Epidemiology of endoparasites of recolonizing European grey wolves in a multi–host predator–prey–system

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Content

Zusammenfassung

Wenn lokal ausgestorbene Großkarnivoren die Kulturlandschaft wiederbesiedeln, stellen sie sowohl neue Beutegreifer als auch neue Wirte im jeweiligen Ökosystem dar. Es kann vorkommen, dass sie hier nicht nur die Nahrungskette, sondern auch die Übertragung von Krankheitserregern innerhalb des Nahrungsnetzes verändern. Solche Ereignisse ermöglichen es, generelle ökologische Aspekte der betroffenen Arten zu untersuchen, wie zum Beispiel ihre Ernährung, ihr Verhalten und ihre Pathogenlast. Gleichzeitig stellt ein solches Ereignis aber auch ein „Eliminations-Experiment“ dar. Es erlaubt uns potentielle epidemiologische Effekte des Rückkehrers auf seine Beutetiere oder verwandte Fleischfresser in seiner An- und Abwesenheit zu untersuchen. Der Europäische Grauwolf (*Canis lupus*) begann in den späten 1990er Jahren Deutschland wieder zu besiedeln und breitet sich seither kontinuierlich aus. Im Rahmen des nationalen Wolf-Gesundheits-Monitorings wird der Ausbreitungsprozess hinsichtlich der Todesursachen und Infektionskrankheiten der Wölfe, inklusive ihrer Endoparasiten, untersucht.

Die Parasitenlast eines Wirts kann durch vielerlei Faktoren, die mit der individuellen Fitness oder auch mit Umweltbedingungen verknüpft sind, beeinflusst werden und ist daher anhand solcher Parameter vorhergesagbar. In **Kapitel I** habe ich untersucht, welche intrinsischen und extrinsischen Faktoren die Parasitendiversität und -artenvielfalt bei Wölfen der zentraleuropäischen Flachlandpopulation (ZEF) während der Populationsausbreitung steuern. Ich habe die Helminthen- und *Sarcocystis*-Fauna mit Hilfe einer Kombination aus klassischen Sequenzier- und modernen *metabarcoding* Methoden charakterisiert. Weiterhin habe ich nach Hinweisen gesucht, ob bei Anwesenheit von Wölfen die Befallsrate mit Metazestoden in ihren Beutetieren ansteigt, da diese als Zwischenwirte für einige Wolfsbandwurmarten fungieren. Ich habe 13 bekannte Helminthen- und 11 bekannte *Sarcocystis*-Arten identifiziert sowie eine geringe Prävalenz zoonotischer Arten (2%). Ich konnte zeigen, dass die Helminthendiversität und -artenvielfalt signifikant mit dem Wolfsalter schwankt und mit wachsender Wolfspopulationsgröße signifikant ansteigt, wobei der Heterozygotiegrad, das Geschlecht und die geographische Herkunft keinen Einfluss hatten. Ich fand keine Hinweise darauf, dass es eine Verbindung zwischen der Befallsrate mit Metazestoden in den Beutetieren und der Anwesenheit der Wölfe gibt. Hieraus lässt sich schlussfolgern, dass die Akkumulation von Wolfsparasiten in der Umwelt sowie eine erhöhte Kontaktfrequenz unter Artgenossen zu einer erhöhten Belastung mit Parasiten innerhalb der Wolfspopulation führen. Beide Faktoren sind durch die wachsende Populationsgröße bedingt. Andererseits scheint derzeit der Einfluss der Wölfe auf ihre paarhufigen Beutetiere bezüglich

Zestodeninfektionen vernachlässigbar zu sein. Dies könnte allerdings mit fortschreitender Wolfspräsenz und -ausbreitung an Bedeutung gewinnen. Außerdem deuten die altersbedingten Schwankungen in der Helminthenlast darauf hin, dass reifende Immunprozesse bei heranwachsenden Wölfen die individuelle Wurmlast senken, während mit fortschreitendem Alter eine Akkumulation eintritt.

Wie in **Kapitel I** gezeigt, sind ZEF Wölfe Endwirt verschiedener *Sarcocystis*-Arten. Allerdings war bisher nicht bekannt, inwieweit zurückkehrende Wölfe die *Sarcocystis*-Infektionsmuster der paarhufigen Zwischenwirte beeinflussen. Diese Parasiten aus dem Stamm der Apikomplexa brauchen zwei Wirte zur Vollendung ihres Lebenszyklus – einen Fleischfresser als Endwirt und einen Zwischenwirt, der als Beutetier des ersten fungiert. In **Kapitel II** habe ich eine Kombination aus Mikroskopie und *metabarcoding* genutzt, um *Sarcocystis*-Infektionen in drei Paarhuferarten und Wölfen zu bestimmen. Ich zeigte, dass Rothirsche ein signifikant höheres Infektionsrisiko haben, wenn sie ihr Habitat mit Wölfen teilen und dass dieser Effekt wahrscheinlich durch die beiden Arten *S. grueneri* und *S. taeniata* hervorgerufen wird. Es lässt sich vermuten, dass diese beiden Parasitenarten sehr gut an Wölfe angepasst sind, da sie häufiger als erwartet, basierend auf den Infektionsmustern in den Beutetieren, in Wölfen detektiert wurden. Sinngemäß verwende ich im Rahmen dieser These den Begriff ‚wolfsspezialisiert' für diese beiden Arten. Die *Sarcocystis*-Artenvielfalt nimmt mit steigendem Wolfsalter signifikant ab, was auf eine mit dem Alter steigende Immunkompetenz hinweist. Die Ergebnisse deuten darauf hin, dass Wölfe als Hauptbeutegreifer der Rothirsche zurückgekehrt sind und nun die Parasitenzyklen in diesem Paarhufer wiederbeleben, während sie einen weniger relevanten Einfluss auf andere Paarhufer haben.

Um die Epidemiologie parasitologischer Infektionen in einem breiteren Kontext beurteilen zu können, ist es wichtig, die Dynamik von Infektionserregern und die Rolle alternativer Wirte besser zu verstehen. Dies ist von besonderer Relevanz, wenn Erreger mehrere Wirtsarten befallen können. Hierdurch ließe sich die Übertragung solcher Pathogene auf bedrohte Tierarten oder auch von Wildtieren auf Haustiere und/oder Menschen reduzieren/verhindern. In **Kapitel III** habe ich ein *metabarcoding* Protokoll angewendet, um herauszufinden, ob Wölfe ebenfalls einen kumulativen Effekt auf die Parasitenlast bei Jagdhunden haben. Es hat sich gezeigt, dass sich Hunde die meisten der bei ihnen festgestellten Parasiten mit Wölfen teilen. Weiterhin gibt es keinen wolfsassoziierten Anstieg der Prävalenz und Artenvielfalt von Helminthen und *Sarcocystis* sp. bei Jagdhunden. Infektionen mit der wolfsspezialisierten Art *S. grueneri* waren allerdings wahrscheinlicher,

wenn Wölfe anwesend waren. Daraus lässt sich ableiten, dass Wölfe einen geringen epidemiologischen Einfluss auf die Parasitenlast von Jagdhunden haben. Naheliegende Gründe für diese Ergebnisse sind, dass Jagdhunde regelmäßig entwurmt werden, wodurch die Wurmlast niedrig gehalten wird. Außerdem erhalten Jagdhunde eine diverse Mischung an Rohfleisch, was die generelle *Sarcocystis*-Last hoch hält. Weiterhin ist denkbar, dass Jagdhunde und Wölfe gleichwertige Wirte für bestimmte Parasiten darstellen, was zu einer Substitution des Endwirtes Wolf durch den Endwirt Jagdhund während der lokalen Wolfsausrottung geführt haben könnte.

Zusammenfassend kann man sagen, dass ZEF Wölfe trotz ihrer diversen Parasitenfauna nur eine untergeordnete Rolle für die Verbreitung zoonotischer Arten spielen. Weiterhin schafft diese These einen wertvollen Beitrag zum Verständnis der Ökologie von Parasiten eines wilden, großen Beutegreifers und dessen Einfluss auf, mit der Nahrungskette assoziierte, Parasiten und deren Verbreitungsmuster in Jagdhunden und ihren gemeinsamen Beutetieren.

Summary

When large carnivores recolonize anthropogenic landscapes after phases of local extinction, they represent a new predator and host that may impact its new ecosystem by changing trophic cascades and altering pathogen transmission dynamics. Such an event allows us to study general ecological aspects such as diet, behaviour and pathogen load, but it is also the equivalent of a 'removal experiment'. Here, potential epidemiological effects of the returnee on its prey or related carnivore species in its presence and absence can be investigated. European grey wolves (*Canis lupus*) returned to Germany during the late 1990's, and have been continuously expanding their range. Within the frame of the *national wolf health monitoring project,* researchers have examined the expansion process regarding causes of death and infectious diseases, including endoparasites.

Host-parasite burden may be influenced by several factors related to individual fitness but also environmental conditions, and may therefore be predicted based on such measures. In **chapter I**, I investigated which intrinsic and extrinsic factors impact parasite diversity and species richness in the Central European lowland (CEL) wolf population during population expansion. I characterized its helminth and *Sarcocystis* fauna using a combination of classical sequencing and current metabarcoding techniques. Furthermore, I sought to find evidence on whether wolves increase metacestode prevalence in their ungulate prey species that serve as intermediate hosts of some wolf cestodes. I identified 13 known helminth and 11 known *Sarcocystis* species in the CEL wolf population, but only a low prevalence (2%) of zoonotic species. I discovered that helminth diversity and species richness vary with wolf age, and significantly increase with growing population size, whereas genetic heterozygozity, sex and geographic origin had no influence. However, I did not find a significant link of ungulate metacestode prevalence and wolf presence. Concluding from these findings, I suggest that the accumulation of wolf endoparasites within the environment as well as increased contact rates amongst conspecifics lead to an increased general parasite burden in wolves linked to population growth. On the other hand, the influence of wolves on their ungulate prey regarding tapeworm infections seems to be negligible but may increase with ongoing wolf presence and range expansion. Furthermore, the detected age-related fluctuations in helminth burden suggest that maturing immune processes control individual helminth burden during early life stages in wolves, while an accumulation of helminths occurs during later life stages.

As shown in **chapter I**, CEL wolves are the definitive host of several *Sarcocystis* species, but it is presently not clear how returning wolves alter *Sarcocystis* infection patterns in ungulate intermediate hosts. This apicomplexan parasite has a two-host life cycle, which

involves a carnivorous definitive host and intermediate hosts that serve as prey to the former. In **chapter II**, I use a combination of microscopy and metabarcoding to investigate *Sarcocystis* infection in three ungulate prey species and wolves. I show that red deer have a significantly increased risk of *Sarcocystis* infection when wolves are present in the same habitat, and that this effect is most likely driven by the two species of *S. grueneri* and *S. taeniata.* These two parasite species seem to be well adapted to wolves as they occurred more often in wolves than expected considering the infection patterns of their prey. Contextually, for the purposes of this thesis I term these parasite species as 'wolf-specialized'. *Sarcocystis* species richness significantly decreased with wolf age, indicating that an age-related increase in immune competence might control *Sarcocystis* burden in wolves. Based on these results, I speculate that wolves represent a returning apex predator of large ungulates like red deer, and therefore are resurrecting the parasite life cycles in this species, while they have a non-significant impact on other ungulates.

To understand the broader picture of parasite epidemiology the role of alternative hosts must not be disregarded. Understanding the infection dynamics of multi-host pathogens is essential to reduce/prevent spillover to threatened wildlife but also from wildlife to domesticated species or even humans. In **chapter III,** I use a metabarcoding protocol to analyse whether wolves also have a cumulative impact on parasite burden in domestic hunting dogs. In this chapter, I present that most species I detected in dogs are shared with wolves, and that there is no wolf-associated increase of helminth or overall *Sarcocystis* prevalence and species richness in hunting dogs. Only the 'wolf-specialized' protozoan *S. grueneri* was more likely to occur in hounds when wolves were present. Therefore, wolves only have a minor epidemiological influence on hounds regarding the parasites they share. The most likely reasons for these findings are that dogs receive regular anthelminthic treatments keeping helminth infection rate low, and they receive a diverse mix of raw meat when fed by their owners, keeping *Sarcocystis* infection rate high. Likewise, hunting dogs and wolves may represent equally suitable hosts for particular parasites leading to a substitution of wolves by dog hosts while wolves were locally extinct.

To conclude, CEL wolves do harbour a diverse endoparasite fauna, but their role regarding the spread of zoonotic species is negligible. Aside from that, this thesis provides valuable insights into the parasite ecology of a recovering large carnivore population as well as its impact on food-web related parasite distribution patterns in their prey species and domestic hunting dogs.

General Introduction

General Introduction

Biodiversity is defined as the variety of organisms that live and share an ecosystem. It includes the diversity within and between species but also of whole ecosystems (Magurran, 2004). Especially in the context of conservation, measuring biodiversity can be a useful tool to evaluate the (health) status of the ecosystem of interest, ranging from a single host, to a population and up to a global scale (Keesing et al., 2010). Despite the ongoing biodiversity crisis (Koh et al., 2004), some endangered species manage to recover well from local extinction events (Chapron et al., 2014). The recolonization of grey wolves in Central Europe is one of such remarkable success stories, where a combination of conservation measures as well as legal and socio-political efforts has permitted the integration of a highly adaptable large carnivore into anthropogenic landscapes (Chapron et al., 2014). However, the presence of large carnivores always comes along with public concerns; ranging from fear of (economical) losses due to predation to disease/health concerns. To address and ease such fears and in order to gain general knowledge on wolves in Central Europe, several research projects have scientifically investigated ecological aspects of the Central European lowland (CEL) wolf population. This thesis was conducted within the frame of the German *wolf health monitoring program* and contributes to our understanding of parasite epidemiology in the context of the ongoing wolf recolonization and population expansion in Central Europe. Applying molecular genetic tools, the endoparasite fauna of CEL wolves is described for the first time, and drivers of parasite diversity are determined in this particular population. This emerging knowledge allows us to further analyse the effect of this returning carnivore on the parasite burden in intermediate and other definitive hosts occurring across respective parasite life cycles. Analyzing parasite epidemiology and transmission dynamics in this multi-host system helps to better understand the ecological role of predators and the consequences of their local, temporal extinction and return to anthropogenic environments.

Wolf recolonization, biology and monitoring

At the beginning of the 20th century, wolf populations all over Europe had been decimated to the point of near extinction, with local extinctions in Scandinavia and large parts of Central and Western Europe (Figure 1A). Wolves were primarily hunted to reduce the predation risk of livestock, but also to protect humans and to make use of their fur (Okarma, 2002). Not until 1979, when the Bern Convention was ratified, did wolves receive a legal protection status in wide parts of Europe, except for, *i.a.* the former German Democratic Republic (GDR)

(Council-of-Europe, 1979). Only with the German reunification in 1990 did wolves eventually become a protected species in the former GDR. This legal amendment was a crucial milestone with respect to their recolonization of Central Europe, as immigrating wolves (most probably originating from the Baltic population) had previously been routinely culled over the years throughout the GDR (Butzeck, 1988).

In 2008, the global IUCN red list status of grey wolves was assessed as being of least concern (http://dx.doi.org/10.2305/IUCN.UK.2010-4.RLTS.T3746A10049204.en). Moreover, European wolf populations had started to recover and recolonize their former habitats (Figure 1 B). However, small populations such as the Sierra Morena and CEL wolf population are still at the edge of local extinction unless they reach the size of a minimum viable population (Reinhardt, 2015).

