, the prevalence of this nematode is estimated to be 70% (Gey 1998), while the North-eastern population lacks this parasite (Lux & Priemer 1995). A few publications on raccoons describe canine distemper virus prevalence (Michler et al. 2009, Nikolin et al. 2012, Anheyer-Behmenburg 2013) and a single survey on selected bacteria (Anheyer-Behmenburg 2013) has been reported from Germany. For the rest of Europe, only one parasitological survey of raccoons from Western Poland has been published (Bartoszewics et al. 2008). In this study, a 3.7% prevalence of *B. procyonis* was reported. (Bartoszewicz et al. 2008, Beltrán-Beck et al. 2012, Michler & Michler 2012, Vos et al. 2012). The main objective of this dissertation was to examine the occurrence of pathological changes and selected infectious pathogens in rural and urban raccoon populations in North-eastern Germany through histo-pathological and molecular biology analyses in order to describe which diseases or infectious agents are present in the North-eastern German raccoon population. Section 3 of this dissertation comprises 4 scientific articles that describe and discuss the results of this study. Article I reports the presence of an European trematode, *Alaria alata* in mostly rural raccoons from this project. Article II and III describe cases of *Sarcoptes scabiei* and canine distemper virus infection respectively in urban raccoons, giving insight of interspecies transmission amongst urban carnivores in the cities. Finally, article IV comprises a cumulative study of pathological findings and selective pathogen screening in rural and urban raccoons, including some information from the previous publications, in order to give a broader insight of raccoon health in rural and urban Germany. The results are also described in the context of the potential risk raccoons may represent for disease transmission in Germany.

2. LITERATURE REVIEW

2.1. Biological and ecological aspects of raccoons

The raccoon is the largest member of the family *Procyonidae*. Other members of this family are the coatis (*Nasua*), ringtail (*Bassariscus*), and olingos (*Bassaricyon*). The North-American raccoon (*Procyon lotor*) shares the *Procyon* genus with two other species, the crab-eating raccoon (*Procyon cancrivorus*) occurring in South-America and the critically endangered Cozumel raccoon (*Procyon pygmaeus*) restricted to the Cozumel Island in South-eastern Mexico. Raccoons are medium size mammals with a variable fur coloration ranging from grey to almost black and some animals display brown or reddish coloration. Raccoons have a ringed tail and a characteristic black “masked” face delimitated by a light frame of fur identifying the genus. The body length ranges from 41.5 to 60 cm excluding the tail, which is 20 to 40.5 cm long and the body weight varies from 2 to 12 kg depending on the animals’ age (Kays 1999). Size and weight average decrease from colder to warmer regions with animals in colder environments being larger than raccoons living in warmer areas (Lotze & Anderson 1979).

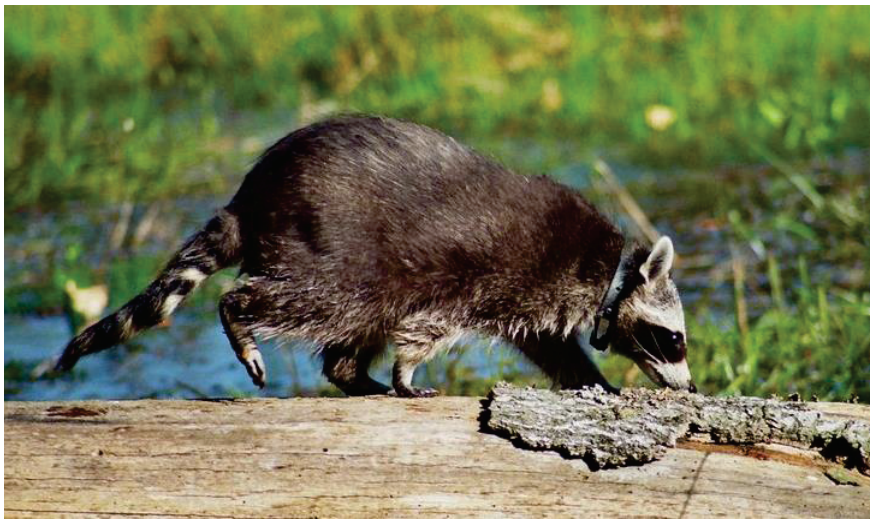

Figure 1.- Radio-collared raccoon from Müritz National Park (Image with kind permission of F-U Michler)

Similarly, reproductive parameters vary by region and habitat (Fritzell 1978). Raccoons are polygamous and mating season starts in January and can last until August, with a peak in March (Lotze & Anderson 1979). However, raccoons from warmer areas begin their mating season earlier (Fritzell 1978). Gestation lasts around 63 days with births occurring between April and June with the most occurring in May (Lotze & Anderson 1979). Litter size varies from 3 to 7 offspring with a mean of 4.8 siblings and a higher number of siblings for animals in colder northern regions (Fritzell 1978) which compensates for higher mortality rates in these areas. Litters are weaned between the 7th week and the 4th month of age (Montgomery 1969) and they start to disperse around the 9th month of age (Stuewer 1943). Life span in free-ranging raccoons

is rarely greater than 5 years with a mean of 3.1 years (Johnson 1970), while captive raccoons can live up to 17 years (Rue 1965).

The social system of raccoons involves limited interaction among individuals. Males form small groups of mostly adult animals and such coalitions may last a year (Gehrt & Fritzell 1998, Gehrt et al. 2008, Pitt et al. 2008, Hirsh et al. 2013). Males and females do not seem to interact outside of the mating season (Hirsh et al. 2013). Females are considered to be solitary but two or more females can sporadically share range areas without close interaction (Pitt et al. 2008). Mother-offspring relationship last until the subsequent mating season and the offspring's home range usually lies within the mother's territory (Muschik et al. 2011). Subsequently, male offspring will leave the maternal territory, a strategy that avoids potential inbreeding. Female offspring, in contrast, remain close to their mother's territory. Besides predation, interspecific contact does sporadically occur between raccoons and other wildlife, although it is rarely documented and tends to take place during the night around feeding areas (Campbell et al. 2013).

Raccoons are omnivorous, nocturnal animals with a diet consisting mostly of invertebrates, plants (including fruits and grains) and small vertebrates; the proportion of each food group varying seasonally (Engelmann et al. 2011). In urban areas, food from anthropogenic sources constitutes part of the raccoon diet (Prange et al. 2004) and its availability has a strong positive influence on the selection of home range in urban raccoons (Bozek et al. 2007). Additionally, increased anthropogenic food sources and shelter reduces the size of the home range of urban raccoons (Prange 2004). Home ranges in the cities are small with an average of 5 to 79 ha per animal in contrast to 50 to 300 ha for animals in rural regions (Gehrt 2004). In sylvatic habitats home ranges can reach up to 702 ha for males and 263 ha for females (Köhneman & Michler 2009).

In natural areas, raccoon habitats are deciduous and mixed forests close to water sources like rivers, swamps and lakes (Pedlar et al. 1997). In rural and sylvatic regions raccoon densities reach up to 8 animals/km^2 or 4.7 animals/km^2, respectively (Rosatte 2000). Proximity to agriculture and rural settlements can increase raccoon density (Pedlar et al. 1997). Raccoons are also present in urban environments. In cities, raccoons occur near parks and residential areas (Rosatte et al. 1992a, 1992b), and urban habitats maintain a high raccoon density, reaching on average 125 animals /km^2 with a range of 66.7 to 333.3 animals /km^2 (Riley et al. 1998).

Causes of death in free-ranging raccoons vary depending on the habitat. Starvation and parasitism play a significant role in rural raccoon mortality (Mech et al. 1968, Rosatte 2000). However, hunting, trapping and road-kills are the major cause of raccoon deads in rural

environments (Sanderson 1987, Rosatte 2000). Predation also occurs in mostly sylvatic areas. For example, in a national park near Cleveland, Ohio, USA, raccoon remnants were found in 18% of coyote scats (Cepek 2004). In urban areas hunting and trapping do not constitute a major risk, whereas vehicle accidents are a considerable cause of mortality (Prange et al. 2003). Additional to road-kills, infectious diseases like distemper, rabies or parvovirus, and dog attacks are the most common causes of mortality for raccoons in the cities (Riley et al. 1998, Rosatte 2000, Hadidian et al. 2010).

Raccoons are considered a pest species in most human populated regions of USA and Canada. Furthermore, raccoon-human conflicts occur frequently as the animals can cause substantial economic loss due to crop damage, especially in cornfields (Beasley & Rhodes 2008). In urban areas raccoons can damage houses and other private property, forage in garbage and attack domestic animals. Public perception of raccoons is mostly negative. For example, residents in the Chicago metropolitan area where property damage was recorded blamed raccoons 26% of the time (Miller et al. 2001). In the late 1990's economic losses in Chicago due to raccoon activity were estimated around 1 million US dollars (Gehrt 2003).

2.2. Geographical distribution

Raccoons are native to North and Central America and occur from southern Canada to Panama. Outside their native range, raccoons have been introduced to Europe, the Caucasus, Belarus, Uzbekistan and Japan (Figure 2). The first of these introductions took place in Europe in the Federal State of Hessen, Central Germany. In the early 1900's, raccoon fur became a popular material for winter clothing in the USA; Europe followed this trend and in the 1920's raccoon coats were imported to Europe. Since importing fully finished coats was expensive, some fur farms in Germany began importing raccoons from the USA for breeding to meet the demand for raccoon fur.

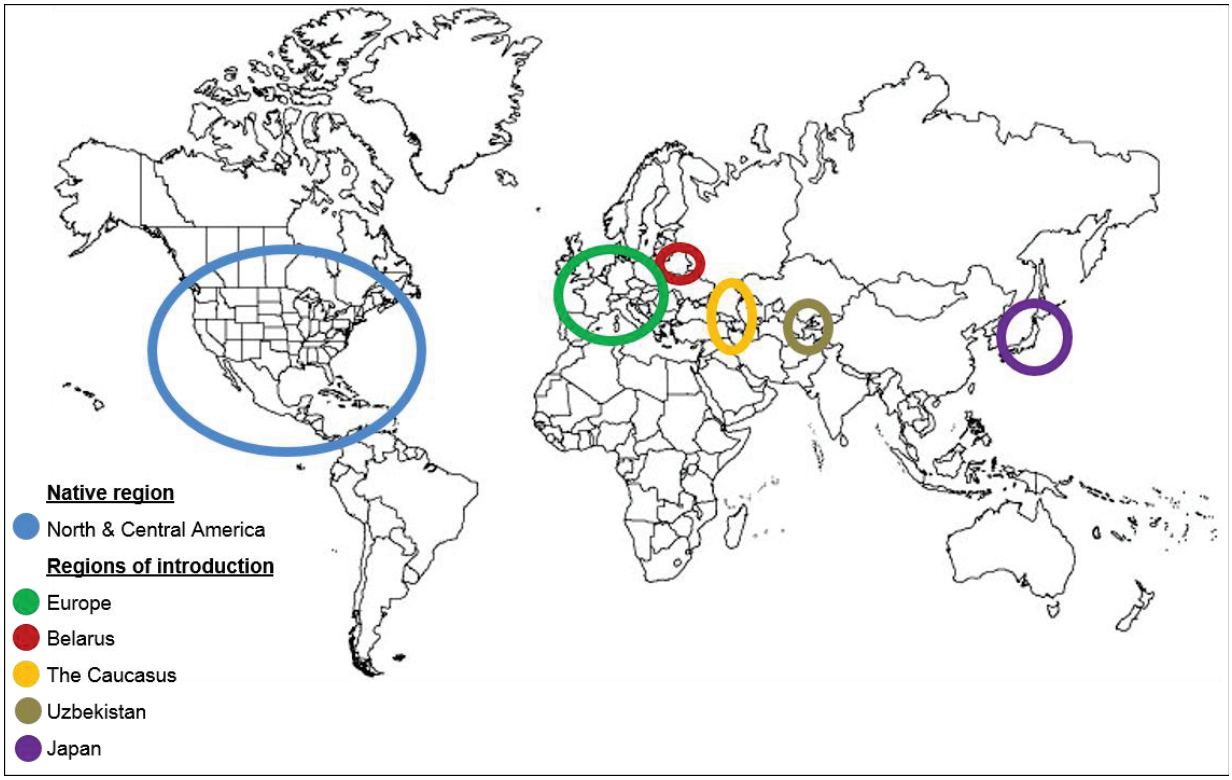

Figure 2.- Regions with raccoon occurrence

The first raccoon release and successful introduction into the wild occurred in 1934 in the Federal State of Hessen, in central Germany. Rolf Haag, a local raccoon breeder, requested from the Forestry Office the permission to release two pairs of raccoons into the wild with the objective to enrich the local fauna. Permission was granted and on April 12th 1934 four raccoons were released in Edersee, Northern Hessen(Hohmann & Bartussek 2005). In 1945, a separate second introduction took place in Wolfshagen, Brandenburg, Northern Germany, where some animals escaped from fur farms that were bombed during World War II.

Even though raccoons are now distributed throughout Germany, both separate introductions are still distinguishable as two density hot spots (Figure 3): one in the centre of the country which originates from the 1934 introduction in Hessen; and the other in the North-eastern region of Germany, which originated from the animals that escaped in 1945. Currently, between 600,000 and 800,000 raccoons are thought to be present in Germany (F.-U. Michler, unpublished data).

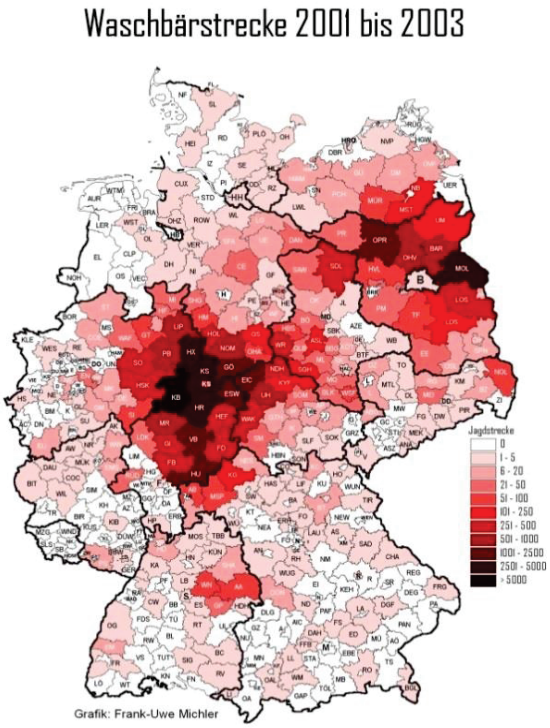

Figure 3. Raccoon hunting bag in Germany during 2001-2003 (Image with kind permission of F-U Michler)

From Germany raccoons have spread to neighbouring countries. Today, a small population of raccoons can be found in Poland (Bartoszewicz et al. 2008), and sporadic to rare occurrence in Austria (Spitzenberger et al. 2001), France (Léger 2001), Luxemburg (Schley et al. 2001), The Netherlands (Broekhuizen et al. 2001) and Hungary (Heltai et al. 2000). Additional, reports of raccoons in Italy (Canova & Rossi 2008) and Spain were recently published, the latter suspected as deliberate raccoon releases by pet-raccoon owners (García et al. 2012).

Independent raccoon introductions also occurred in regions of the former Soviet Union, especially the Caucasus, some animals were released in Belarus and Eastern Uzbekistan (Aliev & Sanderson 1966, Zima 1978). These introductions established small but stable raccoon populations that can be still be found. In Japan raccoon introduction occurred in the late 1970s after the success of an animated TV series called “Rascal raccoon” in 1977, which popularized the animals. As a result, many raccoons were imported to Japan and were kept as pets (Ikeda et al. 2004). When the animals reached puberty and became aggressive, owners irresponsibly abandoned them. Raccoon breeders also released animals into the wild owing to reduce breeding colony sizes. The Japanese raccoon population has expanded and is now distributed throughout 42 of 47 prefectures of the country (Ikeda et al. 2004).