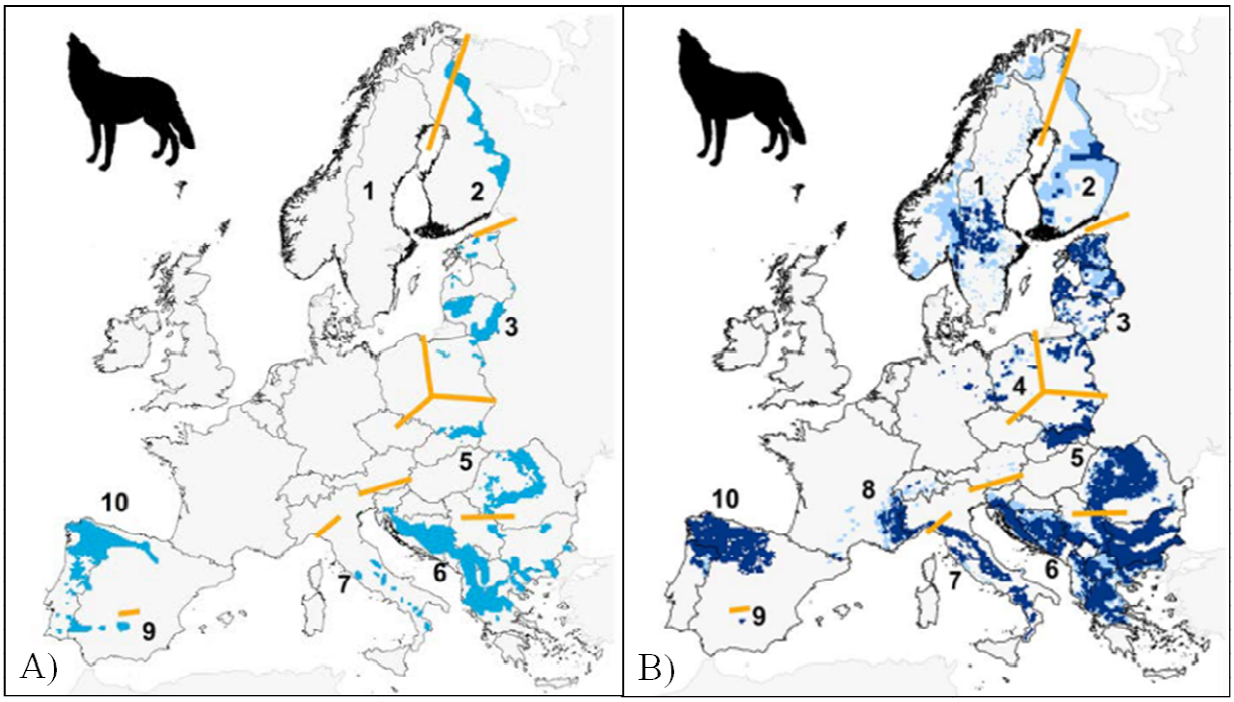

Figure 1: Distribution of grey wolves in Europe at their lowest extend during the 1950–1970s (A) and in 2011 (B). Numbers refer to populations: 1) Scandinavian, 2) Karelian, 3) Baltic, 4) Central European lowland, 5) Carpathian, 6) Dinaric-Balkan, 7) Italian peninsula, 8) Alpine, 9) Sierra Morena, 10) North-West Iberian. Orange lines indicate boundaries between populations as described in Chapron et al., 2014. Figures extracted from Chapron et al., 2014.

Figure 2: Distribution of grey wolf packs (dark green), pairs (light green) and single individuals (white) in Germany (large map) and within the whole CEL wolf population (small map) in 2015/2016. In the monitoring year 2015/2016 altogether 46 packs, 15 pairs and 4 territorial single wolves are recognized in Germany, while 53 packs and pairs are recognized in Western Poland. (Maps provided as courtesy of IFAW - International Fund for Animal Welfare.)

In order to monitor the process of population expansion and to learn about the ecology of the CEL wolf population, several German and Polish research projects have investigated population structure, pedigree and dispersal (Andersen et al., 2015; Ansorge et al., 2010; Nowak and Mysłajek, 2016), feeding habits (Nowak et al., 2011; Wagner et al., 2012), habitat suitability (Fechter and Storch, 2014), and infectious/non-infectious diseases (Szentiks, 2016). Prior to writing this thesis, the endoparasitic fauna of CEL wolves had not been reported. Additional data collected as part of the *German wolf monitoring program* made it possible to investigate the drivers of parasite diversity in this population, and to correlate factors such as individual genetic heterozygosity or individual geographic origin. To analyse relatedness and origin amongst wolves, a combination of field monitoring data and genetic microsatellite analyses are commonly used (Harms V, 2011) (www.wildtiergenetik.de). These data are permanently collected, annually evaluated and published. A 'wolf monitoring year' begins on the 1st May when pups are born and ranges till 30th April of the following year (Reinhardt, 2015). On average, wolf parents sire 4 to 6 pups each year, while some yearlings of the previous litter are still supporting members of the pack. Yearlings between their first and second year disperse to find a partner and roaming territory (Okarma, 2002). The last wolf 'census' in 2015/2016 showed 61 and 53 German and Polish CEL wolf families, respectively (Figure 2).

As microsatellite analyses are a convenient tool to investigate the pedigree of a population, it is simultaneously used to identify the genetic and geographic origin of individuals. There are cases of wolves of German origin that have been found dead in the Netherlands (Gravendeel et al., 2013) and Denmark (Andersen et al., 2015), whereas the most likely origin of the CEL population founders is the Baltic population (Ansorge et al., 2010). These examples show that while most wolves find their new territories within on average 100 km of their natal origin (Gese and Mech, 1991; Kojola et al., 2006), in fact some are long-distance dispersers (Wabakken et al., 2007). Hence, CEL wolves are likely to expand their range to further regions across Germany and Europe. In this context, Fechter *et al*. applied computational habitat modelling and found that approximately 400 wolf packs would find suitable habitat conditions in Germany (Fechter and Storch, 2014). These findings are based on an average home range size of 200 km^2, whereas wolf territories in, i.e. Canada or Alaska tend to be much larger, covering several thousands of square kilometres in extreme cases (Mech and Cluff, 2011). By most measures, the main determinant of wolf home range is prey density/availability linked to latitude (Jędrzejewski et al., 2007). Faecal analyses in CEL wolves have shown that their main prey are the most common wild ungulate species, which

include roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), and wild boar (*Sus scrofa*), whereas Leporidae and domesticated animals (Nowak et al., 2011; Wagner et al., 2012) as well as fallow deer (*Dama dama*) (unpublished data) are rarely detected in wolf faeces. During the first years of wolf recolonization in Lusatia (Germany) also European moufflon (*Ovis ammon musimon*) made up a considerable proportion of the diet of wolves, but as moufflon numbers have declined due to wolf predation their proportion in wolf diet has diminished over time (Wagner et al., 2012). Moufflons are well adapted to mountainous habitats as they are indigenous to parts of Central Asia, the Middle East, the Crimean peninsula and Balkans, and were introduced as game species to Europe (Rezaei et al., 2010). With the return of wolves, moufflons in Lusatia became a likely food source to this predator as they could not apply their species-specific anti-predatory behaviour of escaping into elevated, rocky areas. Although wolves have had a diminishing impact on this neozoan ungulate in Lusatia, indigenous ungulate populations are unlikely to suffer population declines due to wolves (http://www.wolf-sachsen.de/faq/26-berkontaktbuero/340-faq-wolf-und-wild-wolf-und-nutztiere).

Wolf health monitoring and multi-host diseases

Due to their constant population growth and expansion in Central Europe, it is evident that wolves cope well with local habitat conditions. Thus, it is not surprising that their major causes of death are of anthropogenic origin. According to the *wolf health monitoring program* conducted by the Leibniz Institute for Zoo and Wildlife Research, 71% of the examined wolves (n_{total}=142) died in traffic accidents, whereas in 13% of the investigated cases illegal killing was identified as cause of death, and natural causes in 11% (Szentiks, 2016). The screening for underlying infectious diseases revealed that canine distemper (CDV) is the only confirmed viral disease circulating at a low prevalence of 4% (n=5), whereas rabies, pseudorabies, parvovirosis, and hepatitis contagiosa canis have not been documented to date. Sarcoptic mange was detected in 6% (n=8). Even though the prevalence of infectious diseases has been low since the beginning of recolonization, according to the OneHealth concept, a continuous monitoring of pathogens remains crucial in order to conserve the population of interest as well as other fauna like domesticated populations and humans (Day, 2011; Haydon et al., 2002; Taylor et al., 2001; Thompson, 2013).

CDV and Sarcoptic mange are accurate examples of how multi-host pathogens circulate and affect the above mentioned host groups. When wolves were reintroduced to Yellowstone National Park in 1995/1996, healthy founders were vaccinated for a list of viral

diseases including CDV, and were additionally treated with anthelminthics (Fritts et al., 1997). However, offspring generations of wolves were affected by three distinct distemper outbreaks (1999, 2005, 2008) that are most likely a result of spillover from the coyote (*Canis latrans*) population in the national park. The last CDV outbreak coincided with a mange outbreak (of unknown source) that resulted in wolf population decline and eradication of complete packs (Almberg et al., 2012).

Other than viral diseases, parasitic agents such as mange are rather chronic or moderately pathogenic, and their costs of infection have even been shown to be mitigated by social and cooperative behaviour within wolf packs (Almberg et al., 2015). Although the consequences of parasite infection are rarely fatal, such pathogens have a high potential to steadily circulate within packs and populations, and therefore their infection risk towards alternative wildlife or domestic hosts is constantly high.

Determinants of parasite diversity

Parasitism is generally defined as a non-mutual relationship in which one species (parasite) exploits resources of the other (host) (Aspöck et al., 2006). It is a common ecological phenomenon observed across flora and fauna, e.g. in parasitic weeds that obtain water and nutrients from host plants (Dawson et al., 1994; Fernandez-Aparicio et al., 2016), vampire bats that cover their nutritional intake with blood meals from their hosts (Arellano-Sota, 1988; Voigt and Kelm, 2006) and brood parasitic cuckoos (Cuculidae) that exploit the brood care behaviour of other avian species in order to raise their chicks (Martinez et al., 1999). However, when using the term "parasite" people most commonly think of parasitic arthropods, helminths or even smaller microparasites such as protozoa. Depending on the 'habitat' of such parasites, one distinguishes between *ectoparasites* (from Greek "ectos": outside) that live outside of a host like mites, mosquitoes, ticks or fleas, whereas *endoparasites* (from Greek "endon": inside) live within a host (Aspöck et al., 2006). Amongst endoparasites, one furthermore distinguishes between macroscopically visible *macroparasites* (usually helminths) and microscopically visible *microparasites* which include protozoa as well as, in a broader sense, viruses and bacteria.

When analyzing host-parasite interactions, numerous inter-specific studies in mammals have correlated parasite burden and diversity with host body mass, geographical range size (Lindenfors et al., 2007; Torres et al., 2006) and most often host population density (Arneberg, 2001; Arneberg et al., 1998; Lindenfors et al., 2007; Morand and Poulin, 1998; Nunn et al., 2003; Torres et al., 2006). Within-species analyses however, allow stepping

beyond the above-mentioned determinants and enable insights into species-specific ways to cope with parasite infestation.

Several intra-specific mammalian studies ranging from mouse lemurs to large carnivores such as wolves and hyenas have linked resistance/susceptibility of particular endoparasite species to host age (Acevedo-Whitehouse et al., 2006; Guberti et al., 1993; Hamalainen et al., 2015; Segovia et al., 2001), host genetic constitution (Acevedo-Whitehouse et al., 2006; Luikart et al., 2008), and life history traits (e.g. litter size, social status and lactation) (East et al., 2015). It has always been the goal of such studies to uncover general principles in parasite ecology, and many of them succeeded in describing regular patterns in naturally complex ecosystems. However, not every natural set-up allows studying unique questions like, for example, how parasite diversity might be influenced after a host population bottleneck like the one encountered by the CEL wolf population. Such recolonization events are not only a unique opportunity to study how population expansion alters parasite burden in the target host population, but an opportunity to analyse how parasite interactions change in multi-host systems involving definitive and intermediate hosts, as well as alternative definitive hosts.

Canid parasites, life cycles and zoonoses

Nearctic and Palearctic wolves are known to host a long list of helminths (most of which are intestinal parasites), comprising 28 species of nematodes, 27 species of cestodes, and 16 species of trematodes (Craig and Craig, 2005). Furthermore, several protozoan taxa such as *Cryptosporidium* sp. (Hermosilla et al., 2017; Kloch et al., 2005; Stronen et al., 2011), *Cystoisospora* spp. (Hermosilla et al., 2017), *Giardia* spp. (Andersen et al., 2015; Hermosilla et al., 2017; Kloch et al., 2005), *Neospora caninum* (Gondim et al., 2004; Stieve et al., 2010), *Sarcocystis* spp. (Andersen et al., 2015; Hermosilla et al., 2017; Khan and Evans, 2006), and *Toxoplasma gondii* (Stieve et al., 2010) have been reported in wolves as well.

Even though many endoparasites have been described in this canid before, it is not clear which particular helminth and protozoan species are circulating within the recently re-establishing CEL wolf population, whether immigrating wolves import (non-indigenous) species, and more importantly regarding the OneHealth concept, if zoonotic species are involved. Equally, it would be highly informative to investigate to which extent the main prey species of wolves – that usually serve as intermediate hosts of such endoparasites – are affected by an increasing prevalence and diversity of cysts and metacestodes due to wolf presence. Similarly, returning wolves could potentially also increase parasite burden in

domestic hunting dogs (*Canis lupus familiaris*) that live in the same areas and feed on the same infected meat. Given their similar biology and relatedness, helminths and protozoa of wolves can also parasitize dogs, but the endoparasite fauna and prevalence in dogs may deviate from wolves depending on their anthelminthic treatment, diet and environment (Al-Sabi et al., 2013; Barutzki and Schaper, 2003; Barutzki and Schaper, 2011; Bugg et al., 1999; Otranto et al., 2015a; Otranto et al., 2015b).

Irrespective of the definitive host species, some of these canid endoparasites are zoonotic species and may therefore also bear a threat to human health. The fox tapeworm *Echinococcus multilocularis* is probably one of the best-known zoonotic agents, causing alveolar echinococcosis in humans (Hegglin and Deplazes, 2013). While vole species serve as intermediate hosts, foxes are its main definitive host (Oksanen et al., 2016). Domestic dogs and wolves are also possible definitive hosts of this parasite as both canids occasionally prey on small rodents (Bagrade et al., 2009; Karamon et al., 2016; Nowak et al., 2011; Wagner et al., 2012). Nevertheless, cestodes of the genus *Taenia* are the most common tapeworms in wolves as their infective larvae usually occur in typical prey animals of wolves such as (wild or domesticated) ungulates, but also in Leporids and rodents (Bagrade et al., 2009; Friesen and Roth, 2016). In addition, some species like *T. multiceps* or *T. crassiceps* have zoonotic potential and may cause human coenurosis and (neuro-) cysticercosis, respectively (Ambekar et al., 2013; François et al., 1998; Ing et al., 1998; Lescano and Zunt, 2013).

The developmental cycle of *Taenia* begins when proglottids detach from the strobila and infectious eggs are released and excreted by the host during defaecation. Some species even produce mobile gravid proglottids that actively leave their host through the anus or move away from the faecal dropping in order to be ingested by coprophobic intermediate hosts (Hui, 2000). Most parasite eggs are very resistant to weather conditions and can survive broad ranges of temperatures and humidity for several months and even years (Hildreth et al., 2004; Veit et al., 1995). Once the intermediate host ingests an embryonated egg, the physical (light and temperature changes, mechanical pressure) and chemical conditions (pH, CO_2 or trehalose concentration) within the host trigger the first-stage larva to hatch (Perry, 1989; Rogers, 1960; Whittington and Kearn, 1988). Hatched larvae then penetrate the intestinal mucosa and use the blood and lymph system to circulate through the body in order to reach their target organ (Gottstein et al., 2009; Walker and Zunt, 2005). Depending on the species, this destination may vary from muscular (*T. krabbei*, *T. ovis*, *T. serialis*) or connective tissue associated with body cavities (*T. hydatigena*), the liver (*T. hydatigena*, *T. taeniaeformis*) to the brain (*T. multiceps*) (Bürger, 2006). Once the larva reaches its target tissue, it attaches,

encapsulates, forms a cyst and remains in a permanent stage known as *metacestode* – the infectious stage for the definitive host. Depending on the *Taenia* species and the tissues affected, metacestodes can either stay subclinical or cause diverse disease symptoms in its intermediate host, e.g. Coenurus cerebralis in sheep, also known as gid or sturdy, caused by metacestodes called "coenuri" of *T. multiceps* that infest the brain leading to neurological disorders (Achenef et al., 1999; Ozmen et al., 2005). Clinical signs are usually better researched in livestock. Likewise, in the case of other *Taenia* species such as *T. hydatigena*, it is known that its metacestodes called "cysticerci" may impair liver function in sheep (Bamorovat et al., 2014; Singh et al., 2015), but the larval stage has also been reported in wild ungulates (Murai, 1979; Shimalov and Shimalov, 2000). Such metacestodes are also the causative agent in zoonotic infections if a cestode egg is ingested and humans thereby serve as accidental (intermediate) or dead-end hosts (Lescano and Zunt, 2013).