2.3. Selected infectious pathogens of raccoons

2.3.1. Parasites

Raccoons are hosts of *Baylisascaris procyonis*, the raccoon roundworm which is a large nematode from the family *Ascaridida*e. Raccoons are the final host of *B. procyonis*, and the adult parasite resides in the small intestine. *B. procyonis* is prevalent across the United States especially along the west coast with a prevalence of 68-82% (Kazakos 2001). The parasite life cycle begins with unembryonated eggs being shed in raccoon's faeces. After 11-14 days, the eggs transition to an embryonated infective stage represented by the second stage larva. In this stage, the parasite can either re-infect the raccoons orally or enter a paratenic host, usually small mammals or birds. The paratenic host becomes infected by foraging in raccoon latrines while searching for undigested seeds (Kazakos & Boyce 1989). Once infecting the paratenic host, *B. procyonis* larvae hatch from the eggs and aggressively migrate to different organs. The lesions vary by host species and individual but initial haemorrhagic lesions can be seen in the lung at 12-48 hours post infection in mice. Some of the larvae migrate to the central nervous system (CNS) at 3 days post infection and can lead to severe neurological damage or death in about 9 days post infection (Sheppard & Kazacos 1997), facilitating opportunistic predation of the paratenic host by raccoons (Sheppard & Kazacos 1997, Kazakos 2001). Once adult raccoons ingest the paratenic host, the larvae develop into their adult stage in the intestinal lumen after 32 to 38 days (Kazakos & Boyce 1989) and the cycle starts again. As young raccoons do not feed on paratenic hosts they become infected by second stage larvae from the mother's nipples or fur, contaminated dens or latrines (Kazakos 2001). After ingestion, the larvae hatch and enter the mucosa of the small intestines, where they develop for several weeks. The parasite then re-enters the intestinal lumen where it will reaches adulthood (Kazakos 2001). *B. procyonis* has a wide range of paratenic hosts with rodents, lagomorphs, birds, and primates being especially susceptible, whereas infections in reptiles and amphibians have not been recorded (Kazakos 2001). Humans can be infected with the second larval stage of the parasite followed by larval migration to different organs. Cases of human baylisascariosis have been reported, few of them fatal. Human cases are mostly common in infants (Huff et al. 1984, Fox et al. 1985, Cunningham et al. 1994) or patients that have had contact with raccoons (Goldberg et al. 1993, Küche et al. 1993). Raccoons are not the only final hosts of *B. procyonis* and adult parasites have been found in the intestine of dogs (Greve & O'Brien 1989), kinkajou (*Potos flavus*) (Overstreet 1970), Northern olingo (*Bassaricyon gabbii*) (Overstreet 1970) and opossums (*Didelphis virginiana*) (Kazakos & Boyce 1989).

Cooler temperatures and adequate moisture typically found in mountainous areas can benefit parasite infectivity and allow eggs to persist in the environment for years (Kazakos & Boyce 1989). *B. procyonis* prevalence is higher in such areas, while the prevalence decreases in warmer and drier regions (Kazakos 2001). Raccoon ecology also affects *B. procyonis* prevalence. Page et al. (2008) reported a higher prevalence of *B. procyonis* infection of raccoons in rural (65%) compared to urban areas (41%). This difference can be explained by the incorporation of human originated food in the diet of urban raccoons, which also decreases the consumption of paratenic hosts (Page et al. 2008). Because raccoons are attracted to human settlements in both urban and rural areas, the risk of infection to humans is considerable, especially for homeowners with small children and professionals working with raccoons (Page et al. 2009).

Another parasite of zoonotic importance in raccoons is *Trichinella* spp., a nematode from the family *Trichinellidae*. The genus *Trichinella* has eight species and is distributed worldwide. The life cycle of *Trichinella* spp. comprises two developmental stages in one or more hosts. The parasite has a large number of host species, which can be mammals, birds or reptiles. The female nematode deposits larvae in the intestinal mucosa of the host and the larvae subsequently migrate to the lymphatic and blood vessels and then to the skeletal muscles. After penetrating the muscle tissue, the larvae induce capsule formation (Taratuto & Venturiello 1997, Gottstein et al. 2009). Encapsulated parasites can remain in the host muscle over an extended period of time (Fröscher et al. 1988) or until the host is consumed by another potential host. The larvae are released during gastric digestion and reach the duodenum where they embed into the intestinal mucosa and develop into the adult stage within the first two days post infection. Five days post infection, male and female nematodes mate and a new generation of larvae develops (Gottstein et al. 1988).

In humans, acute trichinellosis can result in fever, facial oedema, pyrexia and myalgia, commonly accompanied by myocarditis. Chronic trichinellosis causes encephalitis and secondary infections such as bronchopneumonia and sepsis (Gottstein et al. 1998). Consumption of undercooked meat, especially of porcine or game origin is the most common cause of *Trichinella* spp. infection in humans. Raccoons are known hosts for *Trichinella* (Kobayashi 2007, Hill et al. 2008) although no case of human trichinellosis spread by raccoons has been reported, animals living in close proximity to domestic swine or to game animals could further transmit the parasite functioning as a vector host (Dame et al. 1987).

In addition to *B. procyonis* and *Trichinella* spp., raccoons can carry a considerable number of helminth species. More than 30 species of trematodes, 15 species of nematodes, three cestode

species and one Acanthocephala species (*Macracanthorhynchus ingens*) have been found in raccoons (Jordan & Hayes 1959, Harkema & Miller 1964, Bafundo et al. 1980, Cole & Shoop 1987, Richardson et al. 1992). Among these are trematodes from the genus *Alaria.* Raccoons have been reported as paratenic hosts for the mesocercarial stage of two Alaria species: *Alaria mustelae* and *Alaria marcianae* (Bosma 1931, Shoop & Corkum 1981). The genus *Alaria* belongs to the *Diplostomatidae* family. It consists of six known species, five of which occur in North and South America. *Alaria alata* is the only species present in Europe. The life cycle for *Alaria* spp. includes two intermediate hosts and seven developmental stages. The eggs are shed in the faeces of the final host such as species in the families Canidae, Felidae or Mustelidae. Once in the environment, miracidia hatch from the eggs after two weeks and actively infect the first intermediate host: four genera of fresh water snails (*Planorbis*, *Heliosoma*, *Lymnea* and *Anisus*). Within a year, two generations of sporocysts develop in the snail host. Tailed cercariae are released into the water and infect the second intermediate host: amphibian tadpoles. In the second intermediate host, the cercariae develop into mesocercariae and infect the final host by predation of the secondary intermediate host. In the final host, the mesocercariae migrate to the lungs and mature into metacercariae, which migrate to the intestine and develop into the adult phase. The life cycle can be extended at the mesocercarial stage by addition of a paratenic host in which the mesocercariae stays in a resting phase encysted within the tissue.

Humans can be infected by the mesocercarial stage of *Alaria* spp. by consuming undercooked or raw meat of a paratenic host, including raccoon meat in one case (Beaver et al. 1977, Kramer et al. 1996) or the secondary intermediate host (Shea et al. 1976, Freeman et al. 1976, Fernandez et al. 1976, McDonald et al. 1994). Human alariosis can cause mild respiratory or cutaneous disease (Beaver et al. 1977), unilateral neuroretinitis (Bialasiewcz 2000), or fatal anaphylactic shock in as represented by a single case (Freeman et al. 1976). So far, seven cases of human infection with *Alaria* mesocercariae have been reported, all occurring in North America (Byers & Kimura 1974, Fernandez et al. 1976, Freeman et al. 1976, Shea et al. 1973, Beaver et al. 1977, Mcdonald et al. 1994, Kramer et al. 1996). No cases of human alariosis have been reported in Europe, however, *Alaria alata* has been detected with increasing frequency in meat from wild boar (*Sus scrofa*) in European countries including Germany and France (Möhl et al. 2009, Portier et al. 2011, Riehn et al. 2012, Paulsen et al. 2012, Portier et al. 2013, Riehn et al. 2014). The Federal Institute of Risk Assessment (BfR) in Germany considers meat infected with *A. alata* mesocercariae unfit for human consumption (BfR 2007).

Ectoparasites like lice (Richardson et al. 1994, Nelder & Reeves 2005), ticks (Hamir et al. 1993, Richardson et al. 1994, Nelder & Reeves 2005, Monello & Gompper 2007) and mites

(Richardson et al. 1994, Ninomiya & Ogata 2002, Nelder & Reeves 2005) have been reported in raccoons. One of the mites species found (Fitzgerald et al. 2004) is *Sarcoptes scabiei*, the aetiological agent of Sarcoptic mange. It belongs to the family *Sarcoptidae* that infects the epidermis of more than 100 host species including members of the families Bovidae, Cervidae, Suidae, Canidae, and Mustelidae. It also affects primates such as Gorillas (*Gorilla gorilla*) or humans (Pence & Ueckermann 2002). Currently, the parasite is considered a single species with several varieties and a certain degree of host specificity, the first remains a topic of debate (Walton et al. 2004). *S. scabiei* is distributed worldwide; the life cycle of the mite occurs in a single host. The female burrows tunnels into the epidermis and eggs are laid in these tunnels. After three days, the eggs hatch and the larvae emerge and reach the first nymphal stage (protonymphs). The second nymphal stage, called tritonymph, occurs three days later. Two to four days later the mite matures into the adult stage (Arlian 1989, Bornstein et al. 2001). The disease mange is mainly caused by the mechanical disruption and chewing by the mite when it burrows into the skin, as well as the secretions and excretions of dead *S. scabiei*, mites, egg shells and skin of nymphs and adults which generate hypersensitivity reactions by the host (Arlian 1989, Bornstein et al. 2001, Pence & Ueckermann 2002). Clinical signs start within 2 to 3 weeks post infection and consist of papules and seborrheic dermatitis. Five to seven weeks post infection intensive pruritus, hyperkeratosis and alopecia developed. Changes are progressive and thick encrusted skin lesions and serous exudates develop to cover most of the body. The main histological lesion is severe hyperkeratosis with leucocyte infiltration (Nimmervoll et al. 2013). The host becomes dehydrated, emaciated and dies 2 to 3 months after infection (Bornstein et al. 2001, Pence & Ueckermann 2002). In wildlife populations epizootics have been reported in carnivore species such as red foxes (*Vulpes vulpes*) (Mörner 1992), wolves (*Canis lupus*) (Todd et al. 1981), coyotes (*Canis latrans*) (Pence & Windberg 1994), and a few cases in lynxs (*Lynx lynx*) (Ryser-Degiorgis et al. 2002). Wildlife ungulates are also affected by *S. scabiei*. European populations of chamois (*Rupicapra rupicapra*) and ibex (*Capra ibex*) experience epizootics of sarcoptic mange in a 15-year intervals (Rossi et al. 1995). Even though wildlife populations are able to recover from sarcoptic mange epizootics (Bornstein et al. 2001, Pence & Ueckermann 2002), public health concerns exist since transmissions between wildlife and domestic animals and occasionally between wildlife and humans can occur (Arlian 1989, Bornstein et al. 2001, Pence & Ueckermann 2002). However, the frequency of such interspecies transmissions is still not clarified (Pence & Ueckermann 2002).

2.3.2. Viruses

Canine distemper virus (CDV) is an RNA virus of the family paramyxoviridae, genus Morbillivirus. Examples of other members of the genus are measles virus, peste-des-petits-ruminants virus and rinderpest virus. CDV infects a wide range of carnivore hosts, including domestic and wild canids such as dogs, foxes, coyotes, and wolves. Other carnivores such as mustelids, procyonids, ursids, and large felids can also be infected. Despite vaccination programs in domestic and captive carnivores, CDV is present worldwide. The virus is highly contagious and it affects animals of all ages although juveniles are more susceptible. Infected animals exhibit gastrointestinal and respiratory clinical symptoms that can be accompanied by central nervous system symptoms (Amude et al. 2007). Transmission occurs through body secretions and excretions. Twenty-four hours after infection, CDV replicates in macrophages, spreads to tonsils and bronchial lymph nodes and proliferates. Less than one week after infection, the virus is found in spleen, gastrointestinal lamina propia, mesenteric lymph nodes and Kupffer's cells in the liver (Greene & Appel 2006). By day eight to nine CDV reaches the CNS and epithelial tissues and viral shedding begins (Greene & Appel 2006). CDV invasion of CNS tissue depends on host and viral strain (Deem et al. 2000). It has been reported that CDV reaches the CNS through the cerebrospinal fluid (Higgins et al. 1982), cerebral blood vessels, the olfactory nerve (Rudd et al. 2006) or meningeal cells of the *pia mater* (Baumgärtner et al. 1989). Common histological findings include eosinophilic inclusion bodies in epithelial cells of lung, kidneys, urinary bladder and gastrointestinal tract. The lung tissue usually reveals broncho-interstitial or interstitial pneumonia with proliferation of pneumocytes type II and syncytial cells. In the CNS inclusion bodies are found in glial cells and neurons, demyelination and non-suppurative perivascular cuffing and leptomeningitis are also observed. Chronic cases present severe hyperkeratosis of the nose and footpads (hard pad disease).

Canine distemper is one of the main infectious mortality causes in urban and rural raccoons (Rosatte 2000, Hadidian et al. 2010) with regular reports of outbreaks in raccoon populations (Deem et al. 2000). It has been suggested that raccoons play a role in distemper epidemics in occurring in domestic dogs and other wildlife carnivores (Cranfield et al. 1984, Nakano et al. 2009) especially in threatened species such as the Island fox (*Urocyon littoralis*) in California, USA (Timm et al. 2009).

Rabies is a fatal zoonotic disease that infects all mammals and causes viral encephalomyelitis. It is responsible for approximately 50,000 human deaths worldwide each year (Knobel et al. 2005). The aetiological agent is a single-stranded RNA virus belonging to the order Mononegavirales, family Rhabdoviridae, and genus Lyssavirus. Rabies viruses in this genus include the classical

rabies virus, Mokola virus, Lagos bat virus, European bat lyssavirus 1 and 2, and Australian bat lyssavirus. The most common route of infection is via saliva of a rabid animal transferred by biting or scratching another animal. The virus initiates infection by replicating in the wound tissue, though the incubation period can vary from days to years. From the initial point of entry the virus reaches the peripheral nerves and attaches to nerve endings moving towards the CNS. Once the virus reaches the CNS, it massively increases replication resulting in disease that has a fatal outcome soon after the first clinical signs of the acute neurological phase. The infected individual can die within days. Microscopic lesions in the CNS of infected animals exhibit non-suppurative perivascular cuffing, mononuclear infiltration, Negri bodies, or Babè's nodules of glial cells. Rabies transmission occurs sylvatic and an urban, cycles. The sylvatic cycle is maintained by different wildlife species, for example in North America by raccoons, skunks, coyotes, and foxes. In South America vampire bats are a major reservoir. In Europe, foxes or raccoon dogs are the key reservoir species. The sylvatic cycle can converge with the urban cycle, in which dogs are the main reservoir, by transmission from wildlife, especially urban adapted species like raccoons or foxes to domestic dogs. This circumstance is particularly important in countries where only dog rabies is controlled.