The development of *Taenia* continues once a carnivorous definitive host preys and feeds on cyst-infected intermediate hosts. The destroyed metacestode releases the protoscolex that continues along the digestive tract, and eventually attaches to the intestinal epithelium where it matures into an adult tapeworm. Depending on the *Taenia* species, adult tapeworms can reach a length between 150 cm (*T. pisiformis*) and 250 cm (*T. hydatigena*) (Deplazes et al., 2012), whereas in human taeniasis adult cestodes can reach a length of up to 7 m (*T. solium*) (https://www.cdc.gov/parasites/taeniasis/biology.html). In this stage, cestodes are completely dependent on their hosts to sustain their energetic metabolism. Unlike nematodes, they are hermaphrodites possessing both ovary and testes, and are therefore able to fertilize themselves (Aspöck et al., 2006). Thus, a single tapeworm can produce tens of thousands of eggs per proglottid that are either passively released with the faeces, or actively with mobile gravid proglottids (Ma et al., 2002).

The development of protozoan parasites like *Sarcocystis* follows similar patterns involving an external phase and two hosts that usually live in a predator-prey-relationship (Figure 3). In the predator definitive host *Sarcocystis* cause the intestinal form of the disease, while in the prey intermediate hosts musculature or nervous tissue are affected (Bürger, 2006). In Europe, *Sarcocystis* species have only been reported as zoonotic agents of intestinal sarcosporidiosis in which humans serve as the definitive host (Fayer, 2004). In contrast, recurring outbreaks of acute muscular sarcocystosis have occurred in South-East Asia showing that humans may also (accidentally) function as intermediate dead-end host (Esposito et al., 2012; Esposito et al., 2014; Greve, 1985).

The developmental cycle of *Sarcocystis* begins when the definitive host sheds infectious sporocysts into the environment. These may stay viable for years due to the resilience of coccidian sporocysts in a wide range of environmental conditions (Elsheikha et al., 2004; Langkjær and Roepstorff, 2008; McKenna and Charleston, 1992; Saleque et al., 1990). To continue their development sporocysts need to be ingested by an intermediate host (Figure 3 A). For example, in the case of bovine sarcocystosis caused by *S. cruzi,* raccoons (*Procyon lotor*), dogs, foxes, wolves and coyotes are possible definitive hosts shedding their infectious sporocysts, whereas Bovidae serve as intermediate hosts (Dubey, 1976). Once sporocysts have been ingested, they release sporozoites (Figure 3 B) which undergo several rounds of asexual reproduction on their way through the vascular system, where they then form schizonts that attach to the vascular endothelium (Fayer and Dubey, 1986). In this phase, pathogenic species such as *S. cruzi* cause most clinical signs (Dubey, 1976). When the several phases of asexual reproduction are complete, schizonts dissolve to release merozoites that finally invade the target tissue – usually muscles or neurocytes in case of an infection with *S. cruzi*, respectively (Fayer, 2004). Intramuscular sarcocyst formation is initiated when merozoites round up to form metrocytes which undergo repeated asexual multiplication leading to an accumulation of metrocytes and a size increase of the sarcocyst (Figure 3 C) (Fayer, 2004). Depending on the *Sarcocystis* species, the consequent maturation of non-infectious metrocytes into infectious bradyzoites takes several weeks to months, until the sarcocyst enters and persists in this developmental stage (Fayer and Dubey, 1986). The morphological characteristics of mature sarcocysts such as size and wall structure (thickness, villar protrusions) have typically been used to describe and distinguish *Sarcocystis* spp. isolated from intermediate hosts (Odening et al., 1995; Stolte et al., 1998; Wesemeier and Sedlaczek, 1995a; Wesemeier and Sedlaczek, 1995b). *Sarcocystis* development continues when the mature sarcocyst is ingested by a susceptible definitive host. After cyst rupture, released bradyzoites (Figure 3 D) infest the intestinal *lamina propria* where they develop into male and female stages known as micro- and macrogametes, respectively (Figure 3 E). Next, these gametes fuse and develop into an oocyst containing two sporocysts (Figure 3 F). Mature oocysts migrate into the intestinal lumen, where their thin wall usually ruptures, and released sporocysts are consequentially excreted with the faeces (Figure 3 G) (Poulsen and Stensvold, 2014). In contrast to the intramuscular sarcocyst stage, sporocysts do not vary in their morphology, and are therefore morphologically indistinguishable. Hence, genetic tools that usually combine target enrichment with a sequencing technique are required for *Sarcocystis* spp. identification from definitive hosts (Xiang et al., 2009).

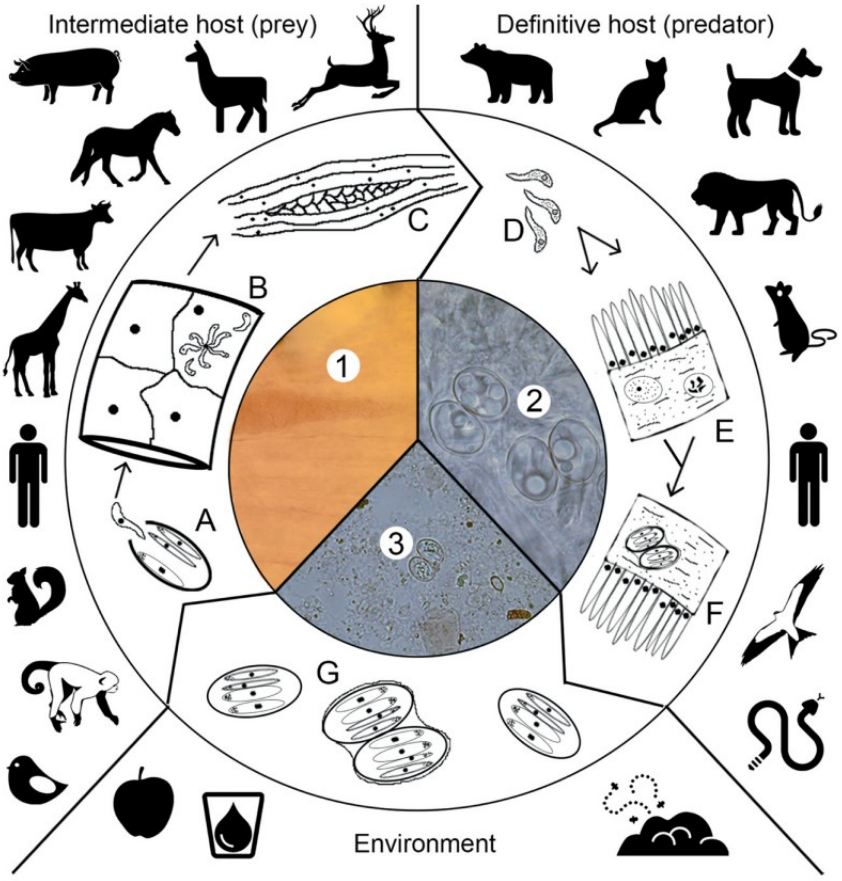

Figure 3: Life cycle of *Sarcocystis* sp. covering the developmental phases in the environment, intermediate and definitive hosts (1-3) and its seven stages (A-G). Infectious oocysts/sporocysts are ingested by intermediate hosts and sporozoites (A) are released that enter the endothelial tissue and (B) undergo several rounds of asexual merogony until merozoites reach muscular or nervous tissue where they develop into mature sarcocysts (C and 1) containing infective bradyzoites. Once definitive hosts ingest sarcocyst-infected meat, bradyzoites (D) are released which invade the intestinal epithelium and *lamina propria* to undergo gametogony in order to produce micro- and macrogametes (E). When gametes fuse, oocysts develop (F and 2) which are excreted in faeces and potentially contaminate soil, water and food (G and 3) whereby they are infectious to the intermediate host. (Figure taken from Poulsen and Stensvold, 2014.)

Sequencing approaches and their use in parasitology

During the last decades, ever-improving sequencing techniques have revolutionized the life sciences. The development of the polymerase chain reaction (PCR) and automated sequencing techniques have lead to sequential milestones in the genomics field with the *Human Genome Project* representing one of the major advances of this period (Hood et al., 1987; International-Human-Genome-Sequencing-Consortium, 2001; Mardis, 2008; Mullis, 1990). These advances have come along with the improvement of automated instrumentation, bioinformatics and computer databases, while simultaneously decreasing sequencing costs per base (Mardis, 2008). Recent achievements in cost reduction are based on the introduction of massively parallel sequencing techniques. So called *next-generation sequencing* (NGS) platforms enable the simultaneous recording of millions of diverse reads, in contrast to the

'old-fashioned' gel or capillary-based techniques such as those employed with the Sanger chain-termination method that were limited to clonal sequences (Mardis, 2013; Sanger et al., 1977). The main principle of NGS lies in sequencing a library of DNA fragments that have a platform-specific, universal adapter. These DNA fragments can either be shotgun genomic libraries or target-enriched libraries, in which genomic regions instead of whole genomes are selectively captured from a DNA sample before sequencing. Most commonly these libraries are enriched by hybrid capture or PCR amplification of specific genes (e.g. prokaryotic 16S rRNA, eukaryotic 18S rRNA) that allow a taxonomic classification of the biodiversity of the investigated sample excluding the need for cultivation (Mamanova et al., 2010; Mertes et al., 2011).

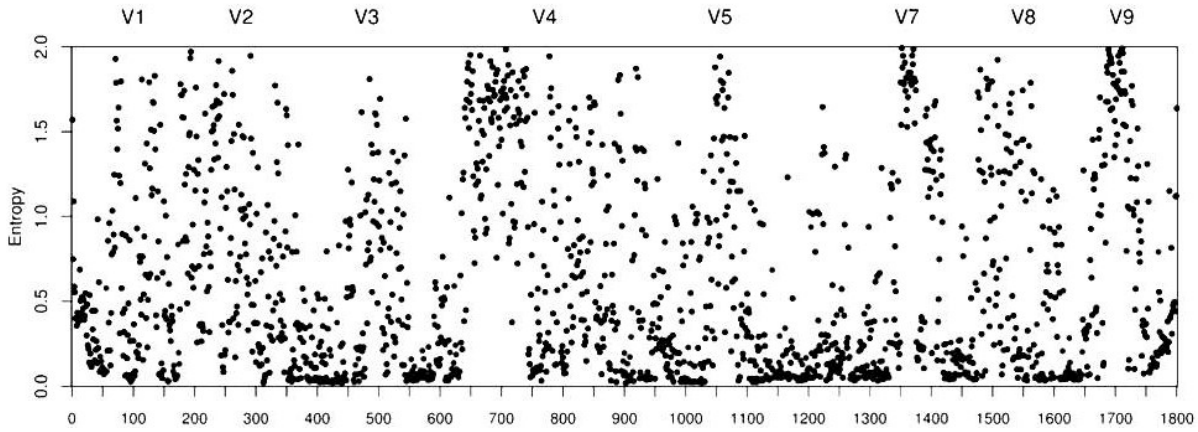

Figure 4: The eukaryote 18S rRNA gene contains nine variable regions V1 – V9. 24,795 eukaryotic 18S rRNA sequences were aligned and nucleotide substitutions used to calculate Shannon entropy values (black dots) that were mapped to the 1,800 bp long yeast (*Saccharomyces cerevisiae*) 18S rRNA gene as described in Hadziavdic et al., 2014. Figure taken from Hadziavdic et al., 2014.

For example, the 18S rRNA gene encodes for a component of the small ribosomal subunit in eukaryotes and consists of alternating variable and conserved gene regions across species (Figure 4). In metabarcoding studies, PCR primers are designed to anneal to the conserved regions and flank the regions of high nucleotide diversity (Hadziavdic et al., 2014; Machida and Knowlton, 2012). Evolved nucleotide substitutions in the target gene allow the taxonomic discrimination of the contained species when sequencing amplicons from a diverse sample.

Sample variety has been reported from soil (Panke-Buisse et al., 2015) and sediments (Hamdan et al., 2013), surface biofilms (Celikkol-Aydin et al., 2016), water samples (Hamdan et al., 2013; Hemme et al., 2015) to human (Alquezar-Planas et al., 2013; Sun et al., 2016) and animal tissues (Alfano et al., 2015; Menke et al., 2014) and faeces (Hino et al., 2016; Kramna et al., 2015; Srivathsan et al., 2016) in order to describe the bacterial, viral and parasitological communities and even dietary variation. Wildlife research and conservation

projects are making increasing use of NGS as poor quality samples, hardly cultivatable when collected under field conditions, have now become investigable by combining target enrichment and high-throughput sequencing. In parasitology, NGS for the first time enables the classification of diverse taxa from intestinal/faecal samples derived from definitive hosts from which helminth eggs or protozoan oocysts are often morphologically indistinguishable at the species level, while simultaneously avoiding labour-intensive dissections and cloning experiments (Moré et al., 2016; Tanaka et al., 2014; Xiang et al., 2009).

Study aims

The current wolf recolonization event in Central Europe and the high complexity of canid parasite life cycles that often involve several hosts, offer a unique opportunity to investigate how parasite epidemiology changes when a large carnivore returns to its former habitat. The aim of this thesis was to investigate several aspects of (i) parasite ecology in an expanding wolf population, and to understand the epidemiological influence of wolves on (ii) their ungulate prey as well as on (iii) domestic hunting dogs in the context of the detected parasitoses.

In **chapter I,** I dissected 53 wolf carcasses collected in Germany and characterized their helminth and *Sarcocystis* spectrum. To do so, I applied classical (Sanger) and modern (NGS) sequencing techniques, respectively, in order to create a baseline for the CEL wolf population endoparasite community, including information on potentially present zoonotic species. Based on these data, I questioned whether parasite diversity and species richness are connected to individual intrinsic (age, sex, genetic heterozygosity) and extrinsic (geographic origin, growing population size) factors and compared the spectra of native and immigrated individuals to ascertain whether wolves introduce parasites to newly (re)colonized environments. Furthermore, I analysed the link between wolf presence and metacestode levels in ungulate prey species.

Roe deer, red deer, and wild boar typically serve as intermediate hosts of *Sarcocystis*, and are commonly preyed on by CEL wolves. In **chapter II**, firstly I analysed whether ungulates from areas that are recolonized by wolves have an increased *Sarcocystis* sp. prevalence compared to animals from wolf-free control areas, as wolves might represent an additional host spreading this protozoan and increasing parasite prevalence in intermediate hosts. I tested this for each ungulate species and used classical microscopy of muscular tissue in this approach. Secondly, I characterized the *Sarcocystis* fauna of ungulates in the attempt to identify ‘wolf-specialized’ *Sarcocystis* species that might be the drivers of the general

Sarcocystis prevalence increase in the respective ungulates. If such species existed, they would be overrepresented in wolves in regard to the available *Sarcocystis* fauna and infection frequencies of ungulates. To achieve this, I applied metabarcoding of the 18S rRNA gene on microscopically-positive specimen. Thirdly, I investigated in each ungulate species whether such 'wolf-specialized' *Sarcocystis* species had a higher prevalence increase in wolf areas compared to wolf-free areas.

Because dogs and wolves are closely related canids with a similar biology, they are both susceptible to the same parasite species. However, unlike wolves, dogs undergo regular anthelminthic treatments that eradicate helminths but have no affect on protozoans like *Sarcocystis*. Moreover, domestic hunting dogs are considered a risk group regarding parasitological infections as they have access to potentially infected game, and because they work in forests where wild carnivores are present. In **chapter III**, I apply metabarcoding on dog faecal samples in order to analyse whether hunting dogs from areas recolonized by wolves have a higher helminth and *Sarcocystis* prevalence or species richness than dogs from wolf-free control areas. Additionally, I investigated whether hunting dogs are more likely to be infected with 'wolf-specialized' *Sarcocystis* species (identified in chapter 2) when living in areas recolonized by the CEL wolf population.

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Chapter I

Population expansion and individual age affect endoparasite richness and diversity in a recolonising large carnivore population

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Population expansion and individual age affect endoparasite richness and diversity in a recolonising large carnivore population

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The recent recolonisation of the Central European lowland (CEL) by the grey wolf (*Canis lupus*) provides an excellent opportunity to study the effect of founder events on endoparasite diversity. Which role do prey and predator populations play in the re-establishment of endoparasite life cycles? Which intrinsic and extrinsic factors control individual endoparasite diversity in an expanding host population? In 53 individually known CEL wolves sampled in Germany, we revealed a community of four cestode, eight nematode, one trematode and 12 potential *Sarcocystis* species through molecular genetic techniques. Infections with zoonotic *Echinococcus multilocularis*, *Trichinella britovi* and *T. spiralis* occurred as single cases. Per capita endoparasite species richness and diversity significantly increased with population size and changed with age, whereas sex, microsatellite heterozygosity, and geographic origin had no effect. Tapeworm abundance (*Taenia* spp.) was significantly higher in immigrants than natives. Metacestode prevalence was slightly higher in ungulates from wolf territories than from control areas elsewhere. Even though alternative canid definitive hosts might also play a role within the investigated parasite life cycles, our findings indicate that (1) immigrated wolves increase parasite diversity in German packs, and (2) prevalence of wolf-associated parasites had declined during wolf absence and has now risen during recolonisation.

Biodiversity describes the variety of organisms sharing an ecosystem and can be measured in levels of genetic variation, the number of occurring species (species richness) or by determining species diversity when accounting for the number of species and their abundance[1]. The respective ecosystems can be of different dimension, ranging from a single individual serving as host ecosystem for a community of microorganisms, to a local population in a distinct environment up to a global scale. In conservation biology, measuring biodiversity is a crucial tool to assess the (health) state of the ecosystem of interest[2].

The factors responsible for the presence and diversity of parasites in free-ranging mammalian host populations have been the subject of an increasing number of investigations in the past two decades[3–9]. These include external factors such as host population density and geographical location, and intrinsic factors such as genetic constitution, life history, and other conditions which may vary between individuals and host populations. Most of these studies have been conducted on rodents[7–9]. Many have investigated the drivers of parasite diversity across several species, while only few intraspecific studies have considered carnivores as hosts[10,11], particularly ecologically important apex predators[12], and even fewer have either been experimental in nature or used natural events that correspond to a quasi-experimental study design[13,14]. The typical framework of these studies has been a

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reasonably stable ecological setting within which the host population(s) under scrutiny has existed at the study site within living memory. The consequences for parasite presence and diversity are thus not clear, should a host population, particularly an apex predator, go extinct and recolonise its habitat almost a century later. Such extinction events correspond to a quasi-experimental set-up. It allows addressing questions such as: How would parasite diversity be affected by a small host founder population; to what extent do extrinsic and intrinsic factors control parasite diversity for individual hosts in an expanding host population; and which role do prey populations play in the re-establishment of parasite life cycles and parasite transmission for predator hosts? Here we use a recent and intriguing case of a recolonising and expanding apex predator, the grey wolf (*C. lupus*) in Central Europe to study these questions.

After having been eradicated for almost a century from Central Europe, grey wolves returned to Germany during the late 1990s and established the first breeding pack in the year 2000[15]. The first individuals immigrated from the Baltic wolf population from North Eastern Poland[15,16]. Since then, the population has rapidly expanded, leading to the establishment of the current Central European lowland (CEL) wolf population across Northern Germany and Western Poland. As of 2015, at least 39 breeding packs and pairs live in Germany, and at least 30 packs and pairs occupy Western Poland[17]. This newly established CEL wolf population provides an opportunity to study some additional and – in the context of conservation management – highly relevant questions on host-parasite relationships. In contrast to study sites in North America or Africa with a minor overlap between predators and people, people and wildlife in Central Europe coexist in an anthropogenically modified cultural landscape with a high human population density[18]. Here, transmission of pathogens between wolves, companion animals, livestock and people may easily occur[19] because free-ranging grey wolf populations are hosts of and vectors for a variety of macro[20]- and microparasites[21] which circulate in sylvatic and domestic cycles. Both pathogen spillover and spillback may occur and affect wild and domestic species, threaten human health[19], and in the case of livestock may even have an economic impact[22].

Such issues are especially accessible to investigation in eukaryotic parasites, establishing more stable host-parasite interactions compared to bacteria and viruses. Amongst helminths, the larval stages of taeniid species are known to cause health problems in people and livestock. They require a two-host cycle, with an intermediate host developing the metacestode/cysticercus and a predator definitive host consuming it and developing the mature tapeworm. Local diet analyses of wolves have demonstrated that roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), and wild boar (*Sus scrofa*) are the main prey species of the newly expanding wolf population in Germany[23], and may therefore serve as intermediate hosts of typical wolf endoparasites. For instance, the cestode *Taenia krabbei* might play a crucial role as intestinal parasite in European wolves[24] and is known to develop metacestodes in cardiac and skeletal muscles in intermediate hosts[24,25]. In contrast to *T. krabbei*, for which no human case of cysticercosis has been reported so far, other tapeworm species have a high zoonotic potential[26] and are responsible for several types of cysticercoses (*T. hydatigena*)[27], coenuroses (*T. multiceps*)[28], and echinococcoses (*E. multilocularis, E. granulosus*)[29]. In addition, nematodes and trematodes spread by carnivores are known to cause trichinellosis (*Trichinella* spp.)[30] and alariosis (*Alaria alata*)[31] in people, livestock and wildlife. The causative agents of all these diseases are known to occur in free-ranging wolves[20]. It would therefore be highly instructive to know which helminth species are circulating within the CEL wolf population. Equally, little is known about protozoan infections in wolves, even though wolves could potentially be the definitive host and vector of microparasite diseases such as neosporosis or sarcosporidiosis, which play a vital role for wildlife, livestock and public health in general[32].

In this study, we therefore tackled the questions (1) which endoparasite species are circulating within the CEL wolf population, (2) whether these parasites are zoonotic, (3) to which extent wolves may have an epidemiological influence on their local prey species, and (4) if and to what degree the endoparasitic load of an expanding wolf population changes within the first years of resettlement. To address these issues, we apply a variety of molecular tools to identify individual wolves, their helminth and protozoan community retrieved from whole carcasses, as well as cysticerci isolated from their prey. We use this information to characterise the parasite infection status of individual wolves and subsequently test the influence of intrinsic factors such as age, sex and genetic constitution, and extrinsic factors such as population size and geographic origin on parasite diversity in an expanding wolf population. By knowing the genetic identity of most wolf packs of the German part of the CEL population, we could also identify 'immigrants' – wolves that were not born in one of the known German packs – and (5) identify the parasite species 'imported' by them.

Results

Genetic structure of wolf sample. As part of the German national wolf monitoring, we dissected and genotyped 53 carcasses between 2007 and 2014. One common mtDNA control region haplotype, HW01 dominated in the 52 successfully analysed individuals, with the exception of a single HW02 wolf (corresponding to haplotypes w1 and w2 described in other studies[33]). By comparing the 53 microsatellite-based genotypes to the German wolf genotype database (unpublished), 36 wolves could be assigned to packs in Germany and thus were considered 'native'. The remaining 17 genotypes showed no first-order relationship to known German packs and were thus considered likely to be 'immigrant' individuals from Western Poland or the Baltic wolf population.

Subsequent Bayesian population clustering suggested five population clusters (see Supplementary Fig. S1). One individual was assigned to the group of reference samples of the Baltic wolf population (CL87/14 haplotype HW02), and three individuals (CL79/12, CL133/12, CL534/12) showed intermediate genotypes. All other wolves formed a single, distinct CEL wolf cluster, indicating a genetic separation of this newly established population from its Baltic source population. Microsatellite allele frequencies from the CEL wolf population were distinctly different from domestic dog reference samples.

Individual microsatellite heterozygosities ranged between 0.36 and 0.86 with a mean value of 0.6 (SEM = 0.02, 95% confidence limits 0.57–0.63, n = 53).

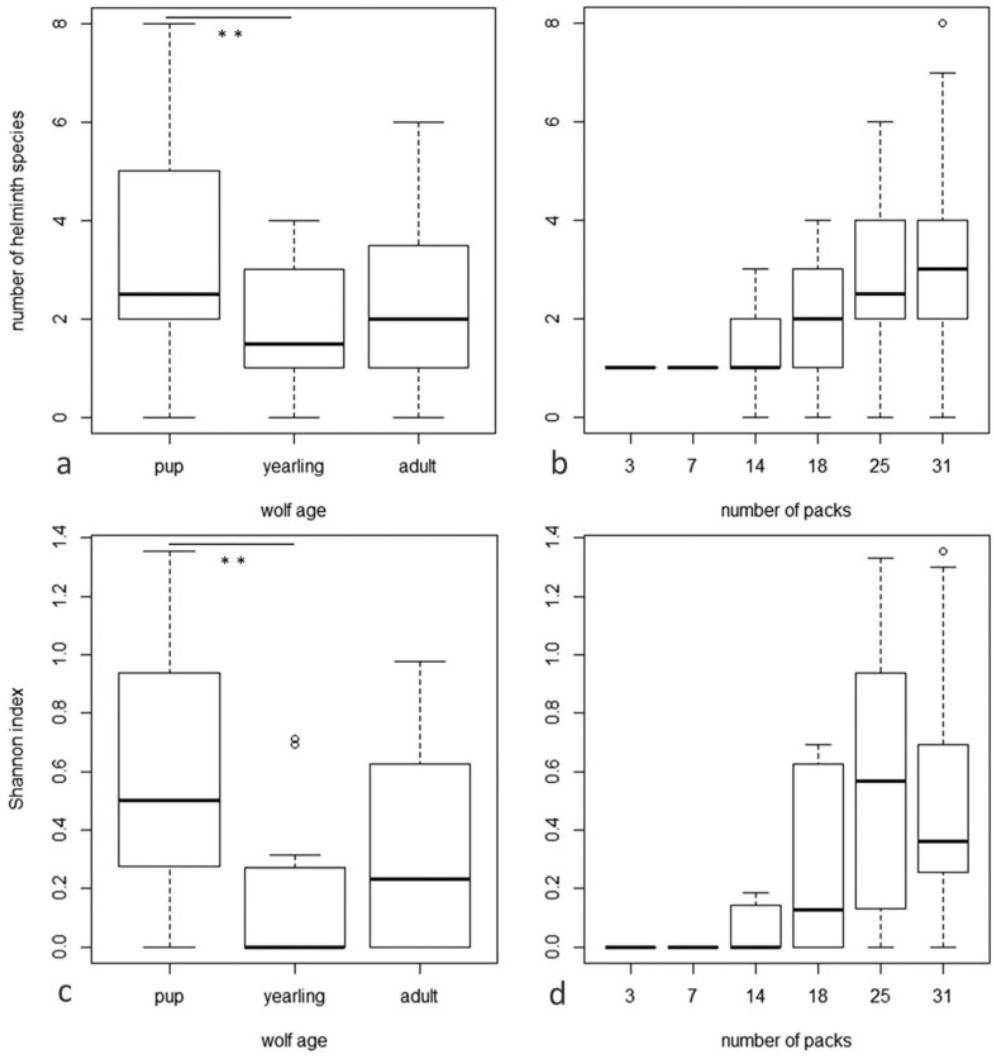

Figure 1. Relevant effectors of helminth species richness and diversity (Shannon index) in wolves from the CEL population. Helminth species richness (**a,b**) and helminth diversity (**c,d**) vary with wolf age significantly decreasing from pups to yearlings ($n_{pup} = 21$, $n_{yearling} = 16$, $n_{adult} = 14$) and increase with wolf population size ($n_{3packs} = 1$, $n_{7packs} = 1$, $n_{14packs} = 8$, $n_{18packs} = 11$, $n_{25packs} = 14$, $n_{31packs} = 16$). Dots represent outliers. Box plot edges depict the quartiles for number of helminths species (**a,b**) and the Shannon index (**c,d**). Whiskers extend to non-outlier extremes. Statistical significance was calculated using a general linear model.

Helminth diversity in wolves. Alpha diversity of the helminth population was determined by species richness and the Shannon index – a measure of diversity considering both the number of occurring species and their abundance. Infection with a single helminth species was recorded in 20.8% of the cases. Co-infection occurred most frequently with two species (22.6%), constantly decreasing to three helminth species (20.8%), four species (11.3%), five, six or seven helminth species (3.8% each). Eight helminth species per wolf were only detected once (1.9%), while 11.3% were helminth negative. Mean species richness over all individuals was 2.57 (SEM = 0.26, 95% C.L. 2.03–3.10, n = 53). Helminth species richness in 'native' individuals was 2.72 species (SEM = 0.34, 95% C.L. 2.03–3.41, n = 36) and in 'immigrants' 2.24 species (SEM = 0.39, 95% C.L. 1.41–3.06, n = 17). Helminth diversity, as measured by the Shannon index, ranged between 0 and 1.35 with a mean value of 0.38 (SEM = 0.06, 95% C.L. 0.27–0.49, n = 51).

Helminth species richness (general linear model, overall likelihood ratio test, $\chi^2 = 23.865$, df = 6, $p < 0.001$, n = 51) significantly increased with population size ($F_{1,46} = 14.58$, $p < 0.001$, Fig. 1b) and significantly changed with wolf age category ($F_{2,46} = 4.688$, $p = 0.014$, Fig. 1a). Pairwise post-hoc tests indicated that helminth species richness significantly declined from pups to yearlings ($p = 0.006$). Similarly, helminth diversity (general linear model, overall likelihood ratio test, $\chi^2 = 25.967$, df = 6, $p < 0.001$, n = 51) significantly increased with population size ($F_{1,46} = 10.77$, $p = 0.002$, Fig. 1d) and significantly changed with wolf age category ($F_{2,46} = 5.230$, $p = 0.009$, Fig. 1c). Pairwise post-hoc tests indicated that helminth diversity significantly decreased from pups to yearlings

Predictor	Direction of effect on chance of *Taenia* abundance being in a given category*			Df	G	p	AIC	ΔAIC	AIC_{qh}	ΔAIC_{qh}
	None	Low	High							
Sex	0.113 ♀ > ♂	−0.304 ♀ < ♂	0.191 ♀ > ♂	2	5.225	0.073	113.299	1.23	2.920	−0.177
Heterozygosity	0.687 ↑ as heterozygosity increases	0.349 ↑ as heterozygosity increases	−1.036 ↓ as heterozygosity increases	2	4.704	0.095	112.777	0.70	2.910	−0.187
Age pup	−0.383 pups < yearlings	0.106 pups > yearlings	0.277 pups > yearlings	4	8.364	0.079	112.44	0.37	2.715	−0.382
Age adult	−0.215 adults < yearlings	−0.018 adults < yearlings	0.234 adults > yearlings							
Geographic origin	−0.302 immigrants < natives	−0.124 immigrants < natives	0.426 immigrants > natives	2	8.989	0.011	117.063	4.99	2.994	−0.103
Population size	−0.010 ↓ as population size increases	0.013 ↑ as population size increases	−0.002 ↓ as population size increases	2	2.322	0.31	110.395	−1.68	2.863	−0.234

Table 1. Multinomial logistic regression of predictors affecting the chance of being in a given *Taenia* abundance category. Tests for significance of each parameter used log-likelihood ratio tests (G). Values for the Akaike Information Criterion (AIC) and the quasi-likelihood information criterion (AICqh) are shown for each alternative model when the specific predictor was removed. For the full model, AIC was 112.074 and AICqh was 3.097. *Global change of the probability of each of the three levels of *Taenia* abundance in response to a change in the value of each predictor variable. The sum of the values for each predictor is 0, as an increase in the probability in one level must be compensated for by a decrease in other levels.

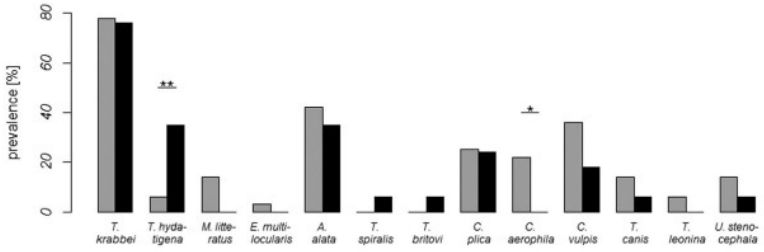

Figure 2. Helminth prevalence of CEL wolves in relation to their geographic origin. 'Native' wolves (grey bars) had a significantly lower prevalence of the tapeworm *T. hydatigena* (p = 0.010) and a significantly higher prevalence of the lung nematode *C. aerophila* than 'immigrants' (black bars) (p = 0.044). Statistical significance was calculated using the Fisher's exact test.

(p = 0.004). Sex, microsatellite heterozygosity, and geographic origin had no significant effect on helminth species richness and diversity.