Feline panleukopenia virus (FPV) is a single-stranded DNA parvovirus from the family *Parvoviridae*, subfamily *Parvovirinae*. The virus is distributed worldwide and can infect felids, especially domestic cats, mustelids, procyonids and viverrids. FPV does not infects canids although it is closely related to canine parvovirus 2 (CPV-2), which was first described in 1978 and spread rapidly among canids.Subtypes of this virus exist: CPV-2a and CPV-2a-derived strains. While CPV-2 is thought to be unable to infect cats, subtypes have been isolated from cats suffering from panleukopenia (Truyen et al. 1996). However, the epidemiology of CPV-2a or derived subtypes in cats has not been studied. Both FPV and CPV are highly contagious and are acquired by direct contact with infected animals or faeces. The virus initially replicates in the pharyngeal lymphoid tissue and spreads from there to other organs via the blood stream (Parrish 2011). Subsequently, the virus replicates in lymphoid organs where it destroys leucocytes causing profound leukopenia. Since the virus replicates in dividing cells, not just leucocytes, epithelial cells of the intestinal mucosa are also affected causing diarrhoea. During post-mortem examinations, petechial haemorrhages on the bowel serosa or segmental congestion of the mucosa can be found. Histology shows attenuation of the epithelium lining and distended intestinal crypts. Intranuclear inclusion bodies are rarely present in crypt cells. CPV pathology is similar to FPV but may additionally cause myocarditis in pups. Raccoons are susceptible to FPV and to a closely related virus called Mink enteritis virus (MEV) (Barker et al.

1983). Barker et al. (1983) experimentally infected raccoons with FPV and MEV. Raccoons infected with FPV had diarrhoea with blood or fibrin, appetite loss, depression and finally died between six to ten days post infection. For Raccoons infected with MEV the virus was highly virulent and the animals showed similar symptoms as FPV infected raccoons. Experimental CPV infections of raccoons did not result in clinical disease or sporadic shed of the virus in the faeces (Barker et al. 1983). However, viral strains closely related to CPV-2 have been isolated from a single raccoon with clinical signs of parvoviral infection (Kapil et al. 2010). In urban areas of the South-eastern USA, FPV sero-prevalence in raccoons ranges from 25% to 89%, suggesting that urban raccoons are commonly exposed to the virus (Junge et al. 2007). Raccoons are able to carry both feline and canine parvovirus, and thus it has been hypothesized that they may have played a role in the evolution of parvoviruses, specifically, the adaptation of parvovirus to canine hosts (Allison et al. 2014).

Canine adenovirus type 1 (CAV-1) is a linear double-stranded DNA virus from the genus *Mastadenovirus*, family *Adenoviridae*. It was first described in wild foxes in the 1920's and later detected in domestic dogs in the 1930's. Since then, the host range of CAV-1 has expanded to include canids, mustelids, ursids and procyonids. Clinically, the disease is characterized by fever, apathy, anorexia, conjunctivitis and discharge from eyes and nose. Initial infection occurs through the oropharynx. Having entered the host, the virus infects tonsils and spreads to lymph nodes and blood. It infects endothelial and parenchymal cells of liver, kidneys, spleen and lungs, causing haemorrhage and necrosis (Knowles 2011). Intranuclear inclusion bodies can be found in hepatocytes and Kupffer cells. CAV-1 has been controlled in domestic dogs in many countries by vaccination. However, CAV-1 transmission from wildlife to dogs can occur since the virus is still present in wild carnivores like foxes (Åkerstedt et al. 2010, Thompson et al. 2010), artic foxes (Åkerstedt et al. 2010), gray foxes (Gerhold et al. 2007), wolves (Åkerstedt et al. 2010), European mink (Philippa et al. 2007), urban raccoons (Junge et al. 2007), and other raccoon species such as the pygmy raccoon (McFadden et al. 2005).

In addition to the viruses most commonly associated with carnivore populations, raccoons can also be infected by suid herpesvirus 1 (SuHV-1). This virus is the aetiological agent of Aujeszky's disease or pseudorabies, which is an important infectious disease in the pork production industry. SuHV-1 is a double-stranded DNA porcine virus from the family Herpesviridae. It is distributed worldwide and its main hosts are domestic pigs and wild boars. Vaccination has successfully eradicated the virus in domestic swine populations form Norway, Finland, Germany, Austria, Sweden, UK, Denmark, Canada, New Zealand and the United States. In some of these countries, however, SuHV-1 is still present in the free-ranging wild boar population (Ketusing et

al. 2012). The virus can occasionally infect non-suid wildlife species that have been in contact with pigs or wild boar. Pseudorabies has been reported in rats (Von Becker & Herrmann 1963), roe deer (Nikolitisch 1954), Florida panther (Glass et al. 1994), black bear (Schultze et al. 1986), coyotes (Raymond et al. 1997), and raccoons (Kirkpatrick et al. 1980, Thauley & Wright 1982, Junge et al. 2007). In swine, the disease presents respiratory infections in adult pigs and abortion in sows. The virus spreads via an oral-faecal or venereal route in pigs. Once in the host,the virus spreads to lymph nodes or CNS. Mortality in pigs is low, while many wild species like raccoons, bears or coyotes succumb to the disease. Carnivores most likely get infected when ingesting infected boar or pig. Other species like deer contract the virus when grazing in proximity to infected swine populations.

2.3.3. Bacteria

Raccoons are known vectors for *Leptospira* spp., which are zoonotic bacteria with a worldwide distribution. *Leptospira* are obligate aerobic spirochetes and the genus *Leptospira* has 17 species (Bharti et al. 2003). Leptospirosis infects humans, domestic and wild mammals. It occurs globally in industrialized and developing countries, although it is more common in tropical regions. *Leptospira* spp. is transmitted through minute skin lacerations or mucosal contact with urine of an infected host or contaminated soil or water. The pathogenic mechanisms of *Leptospira* spp. have not been entirely elucidated (Kelly 1989, Bharti et al. 2003). Once in the host, the bacteria spread via bloodstream to kidneys, liver, meninges, skeletal muscles or placenta. The disease manifestations are variable and include renal failure, febrile illness, pulmonary haemorrhage or subclinical infection (Bharti et al. 2003). Chronically infected hosts are typically asymptomatic although they harbour and shed *Leptospira* for months or longer via urine (Kelly 1989). Histologically, interstitial nephritis is present in many cases. However, chronic carriers of *Leptospira* spp. do not commonly present such lesions (Bharti et al. 2003). Rats and domestic dogs are considered as major vectors followed by cattle, swine, goats (Kelly 1989), and raccoons as a major wildlife host (Kelly 1989, Richardson & Gauthier 2003, Junge et al. 2007, Tan et al. 2014).

Salmonella species are also among the major bacterial pathogens associated with raccoons (Morse et al. 1983, Compton et al. 2008, Jardine et al. 2011, Lee et al. 2011). *Salmonella* are zoonotic Gram-negative motile bacteria of the family Enterobacteriaceae and are distributed worldwide. The genus *Salmonella* consists of more than 2,400 different serotypes, all of them potentially pathogenic (Carter & Wise 2004), infecting a wide range of mammalian, avian, and reptile hosts. The bacteria are transmitted by contaminated food, water, or faeces and primarily colonize epithelial cells of the ileum and colon. Clinical disease occurs primarily in stressed

hosts since stress facilitates *Salmonella* invasion of the intestinal epithelium (Plym Forshell & Wierup 2006). Animals with clinical signs present diarrhoea, fever and in many cases they die from the infection (Jahraus & Philips 1999). Salmonellosis is also of importance in public health as 1.3 billion human cases are reported annually (Coburn et al. 2007). Wildlife is closely associated with *Salmonella* outbreaks in humans (Handeland et al. 2002), domestic animals (Humphrey & Bygrave 1988), and food contamination by *Salmonella* spp. (Orozco et al. 2008).

3. SCIENTIFIC ARTICLES

This cumulative dissertation is based on four scientific articles. Three of these have been published in international peer-reviewed journals. The fourth article is presented as unpublished manuscript.

3.1. Article 1: *Alaria alata* mesocercariae in raccoons (*Procyon lotor*) in Germany

Rentería-Solís ZM, Hamedy A, Michler F-U, Michler BA, Lücker E, Stier N, Wibbelt G, Riehn K (2013) ***Alaria alata* mesocercariae in raccoons (*Procyon lotor*) in Germany**. Parasitol Res, 112:3595-3600. DOI: 10.1007/s00436-013-3547-4.

Contributions: ZMR-S performed raccoon necropsies, tongue tissue sampling, histology and cryotome slides preparation, histo-pathological examinations, *A. alata* isolation and identification, and wrote the paper.

Please purchase this part online.

3.2. Article 2: Genetic epidemiology and pathology of raccoon-derived *Sarcoptes* mites from urban areas of Germany

Rentería-Solís Z, Min AM, Alasaad S, Müller K, Michler F-U, Schmäschke R, Wittstatt U, Rossi L, Wibbelt G (2014) **Genetic epidemiology and pathology of raccoon-derived *Sarcoptes* mites from urban areas of Germany**. Med Vet Entomol, 28(S1):98-103. DOI: 10.1111/mve.12079.

Contributions: ZMR-S performed raccoon necropsies, histo-pathological examination, mite isolation and identification, microsatellite data analyses and wrote the paper.

Please get this part online.

3.3. Article 3: Canine distemper outbreak in raccoons suggests pathogen interaction amongst alien and native carnivores in urban areas from Germany

Rentería-Solís Z, Förster C, Aue A, Wittstatt U, Wibbelt G, König M (2014) **Canine distemper outbreak in raccoons suggests pathogen interspecies transmission amongst alien and native carnivores in urban areas from Germany**. Vet Microbiol, 174:50-59. DOI: 10.1016/j.vetmic.2014.08.034.

Contributions: ZMR-S performed RNA extraction from raccoon tissue samples, RT-PCR assays, cloning of PCR products, histo-pathology and immunohistochemistry examination and wrote the paper.

Please purchase this part online.

3.4. Article 4: Disease investigations in free-ranging raccoons (*Procyon lotor*) from rural and urban settlements in Germany

Rentería-Solís Z, Michler F-U, Michler BA, Förster C, Nöckler K, Mayer-Scholl A, Grobbel M, Aue A, Wittstatt U, König M, Wibbelt G. **Disease investigations in free-ranging raccoons (*Procyon lotor*) from rural and urban settlements in Germany**. Unpublished manuscript.

Contributions: ZMR-S performed raccoon necropsies, histo-pathological examinations, parasitological examinations of the MNP raccoons, virological examinations, and wrote the paper.

Remarks: It is intended to submit this manuscript to BMC Veterinary Research and thus it follows journal's layout and format.

Diseases in free-ranging raccoons (*Procyon lotor*) from rural and urban settlements in Germany

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Abstract

Background

Raccoons (*Procyon lotor*) were introduced into Germany in 1934. Despite the raccoons' successful establishment in Europe, knowledge on their infectious diseases is limited. This study aimed to investigate the occurrence of pathogens and sub- /clinical infectious diseases present in rural/sylvatic and urban raccoons from north-eastern Germany.

Results

Two hundred and forty hunted, road-killed or euthanized raccoons from Müritz National Park (MNP) (n=100) and Berlin metropolitan area (n=140) were collected. Macroscopic findings were mostly traumatic injuries related to the cause of death. Histologically, in more than half of the examined raccoons inflammatory lesions were found (65.6%); the most common inflammatory change was an infiltration by mononuclear cells and eosinophils in various organs. A number of pathogens, known to commonly occur in the raccoons' North American conspecifics, were detected: Canine distemper virus (CDV), parvovirus (PV), *Leptospira* spp. and *L. interrogans*, *Salmonella enterica*, *Yersinia enterocolitica*, *Sarcoptes scabei* and *Metorchis* sp. Other infectious agents were newly described like *Alaria alata* or *Streptococcus canis*. Some pathogens seem to prevail in one habitat, e.g. *A. alata* almost exclusively infected raccoons from the sylvatic MNP, while infection with *Leptospira* spp. and *S. scabei* were associated with the urban habitat. None of the animals was positive for rabies, canine infectious hepatitis, Aujeszky's disease, *Baylisascaris procyonis* or *Trichinella* spp.

Conclusions
Despite being an alien species, raccoons settled well into the different habitat types of Germany and carry a similar range of diseases as in their native North America. Rural/sylvatic and urban raccoons from Germany revealed some distinct differences in their pathogen burden. However, their role as reservoir host for certain pathogens seems currently of lesser importance than known from North America. Whether this will change while their population number in Germany keeps increasing or whether native wildlife species like red foxes will sustain their apparent leading role as reservoir host for certain pathogens has to be seen. In any case, the exploitive behaviour of raccoons of human settlements should prompt applied measures to avoid transmission of zoonotic pathogens. Interdisciplinary work between wildlife ecologists and veterinarians will help to establish balanced solutions for both wildlife and human perspectives.

Background

The introduction of foreign species into a new geographical region can influence the local biodiversity or the occurrence and distribution of pathogens. In Europe 71 non-European mammal species were introduced, 37 species have established populations in one or more European countries [1]. One of these species is the North-American raccoon (*Procyon lotor*), a medium-sized mammal whose adaptability to different environments facilitated its wide distribution from Canada to Panama. Raccoons occur in sylvatic, rural, and urban areas. In the cities and rural regions, raccoons tend to approach human settlements to exploit anthropogenic resources such as shelter and food. They are known as successful urban adapters with population densities that can exceed 100 animals /km^2 [2] and they are well-known carriers of pathogens of public health importance [2, 3]. For example, in North America raccoons are an important reservoir species for rabies and canine distemper [4, 5]. Raccoons are also the final host of the nematode *Baylisascaris procyonis*, a zoonotic parasite that can cause severe to fatal neurological damage in birds and mammals including humans [3]. Between 1975 and 2000 23 cases of human baylisascariosis were registered in the USA, six of them fatal [3].
In Germany, the raccoon was introduced in 1934 to the Federal State of Hessen in central Germany. A second separate introduction took place in 1945 in the north-eastern part of the country. Today, raccoons are widespread throughout Germany but the two initial introduction areas can still be differentiated as hotspots of these populations [6, 7]. In 2011/2012 the hunting bag for raccoons counted 70,000 animals [8] and 500,000 raccoons are estimated to live in Germany today. They also occur in the neighbouring countries although with distinctly lower population numbers. Raccoons are sporadically sighted and estimated populations of a few hundred animals are assumed for Austria (personal communication, Walzer, Vienna University), Belgium (personal communication, Van den Berge, Instituut voor Natuuren Bosonderzoek), the Netherlands [9] and Switzerland (personal communication, Bontadina, stadtwildtiere.ch). For France there is a local population of estimated 300-500 raccoons in the Ardennes and a few sightings are reported mostly from the north-eastern part of the country[10]. In the Czech Republic and in Denmark only a few animals are regularly sighted [11, 12] and in Poland they are reported to be locally abundant [13].
Similarly to their American counterparts, raccoons are thriving in German urban areas with densities of 100 animals /km^2 reported for the city of Kassel, Federal State of Hessen [14]. But despite their successful establishment in the Europe, there is still a lack of information regarding diseases and infectious pathogens present in these animals [15]. Raccoons in central Germany are known to carry *B. procyonis* with a prevalence of 71% for Hessen [16], which is comparable to prevalences in the US (68 - 82%) [3]. In the German Federal States adjacent to Hessen the prevalence of *B. procyonis* prevalence is lower with 60% in Lower Saxony and 39% in Saxony-Anhalt [17, 18], while a previous study in raccoons in north-eastern Germany reported the absence of the in this region [19]. Canine distemper virus (CDV) was found in rural [18, 20, 21] and urban [22] raccoons from northern Germany. In 2008 Germany was officially declared free of rabies. Before this, between 1960 and 1977 15 rabies cases were reported in raccoons in former West-Germany [23]. Five additional cases, with the last case in 2000, occurred between 1990 and 2006; for comparison there were 10534 rabid foxes in the same 16 years' period [24]. In contrast to North America the importance of raccoons for this disease's ecology seem to be rather minor [6, 25].