The genus *Taenia* was the most prevalent and most abundant genus of helminths (0–109 parasites per individual). *Taenia* spp. abundance category (multinomial logistic regression, overall likelihood ratio test, $\chi^2 = 22.635$, df = 12, p = 0.031, n = 51, Table 1) changed significantly with geographic origin of wolves, with 'immigrants' significantly more often showing a high level of *Taenia* abundance than 'natives'. Sex (p = 0.073) and age (p = 0.079) marginally affected *Taenia* abundance, in that females were more likely to either have high or no *Taenia* abundance than males, and yearlings had lower levels of *Taenia* abundance than pups or adults. Genetic heterozygosity and population size had no influence.

Helminth fauna and prevalence in wolves. Thirteen helminth species were identified based on 18S rRNA and cytochrome c oxidase subunit I genes (Supplementary Table S1), while in 11% and 8% of the cases the isolated lung and intestinal nematodes could not be determined. Nematodes were the most diverse class (eight species), followed by cestodes (four species), and trematodes (one species). Infestations with the highly zoonotic *Trichinella* species *T. britovi* and *T. spiralis* (in muscular tissue) and with the fox tapeworm *E. multilocularis* were documented in three single cases, each representing a rare species in wolves, with a prevalence of 2%. The cestode *T. krabbei* was the most common (77%) helminth species in wolves, and is therefore considered the core species (by definition > 60% prevalence[34,35]) in this population. *T. hydatigena* and *Mesocestoides litteratus* were identified in 15% and 9% of wolves, respectively. *A. alata*, the only trematode, was detected in 53% of all wolves and can therefore be considered a secondary species (by definition 40–60% prevalence[34,35]). The three intestinal nematodes *Uncinaria stenocephala* (11%), *Toxocara canis* (11%) and *Toxascaris leonina* (4%) were isolated less frequently than cardio-pulmonary parasites. The two lung nematodes *Crenosoma vulpis* and *Capillaria aerophila* were found in 25% and 15% of all cases. *C. plica* was isolated from the urinary bladder of 25% of all wolves.

In total, 89% of investigated wolves carried endoparasites. Differences in the helminth fauna of all 53 individuals as a function of their geographic origin are depicted in Fig. 2. The cestode *T. hydatigena* occurred significantly less frequently in 'natives' born in Germany (3%) than in 'immigrants' (37%) (Fisher's exact test, p = 0.010, 95% C.L. 1.33–101.54, n = 53). In contrast, 'native' wolves had a significantly higher prevalence of the lung nematode *C. aerophila* (24%) than 'immigrants' which were not infected with this helminth at all (Fisher's exact

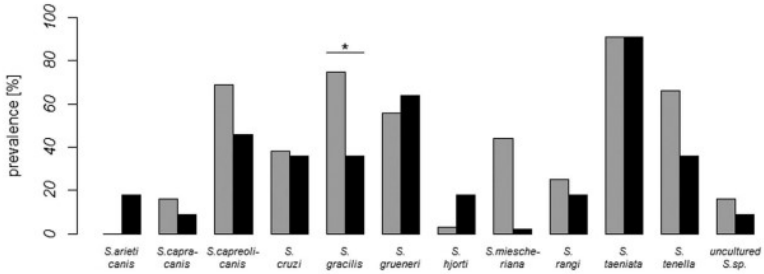

Figure 3. *Sarcocystis* spp. prevalence of CEL wolves in relation to their geographic origin. 'Native' wolves (grey bars) had a significantly higher *S. gracilis* prevalence than 'immigrant' wolves (p value = 0.031) (black bars). Statistical significance was calculated using the Fisher's exact test.

Intermediate host	Sample sizes		Cestode species	Number of cases (%)		Fisher's exact test
	n_{wt}	n_{ca}		Wolf territories	Control area	p value
Fallow deer (*D. dama*)	7	28	*T. krabbei*	0 (0.0%)	0 (0.0%)	1.0
			T. hydatigena	0 (0.0%)	0 (0.0%)	1.0
Red deer (*C. elaphus*)	82	20	*T. krabbei*	3 (3.7%)	1 (5.0%)	1.0
			T. hydatigena	5 (6.1%)	0 (0.0%)	0.58
Roe deer (*C. capreolus*)	105	72	*T. krabbei*	5 (4.8%)	1 (1.4%)	0.40
			T. hydatigena	2 (1.9%)	1 (1.4%)	1.0
Wild boar (*S. scrofa*)	88	38	*T. krabbei*	0 (0.0%)	0 (0.0%)	1.0
			T. hydatigena	5 (5.7%)	0 (0.0%)	0.32

Table 2. Cysticercoses prevalence in ungulates recovered from wolf territories (sample sizes n_{wt}) and the control area (sample sizes n_{ca}).

test, p = 0.044, 95% C.L. 0.00–1.12, n = 53). There were no significant prevalence differences for other helminths between immigrated and native individuals.

Sarcocystis fauna and diversity. Each of the 15 used primer sets successfully amplified *Sarcocystis* spp. DNA on the integrated fluidic circuit. For brevity we use the term 'species' to refer to 'operational taxonomic units' (OTUs)[36] from our molecular identification approach. Metabarcoding of the *Sarcocystis* spp. 18S rRNA gene revealed the presence of at least 12 different potential species of the genus *Sarcocystis* with a total prevalence of 95% (n = 43). The most prevalent OTU had an 18S rRNA sequence identical to *S. taeniata* (91%), *S. gracilis* (65%), *S. capreolicanis* (63%), *S. grueneri* (58%) and *S. tenella* (58%). Less than half of the wolf population was infected with *S. miescheriana* (40%), *S. cruzi* (37%), *S. rangi* (23%), *S. capracanis* (14%), *S. hjorti* (7%), and *S. arieticanis* (5%). In 14% of all cases, the isolated sequence was assigned to an undetermined *Sarcocystis* species. *S. gracilis* was the only species that occurred with a significantly higher prevalence in 'native' wolves than in 'immigrants' (Fisher's exact test, p = 0.031, 95% C.L. = 0.03–1.02, Fig. 3). Sarcocystis species richness could not be predicted from sex, age, microsatellite heterozygosity, geographic origin, and population size (general linear model, overall likelihood ratio test, $\chi^2 = 5.525$, df = 6, p = 0.478, n = 43).

Cysticercoses in ungulate intermediate hosts. In both study areas, *T. krabbei* and *T. hydatigena* were the only metacestodes detected in three of the four ungulate species (Table 2). In our limited sample of fallow deer, no individual was infected with any kind of cysticercus, while in red deer and roe deer *T. krabbei* and *T. hydatigena* prevalences were low, ranging from 0% to 6.1%. Wild boar were solely infected with Cysticercus tenuicollis. There was no significant cyst prevalence difference between wolf territories and the control area for a single ungulate species (Table 2). Using a general linear model (overall likelihood ratio test, $X^2 = 10.219$, df = 6, p = 0.069, n = 440) we were not able to show a significant effect of wolf presence on the cysticercosis prevalence across all ungulates. However, there was still a trend (p = 0.084) indicating that ungulates from wolf areas have a marginally higher cysticercosis prevalence.

Discussion

The recent recolonisation of large carnivore populations in Europe is a remarkable success of conservation efforts based on legislative decisions, increased public awareness, and scientific knowledge[18]. Wolves had been eradicated from Central Europe for about a century. The CEL wolf population has grown from one pack in the year 2000 to approximately 60 packs by 2015, and continues to expand and increase[17,37]. We used this unique quasi-experimental environment to investigate how endoparasite diversity is affected by founder events, how prey populations interact in parasite transmission to predator hosts and vice versa, and which intrinsic and extrinsic

factors control parasite diversity. To address these questions, we dissected entire wolf carcasses, applied classical and molecular genetic techniques to identify individuals and their helminth community, and used a metabarcoding approach to analyse whole gut sediments to screen for protozoan parasites. With this study we intended to generate appropriate evidence to clarify potential public health issues, which frequently arise during recolonisation events of large carnivore populations.

We applied mtDNA sequencing to haplotype wolf individuals and to identify their geographic origin. This revealed two haplotypes, HW01 and HW02, which are widespread across Europe[33] and commonly found in the CEL wolf population, including German wolves (ref. 16; German wolf genotype database, unpublished). Microsatellite-based structure analysis suggested that all but one carcass were likely to come from the CEL wolf population, which is clearly differentiated from the Baltic, Carpathian, and Alpine populations. Three individuals could not be clearly assigned to either the CEL or the Baltic wolf population, suggesting the existence of a contact zone or the possibility of long-distance dispersal with successful admixture. Individual CL87/14 was identified as the second male introducing haplotype HW02 to Germany and thus this individual provides the only obvious case of gene flow from an adjacent source population in our dataset. Using the German genetic wolf database, almost three-quarter of the wolf carcasses were assigned to German packs, providing evidence of their native origin. Seventeen wolves could not be assigned to any genetically known German pack and were thus considered most likely to be immigrants from Western Poland.

To investigate the endoparasite fauna in these wolves we avoided – commonly conducted but inadequate – parasitological scat analyses, which often underestimate parasite diversity[38], owing to intermittent egg shedding, biases towards hermaphroditic or female parasites, and limitations towards species that excrete their eggs through the intestine. But still our approach of isolating parasites from dead and partially decomposed wolves implies a minor drawback. While we were able to collect data on helminth species richness using molecular techniques in each case, counting of cestodes was not possible in 22 cases due to decomposition. To overcome this problem when calculating the Shannon index, we used mean values depending on the infestation level and based the statistical model regarding helminth diversity on these approximate values.

Furthermore, a sample size of 53 wolves might appear to be relatively small, but despite the limited availability of carcasses it is still a well-represented sample considering the current population size of approximately 39 known wolf packs in Germany. Based on these 53 individuals, we investigated the drivers of parasite species richness and diversity in wolves during their recolonisation of Central Europe by using individual characteristics (age, sex, genetic heterozygosity, parasite load) as well as geographic background data ('native' versus 'immigrant') of each individual. Interspecies studies in mammals have shown that parasite richness and diversity in free-ranging wildlife can generally be influenced by biogeographical, ecological, immunological, life-history traits, and individual characteristics[3–6]. In concordance with previous intraspecific parasite ecology studies in European wolves[10,39,40], we analysed the correlation of sex, age, geographic origin, and genetic constitution with parasite diversity in wolves, and additionally investigated the effect of a growing host population size – given the circumstances of the current CEL wolf population expansion. None of the previous studies found an effect of sex[10,40] or geographic origin[10,40], while the prevalence of particular helminth species was correlated with age[10,39] and year/season of death[39]. In contrast to these studies, we analysed the correlation of host parameters with parasite alpha diversity instead of single helminth species, and confirm that age significantly affected the level of parasite alpha diversity, which is also consistent with helminthological findings in domestic dogs[41]. Helminth diversity, parasite species richness and *Taenia* spp. abundance decreased from pups to yearlings, then tended to increase from yearlings to adults, suggesting two separate processes to be responsible for these changes. Age-intensity relationships in helminth disease etiopathology have also been described in other species[42–45] but the interpretation of such data currently remains vague, though opening room for speculation about adaptive immune processes during early life and posterior accumulation effects.

As wolves – and potentially their parasites – had been eradicated from Central Europe for more than a century, we investigated the effect of an increase in wolf population size on parasite alpha diversity. Helminth diversity and helminth species richness increased with the annually growing number of wolf packs, but not Sarcocystis species richness or Taenia abundance. While density-dependent effects of parasite diversity have been repeatedly discussed in cross-species approaches[4,5,46], host population size has – to our knowledge – not been previously considered in an intraspecific study focussing on wolves in Europe. Our work provides principal evidence that wolf helminth diversity increases during wolf population expansion, indicating that density-dependent parasite transmission amongst conspecifics and between wolves and their prey might play a major role in this carnivore. As wolves share their parasites with other predator and prey species, it is currently not clear to what extent alternative carnivore hosts transmit typical canine endoparasites in the area currently occupied by the German wolf population. For Central Europe in particular, anthropogenic factors such as tourism and hunting are likely to influence endoparasite communities of wildlife, since domestic dogs, particularly hunting dogs, share a similar diet with wolves and may serve as an additional parasite reservoir.

'Immigrants' had a higher abundance of *Taenia* cestodes than 'residents', potentially indicating an effect of the geographic origin in terms of either former habitat, *Taenia* metacestode infection in prey in the local habitat, or potential immunogenetic differences between 'immigrants' and 'natives'. Individual heterozygosity as measured by microsatellites did not correlate with parasite alpha diversity in our wolves, although heterozygosity has been associated with individual and population fitness and stress resistance, including parasite and disease susceptibility[47]. The mean heterozygosity of 0.6 in our wolves was lower than that of other European populations, but higher than in some small populations with a recent bottleneck history such as the Italian one[48]. This moderate level in combination with the fact that microsatellite markers may not appropriately reflect functional or genome-wide heterozygosity[49] might explain the lack of a significant association with parasite diversity and richness. The relatively low number of non-coding genetic markers might not be linked to functional immunogenetic loci, so potential associations between genome-wide heterozygosity and parasite load would become indistinct. Such loci play a

fundamental role for pathogen resistance and are important indicators in evolutionary ecology and conservation[50]. Hence, genetic diversity of loci under balancing or positive selection, such as the major histocompatibility complex[51] should be studied to evaluate the CEL wolf population's genetic potential to cope with parasites and reveal whether a founder effect has created a potentially impaired immune competence.

When analysing helminth communities and prevalence in wolves relative to their geographic origin, we found further implications potentially arising from immunogenetic or habitat effects. The lung nematode *C. aerophila* was exclusively found in 'native' wolves, while 'immigrants' had a significantly higher prevalence of *T. hydatigena*, so 'immigrants' can be considered importing this cestode into German wolf territories. Despite some ungulate intermediate hosts of *T. hydatigena* being uncommon in Germany, e.g. moose (*Alces alces*)[52], our cysticercosis screening in German ungulates demonstrates the presence of Cytsticercus tenuicollis in different parts of Germany (see below).

General helminth prevalence in our sample of wolf carcasses was 89%, which is similar to what several other studies found in wolves from the Baltic population (see Supplementary Table S3 for literature comparison of all species). With 13 genetically distinguishable helminth species and a mean of 2.57 ± 0.26 (SEM) species per individual, our sample had a significantly lower helminth species richness than Latvian[10] or Polish[38] wolves. At least 11 out of 13 isolated species have also been diagnosed in their Eastern relatives from the Baltic population. Presumably, the founders of the CEL wolf population had introduced a subset of the 'original' helminth community of the Baltic population, while at the same time intermediate hosts of some parasites are potentially absent in Central Europe such as moose or European bison (*Bos bonasus*). Our study suggests that parasite prevalence and diversity in the CEL wolf population will increase over time with ongoing expansion and immigration of new individuals.

Among the 13 helminth species, the three highly zoonotic parasites *T. spiralis*, *T. britovi* and *E. multilocularis* occurred in one case each (prevalence 2%). Hence, wolves play a minor role as reservoir of *Trichinella* larvae. Likewise, their role as vector and reservoir of *E. multilocularis* in Europe is insignificant compared to foxes, which occur in higher numbers and can reach a local prevalences between 0% and 60%[53].

Further results of our literature comparison of helminth prevalences with Baltic wolves sampled in Poland, Latvia, Estonia and Belarus are depicted in Supplementary Table S3. Most likely, significant differences can be explained by (1) higher or lower general prevalences of the particular parasite in the alternative habitat, (2) higher or lower prevalence of intermediate or additional definitive hosts in that habitat or (3) differing wolf diet and thus avoidance of the particular parasite.

An illustrative example where all hypotheses could be tested is the detection of the trematode *A. alata* which occurred more frequently in German wolves than in Belorussian ones, but less often than in Latvian and Estonian wolves, where it is the most frequent helminth. Since *A. alata* infection in carnivores depends on the consumption of infected wild boar meat, prevalence differences might either occur due to differing regional trematode abundances resulting from (1) varying environmental conditions for parasite development, (2) varying abundance of primary (snails) and secondary hosts (wild boar), or (3) due to regional differences in wolf diet.