In the past few years, a number of reviews were published aiming to increase awareness in Europe regarding the potential impact of raccoons in disease transmission [15, 25, 26]. However, as they are mostly based on the situation of North America the information presented may not be representative for raccoons in Germany or Europe.
To elucidate the occurrence of diseases in raccoons from Germany, we investigated raccoon carcasses from a sylvatic and an urban population by histopathology and molecular methods to determine possible sub- /clinical lesions as well as the presence of selected pathogens. Moreover, it was the aim to evaluate the potential risk of infectious disease transmission from raccoons to native wildlife, domestic animals or humans in this region.

Methods

Study Area

The Müritz National Park (MNP) is located in the north-eastern Federal State of Mecklenburg-Western Pomerania. With an extension of 322 km^2, MNP consists of broadleaf tree forests and ramified peatbogs and swamps. The area is very rich in wildlife species and it is also popular for recreational tourism and hunting. Raccoon carcasses collected from MNP were part of a long-term study designed to investigate raccoon ecology in this area [20].
Berlin is the most populated and largest city in Germany with around 3.4 million inhabitants in 892 km^2. The city outskirts are covered with woodlands, lakes, numerous parks, and green areas are distributed throughout the urban landscape. Buildings and urbanized areas comprise 56.1% of the city area, forest, public green areas and agricultural land represent 18.3%, 14.5% and 4.4 % respectively. Besides humans and domestic animals, mostly pets, local wildlife species such as red foxes (*Vulpes vulpes*), wild boars (*Sus scrofa*), and wild rabbits (*Oryctolagus cuniculus*) are commonly spotted in the Berlin landscape.

Raccoon samples

From 2006 to 2013, a total of 240 raccoon carcasses were collected. One hundred of them were derived from MNP and 140 animals originated from Berlin metropolitan area. The latter included 97 animals from a distemper outbreak in Berlin. The age determination of the animals was based on size, weight and dental appearance according to Grau et al [27]. Animals collected from MNP were frozen at -20°C and later sent to the Leibniz Institute for Zoo and Wildlife Research for post-mortem examination. Raccoon carcasses retrieved from Berlin were immediately examined macroscopically at the Berlin-Brandenburg State Laboratory.

Histo-pathology examinations

A full necropsy was performed on all raccoons. For histology, tissue samples of brain, tongue, tonsils, thyroids, submandibular and mesenteric lymph nodes, lung, heart, liver, stomach, intestine, kidney, urinary bladder, adrenal glands, and reproductive organs were retrieved from 148 (MNP: 100, Berlin: 48) raccoon necropsies including 5 raccoons belonging to the CDV outbreak, whereas tissue samples of 92 Berlin raccoons collected during the CDV outbreak (outbreak group) in winter 2012/2013 [22] were limited to central nervous system (CNS), lung, liver, spleen and kidney . Tissue samples were fixed in 4% formalin, processed routinely and embedded in paraffin. Sections of 4µm were cut, stained with haematoxylin-eosin (HE) and examined by light microscopy. Depending on histological findings, Azan, Giemsa, Sudan red, or silver staining was additionally used when necessary. To investigate the ultrastructure of specific cells transmission electron microscopy was performed. Formalin fixed tissue samples from lymph nodes were transferred in 3% glutaraldehyde solution (w/v). After a washing step in phosphate buffered saline solution (pH 7.2) samples were fixed again in 2% osmium tetroxide, dehydrated via increasing ethanol concentrations and samples were embedded in EPON 812. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a Tecnai G2 electron microscope (120 kV; Nijmegen, Netherlands).

Parasitology examinations

When possible, ectoparasites were collected and classified by their taxonomic order following Wall and Shearer [28]; no species differentiation was made except for mites. Mite species were identified bytheir characteristic morphology according to Fain [29]. Investigations on endoparasites were mainly focused on the zoonotic nematodes *B. procyonis* and *Trichinella* spp. During necropsy, intestines were dissected and

carefully searched for adult *B. procynis* and other helminths. A broad classification of helminths in nematodes, trematodes and cestodes in the intestines followed Castro [30]; *B. procyonis* identification followed the morphological description of Sprent [31]. For *Trichinella* spp. analyses, the diaphragms and skeletal muscles from the lower extremities were stored at -20°C and were later submitted to the National Reference Laboratory for *Trichinella*, Federal Institute for Risk Assessment, Berlin, Germany. Muscle samples were analysed by artificial digestion using the magnetic stirrer method following regulation (EC) No. 2075/2005 [32].

Virology examinations

For virology screening, five viral agents previously reported in raccoons were selected: lyssavirus (rabies), canine distemper virus (CDV), parvovirus (PV), canine adenovirus 1 (CAV-1), and suid herpesvirus 1 (SuHV-1) [4, 21, 33, 34]. Tissue samples of thirty raccoons from MNP and thirty animals from Berlin were selected based on sufficient tissue preservation determined by histological examination. The integrity of these samples was also tested by amplifying a house keeping gene (beta-actin) (Table 1S additional files). From each animal small pieces of cerebrum, cerebellum, liver, spleen, intestines, tonsils and lymph nodes were frozen at -80°C. For molecular investigations these samples were pooled as follows: lymph nodes and tonsils, liver and kidney, lung and spleen, cerebrum and cerebellum, small and large intestine. DNA and RNA were extracted from these sample pools using the RNeasy Kit (Qiagen GmbH, Germany) following manufacturer's instructions. For lyssavirus, only the CNS was tested. Brain and lung samples from the outbreak group (n=97) were tested for CDV [22]. For lyssavirus, the reverse transcription nested-polymerase chain reaction (RT-PCR) assays followed instructions by Vázquez-Morón et al. [35]. Canine distemper virus RT-PCR was conducted as described by Becher et al. [36] with two sets of primers that target a conserved region of the N-gene. Conventional nested PCR assays were performed for the DNA viruses: PV, CAV-1 and SuHV-1. Every set of PCR used 50µl of reaction including 0.5 µl of each primer (Table 1S additional filesl) and 2.5µl of extracted DNA. Primer sequences are shown in Table 1. PCR products were analysed in 1.5% agarose gel and checked for bands of the respective amplicon size.

Bacteriology examinations

Kidney samples of the 60 animals selected for virological screening including five raccoons with histologic evidence of interstitial nephritis as well as five additional animals with interstitial nephritis were used for screening for *Leptospira* spp. (MNP: 31, Berlin: 34). DNA was extracted from these kidneys using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. For identification of the genus *Leptospira*, *Lipl32*, a highly conserved gene in all pathogenic *Leptospira* strains was targeted. The *Lipl*32 PCR protocol was performed according to Mayer-Scholl et al. [37]. Positive samples were consequently submitted for species identification through sequencing of a 254 bp segment of the *secY* gene following Victoria et al. [38]. The sequence type of each isolate was obtained using the multilocus sequence typing (MLST) scheme adapted from Thaipadungpanit et al. [39].

Further bacteriological investigations were restricted to organs with macroscopic lesions suspicious for bacterial infection. Respective tissue samples were cultured on Columbia agar with 5% sheep blood, Gassner agar, chocolate agar and/or Brilliance UTI clarity agar (all Oxoid Deutschland GmbH, Wesel, Germany) under aerobic, capnophilic (5% CO_2) or and anaerobic conditions. Dependent on the results of the Gram stain as well as the catalase and oxidase test (Becton Dickinson GmbH, Heidelberg, Germany), biochemical identification of the bacteria was performed by traditional biochemical analysis[40] or by using the respective API® Identification System (BioMérieux Deutschland GmbH, Nürtingen, Germany). For culture isolation of *Yersinia* sp. samples were directly streaked out on CIN agar and additionally, cold enrichment was performed (1ml Phosphate buffered saline (PBS), 4° C, 28 days). Colonies with suspicious morphology were biochemically identified via API 20E System (BioMérieux Deutschland GmbH, Nürtingen, Germany).

Clostridium perfringens toxins were identified by a multiplex PCR on *C. perfringens* toxins alpha, beta, epsilon, iota and enterotoxin was performed using the protocol by Meer and Songer [41].

Results

Of the 240 raccoons examined in this study, 130 (54.1%) were male (MNP: 56, Berlin: 74), 102 (42.5%) were female (MNP: 44, Berlin: 58), while in 8 cases (3.3%) the sex could not been determined due to severe body destruction (road kills). Adults comprised 67.9% (MNP: 51, Berlin: 112) of the total, 28.3% (MNP: 46, Berlin: 22) were juveniles; neonates and sub-adults represented 0.4% (MNP: 1, Berlin: 0) and 1.2% (MNP: 2, Berlin: 1) respectively, in five animals age was not determined due to severe body destruction (road kills). The collected animals were hunted (MNP: 52, Berlin: 47), road-killed (MNP: 46, Berlin: 43), found dead (MNP: 1, Berlin: 38) or euthanized (MNP: 1, Berlin: 12).

Macroscopic findings

During necropsy in all animalsa peculiar, but for raccoons physiologic, finding was a characteristically firm texture of the liver, which after exenteration prevented the liver to flatten but instead to keep a rather bulged shape instead (Figure 1A). The pancreatic tissue was of similar texture and cross-sectioning of the pancreas' lobes showed a firm almost triangular cut surface.

Traumatic injuries associated with the cause of death were found in 76 out of the 148 animals necropsied. In these raccoons, severe haemorrhages and tissue destruction caused by gunshots were detectedas well as lesions due to road kills like skin lacerations or abrasions, closed or open fractures, diaphragmatic hernia with thoracic displacement of the stomach, or rupture of the abdominal wall with evisceration of liver and intestines.

Besides traumatic injuries, other macroscopic changes were found in 32% (n=48) of the 148 animals necropsied. In these animals, a main gross finding unrelated to trauma was multifocal capsular fibrosis of the liver (MNP: 32/100, Berlin: 10/48) (Figure 1A). Five raccoons (MNP: 1/100, Berlin: 4/48) collected between late autumn and late winter presented yellowish coloration of the liver consistent with fatty degeneration. Other macroscopic lesions detected were mild to moderate follicular hyperplasia of spleen or lymph nodes (MNP= 3/1010, Berlin: 5/48), large renal medullary cysts (MNP: 1/100, Berlin: 1/48), and left kidney hypoplasia (Berlin: 1/48). Finally, multifocal small white abscesses in the liver (MNP: 1/100), haemorrhagic enteritis (Berlin: 1/48), and purulent vaginitis (MNP: 1/100) were also found in these animals.

Histo-pathological findings and related aetiologies

In total, organs of 224 out of 240 animals (MNP: 84, Berlin: 140) were histologically examined (Table 2S additional files). Organ samples of 16 MNP animals were not considered due to severe autolysis of the carcasses (n=15) or complete organ destruction in one road-killed animal.

As a physiologic finding all livers exhibited distinct fibrous connective tissue scaffolding (Figure 1B) which separating the hepatic lobules and which is responsible for the bulged macroscopic appearance of the organ. Similar connective tissue scaffolding was found in the pancreas.

Overall, histo-pathological changes were found in 84.8% (n=190) of the 224 raccoons and are summarized in table 2S, additional files. Inflammatory lesions mostly of mild degree, occurred in one or more organs in 147 (65.6%) of the raccoons. In general, the predominant cell type were eosinophilic granulocytes, while surprisingly sparse numbers of neutrophils were found. Because of the brightly stained cytoplasm of neutrophils and the unexpected high number of eosinophils in all organs, electron microscopy was used to confirm that eosinophils were not accidentally confused with neutrophilic granulocytes. The typical ultrastructure morphology of the granules verified these cells as eosinophils [42]. Mild to moderate follicular hyperplasia of the spleen was found in 112 raccoons (MNP: 44, Berlin: 68), of the lymph nodes in 26 animals (MNP: 17, Berlin: 9) and of the tonsils in 13 individuals (MNP: 7, Berlin: 6). Furthermore in 97 of the animals with follicular hyperplasia, concurrent inflammatory lesions in one (MNP: 15, Berlin; 49) or more (MNP: 26, Berlin: 11) organs were also detected. In 68 of the 224 raccoons histopathological changes were suggestive of or could be attributed to a specific aetiology, these findings are summarized below..

The most common non-inflammatory findings were seasonal hepatic fatty degeneration (MNP: 10/84, Berlin: 26/140) confirmed by positive Sudan red staining (Figure 1C), and multifocal mild anthracosis in the lung (MNP: 3/84, Berlin: 23/140).

Histopathology findings with parasitic aetiology

Histo-pathologic lesions caused by endoparasites were a major finding and were detected in 68 out of 224 cases, which comprised 48/84 of the MNP raccoons and 20/140 of Berlin raccoons. In general, parasite related histo-pathological changes most often included granulomatous inflammation with occasional cross-sections of nematode larvae (Table 2S, additional files). Severe nematode infection and larval migration tracts in more than four organs were recorded in four MNP raccoons.

Lymph nodes of 53/132 raccoons (MNP: 40/84, Berlin: 13/48) were often severely infiltrated by eosinophilic granulocytes, either generalized or restricted to the subcapsular space or the eosinophils demarcated focal granulomas (MNP: 20/84, Berlin: 2/48). The granulomas were mostly localized adjacent to the capsule or within the cortex close to the capsule. In the liver confluent tracts of hepatocellular necrosis caused by nematode larval migration were recorded in 32 animals (MNP: 20, Berlin12) (Figure 2a, b). These lesions were sometimes associated with mild or moderate periportal infiltration by mononuclear cells and eosinophils. Additionally, irregular distributed hepatic granulomatous lesions were found in 14 raccoons (MNP: 12, Berlin: 2). Generalized mild to severe infiltrations by eosinophilic granulocytes were found in the lamina propia of the stomach (MNP: 9, Berlin: 8) and of the intestines (MNP: 45, Berlin: 35). Many of these were accompanied by mild to moderate multifocal granulomatous lesions with sporadic cross-section of nematode larvae (stomach, MNP: 15, Berlin: 1; intestines, MNP: 14, Berlin: 3) (Figure 2C) (Table 2S, additional files). One raccoon from MNP had trematode larvae in its intestinal crypts (Figure 2d).

Within the tongues had encysted mesocercariae were detected in 36 MNP raccoons and in one Berlin raccoons (Figure 2E). Recently, the encysted trematode stage was identified as *Alaria alata*. The microscopic description and the results of these investigations were published separately [43].

Lungs and kidneys were less affected by endoparasitic infection. Nematode larvae were noted within alveolar spaces in one Berlin animal (Figure 2F) and in lung of one MNP raccoon intravascular nematode larvae were found. None of the animals had parasites in kidneys or urinary bladder.

Of all 240 animals, histologic examinations of the skin revealed mild to severe parakeratotic or orthokeratotic hyperkeratosis with moderate to severe suppurative dermatitis associated with numerous intracorneal mites in four Berlin animals (Figure 3C, D). Isolated mites isolated presented the characteristic morphology of *Sarcoptes scabiei* (Figure 3B). Detailed pathologic findings and performed genetic investigations of two of these raccoons were published elsewhere [44].

Histopathology findings with viral aetiology

Exclusively in raccoons from Berlin the major findings were detected in lung and brain tissue and were associated with a regional CDV outbreak. In 58 Berlin raccoons moderate to severe non-suppurative broncho-interstitial pneumonia was found. Intracytoplasmic and intranuclear inclusion bodies were detected mostly in epithelial cells of the respiratory tract as well as in syncytial cell formation of pneumocytes. Immunohistochemistry confirmed CDV antigen in 60 lung samples [22]. Nineteen raccoons from Berlin presented sparse intracytoplasmic and intranuclear inclusion bodies in neurons, astrocytes, and granular cells of the cerebellum, and mild to moderate perivascular mononuclear cell cuffing was detected in seven animals. A single raccoon had mild non-suppurative leptomeningitis. Immunohistochemistry assays for CDV antigen were positive in all cases. A detailed description of the microscopic and molecular investigations of these raccoons was recently published [22].