Taenia represents another important helminth genus in our wolf sample that requires a two-host-cycle (herbivore/omnivore and carnivore). Wolves from the Baltic population were infected with a higher diversity of *Taenia* species than our wolves, in which *T. krabbei* (prevalence 77%) and *T. hydatigena* (prevalence 15%) were the only two detected species. This loss of *Taenia* spp. richness suggests that the founders of the CEL wolf population started with a reduced parasite community and/or that German wolves fed on a lower diversity of prey and therefore acquired fewer cestode species. This in turn might change during a longer presence of wolves as definitive hosts altering transmission dynamics.

Our cysticercosis screening in wild ungulates was intended to assess whether metacestode prevalence differed in the four main prey species of wolves in Germany between areas with and without wolves. Both detected cestode species *T. krabbei* and *T. hydatigena* are known to cause cysticercoses in wild and domestic ungulates. Contrasting our hypothesis, metacestode infection rates did not differ significantly between the two study areas, but still we found a trend of prevalences being slightly higher when wolves are present. Given the relatively low metacestode prevalence in both study areas, it was not feasible to sample an appropriate number of individuals in order to increase the statistical power of the analysis. Furthermore, it is not only wolves that shed their parasites into the environment. So the role of alternative definitive hosts such as domestic dogs, red foxes or racoon dogs must not be underestimated and should be investigated in future studies before final conclusions can be drawn.

Unfortunately, comprehensive cysticercoses data from Central and Eastern European wild ungulates are scarce. However, a recent Danish study reported the reoccurrence of *T. krabbei* cysticerci in roe deer after more than 60 years of absence in this species[25] and suggested that wolves may be responsible, since a *T. krabbei* infected individual had been documented in the same area[54]. Underlining the need to evaluate the role of alternative hosts, notably higher *T. krabbei* metacestode prevalences were reported during the 1970s, with 33% in roe deer and 19% in red deer[55], even though wolves have not been resident in Hungary (see Supplementary Table S4 for literature comparison). *T. hydatigena* prevalence was also significantly higher in all four ungulate species compared to Germany. Hydatid disease caused by *E. granulosus* was not detected in German ungulates but found at remarkably high rates in wild boar and red deer in Eastern Europe[55,56].

We could not include skeletal muscle tissue of ungulates in our screening, so the only muscular tissues analysed macroscopically were tongue, heart and diaphragm. This might have made us underestimate *T. krabbei* prevalence and miss the zoonotic *A. alata* mesocercariae and *Trichinella* larvae in the diaphragm of wild boars. Nevertheless, the low species-specific total *Taenia* prevalences between 0% and 5% (see Supplementary Table S4) suggest that larval cestode infections have a minor health impact on the analysed ungulate populations in Germany.

The protozoan parasite *Sarcocystis* is known to cause sarcocystosis and sarcosporidiosis in its intermediate and definitive hosts, respectively. Identification of sarcocysts from the intermediate host's musculature has been

conducted for decades, while identifying *Sarcocystis* sporocysts from the definitive host usually requires complex infection experiments[57] or laboratory methods[58], and has therefore been rarely performed, especially in wild large carnivores. While morphological studies in Europe have not provided any data on *Sarcocystis* prevalence in wolves, *Sarcocystis* spp. prevalence in Canada ranged from 38%[59] to 100%[60]. Using metabarcoding on whole gut sediments to analyse *Sarcocystis* spp. diversity in free-ranging wolves, we found that 95% of our wolves were *Sarcocystis* positive.

Technically, our metabarcoding approach enables us to determine 'operational taxonomic units' as clusters of similar sequences[36]. For brevity, we use the term 'species' instead, accepting the limitations of our method. The species identified via database entries – as sharing highest sequence similarity with our data – have been previously described from various wild and domesticated ungulate intermediate hosts. *S. taeniata* and *S. hjorti* are known in moose[61,62] and red deer[63,64], while *S. capreolicanis* and *S. gracilis* usually occur in roe deer[62]. In our sample of wolves *S. gracilis* was significantly more prevalent in 'natives' than 'immigrants'. As for helminths, such differences could occur due to potential habitat, immunogenetic or diet differences. These findings are independent of our potentially limited species resolution capacities, and whether or not a particular parasite strain with prevalence differences is granted species status.

S. grueneri sarcocysts develop in reindeer[65], red deer[66] and fallow deer[67]; the latter two being the most likely source of infection for our wolves. *S. miescheriana* – known from wild boars[68] and domestic pigs[69] – had a prevalence of 40% in German wolves, consistent with the fact that wild boars contribute 18% of biomass to the German wolf diet[23]. In contrast, sequences sharing highest similarity with *S. rangi*, *S. tenella*, *S. arieticanis*, *S. cruzi*, and *S. capracanis* were detected more often than expected, since wolves usually do not commonly prey on reindeer[65], mufflon[70], domestic sheep[62], cattle[71] or goats[72], respectively. This discrepancy suggests either a lack of resolution in the sequenced gene fragment and that those sequences represent different – yet to be described – species, or that these described species have a broader intermediate host spectrum than previously thought.

While the incidence of emerging infectious diseases has increased in recent decades[73], the presence and impact of wildlife has often been neglected. Wild carnivores may play a major role for the distribution of infectious disease and different host species sharing the same parasites may have an epidemiological influence on each other which is often of complex nature and hard to capture when only focusing on one target species. Our findings suggest that wolves from Central Europe currently have a minor relevance as reservoir of zoonotic parasites. Since we also show that parasite alpha diversity changes with growing wolf population size, the situation might best be described as being in a dynamic state. Thus, it might be useful to implement an endoparasite screening as a future monitoring tool to ease public and veterinary health concerns, since parasite life cycles are complex and some are flexible and may therefore change with time and expanding host population range. In fact, especially domestic dog owners and hunters in wolf habitats are likely to benefit from our findings, helping to make well-informed decisions on anthelminthic dog treatment and ungulate meat hygiene. Since hunters periodically feed their dogs with potentially infected meat, our results suggest that a routine anthelminthic treatment of hunting dogs would be highly advisable as recommended by the European Scientific Counsel for Companion Animal Parasites (ESCCAP).

Material and Methods

Sample collection. Between 2007 and 2014, we examined 53 wolf carcasses, collected as roadkill or poached, originating from five federal states in North and East Germany (50°10′–54°54′N and 6°41′–15°2′E) for endoparasites. Depending on recovery conditions (mostly time period between death and recovery of the carcass, outside temperature) we received the carcasses in different states of decomposition. Wolf sex and age category were determined by computed tomography and during necropsy by two specialised veterinarians for radiology and pathology. Age was estimated by assessing body size and mass, tooth replacement, tooth abrasion, state of thymus involution, state of reproductive organs as well as size and state of growth plates. Age class estimates were furthermore cross-checked and validated with the German wolf monitoring database (www.wildtiergenetik.de) by knowing the individual genetic identity. According to the joint monitoring standards for the CEL wolf population, day of birth was set to the 1st of May by default[17]. Individuals were considered as 'pup' within their first year of life, 'yearlings' within their second year, and 'adults' were older than 2 years. Helminths were isolated from all inner organs by conventional parasitological dissection[74]. When carcasses were fresh, we were able to recover and count all helminths ($n_{wolves} = 29$). Taenia spp. abundance was additionally classed into the categories 'no', 'low' or 'high' load. However, when carcasses were in an advanced stage of decomposition, cestodes were partially degraded and could therefore not be counted, but still their abundance was estimated using the above mentioned categories ($n_{wolves} = 22$). Detection of *Trichinella* larvae from muscular tissue was carried out by the National Reference Laboratory for *Trichinella* (Federal Institute for Risk Assessment, Berlin, Germany). We collected intestinal protozoa by washing and sieving the whole gut to eliminate food remains and collected the flow-through ($n_{wolves} = 43$).

Between 2012 and 2014, we collected 440 individuals of ungulates shot during hunts and screened their inner organs for cysticerci. To do so, we inspected the surface (including connective tissue) of lung, heart, diaphragm, spleen, liver, kidneys, intestines and mesentery, and sliced the tissues into 1 cm thin layers to inspect their interior parts. Isolated metacestodes were stored at −20 °C until DNA extraction. We compared cysticercosis prevalence in these ungulates between wolf territories (German federal states of Brandenburg and Saxony, 50°10′–53°33′N and 11°14′–15°2′E) and a control area where no territorial wolves were known at the time of sampling (German federal state of Schleswig-Holstein, 53°20′–54°55′N and 8°36′–11°7′E). The screening comprised fallow deer (*Dama dama*, $n_{wolf\ territories} = 7$, $n_{control} = 28$), roe deer (*C. capreolus*, $n_{wolf\ territories} = 105$, $n_{control} = 72$), red deer (*C. elaphus*, $n_{wolf\ territories} = 82$, $n_{control} = 20$), and wild boar (*S. scrofa*, $n_{wolf\ territories} = 88$, $n_{control} = 38$).

DNA extraction. For wolf genotyping, ethanol-preserved tissue samples were extracted using the QIAamp DNA Blood & Tissue Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. Helminth DNA was extracted from minced and proteinase K digested tissues using phenol-chloroform-isoamyl alcohol and a standard protocol (Carl Roth, Karlsruhe, Germany). Extraction success and DNA concentrations were determined using the NanoDrop® 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

Wolf protozoan (microparasite) DNA was extracted from 500 µl of pelleted intestinal filtrate suspended in 700 µL Buffer SL2 using the NucleoSpin® Soil Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Extraction success and DNA concentrations were determined using the Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, California, USA).

Microsatellite PCR and sequencing. We used 13 variable microsatellites and two sex markers (DBX6 and DBY7[75]) to assess relatedness and origin of the wolves. Markers CPH5[76], FH2001, FH2010, FH2017, FH2054, FH2087L, FH2088, FH2096, FH2137, FH2140 and FH2161[77], vWF[78], and PEZ17[79] were amplified in three 10 µl multiplex PCRs containing HotStarTaq Master Mix (Qiagen, Hilden, Germany), 0.2 µM of each primer, 2 ng BSA and ~5 ng genomic DNA. PCR started with initial denaturation at 95 °C (15 min), 4 cycles of 94 °C (30 s), 60 °C (90 s) and 72 °C (60 s); another 5 cycles of 94 °C (30 s), 58 °C (90 s) and 72 °C (60 s), 5 cycles of 94 °C (30 s), 58 °C (90 s) and 72 °C (60 s); another 5 cycles of 94 °C (30 s), 54 °C (90 s) and 72 °C (60 s), 25 cycles of 94 °C (30 s), 50 °C (90 s) and 72 °C (60 s), and a final elongation at 72 °C (30 min). PCR products were size-measured on an ABI 3730 DNA Analyzer (Life Technologies, Carlsbad, California, USA) and scored using GeneMarker v1.90 (SoftGenetics, State College, Pennsylvania, USA) by comparison to LIZ600 as an internal size-standard. For mitochondrial DNA control region sequencing, primers L15995[80] and H16498[81] were used. PCRs were performed in 15 µl containing 3 mM $MgCl_2$, 1.5 µl 1 × PCR buffer, 0.13 mg/µl BSA, 0.2 mM dNTPs, 0.333 µM of each primer, 1 U *Taq* polymerase (New England Biolabs Inc., Ipswich, Massachusetts, USA), and 3 µl DNA extract. PCR protocol started with initial denaturation at 95 °C (3 min), 35 cycles of 94 °C (30 s), 54 °C (30 s) and 72 °C (60 s), and a final elongation of 72 °C (10 min). PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA) following the manufacturer's protocol. Sequencing was carried out on an ABI 3730 DNA Analyzer (Life Technologies, Carlsbad, California, USA). Sequencing results were analysed in Geneious v7.1.9 (Biomatters Ltd, Auckland, New Zealand) and compared to sequences deposited in the NCBI database.

Macroparasite PCR and sequencing. Cestodes, trematodes and intestinal nematodes were identified targeting a 450 bp fragment of the cytochrome c oxidase subunit 1 gene (cox1). The cox1 PCR was carried out using the primer set JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (5′-TAAAGAAAGAACATAATGAAAATG-3′) previously described[82,83]. Additionally, we used the primers 18S_965F (5′-GGCGATCAGATACCGCCCTAGTT-3′) and 18S_1573R (5′-TACAAAGGGCAGGGACGTAAT-3′)[84,85] to amplify and sequence a 620 bp fragment of the 18S rRNA gene to identify cardiopulmonary and urinary helminths.

PCRs were performed in an epGradient S thermocycler (Eppendorf, Hamburg, Germany) and had a total volume of 25 µl per sample, including 1 µl DNA template. The reactions contained 1 × FastStart High Fidelity Reaction Buffer without $MgCl_2$, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.2 µM of each primer (cox1) or 1 µM of each primer (18S rRNA) 0.4 µg/µl BSA (only cox1 PCR) and 0.5 U FastStart High Fidelity Enzyme Blend (all components from Roche, Basel, Switzerland). PCRs were run in 40 cycles, starting with an initial denaturation step at 95 °C (10 min), and ending with a final elongation step at 72 °C (10 min). Thermal cycling of the cox1 PCR took place as follows: 95 °C (45 s), 55 °C (45 s), 72 °C (60 s). Thermal cycling of the 18S rRNA PCR took place as follows: 95 °C (30 s), 53 °C (30 s), 72 °C (60 s).

Of each helminth PCR product, we purified 1 µl using 1 U FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 3 U Exonuclease I (Thermo Fisher Scientific, Waltham, Massachusetts, USA) due to the manufacturer's protocol. Sequencing PCR and clean-up were performed under standard conditions using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the BigDye Xterminator® Purification Kit (Life Technologies, Carlsbad, California, USA) before loading them on the Applied Biosystems® 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA).

Microparasite library preparation and sequencing. The eukaryote 18S rRNA gene is typically grouped into nine variable regions V1–V9 suitable for diversity studies of several taxa[86]. In order to design primers that flank the most variable regions of the *Sarcocystis* spp. 18S rRNA gene (see Supplementary Fig. S2), we used primer3 version 0.4.0[87] and oligonucleotides previously described. Sensitivity of oligonucleotide binding to the targeted Sarcocystis spp. sequences was assessed using the tool TestPrime 1.0 in the Silva web interface[88]. Metabarcoding PCR on an integrated fluidic circuit (48.48 Access Array™ IFC by Fluidigm, San Francisco, California, USA) was performed as a duplicate experiment using 15 ng DNA from wolf intestinal contents and the 15 primer sets (Supplementary Table S2). As the assay is limited to 48 wells, we decided to use 43 wolf samples and included 5 quality controls into each run. All amplification and barcoding PCR steps, as well as library preparation steps were carried out according to the manufacturer's user guide (Access Array™ System for Illumina Sequencing Systems, Chapter 6, Fluidigm, San Francisco, California, USA). After running the 48.48 Access Array IFC, we used a 10-fold dilution of the harvested PCR products to perform the barcoding step using the Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) (Fluidigm, San Francisco, California, USA). Post-PCR quality control steps included amplicon quality and length check using the 2200 TapeStation Instrument with D1000 ScreenTapes and D1000 Reagents (Agilent Technologies, Santa Clara, California, USA). Afterwards, PCR fragments between 400 and 1000 bp were purified by PippinPrep using the 1.5% agarose DNA gel cassettes (Sage Science Inc., Beverly, Massachusetts, USA). Then, we pooled the samples and authorized a next-generation sequencing service using the MiSeq Reagent Kit v3 on the MiSeq sequencing system (Illumina, San Diego, California, USA).

Bioinformatics. In order to identify the isolated macroparasites from wolves and ungulates at the species level, we merged the Sanger sequenced forward and reverse reads using the programme SeqMan implemented in Lasergene (DNASTAR Inc., Madison, Wisconsin, USA). Additionally, sequences and the corresponding electropherograms were verified by eye, and sequences corrected manually where necessary. Subsequently, we searched for these sequences in the GenBank nucleotide collection from the National Center for Biotechnology Information (NCBI) using BLAST®[89] and stored the best hit species and the alternative species as a table (Supplementary Table S1). If results were not distinctly clear, we used further information of alternative genetic markers, helminth morphology, and organ of isolation to determine the most likely species.

In order to identify gastrointestinal microparasites from the Illumina metabarcoding data set, we first stratified sequencing reads by amplicon searching for fully identical matches to target specific primer pairs, starting exactly at the first sequence position (behind the removed adapter sequences) in both forward and reverse reads. This resulted in two types of sequence data: (1) Amplicons shorter than 500 bp with overlapping forward and reverse reads. Those were merged using FLASH version 1.2.8[90]. (2) Amplicons larger than 500 bp without forward and reverse read overlap (because of Illumina MiSeq Reagent Kit v3 maximum read length of 300 bp) were not merged, but quality trimmed with Trimmomatic version 0.36[91]. We searched remaining reads in an apicomplexan subset of the NCBI nucleotide database using BLAST®[89]. Only hits with a biunique best bit score to one species were further processed and we applied a criterion of 98% identity for the whole length of the query to assign species names. Additionally, a minimum hit length criterion of 200 bp for trimmed reads and 300 bp for merged reads was applied. Thus, our OTUs can be considered clusters of at least 98% sequence identity with respective database sequences over the whole amplicon.