Histopathology findings with bacterial aetiology

A limited number of raccoons had pathological changes indicating a bacterial infection. In 22 (MNP: 8/84, Berlin: 14/140) out of 224 raccoons multifocal mild non-suppurative interstitial nephritis was recorded (Figure 4A). Silver staining (Warthin-Starry) revealed numerous long slender spiral-shaped bacteria within the renal tubules as well as the tubules' lining epithelial cells in four out of these 22 kidneys (Figure 4B) The kidneys of one raccoon from Berlin had severe multifocal interstitial infiltration with predominantly eosinophilic granulocytes. Multiple small abscesses were found in the liver of a raccoon from MNP, *Yersinia enterocolitica* was isolated in pure culture from this tissue. In another raccoon from Berlin multifocal necrotizing hepatitis demarcated by suppurative inflammation reaction was noted. From this

liver *Salmonella enterica* ssp. *enterica* serovar Typhimurium was isolated. In one further raccoon from Berlin severe haemorrhagic enteritis with loss of villous lining enterocytes was found which was associated with *Clostridium perfringens* Type A. Lastly, an adult female raccoon from MNP had extensive neutrophilic inflammation of the vaginal mucosa and *Streptococcus canis* was isolated from this organ in pure culture.

Results of screening for selected pathogens

Ticks (n=20), mallophages (n=4) and fleas (n=3) were rarely found on the raccoon carcasses. During necropsy. However, mild to severe infestation of cestodes was found in the small intestine of 20 animals. But as most of the endoparasites were either in advanced decomposition or some body parts were missing, a morphologic identification of the species was not performed. In one raccoon the gall bladder contained numerous trematodes, which were identified as *Metorchis* spp. none of the intestines of any animal examined (n=240) contained adult *Baylisascaris procyonis* nematodes.Tissue samples of diaphragms and skeletal muscle of the lower extremities submitted for *Trichinella* examination proved negative for this parasite (n=240).

Before the screening of 30 MNP and 30 Berlin raccoons for selected viruses the DNA quality of the respective organ pools was assessed and only 24 out of 30 initially selected animals from MNP were found suitable for RT-PCR/PCR assays to test for rabies virus, CAV-1, SuHV-1 and CDV. In contrast, after assessing the DNA quality of 30 intestine pools from MNP only ten raccoons were considered useful for PV PCR. All samples (24 MNP, 30 Berlin) were negative for rabies virus, for CAV-1 and for SuHV-1, while three animals from Berlin, two adult road killed males and one shot juvenile female, were positive for PV. The juvenile had haemorrhagic enteritis from where, as mentioned above, also *C. perfringens* was isolated, while the two males had no obvious enteric lesions. Ninety-two raccoons from Berlin were positive for CDV; a detailed description of some of the CDV cases was already published [22].

Sixty-five raccoons (MNP: 31/84, Berlin: 34/140) were tested for *Leptospira* spp., eight of these were positive. From these eight raccoons, seven originated from Berlin, one animal was from MNP. *Leptospira* species identification by *secY* sequencing was possible in five of the 8 raccoons, confirming *L. interrogans* in these cases. Four animals from Berlin carried sequence type 17, which corresponds to the serogroup Icterohaemorrhagiae while the only positive raccoon from MNP was infected with sequence type 24 (serogroup Australis) (Table 3). Interestingly, the kidneys of three Berlin raccoons and the one MNP raccoon positive for *L. interrogans* revealed spiral-shaped bacteria in their corresponding histology sections, while the remaining four *Leptospira* spp./*L. interrogans* positive animals were histologically negative. Moreover, one of the Berlin raccoons PCR positive for *L. interrogans* had no interstitial nephritis.

Discussion

We investigated the occurrence of sub- /clinical infectious diseases and selected pathogens in free-ranging raccoons from north-eastern Germany combining histo-pathology, parasitology, virology and bacteriology. The results of this study allow insight in the diseases and pathogen burden of these rural/sylvatic and urban raccoon populations.

Because most ectoparasites dismount their host soon after its death, the low number of recorded ticks or fleas is considered not to represent true infection rates. However, the mite *Sarcoptes scabei* dwells in the upper layers of the epidermis and does not only cause distinct pathologic changes but remains on its host for some time. *Sarcoptes scabiei* has been reported in raccoons only once in the USA [45]. But *S. scabiei* infections might be underreported in the literature since *Sarcoptes* outbreaks are known to occur in a broad range of other wildlife species in the USA or in Europe [46, 47]. In our study only urban animals were infected with this parasite. Previous microsatellite investigations on mites isolated from these raccoons showed that raccoon-derived mites are closely related to fox-derived *S. scabiei* (carnivore host) and differentiated from chamois (herbivore host) and wild boars (omnivorous host) [36]. In Berlin prevalences in red foxes infected with *S. scabiei* range between 10% and 25% [48], supporting the hypothesis that foxes could be the source of the infection.

In contrast, mesocercariae of the trematode *A.a alata* were almost exclusively found in raccoons from MNP, where the parasite's intermediate hosts, tadpoles and amphibians, exists abundantly. Urban raccoons

in Berlin, despite the many streams, ponds and rivers, possibly neglect these intermediate hosts in their diet while other food sources, e.g. of anthropogenic origin, are freely available. Likewise, the single raccoon containing *Metorchis* sp. in its gallbladder lived in MNP. So far *Metorchis* spp. has not been described in raccoons in Europe, but they were detected in red foxes from Berlin [49]. Slight differences were found between MNP and Berlin raccoon populations regarding the occurrence of endoparasites in the gastro-intestinal tract or larval migration within liver or lymph nodes with relatively more animals affected in MNP. But most importantly, the zoonotic nematodes *B. procyonis* and *Trichinella* spp. were absent from all investigated raccoons, confirming investigations from 1995, where the search for *B. procyonis* in raccoons from north-eastern Germany was also negative [19]. Since the raccoon populations in Germany originate from two separate introductions, with high B procyonis prevalence in the western part [16, 17],it appears that the founders of the north-eastern population were free from *B. procyonis* and equally their offspring seem to remain free. Frantz et al. [7] investigated the genetic haplotypes of raccoons in Germany and found that both MNP and Berlin populations have the same haplotype (PLO2b) indicating the same founder group in contrast to animals from the central part of Germany. We had expected that over time the expanding raccoons populations (centre and North-east) would have started to overlap at their margins and subsequently transmit and share *B. procyonis* between the two populations. Whether this might have occurred in geographic regions more distant to our study area is unknown.

There are various reports on raccoons positive for *Trichinella* spp. in the United States of America [50, 51] as well as the Japanese raccoon population [52]. In Germany sporadic human infections with *Trichinella* sp. occur through the consumption of undercooked infected wild boar. The prevalence of the parasite in German wildlife species ranges from 0.009% in wild boar, 0.08-0.22% in foxes and 5% in raccoon dogs [53]. The negative result on German raccoons could be due to this low prevalence and the number of investigated raccoons might have been too small to detect an animal infected with *Trichinella* sp. But as scavenging behaviour has been described for raccoons in North America [54] it can also be assumed for raccoons in Germany along with potential transmission of *Trichinella* spp.

During the course of this study, a large canine distemper outbreak occurred amongst Berlin wildlife carnivores and 76 out of 97 investigated raccoons were confirmed positive for CDV by PCR and immunohistochemistry. The raccoon population in Berlin is estimated to comprise about 1000 individuals and the number of raccoons found succumbed to CDV indicate a marked impact on the population. Canine distemper is endemic in North American raccoons with outbreaks commonly occurring both in rural and urban populations [5, 55]. In Germany, a small localised CDV outbreak in sylvatic raccoons from MNP occurred between April and July 2007 [20, 21]. A study on 206 raccoons collected between 2011 and 2013 from rural areas in North-western Germany, Federal State of Lower Saxony, found 30 raccoons positive for CDV by PCR assays [18]. Reduced home ranges and more frequent contact between raccoons and other carnivore species like red foxes or domestic dogs in the urban setting of Berlin might have facilitated the pathogen's transmission to the raccoons. The virus strain isolated from the outbreak in this study belongs to the "Europe" lineage of CDV commonly found in domestic dogs or red foxes [22]. As CDV is known to cycle amongst Berlin red foxes with recent peaks in 2008 and 2011[48] they seem a likely source of infection. Müritz National Park raccoons were spared from the 2012/2013 outbreak. Moreover, the sylvatic CDV outbreak in 2007 in MNP was caused by a CDV strain of the "Europe Wildlife" lineage and was limited to raccoons while no other carnivore species was detected to be affected [21]. It seems noteworthy that in contrast to red foxes CDV-related histo-pathologic changes in raccoons were mostly found in the lung and not the brain. Similarly, molecular detection of CDV was also more often successful from the lungs. Whether this depends on the particular CDV strain or the genetic background of raccoon population living in Germany is not clear, but a similar disease pattern was found in the 2007 outbreak in MNP [21] making the influence of the CDV strain unlikely. Because diseased raccoons could likewise act as vectors for CDV pet owners sharing their premises with wildlife carnivores should be adviced to vaccinate their dogs.

Besides the CDV one further viral pathogen was found in Berlin raccoons. Parvovirus was isolated from three raccoons and while this was associated with haemorrhagic enteritis in the juvenile animal, no obvious enteric lesions were found in the two adult raccoons, but early decomposition might have

rendered these tissues. Experimental infections showed that raccoons are susceptible to feline panleukopenia virus (FPV) and mink enteritis virus [33], and strains of CPV-2-like virus have been isolated from a raccoon with clinical signs of disease [56]. Recent phylogenetic investigations on parvovirus strains from various animal species from North America revealed that currently raccoons harbour parvoviruses, which are in phylogenetic intermediate location to canine parvovirus 2 (CPV) and CPV-2a [57], while parvoviruses isolated from raccoons more than 20 years ago clustered with FPV. It is assumed that raccoons played an important role for the transition from CPV-2 to CPV-2a, -2b and -2c. At the same time there are phylogenetic subclusters of CPV-like raccoon viruses that are sustained and circulate amongst raccoons [57]. Unfortunately, the phylogenetic relation of parvoviruses detected in the German raccoons remains open, as they could not be characterized further due to poor tissue preservation. Therefore, the epidemiologic potential of these viruses, i.e. source of infection and possible further transmission, likewise remains elusive. Serologic investigations on antibodies against parvovirus in raccoons in North America showed 22.3% positives in 112 free-ranging raccoons from southern Ontario, Canada, in 1983 [33] and 49.7% from 159 raccoons from a high density raccoon population inhabiting a large zoological park in Missouri, USA, in 2007 [58]. For raccoons in Europe nothing is known in regard to parvoviruses and respective antibody prevalences.

Rabies, canine contagious hepatitis and Aujeszky's disease were not found in any of the investigated raccoons. In the USA raccoons are the main wildlife reservoir for terrestrial rabies. In 2013, 1898 rabies positive raccoons were reported representing 32% of all rabid wildlife cases equating 92% of the total rabid animals in that year [4]. The raccoon rabies virus variant is distinct and belongsto one of the endemic bat lineages, in contrast to the six canine rabies lineages, and it was the main reported rabies variant in the USA for 2013 [4]. Knowledge on canine contagious hepatitis and Aujeszky's disease in USA raccoons is limited. Serologic investigations on canine contagious hepatitis in a high density urban raccoon population found 7% prevalence amongst 159 individuals and positive cases showed a significant association with antibodies against CDV [58]. Another serology study found 17% out of 479 raccoons carrying antibodies against Aujeszky's disease. However, during this time a farm with Aujeszky's disease amongst swine was located close to the study area and as raccoons were seen to feed on garbage as well as swine carcasses it remained uncertain whether raccoons serve as a potential reservoir species or a dead end host [59].

Raccoons from USA and Canada carry *Leptospira* spp. and several investigations found either antibody titer against these pathogens or detected leptospiral organisms in the raccoons' kidneys [58-60]. For Germany, a study on rural raccoons from the Federal State of Lower Saxony reported six (1.3%) out of 457 rural raccoons PCR-positive for *Leptospira* spp. [18]. In our study seven of the eight raccoons positive for pathogenic *Leptospira* spp. originated from Berlin and only one animal lived in MNP. As urban environments are easily exposed to urine from rats, a major reservoir for some pathogenic *Leptospira* [61], this could explain such marked difference. However, an extensive study on up to 3000 rodents from Germany found leptospiral DNA in 10% of all examined animals with *L. kirschneri*, *L. interrogans* and *L. borgpetersenii* as the most common *Leptospira* spp., but without significant differences between rural and urban areas. Besides rodents also wild boar can carry *Leptospira* spp. and investigations on 141 wild boars from Berlin found 18% prevalence of *Leptospira* spp. in these animals [62]. A case report on human leptospirosis in Berlin with wild boar as the suspected source of infection [63] points out another possible cross-species transmission of *Leptospira* spp. also for raccoons. Infected wild boar may contaminate water and water holes with urine during their wallowing behaviour. While 5000 wild boars are estimated to roam within Berlin city limits [63] and raccoons favour habitats with freshwater an overlap of territories with contact zones seems rather likely. The close proximity of urban raccoons to the dwelling of wild boar and rodents as well as to anthropogenic food sources is likely to increase the chance for new interspecies transmission pathways in either direction. And at least one case of human leptospirosis associated with raccoon urine is reported from North America [64]. Shedding of *Leptospira* spp. via urine is likely to occur in animals with evidence of interstitial nephritis associated with leptospiral organisms within the renal tubules. In our study raccoons revealed interstitial nephritis alongside with microscopic evidence of abundant bacterial colonisation, although they only comprised a subset of the

total number of PCR positive animals. Similar histological findings are reported on raccoons from Quebec [65].
Single cases of bacterial infections (*S. enterica* ssp. *enterica* serovar Typhimurium, *C. perfringens*, *Y. enterocolitica*, *S. canis*) were found in four raccoons. Amongst other serovars, *Salmonella enterica* ssp. *enterica* serovar Typhimurium was described in raccoons from North America and Japan [66, 67]. Raccoons are likely to acquire salmonellae from their direct surroundings and investigations on *Salmonella* serotypes revealed that seven serotypes isolated from raccoons matched *Salmonella* serotypes isolated from humans [66]. The animal infected with *Salmonella* in this study originated from the city of Berlin with abundant anthropogenic sources. In contrast to most studies on enterogenic bacteria examining fecal swabs from apparently healthy live-trapped animals, *S. enterica* in this study was isolated from liver necroses but not the intestines. *Yersinia enterocolitica* was also isolated from abscesses in the liver of a MNP raccoon. Only a few reports on raccoons infected by *Yersinia* spp. exist in the literature describing similar hepatic lesions caused by *Y. enterocolitica* [68]. Investigations on bacterial pathogens in faecal samples from feral raccoons in Japan found pathogenic *Y. pseudotuberculosis* in seven (1,5%) out of 459 raccoons [67]. *Y. enterocolitica* occurs ubiquitously in the environment as well as in freshwater bodies and is known to infect a wide variety of animals worldwide [69]. In contrast to the bacterial infections described above *Clostridium perfringens* were isolated from a raccoon with concurrent parvovirus infection. Although it was not possible to determine which of these two pathogens was responsible for the haemorrhagic enteritis of the raccoon, it could be possible that *C. perfringens* followed an initial impact of parvovirus infection. Like parvovirus *C. perfringens* is well-known to cause haemorrhagic enteritis in carnivore species [69]. *Streptococcus canis* belongs to the physiologic microflora of the muscous membranes of dogs and cats [70, 71], but under certain circumstances *S. canis* can cause opportunistic e.g. reproductive tract infections even in humans [72]. All these four bacterial infections are considered coincidental and, except for *S. enterica*, regarded as independent from the respective habitat background. However, some of these bacteria like *Leptospira* spp. are of importance for public health particularly at the interface of human-wildlife contact zones. Wildlife ecology studies on invasive animal species and interdisciplinary approaches are crucial to find ways for a trouble-free coexistence of e.g. raccoons and humans.