Statistics – wolf genetics. Bayesian population clustering implemented in Structure software version 2.3.4[92] was used to test for population origin and potential domestic dog introgression of the 53 wolves. Genotypes of our wolf samples were run together with a set of randomly picked 22 wolf genotypes collected during the German state-based genetic wolf monitoring, reference genotypes from 39 domestic dogs, 16 wolves from the Baltic[18], 15 wolves from the Carpathian[18], and 16 wolves from the Alpine region[18], available from our internal genetic reference database for German wolf monitoring (www.wildtiergenetik.de). Ten independent runs were performed with a K from 2 to 8, a burn-in of 200,000 and 500,000 Markov chain Monte Carlo iterations. We applied an admixture model with correlated allele frequencies. The most probable number of populations was determined based on the second order rate of change of the likelihood[93] using the web-based programme Structure Harvester version 0.6.94[94]. To compute the optimal assignment to the individual clusters for every individual, the cluster output from the independent runs was permuted by Clumpp version 1.1.2[95] using the 'Greedy' algorithm for aligning replicates.

To reconstruct genetic relatedness we compared all individual genotypes to our internal wolf reference database with >350 individual wolf genotypes, covering most German packs. Genotypes that could be assigned to packs in Germany were considered 'native', whereas those with no first-order relationship to a known German pack were considered to be 'immigrants'. Reconstruction of genetic relatedness was done manually by direct genotype comparison, occasionally supported by use of Coancestry software version 1.0.1.5[96]. Individual heterozygosity was calculated in GenAlEx version 6.5[97].

Statistics - parasite diversity. In order to investigate host-parasite interactions, we calculated species richness as the number of endoparasite species, and species diversity using the Shannon index to account for the number of species and their abundance in each individual wolf. We chose to analyse species richness because our methodological approach allows us to extract this information from both presented datasets on helminths and *Sarcocystis*. Moreover, being the most commonly used measure of biodiversity[98], species richness is easy to compare with available wolf parasite literature[10,38,99,100] (Supplementary Table S3). Still, the deductions it allows are rather limited to environmental/geographical information in a sense of "parasite availability in a certain habitat". In this study, we indirectly address such questions by analysing the effect of 'wolf population size' and 'wolf geographic origin'. However, we also included the Shannon index into our analyses, as it accounts for heterogeneities within the parasite community that might potentially be driven by individual host characteristics such as immune capacities which we indirectly intend to correlate by investigating the effect of e. g. 'wolf genetic heterozygosity' or 'wolf age'.

The Shannon index was calculated only for helminths but not protozoan parasites because quantitative measures of *Sarcocystis* presence were not available. Even for helminths, an accurate count of cestodes was not possible in the case of 22 wolf carcasses as they were recovered in an advanced stage of decomposition and the retrieved tapeworms were often highly rotten and fragile. We therefore proceeded as follows: For 51 wolves, *Taenia* spp. abundance could be classified into the three categories 'no', 'low' and 'high' abundance during dissection. We then calculated the means for each category from those wolves where a count was possible and used these as a quantitative estimate of *Taenia* spp. abundance for the 22 wolves with missing *Taenia* spp. count data to calculate a Shannon diversity index for them.

We tested the influence of wolf sex, age, microsatellite heterozygosity, geographic origin of the individual, and wolf population size as defined in Table 3 on helminth and *Sarcocystis* species richness and helminth diversity using general linear models, and *Taenia* spp. abundance using a multinomial logistic regression. In preliminary exploratory analyses we had checked for but found no effect of body mass and carcass recovery location and therefore excluded both predictors from the final analysis. We also excluded year of carcass finding as a high general variance inflation factor (GVIF = 31.97, df = 5) indicated strong collinearity with the predictor population size. For the multinomial logistic regression we report the global summary of the effect of each predictor on the probability of occurrence of each *Taenia* abundance category. We tested the effect of wolf presence on ungulate

Variable in statistic model	Explanation	Units
A) helminth species richness	count of genetically confirmed helminth species per wolf	continuous data [number of species]
B) helminth diversity	diversity of helminths per wolf $H' = -\Sigma_i p \times lnpi$ mit $p_i = \frac{ni}{N}$	continuous data [Shannon index]
C) *Taenia* spp. abundance category	estimate of *Taenia* spp. abundance documented during dissection per wolf	categorical data ('none', 'low', 'high' abundance)
D) *Sarcocystis* species richness	count of genetically confirmed *Sarcocystis* species per wolf	continuous data [number of species]
E) metacestode infection status	presence of *Taenia* spp. cysts in ungulates	categorical data ('infected', 'not infected')
F) age	wolf age classed in ecologically relevant and commonly used categories: 0–12 months: 'pup'; >12 months – 24 months: 'yearling'; >24 months: 'adult'[110]	categorical data ('pup', 'yearling', 'adult')
G) heterozygosity	individual heterozygosity as proportion of heterozygous loci (nH) and analysed loci (nL) of microsatellite ($H_{indiv} = nH/nL$)	continuous data (0–1) [-]
H) geographic origin	genetic affiliation to a known German pack ('native') or unknown pack ('immigrant')	categorical data ('immigrant', 'native')
I) population size	annually recorded number of reproducing wolf packs in Germany	continuous data [number of packs]
J) sex	wolf sex determined by dissection	categorical data ('male', 'female')
K) ungulate species	ungulate species known to be preyed on by wolves in Germany	categorical data ('roe deer', 'red deer', 'fallow deer', 'wild boar')
L) study area	ungulate sample collection sites depending on permanent wolf presence or absence	categorical data ('present', 'absent')
M) metacestode species	Taenia species determined by PCR and sequencing isolated from ungulates	categorical data ('*T. krabbei*', '*T. hydatigena*')

Table 3. Response (A–E) and predictor variables used in statistical models regarding wolves (F–J) and ungulates (K–M).

metacestode infection status using a general linear model and added ungulate species and cestode species as predictors to control for potential species-specific differences (definition of variables see Table 3).

For each multinomial logistic regression, we used log-likelihood ratio tests and information criteria, the Akaike Information Criterion (AIC) and the quasi-likelihood Information Criterion (AICqh) introduced by Hannan and Quinn[101], to check whether the full model was superior to an intercept-only or a reduced model. Models were considered similar, if differences in AIC were less than 2.5, and preferable, if the difference exceeded 6.0[102]. We also report AICqh values, since they can be of interest in the case of substantial dispersion of data. The significance of each predictor variable was assessed as the marginal contribution of each parameter to the full model by subtracting from the full model the log-likelihood of a second model with each specific predictor removed and testing the difference against a chi-square distribution with the appropriate degrees of freedoms (see refs 103 and 104).

The significance threshold of tests was fixed at 5% and all tests were two-tailed. Statistical analyses were performed in Systat 13 (Systat Software Inc., Richmond, VA, USA) and R version 3.2.1[105]. The Shannon index was calculated using R package *vegan* version 2.3-0[106]. Possible co-linearity of predictor variables was tested with R package *car* version 2.0-26[107]. Multinomial logistic regression was performed in Systat 13. Overall likelihood ratio of the general linear models and multinomial logistic regression was tested using R package *lmtest* version 0.9–34[108]. Pairwise post-hoc comparison was performed with R package *mulcomp* version 1.4–5[109].

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Author Contributions

This study was designed by O.K. and supplemented by H.H. and I.L. O.K., C.A.S., I.R., G.K. and I.L. collected the carcasses. O.K., C.A.S. and I.L. dissected the carcasses. I.R., G.K., C.N., V.H. and A.J. assigned the genetic background of wolves based on monitoring and genetic data. E.H. and I.L. performed molecular parasite analyses. I.H., E.H. and I.L. analysed sequencing datasets. H.H. and I.L. did statistical analyses. H.H., C.N., A.J. and I.L. wrote the manuscript. All authors read and commented on the manuscript.

Additional Information

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Supplementary Material

Population expansion and individual age affect endoparasite richness and diversity in a recolonising large carnivore population

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Supplementary Information

Population expansion and individual age affect endoparasite richness and diversity in a recolonising large carnivore population

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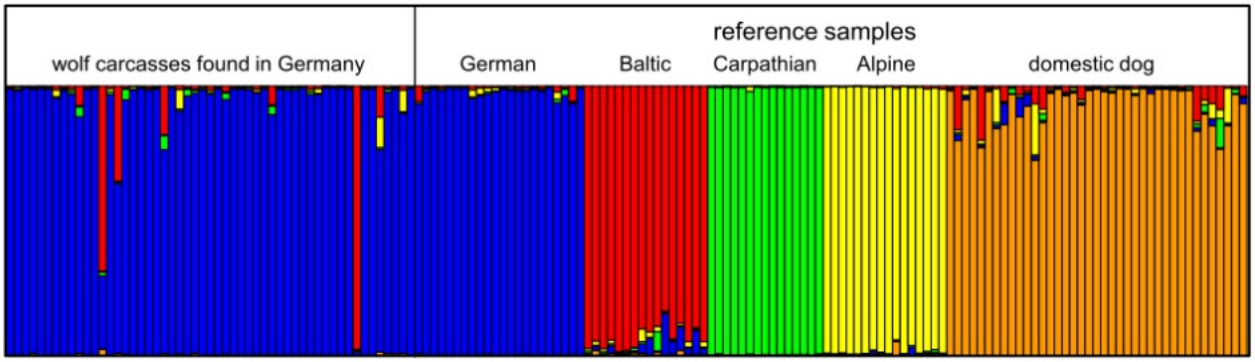
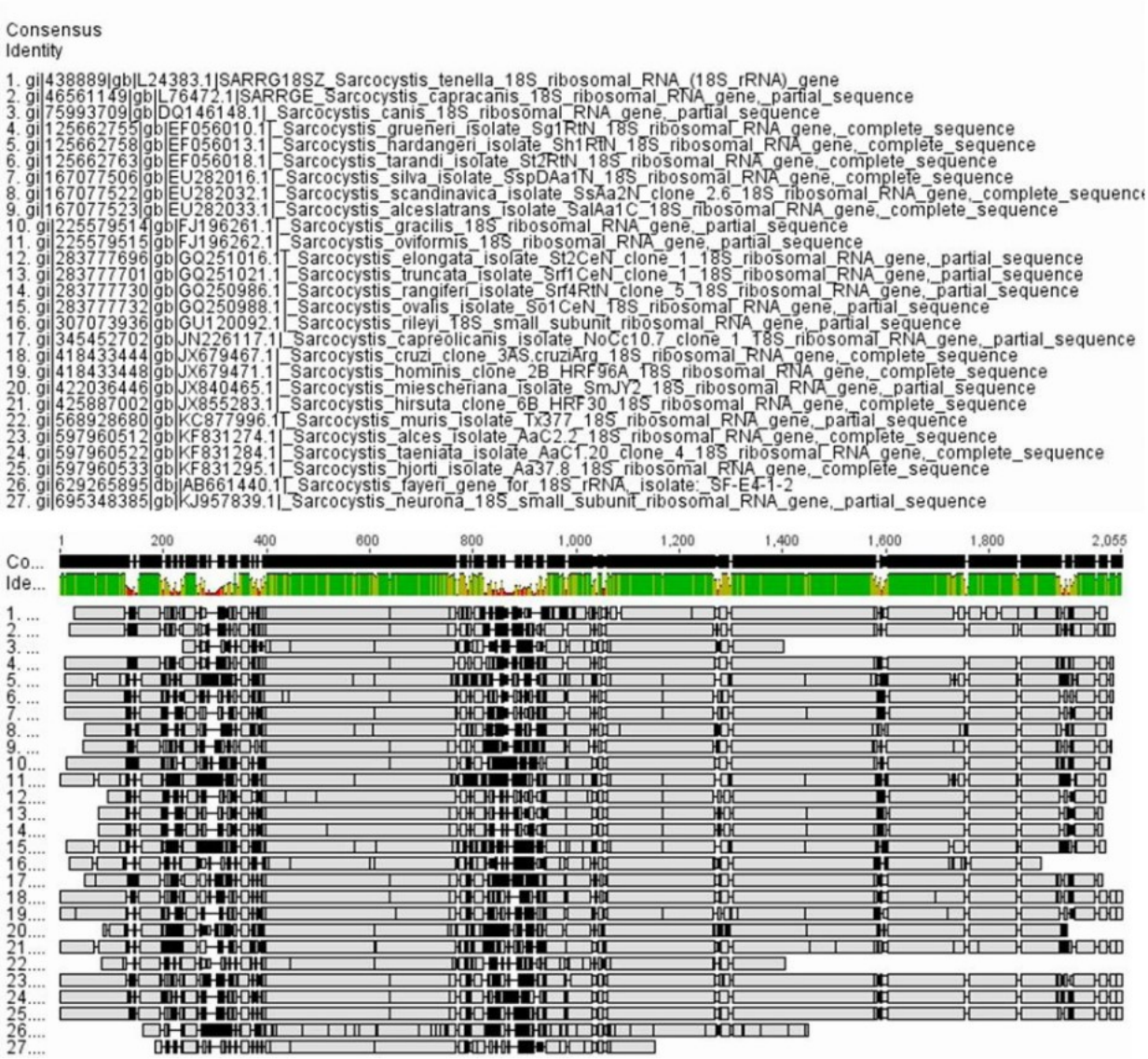


Supplementary Figure S1: Bayesian clustering of 53 wolf carcasses found in Germany in comparison to reference wolf genotypes from Germany (blue), the Baltic region (red), the Carpathians (green), the Alpine region (yellow), and domestic dogs (orange). Each individual is represented by a vertical line divided into five coloured segments that represent the individual's estimated membership fractions in each cluster (K=5).



Supplementary Figure S2: The *Sarcocystis* spp. 18S rRNA consensus sequence used for the amplicon design in this study is based on the alignment of 27 different mammalian *Sarcocystis* species.

Supplementary Table S1 Genetic identification of wolf helminths was performed for a subset of parasites. Helminth species were determined based on cox1 and 18S rRNA sequences which were searched in the NCBI nucleotide database. If there was no distinct best hit species, the search was either repeated using an alternative marker gene or morphological indicators were used to determine the species. *Trichinella* spp. were identified by the National Reference Laboratory for *Trichinella* and are not included in this table.