Conclusions

We investigated raccoon populations from a rural/sylvatic and an urban habitat in Germany to understand which pathogens and sub-/clinical diseases can be expected in this non-native animal species. A number of pathogens were found known to commonly occur in the raccoons' North American conspecifics: CDV, PV, *Leptospira* spp., *S. enterica* serovars, *Y. enterocolitica*, *S. scabei* and *Metorchis* sp. Other infectious agents were newly described like *A. alata* or *S. canis*. Some pathogens seem to prevail one habitat form, e.g. *A. alata* infecting almost exclusively raccoons from the sylvatic MNP, while infection with *Leptospira* spp. were distinctly connected to the urban habitat in Berlin.
Despite being an alien species, raccoons settled well into the different habitat types of Germany comprising a similar range of diseases as in their native North America. However, their role as reservoir hosts for certain pathogens seems currently of lesser importance as compared to North America. Whether this will change while raccoon population numbers keep increasing in Germany or whether native wildlife species like red foxes or wild boar will sustain their apparent leading role as reservoir host for certain pathogens will be seen. However, the intrusion of raccoons into human settlements should lead to some precautionary measures to avoid a transmission of zoonotic pathogens. Interdisciplinary work between wildlife ecologists and veterinarians will help to find smart solutions for both wildlife and human perspectives.

Authors' contributions

Designed and conceived the study: GW, F-UM. Performed post-mortem examinations: ZR-S, AA, UW, GW. Performed pathological analyses: ZR-S. Developed and performed bacteriological analyses: MG. Developed and supervised virology analyses: MK, CF. Performed virology analyses: ZR-S, CF. Developed and performed *Trichinella* and *Leptospira* examinations: KN, AMS. Analysed the data and wrote the paper: ZR-S, GW. All authors read and approved the final version

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Figures

Figure 1.

A) Gross appearance of a raccoon liver with domed shape due to fibrous connective tissue scaffolding.
B) Microscopic image of a normal raccoon liver with perilobular fibrous connective tissue, Azan staining.
C) Microscopic image of a raccoon liver with fatty degeneration, Sudan red staining.

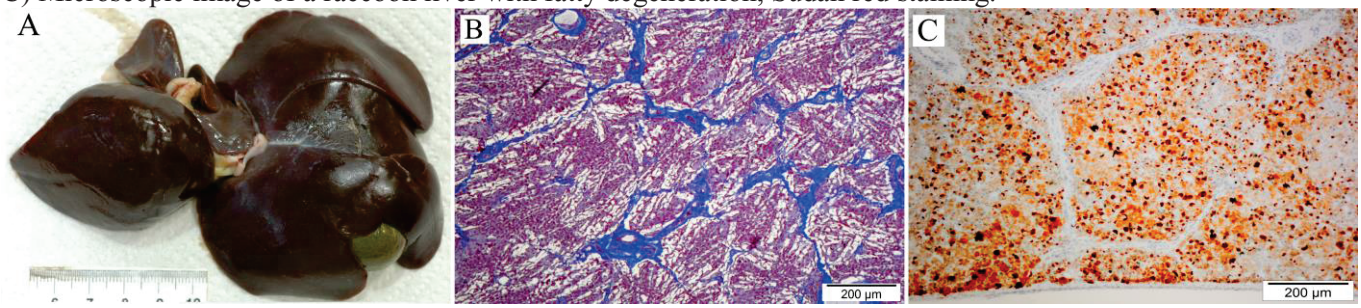


Figure 2

Microscopic images of parasitic lesions in raccoons: A) Liver: Acute hepatic necrosis and haemorrhages caused by larval migration, HE staining. B) Liver: Small pyogranuloma with an intralesional nematode larvae. C) Stomach: Chronic granuloma with intralesional nematode larvae in submucosa. D) Small intestine: Intraluminal nematode larvae of intestinal crypts. E) Tongue: Encysted *Alaria alata* mesocercariae. F) Lung: Intra-alveolar nematodes. All images: HE staining.

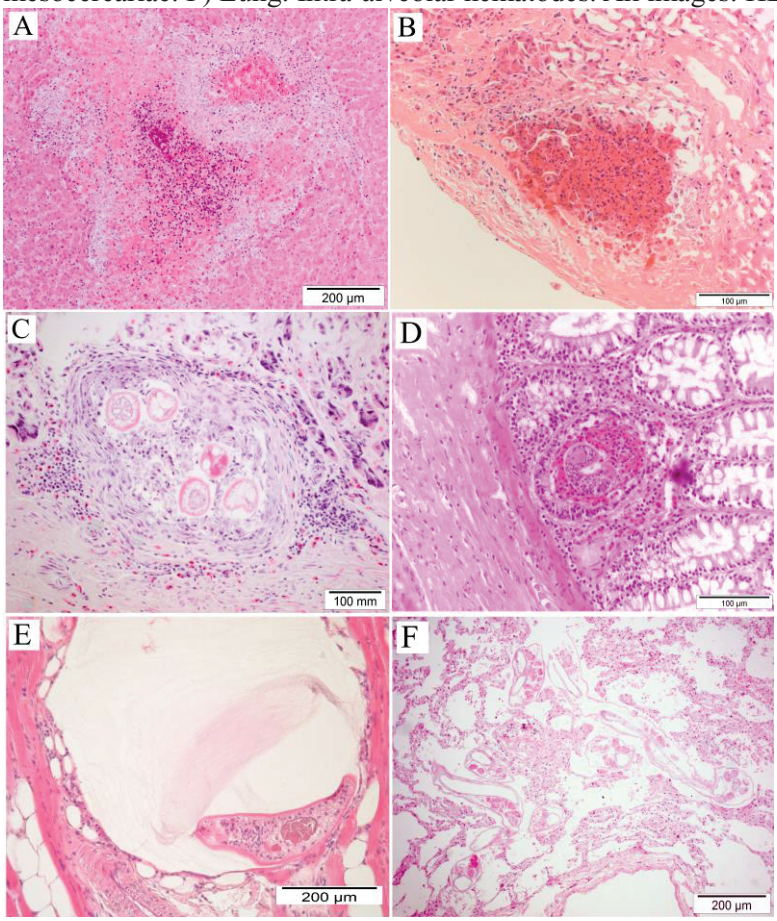


Figure 3
Images of *Sarcoptes scabiei* mange in raccoons: A) Gross lesions in a male raccoon. B) Scanning electron microscopy: Dorsal view of an adult female *Sarcoptes scabiei* mite. C) Microscopic image of a raccoon's skin infected with *S. scabiei*: Severe ortho- and parakeratotic hyperkeratosis, mild to moderate inflammation and intracorneal mites, HE staining. D) Close-up of *S. scabiei* from C), HE staining.

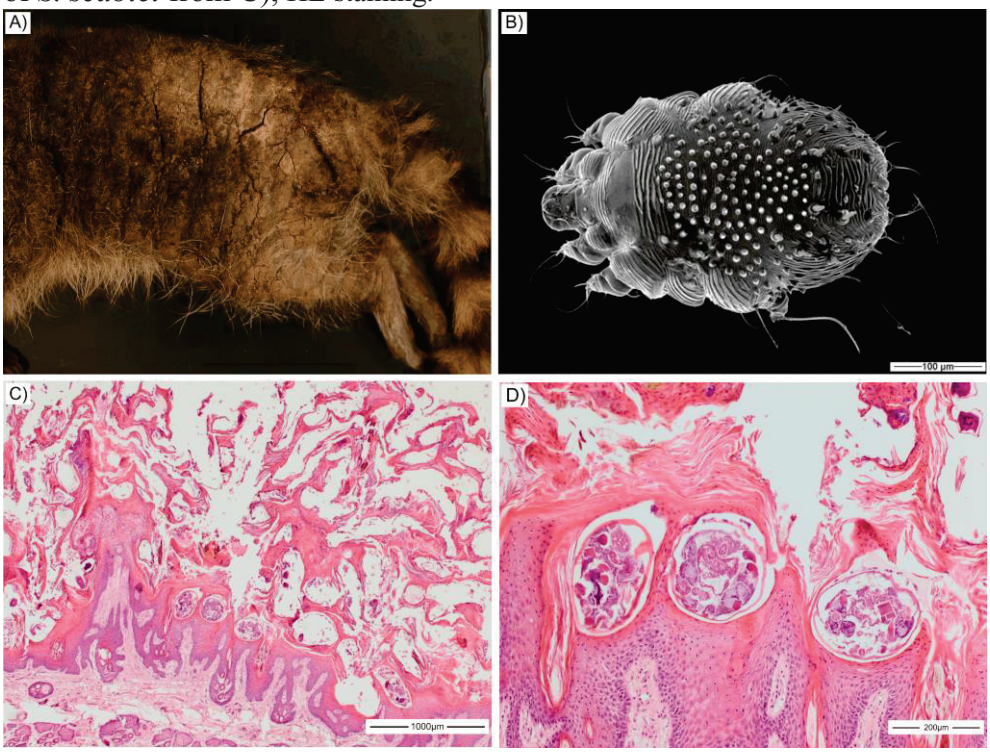
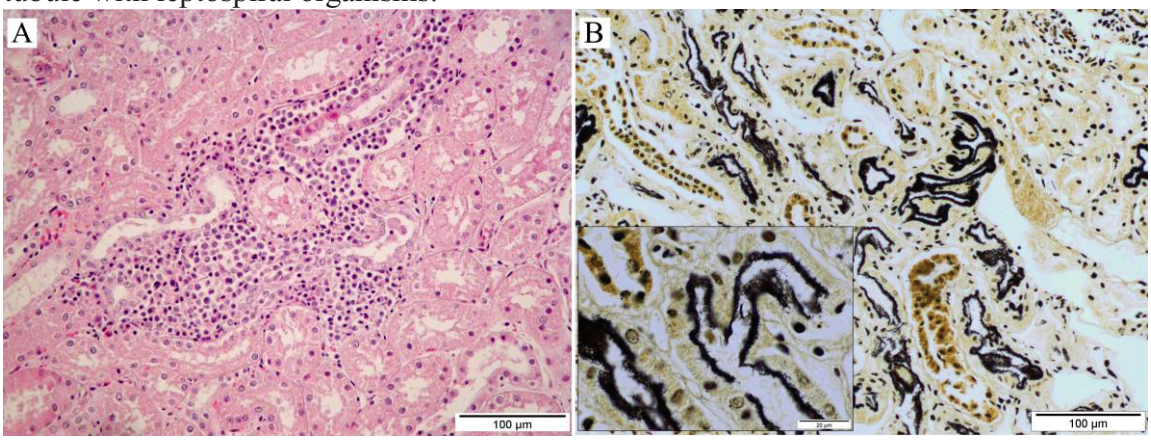


Figure 4
Microscopic images of renal lesions in raccoons: A) Focal non-suppurative interstitial nephritis intermixed with a few eosinophils, HE staining. B) Multiple renal tubules with extensive intraluminal spiral-shaped bacteria consistent with *Leptospira* sp., Warthin-Starry staining. Inset: Close-up of a renal tubule with leptospiral organisms.



Tables

Table 1 - Primer sequences and resulting amplicon sizes (=base pairs). CDV: canine distemper virus, PV: parvovirus, CAV-1: canine adenovirus-1, SuHV-1: suid herpersvirus-1.

Virus	Primer	Sequence	Amplicon size
CDV	CDV-N6-F	CGACTCGGAGATGAGAAGGTG	427
	CDV-N7-R	CAATGGGTAGGACCCTGCAC	
	CDV-N8-F	GATCCCCAGGGAACAAGCC	149
	CDV-N9-R	TCCGGAAAACTCATGCAACC	
PV	PV_VP2 1	GAAAACGGATGGGTGGAAATC	709
	PV_VP2 4	AGAAATGGTGGTAAGCCCAATG	207
	PV_VP2 5	ATACTGGAACTAGTGGCACACC	
CAV-1[59]	CAV-VP1	CTGGGCGGGATTTAGAGGGTGG	704
	CAV-VP2	CAAGGGCGTGGGCGGAGTTAGA	
	CAV-3	TTTGAGCCCATGTGCAGACA	121
	CAV-4	GCGGGTTAAAGGCCGAAA	
SuHV-1[60]	PRV_gB1	ATGGCCATCTCGCGGTGC	334
	PRV_gB2R	ACTCGCGGTCCTCCAGCA	
	PRV_gB3	ACGGCACGGGCGTGATC	195
	PRV_gB4R	GGTTCAGGGTACCCCGC	
Lyssavirus[29]	LV1F	AARATNGTRGARCAYCACAC	374
	LV1R	GCRTTSGANGARTAAGGAGA	
	LV2F	AARATGTGYGCIAAYTGGAG	260
	LV2R	TCYTGHCCIGGCTCRAACAT	
β-Actin	bAct_1-F	AAGGCCAACCGYGAGAAGATG	248
	bAct_2-R	TCCGTGAGGATCTTCATGAGG	

Table 2 - List of pathogens detected in this study and number of positive raccoons.

Pathogen	Positive raccoons			
	MNP	Berlin	Male/female	Juvenile/sub-adult/adult
Canine distemper virus	0/30	77/127	44/33	3/0/74
Parvovirus	0/30	3/30	2/1	1/0/2
Canine adenovirus-1	0/30	0/30	n.a.	n.a.
Suid Herpersvirus-1	0/30	0/30	n.a.	n.a.
Baylisascaris procyonis	0/100	0/140	n.a.	n.a.
Trichinella	0/100	0/140	n.a.	n.a.
Leptospira interrogans	1/31	4/34	2/3	3/0/2
Leptospira spp.	0/31	3/34	2/1	1/1/1
Sarcoptes scabiei	0/100	4/140	1/3	1/0/3
Alaria alata	9/100	1/140	6/4	3/0/7
Metorchis sp.				
Streptococcus canis	1/100	0/140	0/1	0/0/1
Clostridium perfringens Type A	0/100	1/140	0/1	0/0/1
Yersinia enterocolitica	1/100	0/140	1/0	0/0/1
Salmonella enterica ssp. enterica serovar Typhymurium	0/100	1/140[a]	1/0	1/0/0

[a]Concurrent distemper infection

Additional files

Additional file 1 –RT-PCR/PCR cycle conditions for screening selected pathogens in racoons.

RT-PCR/PCR	Reverse Transcription		PCR cycle (1st round, 40 cycles)			Semi-nested PCR (2nd round, 30 cycles)		
	Initial denaturation	Reverse transcription	Denaturation	Annealing	Extension	Denaturation	Annealing	Extension
Lyssavirus	3 min/ 94°C, 2 min/ 4°C	30 min/ 45°C, 80°C/2 min	30 sec/ 94°C	30 sec/ 55°C	30 sec/ 72°C	30 sec/ 94°C	30 sec/ 50°C	30 sec/ 72°C
CDV	3 min/ 94°C, 2 min/ 4°C	30 min/ 45°C, 80°C/2 min	30 sec/ 94°C	30 sec/ 56°C	30 sec/ 72°C	30 sec/ 94°C	30 sec/ 56°C	30 sec/ 72°C
SuHV-1	n.a.	n.a.	30 sec/ 94°C	30 sec/ 60°C	30 sec/ 72°C	30 sec/ 94°C	30 sec/ 65°C	30 sec/ 72°C
CAV	n.a.	n.a.	30 sec/ 94°C	30 sec/ 60°C	30 sec/ 72°C	30 sec/ 94°C	30 sec/ 60°C	30 sec/ 72°C
PV	n.a.	n.a.	30 sec/ 94°C	30 sec/ 60°C	30 sec/ 72°C	30 sec/ 94°C	30 sec/ 60°C	30 sec/ 72°C
β -actin	3 min/ 94°C, 2 min/ 4°C	30 min/ 45°C, 80°C/2 min	30 sec/ 94°C	30 sec/ 56°C	30 sec/ 72°C	n.a.	n.a.	n.a.

Abbreviations: CDV: canine distemper virus, PV: parvovirus, CAV-1: canine adenovirus-1, SuHV-1: suid herpersvirus-1. n.a.: no applicable.

Additional file 2 – Histo-pathological findings in raccoons from rural and urban populations in north-eastern Germany.

Table 2, supplementary material. Histo-pathological findings

Organ	Histo-pathological finding	Predominant cell infiltrate/s	Distribution	Degree (mild/moderate/severe)	Total (n)	MNP[b] (%)	Berlin[c] (%)
Liver							
	Inflammatory cellular infiltrates	Mononuclear, eosinophils	Periportal	39/27/7	73	51,2	21,4
			Intrasinusoidal	25/8/8	41	25,0	14,3
	Hepatocellular necrosis	Mononuclear, eosinophils	Focal/multifocal	22/4/6	32	23,8	8,6
	Granulomatous inflammation	Mononuclear, eosinophils, giant cells	Focal/multifocal	16/4/1	21	17,9	4,3
	Bile duct hyperplasia		Focal/multifocal	2/6/1	9	7,1	2,1
	Fatty degeneration		Diffuse	18/10/0	28	10,7	13,6
			Perilobular	1/5/2	8	1,2	5,0
	Microabsesses	Mononuclear, eosinophils	Focal/multifocal	2/2/0	4	2,4	1,4
	Necrotizing hepatitis	Mononuclear, neutrophils	Focal/multifocal	1/0/0	1	0,0	0,7
Stomach							
	Granulomatous inflammation associated with nematode larvae	Eosinophils, mononuclear, giant cells	Focal/multifocal	6/7/3	16	17,9	2,1
	Inflammatory celular infiltrates in lamina propia and submucosa	Eosinophils	Diffuse	10/6/1	17	10,7	16,7
		Mononuclear, eosinophils	Focal/multifocal	1/4/1	6	6,0	2,1
Intestines							
	Inflammatory cellular infiltrates in lamina propia	Eosinophils	Diffuse	35/40/5	80	53,6	72,9
			Focal/multifocal	0/2/1	3	2,4	2,1
		Mononuclear	Focal/multifocal	3/1/0	4	3,6	2,1
	Granulomatous inflammation associated with nematode larvae	Eosinophils, mononuclear, giant cells	Focal/multifocal	14/2/1	17	16,7	6,3
Tongue							
	Inflammatory cellular infiltrates, subepithelial	Mononuclear	Focal/multifocal	13/0/2	15	11,9	10,4
	Granulomatous inflammation associated with nematode larvae	Mononuclear, eosinophils, giant cells	Focal/multifocal	1/0/0	1	1,2	0
	Mesocercarial cyst/s (*A. alata*)	Mononuclear, eosinophils	Focal/multifocal	15/9/11	37	42,9	2,1

Organ	Histo-pathological finding	Predominant cell infiltrate/s	Distribution	Degree (mild/moderate/severe)	Total (n)	MNP[b] (%)	Berlin[c] (%)
Skin							
	Ortho- and parakeratotic hyperkeratosis with intracorneal mites	Mononuclear	Diffuse	1/0/3	4	0	2,8
Spleen							
	Follicular hyperplasia			56/59/6	121	53,6	54,3
	Inflammatory cellular infiltrates	Eosinophils	Diffuse	33/1/0	34	13,1	16,4
			Focal/Multifocal	2/1/0	3	3,6	0
Lymph nodes							
	Follicular hyperplasia			19/17/1	37	26,2	31,3
	Inflammatory cellular infiltrates	Eosinophils	Diffuse	22/24/8	54	40,5	41,7
			Focal/multifocal	3/7/18	28	25,0	14,6
			Perivascular	0/0/3	3	3,6	0
		Macrophages	Focal/multifocal	2/0/0	2	0	4,2
	Granulomatous inflammation associated with nematode larvae	Eosinophils, mononuclear, giant cells	Focal/multifocal	15/7/4	26	27,4	6,3
Tonsiles							
	Follicular hyperplasia		Diffuse	3/9/0	13	8,3	12,5
	Inflammatory cellular infiltrates	Eosinophils	Focal/multifocal	4/0/0	4	2,4	4,2
Lung							
	Non-suppurative interstitial pneumonia	Mononuclear, eosinophils	Diffuse	23/13/3	37	17,9	17,1
			Focal/multifocal	11/4/2	17	14,3	3,6
	Non-suppurative broncho-interstitial pneumonia with eosinophilic intracytoplasmic/intranuclear inclusion bodies in syncytial cells and epithelial cells	Mononuclear, eosinophils, pneumocyte type II proliferation, syncytial cell formation	Diffuse	24/23/11	58	0	41,4
	Suppurative interstitial pneumonia	Neutrophils, mononuclear, eosinophils	Focal/multifocal	2/1/1	4	1,2	2,1
			Diffuse	1/1/1	3	3,6	0
	Granulomatous pneumonia associated with nematode larvae	Mononuclear, eosinophils	Interstitial	1/1/0	2	1,2	0,7
	Inflammatory cellular infiltrates	Eosinophils, mononuclear	Diffuse	17/3/1	21	17,9	4,3
			Perivascular	3/2/2	7	6,0	1,4
			Peribronchial	4/3/0	7	4,8	2,1
	Anthracosis		Focal/multifocal	24/2/0	26	3,6	16,4
	Adult nematodes	Mononuclear[d]	Alveolar	0/1/0	1	0	0,7
	Nematode larvae	Eosinophils	Intravascular	1/0/0	1	1,2	0

Organ	Histo-pathological finding	Predominant cell infiltrate/s	Distribution	Degree (mild/moderate/severe)	Total (n)	MNP[b] (%)	Berlin[c] (%)
Brain							
	Inflammatory cellular infiltrates		Focal/multifocal	3/0/0	3	0	6,3
		Mononuclear, eosinophils	Perivascular	4/3/0	7	6,7	10,4
	Non-suppurative leptomeningitis	Mononuclear	Diffuse	1/0/0	1	0	0,7
	Eosinophilic inclusion bodies in neurons, astrocytes and cerebellar granular cells		Intracytoplasmic/intranuclear	17/1/1	19	0	13,6
Kidney							
	Interstitial nephritis	Mononuclear, eosinophils, neutrophils	Focal/multifocal	37/3/4	21	9,5	9,3
		Eosinophils	Focal/multifocal	0/0/1	1	0	0,7

[a]In order of predominance

[b]Samples from Müritz National Park (MNP) n=84 (in percentage)

[c]Samples from Berlin: n=140 (in percentage); n=92 limited to liver, lung, spleen and kidney

[d]Mild infiltration

4. GENERAL DISCUSSION

Raccoons have been present in Germany for the past 80 years and have become part of the faunal assemblages in several regions particularly in Central and North-eastern Germany. Despite their successful establishment, only a few studies have been performed to investigate pathogen occurrence in raccoons and they focused primarily in sylvatic or rural populations (Lux & Priemer 1995, Gey 1998, Michler et al. 2009, Anheyer-Behmenburg 2013).

In this doctoral project, pathological changes and infectious agents from raccoons in Müritz National Park (MNP) and the Berlin metropolitan area were studied. This project involved the first investigations of both rural/sylvatic and urban environments, which are typical habitats for raccoons. Endoparasitic infections were the major disease finding in rural/sylvatic animals, while infectious pathogens such as CDV or *Leptospira* spp. were most commonly found in urban raccoons. Firstly, histological and molecular investigations in the raccoons identified the mesocercarial stage of the trematode *Alaria alata* in the tongue tissue of 10 raccoons. Nine of the affected animals were from MNP and a sole animal originated from Berlin. The occurrence of encysted mesocercariae in the tongues shows that raccoons can act as a paratenic host for *A. alata* which extends the parasites host range (Figure 4).

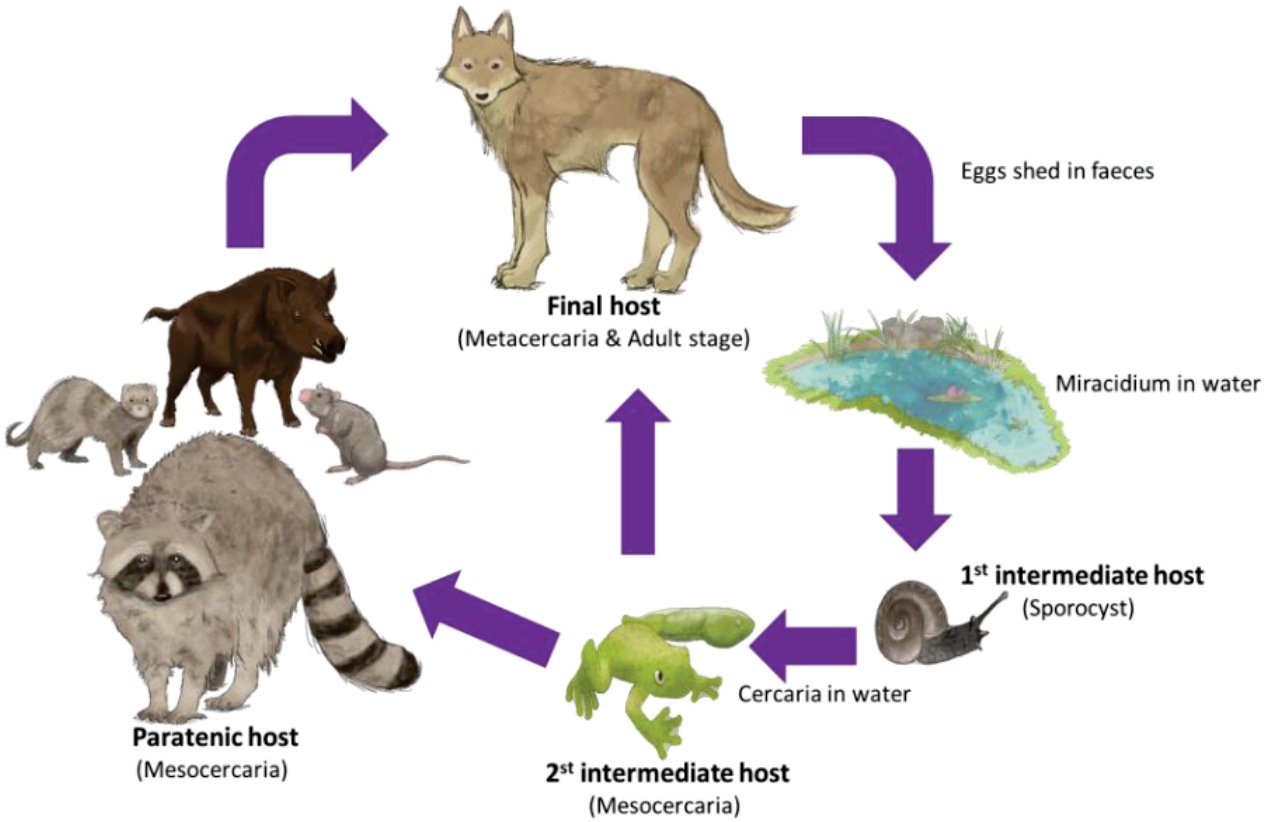


Figure 4.- *Alaria alata* life cycle with the raccoon included as a paratenic host

Secondly, four cases of sarcoptic mange were diagnosed in the course of the study (**Article 2 and 4**). Genetic investigations of *Sarcoptes scabiei* mites from 3 of these raccoons revealed a close relationship to *Sarcoptes scabiei* isolated from foxes (**Article 2**) indicating a vulpine origin of the mite infection in the raccoons. A similar fox to raccoon transmission scenario was hypothesized for 74 raccoons positively diagnosed with CDV infection. The animals were part of a large CDV outbreak in the wild carnivore population of Berlin (**Article 3 and 4**). Phylogenetic analyses of the isolated CDV strains from four of these infected raccoons showed that they were identical and clustered within the "Europe" lineage of the virus and were closely related to CDV from German foxes and domestic Hungarian dogs (**Article 3**). This suggests a fox or canid origin for this outbreak. Since Berlin foxes, which share their habitat with the raccoons, were also infected during this outbreak, they are a likely source of infection. However, introduction of the virus via unvaccinated domestic dogs also needs to be considered (**Article 3**). The urban habitat of Berlin is most likely the main driver for this interspecies contact as habitat defragmentation and a higher availability of anthropogenic resources, such as shelter and food, not only allow for higher raccoon densities and a reduced home range of individual animals but also overlap with neighbouring raccoon territories and with territories of other urban wildlife such red foxes, and domestic animals. These circumstances permit infectious pathogens to spread more easily in urban settings in contrast to rural or sylvatic environments with lower contact rates. Raccoons and other carnivores could interact while exploiting anthropogenic resources such as food in garbage containers, particularly as raccoons are known to concentrate near such places (Prange et al. 2003).

Finally, histological examinations showed the number of nematode-caused granulomas in lymph nodes was constantly higher in MNP animals than in Berlin raccoons. Granulomatous inflammation of the liver or hepatocellular necrosis suggestive of parasite migration also occurred more frequently in MNP raccoons. The higher prevalence of such parasite-originated lesions including *A. alata* mesocercarial cysts in MNP raccoons in comparison with Berlin animals, (**Article 1 and 4**) could be explained by the readily available intermediate hosts consumed by the raccoons in sylvatic areas (Ortmann et al. 2011, Engelmann et al. 2012). In contrast, urban raccoons can either exploit other human derived food sources, which reduce the amount of intermediate hosts in the raccoons' diet, or they do not encounter the intermediate hosts in urban environments as frequently as in MNP which lowers the possibility of parasitic infection. Separately, it could be expected that the prevalence of *B. procyonis* would be similar to the other helminths found in the raccoons, meaning a higher number of infected raccoons in rural settlements than in urban areas (Prange et al. 2008). However, none of the animals

examined was positive for *B. procyonis*, an outcome consistent with a study performed 20 years ago (1995). In contrast, the central German raccoon population exhibited a prevalence of 71% (Gey 1998). For this study, it was hypothesized that *B. procyonis* moved north-eastwards and that the central and the northern raccoon populations started to overlap at their borders allowing them to share the parasite. However, as none of the MNP or Berlin raccoons carried *B. procyonis* such a transmission event is unlikely (**Article 4**). The prevalence of *B. procyonis* diminishes as one move to the northern boundary of the central raccoon population (Winter 2005, Anheyer-Behmenburg 2013). Genetic diversity studies of German raccoons confirm that the central and north-eastern populations have remained distinct (Frantz et al. 2013). Therefore, it is likely that the founding members of the north-eastern raccoon population were not infected with *B. procyonis* and therefore, raccoons residing in this area remain free of this parasite.

Besides CDV (**Article 3 and 4**), PV was found in 3 animals from Berlin while rabies, infectious canine hepatitis or Aujeszky's disease were not found. PV phylogeny was not elucidated, thus the origin of the viral strain in this case remains unknown. However, raccoons in the USA can carry feline panleucopenia and canine parvovirus, as well as a raccoon parvovirus strain; although no information exists for German raccoons regarding parvovirus occurrence. Neither pathologic lesions suggestive of a viral aetiology were found nor were viruses isolated or viral nucleic acids detected in raccoons from MNP (**Article 4**). Unfortunately, no further investigations could be performed on this virus due to poor viral DNA quality. The condition of the samples, i.e. hunted animals without apparent signs of disease or the degree of autolysis in road-killed animals might have hampered the outcome of these investigations (**Article 4**). In 2007, a small outbreak of CDV occurred in raccoons from MNP (Michler et al. 2009, Nikolin et al. 2012). The detection of this outbreak was only possible due to the close radio-tracking of these animals at the time (Michler et al. 2009). During the course of this study, no such similar events were reported in this closely monitored population. As MNP raccoons have large home ranges (Köhnemann & Michler 2009) and contact with other species is only to be sporadic (F.-U. Michler & B.A. Michler, personal communication 2013), the transmission of viruses or bacteria depending on close contact seems to occur infrequently in this sylvatic population.

Leptospira interrogans was detected in five animals and pathogenic *Leptospira* spp. were detected in three examined raccoons. Seven of these raccoons were from Berlin. Rats (*Rattus* spp.) and domestic dogs can carry these bacteria and a study on 329 dogs with renal or liver disease suspected as leptospirosis cases revealed 25% of the dogs were seropositive for *Leptospira* spp. circulating in the environment (Mayer-Scholl et al. 2013). Another study on wild boars from Berlin detected *Leptospira* spp. in 18% of the 141 examined animals (Jansen et al.

2007). Since raccoons thrive throughout Berlin, exposure to urine or contaminated water/soil from infected rats, wild boar or domestic dogs increases the likelihood of infection with *Leptospira* spp. than in MNP. However, the current data does not allow for a firm conclusion to be drawn on the possible participation of raccoons in the transmission of leptospirosis. However, urban raccoons in closer contact with humans and domestic animals can increase the risk of leptospirosis.

Overall, the number of infectious diseases found in these raccoons is lower than other well-known disease vectors such as the fox (Denzin et al. 2013), which suggest raccoons are not playing a major role in disease transmission in Germany yet. Nevertheless, they maintain closer contact with humans and domestic animals than foxes, which could increase pathogen transmission amongst these species.

In conclusion, European raccoon health information is needed to evaluate the impact of this introduced species to the continent and Germany in particular, as it hosts the largest raccoon population in Europe. Similar studies to this one in other German regions and other European countries would help to understand the differences in pathogen occurrence within Germany and across the raccoon's complete range. Additionally, information for veterinarians and wildlife professionals regarding the potential hazards carried by raccoons will be of continuing importance as the raccoon population increases. Moreover, members of the public, which often are uncertain how to perceive raccoon novelty in their vicinity, can be better educated by regional information about this introduced species.

5. SUMMARY

Disease occurrence in free-ranging raccoons (*Procyon lotor*) from rural and urban populations in North-easten Germany

The North-American raccoon was first introduced to Germany in 1934. Currently, racoons occur throughout Germany with two major populations: one in the centre (Hessen), and the other in the northeast (Mecklenburg- Western Pomerania, Brandenburg). In their native North America raccoons are a well-known vector for infectious pathogens such as rabies, canine distemper virus or the zoonotic nematode *Baylisascaris procyonis*. Despite more than 70 years of successful introduction, there is a lack of knowledge regarding infectious diseases present in German raccoons. In order to provide information, two subpopulations of raccoons in north-eastern Germany were selected: one from a rural/sylvatic area (Müritz National Park (MNP) in Mecklenburg-Western Pomerania) and one from an urban settlement (Berlin metropolitan area). In total, 240 road-killed, hunted or euthanized animals were retrieved: 100 raccoons from MNP (2007-2011) and 140 animals from Berlin (2011-2013). Post-mortem examinations, histological, microbiological and molecular examinations of selected pathogens were performed on these animals. The results were published in four scientific articles:

Article 1: *Alaria alata*, a previously detected parasite encysted in raccoon tongue tissue was investigated and identified. Mesocercariae were isolated from eleven selected raccoons and PCR assays successfully identified *A. alata* in nine animals from MNP and one raccoon from Berlin, the parasite was successfully identified as *A. alata*. The mesocercariae were only found in tissue of the tongue and it was absent from other organs during histological examinations suggesting that raccoons are acting as paratenic host for this trematode. The higher number of MNP positive cases in comparison with Berlin indicates that differences in food resources allow raccoons in MNP to more likely ingest *A. alata* intermediate hosts such as amphibians than their urban counterparts. Moreover, since raccoons are an introduced species they can extend the host range of some endemic European parasites.

Article II: The second article of this project investigates sarcoptic mange in urban raccoons with three cases from Berlin and two cases from Kassel. Macroscopic skin lesions, histopathological findings and the morphology of the mites were described. In order to elucidate the infections possible origin, nine microsatellite markers were used to genotype isolated mites from these raccoons to compare the mites with *S. scabiei* derived from foxes, wild boar and chamois. The mites from the raccoons clustered together with fox-derived *S. scabiei*, suggesting a vulpine origin for the infection. These results indicate a cross-species transmission of *S. scabiei* between urban foxes and raccoons.

Article III: The first major canine distemper outbreak in German raccoons is described in this article. It occurred in Berlin metropolitan area from winter 2012/2013. During this time, 97 raccoon carcasses suspicious for CDV infection were collected as part of this thesis. Histology, immunohistochemistry and RT-PCR assays were performed on organ tissue and 74 animals were confirmed positive for CDV. Additionally, phylogenetic analyses of the haemagglutinin gene of CDV strains isolated from 4 of these raccoons were conducted. These CDV strains clustered within the Europe lineage and were closely related to those of German foxes and Hungarian dogs. The results suggest interspecies transmission amongst raccoons and other carnivores, in this case most likely foxes or an unvaccinated dog.

Article IV: This article is the summary of all investigations on the 240 raccoons examined in total. Besides post-mortem and histo-pathology investigations of all animals, screening for selected pathogens including the zoonotic nematodes *B. procyonis* were performed. Macrospcopic findings were mostly related to traumatic injuries as the causes of death. Histological examinations showed that 65.6% of all raccoons had inflammatory lesions, with gastro-intestinal-tract, liver, spleen and lymph nodes as the most commonly effected organs. Berlin animals more frequently carried viral or bacterial infections (CDV, PV, *Leptospira* spp*.)*. Meanwhile, lesions caused by nematodes or *A. alata* were most often found in raccoons from MNP. *B. procyonis, Trichinella* spp., Lyssavirus, CAV-1, and SuHv-1 were not detected in any animal. The differences in pathogen occurrence are most likely driven by the respective environment. For urban raccoons habitat defragmentation, reduced home ranges, abundance of anthropogenic food and shelter as well as higher interspecies contact rate can result in easier pathogen transmission between raccoons and other urban wildlife or domestic animals. While in MNP ideal habitat, wide home ranges, diverse food resources with abundant intermediate hosts on the one side and rare interspecies contact on the other reduce the spectrum of infectious pathogens.

Overall, the low number of infectious diseases in raccoons suggests that this animal species does not play an important role in disease transmission in North-eastern Germany. However, because raccoons explore human settlements closer than other wild species, their potential in pathogen transmission highlights the need for continuous investigation of urban raccoons with regard to public health.

6. ZUSAMMENFASSUNG

Vorkommenden Krankheitsfälle bei frei lebenden Waschbären (Procyon lotor) aus ruralen und urbanen Populationen in Nord-Ost Deutchland

Seit seiner ersten, 1934 erfolgten, Einbürgerung ist der Nordamerikanische Waschbär (*Proyon lotor*) eine invasive Tierart in Deutschland. Waschbären sind in Deutschland weit verbreitet, können aber in zwei Hauptpopulationen differenziert werden: Eine im Zentrum (Hessen), eine andere im nordöstlichen Landesteil (Mecklenburg-Vorpommern, Brandenburg). In Nordamerika gilt der Waschbär als bekannter Überträger von Infektionserregern wie Tollwut, Staupe oder dem zoonotischen Nematoden *Baylisascaris procyonis*. Aber trotz ihrer 70 Jahre währenden, erfolgreichen Einbürgerung gibt es wenig Kenntnis zu Infektionskrankheiten bei Waschbären in Deutschland. Um zu untersuchen, welche Krankheiten oder Krankheitserreger bei diesen Tieren vorkommen, wurden zwei Teilpopulationen in Nordostdeutschland ausgewählt: eine in einem ländlichen Waldgebiet (Müritz Nationalpark (MNP), Mecklenburg-Vorpommern), ein urbane im Großraum Berlin. Insgesamt wurden 240 Verkehrsopfer, jagdlich erlegte oder eingeschläferte Waschbären untersucht: 100 aus dem MNP (2007 bis 2011) und 140 aus Berlin (2011-2013). Tierkörpersektionen, histologische, mikrobiologische und molekularbiologische Untersuchungen von ausgewählten Erregern wurden mit diesen Tieren durchgeführt. Die Ergebnisse sind in vier wissenschaftlichen Artikeln veröffentlicht:

Artikel I: In vorangegangenen Studien histologisch entdeckte Parasitenzysten im Zungengewebe von Waschbären wurden untersucht und ihre Artzugehörigkeit identifiziert. Mesozerkarien konnten aus neun Tieren vom MNP und einem Tier aus Berlin isoliert und mittels PCR als *Alaria alata* in identifiziert werden. In histologischen Untersuchungen wurden *A. alata* Mesozerkarien nur in Zungengewebe detektiert, jedoch nicht in anderen Organen. Das deutet darauf hin, dass Waschbären für diesen Trematoden als paratenische Wirte auftreten. Die höhere Anzahl positiver *A. alata* Fälle im MNP im Vergleich zu Berlin läßt sich durch Unterschiede in der Nahrungszusammensetzung erklären, da den Waschbären im MNP häufiger Zwischenwirte von *A. alata*, wie Amphibien, zur Verfügung stehen als den urbanen Waschbären. Es konnte hier gezeigt werden, dass eine neueingebürgerte Art wie der Waschbär das Wirtsspektrum endemischer Parasiten erweitern kann.

Artikel II: Der zweite Artikel aus diesem Projekt beschreibt Sarcoptesräude in urbanen Waschbären mit drei Fällen aus Berlin und zwei Fällen aus Kassel. Makroskopische Hautläsionen, histo-pathologische Befunde und die Morphologie der Milben werden beschrieben. Um den möglichen Ursprung der Infektionen zu finden, wurden neun Mikrosatellitenmarker für die Genotypisierung der von Waschbären isolierten Milben verwendet,

um sie mit *S. scabiei* von Füchsen, Wildschweinen und Gämsen zu vergleichen. Die Milben der Waschbären lagen in einem Cluster mit *S. scabiei* von Füchsen, was für einem Infektionsursprung aus Füchsen spricht. Diese Ergebnisse deuten auf eine zwischenartliche Übertragung von *S. scabiei* zwischen urbanen Füchsen und Waschbären hin.

Artikel III: Der erste große Staupeausbruch von Waschbären in Deutschland wird in diesem Artikel beschrieben, der im Winter 2012/2013 im Großraum Berlin stattfand. Während dieser Zeit, wurden im Rahmen dieser Doktorarbeit 97 Waschbärkadaver gesammelt. Histologische, immunhistochemische und RT-PCR Untersuchungen wurden mit Organproben durchgeführt und 74 Tiere als Staupe-positiv bestätigt. Zusätzlich erfolgten phylogenetische Analysen des Hämagglutiningens von Staupevirusisolaten aus vier dieser Waschbären. Alle diese Virusisolate lagen im phylogenetischen Cluster der „Europe"-Linie und sind eng verwandt zu Isolaten von Füchsen aus Deutschland und Hunden aus Ungarn. Diese Ergebnisse lassen eine Übertragung zwischen Waschbären und anderen Fleischfressern vermuten, in diesem Fall Füchsen oder möglicherweise auch einem ungeimpften Hund.

Artikel IV: Dieser Artikel beinhaltet die Untersuchungen aller 240 Waschbären der gesamten Studie. Neben Sektionen und histo-pathologischen Untersuchungen wurden Organproben auf ausgewählte Krankheitserreger, die beim Waschbären beschrieben wurden, untersucht. Diese umfassen die zoonotischen Nematoden *Baylisascaris procyonis*, *Trichinella* spp., Tollwutvirus, Canines Adenovirus 1 (CAV-1), Suis herpesvirus 1 (SuHV 1, Aujeszkysche Krankheit), Parvovirus (PV), Canines Staupevirus (CDV) sowie *Leptospira* spp.. Makroskopische Befunde waren meist traumatischen Verletzungen, die in direktem Zusammenhang mit der Todesursache standen. Histologische Untersuchungen wiesen bei 65,6% der Waschbären entzündliche Läsionen auf, wobei Magen-Darm-Trakt, Leber, Milz und Lymphknoten am stärksten betroffen waren. Bei Berliner Waschbären fanden sich häufiger virale oder bakterielle Pathogene: CDV, PV, oder *Leptospira spp*., während Tiere aus dem MNP am häufigsten parasitäre Infektionen aufwiesen. In keinem der untersuchten Waschbären fanden sich weder *B. procyonis* oder *Trichinella* spp. noch Tollwutvirus, CAV-1, oder SuHv-1. Die Unterschiede im Pathogenvorkommen könnten durch die unterschiedlichen Habitate erklärt werden. Für urbane Waschbären kann es durch zersiedelte Habitat, reduzierte Streifgebietsgrößen, reiches Angebot sowohl anthropogener Nahrungsquellen als auch Zufluchtsorte zu erhöhten inner- als auch zwischenartlichen Kontaktraten kommen, die eine Pathogenübertragung erleichtern – nicht nur zwischen Waschbären als auch zwischen Waschbären und anderen urbanen Wildtieren sowie Haustieren oder auch Menschen. Im Unterschied dazu scheinen im MNP ideale Habitate und große Streifgebiete, verschiedenartige Futterressourcen, inklusive zahlreicher Zwischenwirte auf

der einen Seite und seltene zwischenartliche Kontakte auf der anderen Seite zu einem geringerem Pathogenspektrum aber mit Schwerpunkt auf Parasiten zu führen.
Generell deuten die geringen Zahlen von Infektionskrankheiten bei Waschbären im Nordosten Deutschlands daraufhin, dass die Waschbären gegenwärtig keine herausragende Rolle in der Verbreitung oder Übertragung von Pathogenen zu spielen scheinen. Dennoch sollte, gerade aufgrund der Tatsache, dass Waschbären menschliche Siedlungen intensiver nutzen können als andere Wildtiere, die potenzielle Übertragung von Krankheitserregern nicht außer Acht gelassen werden und im Interesse des Gesundheitswesens urbane Waschbären kontinuierlich untersucht werden.

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8. PUBLICATIONS LIST

The publications in section 3 of this dissertation are enlisted below. An unpublished manuscript that will be submitted to a peer-review journal comprises number 4 of this list.

1) Rentería-Solís ZM, Hamedy A, Michler F-U, Michler BA, Lücker E, Stier N, Wibbelt G, Riehn K (2013) ***Alaria alata*** **mesocercariae in raccoons (*Procyon lotor*) in Germany**. Parasitol Res, 112:3595-3600. DOI: 10.1007/s00436-013-3547

2) Rentería-Solís Z, Min AM, Alasaad S, Müller K, Michler F-U, Schmäschke R, Wittstatt U, Rossi L, Wibbelt G (2014) **Genetic epidemiology and pathology of raccoon-derived *Sarcoptes* mites from urban areas of Germany**. Med Vet Entomol, 28(S1):98-103. DOI: 10.1111/mve.12079.

3) Rentería-Solís Z, Förster C, Aue A, Wittstatt U, Wibbelt G, König M (2014) **Canine distemper outbreak in raccoons suggests pathogen interspecies transmission amongst alien and native carnivores in urban areas from Germany**. Vet Microbiol, 174:50-59. DOI: 10.1016/j.vetmic.2014.08.034.

4) Rentería-Solís Z, Michler F-U, Michler BA, Förster C, Nöckler K, Mayer-Scholl A, Grobbel M, Aue A, Wittstatt U, König M, Wibbelt G. **Disease investigations in free-ranging raccoons (*Procyon lotor*) from rural and urban settlements in Germany**. Unpublished manuscript.

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10. SELBSTSTÄNDIGKEITSERKLÄRUNG

Ich erkläre, dass ich die vorliegende Dissertation selbständig, ohne unzulässige fremde Hilfe und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Zaida Melina Rentería Solís
Berlin, 2015