This table is provided as separate online csv file

Supplementary Table S2 Target-specific primers for the amplification of the variable regions of the *Sarcocystis* spp. 18S rRNA gene. *designed for this study

#	primer name	sequence (5'-3')	source	primer position	~product size [bp]
1	G18S4	GCTTGTCTCAAAGATTAAGCCN	1	forward	377
	Euk360_CR	TCTCAKGCKCCYTCTCCG	2	reverse	
2	G18S4	GCTTGTCTCAAAGATTAAGCCN	1	forward	437
	SSU22R	GCCTGCTGCCTTCCTTGG	1	reverse	
3	proti15	TGCCAGTAGTCATATGCTTGTYT	*	forward	378
	proti440_R	CAGGCYCSCTCTCCGGA	*	reverse	
4	proti15	TGCCAGTAGTCATATGCTTGTYT	*	forward	421
	proti482_R	KTTSCGCGCCTGCTGCC	*	reverse	
5	proti15	TGCCAGTAGTCATATGCTTGTYT	*	forward	572
	proti643_R	GAGCTGGAATTACCGCGG	*	reverse	
6	proti89	CKGCGVATGGCTCATTAMAWC	*	forward	485
	proti628_R	GGSTGCTGGCACCAGAC	*	reverse	
7	proti89	CKGCGVATGGCTCATTAMAWC	*	forward	501
	proti643_R	GAGCTGGAATTACCGCGG	*	reverse	
8	F-556	CAGCAGCCGCGGTAATTCC	3	forward	383
	Nem_0425_4R	ARACATTCTTGGCAAATGCYTTC	*	reverse	
9	18S rRNA#1	TGGTGCCAGCAGCCGC	4	forward	577
	Mach2Gen	TCCGTCAATTTCTTTAAGTTTCAG	*	reverse	
10	18S rRNA#1	TGGTGCCAGCAGCCGC	4	forward	389
	Nem_0425_4R	ARACATTCTTGGCAAATGCYTTC	*	reverse	
11	18S rRNA#1	TGGTGCCAGCAGCCGC	4	forward	391
	Nem_0425_2	GAARACATTCTTGGCAAATGCY	*	reverse	
12	18S r DNA549-566	GAGGGCAAGTCTGGTGCC	5	forward	637
	R-1200	CCCGTGTTGAGTCAAATTAAGC	3	reverse	
13	proti628	GTCTGGTGCCAGCASCC	*	forward	604
	18S rDNA 1141-1161	GGTGCCCTTCCGTCAATTC	5	reverse	
14	18S r RNA549-566	GAGGGCAAGTCTGGTGCC	5	forward	588
	Mach2Gen	TCCGTCAATTTCTTTAAGTTTCAG	*	reverse	
15	18S r DNA573-589	GCCGCGGTAATTCCAGCT	5	forward	378
	Nem_0425_4R	ARACATTCTTGGCAAATGCYTTC	*	reverse	

Supplementary Table S3 Comparison of helminth species richness (HSR) and helminth prevalence (%) between wolves from the CEL wolf population (this study) and the Baltic wolf population. Red p-values indicate significant differences.

	this study	Shimalov *et al.*[6]			Bagrade *et al.*[7]			Szczęsna-Staśkiewicz[8]			Moks *et al.*[9]		
	Germany	**Belarus**	**p-value**	**t**	**Latvia**	**p-value**	**t**	**Poland**	**p-value**	**t**	**Estonia**	**p-value**	**t**
n wolves	53	52			34			18			26		
HSR	13	24			17			17			13		
mean $HSR_{individual}$	2.57±0.26	NA			4.6±0.35	**0.002**	-3.422	4.6±0.54	**$2.024e^{-5}$**	-4.5796	NA		
max. $HSR_{individual}$	8	NA			8			9			NA		
Taenia spp richness	2	5			7			5			5		
	%	**%**	**p-value**	**X^2**	**%**	**p-value**	**X^2**	**%**	**p-value**	**X^2**	**%**	**p-value**	**X^2**
C. plica	25	14	0.232	1.429	41	0.161	1.961	NA	NA	NA	0	**0.015**	5.954
C. vulpis	25	8	**0.038**	4.312	9	0.118	2.438	17	0.716	0.132	0	**0.015**	5.954
C. aerophila	15	0	**0.011**	6.487	36	0.054	3.701	17	1.000	$3137e^{-30}$	NA	NA	NA
U. stenocephala	11	15	0.745	0.106	41	**0.003**	8.810	72	**$2.196e^{-6}$**	22.415	77	**$2.46e^{-8}$**	31.093
T. canis	11	21	0.270	1.126	6	0.634	0.227	11	1.000	$5.480e^{-30}$	8	0.916	0.011
T. leonina	4	14	0.154	2.029	0	0.680	0.170	6	1.000	$5.304e^{-30}$	8	0.841	0.040
Trichinella spp.	4	19	0.052	3.785	70	**$5.103e^{-6}$**	20.798	33	0.371	0.800	50	**$3.887e^{-6}$**	21.320
T. krabbei	77	8	**$2.298e^{-12}$**	49.212	9	**$1.756e^{-9}$**	36.227	NA	NA	NA	15	**$6.171e^{-7}$**	24.858
T. hydatigena	15	27	0.212	1.561	41	**0.013**	6.141	56	**0.002**	9.584	12	0.934	0.007
M. litteratus	9	0	0.070	3.281	0	0.170	1.884	NA	NA	NA	0	0.260	1.269
E. multilocularis	2	0	1.000	$1.262e^{-29}$	6	0.693	0.156	0	1.000	$4.819e^{-29}$	0	1.000	$1.478e^{-29}$
A. alata	53	17	**0.001**	12.998	85	**0.004**	8.279	33	0.247	1.340	89	**0.004**	8.818
total	89	80	0.392	0.733	100	0.110	2.559	100	0.317	1.003	100	0.183	1.777

Supplementary Table S4 Comparison of cysticercoses prevalence (%) between ungulates from Germany and Eastern Europe. Red p-values indicate significant differences.

		this study	Murai *et al.*[10]			Kuzmina *et al.*[11]			Onac *et al.*[12]		
host species		**Germany**	**Hungary**			**Ukraine**			**Romania**		
n roe deer (*Capreolus capreolus*)		177	345			92			NA		
n red deer (*Cervus elaphus*)		102	70			NA			21		
n wild boar (*Sus scrofa*)		126	44			NA			267		
n fallow deer (*Dama dama*)		35	35			NA			NA		
	cestode species	**%**	**%**	**p-value**	X^2	**%**	**p-value**	X^2	**%**	**p-value**	X^2
roe deer	*T. krabbei*	0	33	**$8.64e^{-14}$**	55.654	0	0.177	1.825	NA	NA	NA
	T. hydatigena	2	18	**$2.89e^{-07}$**	26.325	2	1	$1.64e^{-29}$	NA	NA	NA
red deer	*T. krabbei*	4	19	**0.004**	8.4254	NA	NA	NA	NA	NA	NA
	T. hydatigena	5	36	**$4.98e^{-07}$**	25.271	NA	NA	NA	NA	NA	NA
	E. granulosus	0	0	NA	NA	NA	NA	NA	12	**0.0282**	4.818
wild boar	*T. hydatigena*	4	43	**$6.42e^{-10}$**	38.189	NA	NA	NA	NA	NA	NA
	E. granulosus	0	36	**$9.63e^{-12}$**	46.403	NA	NA	NA	10	**$8.55e^{-05}$**	15.432
fallow deer	*T. krabbei*	0	6	0.4731	0.51471	NA	NA	NA	NA	NA	NA
	T. hydatigena	0	20	**0.017**	5.7143	NA	NA	NA	NA	NA	NA

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Chapter II

Recolonizing grey wolves increase parasite infection risk in their prey

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Recolonizing grey wolves increase parasite infection risk in their prey

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Key Words

apicomplexa, coccidia, endoparasites, epidemiology, metabarcoding, protozoa, *Sarcocystis*, ungulates

Abstract

The recent recolonization of Central Europe by the European grey wolf (*Canis lupus*) provides an opportunity to study the dynamics of parasite transmission for cases when a definitive host returns after a phase of local extinction. It resembles a "removal experiment" and allows for the study of parasite epidemiology in wild ungulates in comparable ecological settings in the presence or absence of wolves as an apex predator.

We investigated whether a newly established wolf population increased the prevalence of those parasites in its ungulate prey which could serve as intermediate hosts, whether some parasite species are particularly well adapted to wolves, and what the basis for such adaptations might be.

We recorded ungulate *Sarcocystis* prevalence and diversity in wolves and their ungulate prey in study sites with and without permanent wolf presence in Germany using microscopy and DNA metabarcoding.

Sarcocystis prevalence in red deer (*Cervus elaphus*) was significantly higher in wolf areas (79.7%) than in control areas (26.3%) but not in roe deer (*Capreolus capreolus*) (97.2% vs. 90.4%) or wild boar (*Sus scrofa*) (82.8% vs. 64.9%). Of 11 *Sarcocystis* species, *S. taeniata* and *S. grueneri* occurred more often in wolves than expected from the *Sarcocystis* infection patterns of ungulate prey. Both *Sarcocystis* species showed a higher increase in prevalence in ungulates in wolf areas than other *Sarcocystis* species, suggesting that they are particularly well adapted to wolves, and are examples of 'wolf specialists'. *Sarcocystis* species richness in wolves was significantly higher in pups than in adults. 'Wolf specialists' persisted during wolf maturation.

The results of this study demonstrate that (1) predator–prey interactions influence parasite prevalence, if both predator and prey are part of the parasite life cycle, (2) mesopredators do not necessarily replace the apex predator in parasite transmission dynamics for particular parasites of which the apex predator is the definitive host, even if meso– and apex predators were from the same taxonomic family (here: Canidae, such as red foxes *Vulpes vulpes*), and (3) age–dependent maturation of immune competence contributes to the control of protozoan infection in wolves.

Introduction

Apex predators play a critical role in shaping food webs (Estes et al., 2011). When a predator is a definitive host of a parasite and disappears from its habitat, it may leave a gap in the food web. Potential consequences of such disappearances for parasite–host relationships are still poorly understood. Even for well–studied temperate ecosystems, where the grey wolf (*Canis lupus*) is an apex predator and ungulates are its main prey, little is known about the parasitological consequences of a transient wolf removal/extinction (East et al., 2011). Transmission dynamics of trophically transmitted pathogens and parasites that are well adapted to a specific host might change. Parasites could either adapt to alternative hosts or disappear over time (Farrell et al., 2015), which we call the 'host flexibility' hypothesis and the 'fading out' hypothesis, respectively. Wolves are definitive hosts for a wide range of endoparasites (Craig and Craig, 2005), but little is known about their possible influence on parasite prevalence in their ungulate prey if these serve as intermediate hosts, as in the case of helminths or apicomplexa (Lesniak et al., 2017). In particular, it is unclear whether infection risk increases when a definitive host returns after being absent from a specific area for some time, and how infection risk varies amongst different prey species. It is also unclear which factors control parasite etiopathology and whether these factors favour specialization of parasites for specific hosts. The process of the current wolf recolonization of Central Europe provides an excellent opportunity to investigate parasite transmission dynamics in a predator–prey system as the same prey species can be examined in the presence and absence of the predator in the same habitat type.

Grey wolves have recolonized parts of Germany and Western Poland since the year 2000 after an absence of nearly 100 years (Reinhardt, 2015). In Germany, the first wolf packs settled in the eastern state of Saxony. Since then, the population spread in a northwesterly direction. By 2015, almost 40 packs were recognized in Germany and approximately 70 packs were identified within the entire Central European lowland (CEL) wolf population (Reinhardt, 2015). For this population, population structure and dynamics (Ansorge et al., 2010; Nowak and Mysłajek, 2016), infectious diseases and causes of death (Szentiks, 2016), dispersal (Andersen et al., 2015; Reinhardt, 2015) and feeding habits (Nowak et al., 2011; Wagner et al., 2012) have been investigated since recolonization started. The diet analyses demonstrated that red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*) are the three main prey species of the resident wolf population. They may therefore serve as potential intermediate hosts of wolf–transmitted endoparasites such as helminth metacestodes

of *Taenia* spp. (Lesniak et al., 2017) or *Echinococcus* spp. (Onac et al., 2013), and protozoan cysts of *Neospora* spp. (Rocchigiani et al., 2016) or *Sarcocystis* spp. (Kolenda et al., 2014).

Host–pathogen interactions and epidemiology are well understood in apicomplexan taxa such as *Toxoplasma* – a parasite occurring within a domestic and a sylvatic cycle with zoonotic potential (Shaapan, 2016). However, the links between *Sarcocystis* of wild intermediate and definitive hosts are currently unclear, and the prevalence and distribution of *Sarcocystis* species in ungulates, and the potential impact of the removal and then the return of the apex predator are at present unknown.

Free–ranging wolves from the CEL population host at least 12 different *Sarcocystis* species (Lesniak et al., 2017). The genus *Sarcocystis* has an obligatory two–host life cycle involving (partially) carnivorous definitive hosts and a broad range of intermediate hosts such as reptiles, birds or mammals (Dubey and Lindsay, 2006; Munday et al., 1979). *Sarcocystis* are known to be more host–specific in terms of their intermediate than definitive host range, although the current state of knowledge is far from complete, as new *Sarcocystis* species and new hosts continue to be described (Dahlgren, 2010; Gjerde, 2014b). *Sarcocystis* sexually reproduce in the intestines of their definitive host and form (sarco–)cysts during the asexual developmental phase within their intermediate host's muscular or nervous tissue (Dubey, 2015; Dubey and Lindsay, 2006). During the early infection phase, pathogenic species may cause clinical symptoms such as weight loss, anaemia, fever, and abortion in pregnant intermediate hosts (Buxton, 1998; Dubey and Lindsay, 2006) – otherwise sarcocystosis usually has an asymptomatic etiopathology and minor impact on its host.

In this study, we investigated the prevalence of *Sarcocystis* in ungulates and wolves. Under the 'fading out' hypothesis returning wolves would re–import temporarily faded parasites, thereby increasing parasite infection risk in ungulate intermediate hosts. It assumes that at least some *Sarcocystis* species are 'wolf specialists' and are too host–specific to use alternative hosts as definitive hosts. If an increase in *Sarcocystis* prevalence occurred, it should therefore be driven by 'wolf–specialized' parasites, i.e. *Sarcocystis* species that are particularly well adapted to wolves. 'Wolf–specialized' parasites should then be overrepresented in wolves and show the strongest prevalence increase in ungulate intermediate hosts in wolf inhabited areas, and there should be a 'mismatch' in relative parasite frequencies between wolves and their prey. Under the 'host flexibility' hypothesis returning wolves serve as an additional definitive host for endemic parasites also spread by other carnivores (spillback) which had resumed the function of alternative hosts (Kelly et al., 2009; Moré et al., 2016). In this case we do not expect to find *Sarcocystis* species that should

be considered ‘wolf specialists’. Without ‘wolf–specialized’ parasites, relative parasite frequencies in ungulate prey species and in wolves would match as wolves would be non–selectively infected with the *Sarcocystis* they consume.

The ‘fading out’ hypothesis also predicts that if parasites are particularly well adapted to a specific host (‘wolf specialists’), we would expect them to prevent clearance by the host immune system. Young wolves are likely to have a weaker immunity towards apicomplexan *Sarcocystis* parasites than older animals. Younger wolves would therefore be expected to exhibit a higher *Sarcocystis* species richness than adult wolves. In adults an improved immune competence should allow them to clear parasites that might infect pups, except for ‘wolf specialists’ which might employ adaptations that allow them to circumvent the host immune system and persist in older individuals. If no age–related immune processes controlled parasite resistance in wolves, wolves of all ages should host the same *Sarcocystis* community since each pack member is exposed to the same *Sarcocystis* species when they share an infected kill.

Material and methods

Sample collection

Ungulate muscle tissue samples (tongue, diaphragm, heart) originating from wolf territories (WT, German federal states of Brandenburg and Saxony, 50°10′–53°33′ N and 11°14′–15°2′ E) or the control area (CA, German federal state of Schleswig–Holstein, 53°20′–54°55′ N and 8°36′–11°7′ E) where no territorial wolves occurred during the sampling period, were collected between November 2012 and December 2014. Red deer (n_{WT} = 75, n_{CA} = 18), roe deer (n_{WT} = 99, n_{CA} = 72), and wild boar (n_{WT} = 83, n_{CA} = 37), shot during hunts and intended for food consumption, were screened. Ungulate age classes (juveniles, subadults, adults) were estimated by hunters.

Forty three wolf carcasses collected between 2007 and 2014 were examined for the presence of intestinal *Sarcocystis* spp. Wolves were collected as roadkills or as confiscated poached animals originating from five federal states in northern and eastern Germany (50°10′–54°54′ N and 6°41′–15°2′ E). Wolf age classes (pup, yearling, adult) were determined as previously described (Lesniak et al., 2017).

Ungulate muscle histology

Fresh ungulate muscle tissue samples were fixed in 4% formalin and then embedded in paraffin blocks. Paraffin–embedded blocks were sectioned at 3 µm, stained with haematoxylin–eosin and examined by light microscopy to determine *Sarcocystis* sp. presence.

DNA extraction, PCR and library preparation

DNA from ungulate specimen was isolated using the Invisorb® Spin DNA Extraction Kit (STRATEC Molecular, Berlin, Germany) according to the manufacturer's instructions. DNA eluates (tongue, diaphragm, heart) were pooled per individual for subsequent PCR screening. *Sarcocystis* 18S rRNA gene amplification was performed using a set of three primer pairs (proti15F: 5′–TGCCAGTAGTCATATGCTTGTYT–3′, proti440R: 5′–CAGGCYCSCTCTCCGGA–3′ (Lesniak et al., 2017), SarAF: 5′–CTGGTTGATCCTGCCAGTAG–3′, SarAR: 5′–TTCCCATCATTCCAATCACT–3′, SarBF: 5′–GGGAGGTAGTGACAAGAAATAACAA–3′, SarBR: 5′–GGCAAATGCTTTCGCAGTAG–3′ (both primer pairs taken from Kutkiene *et al*. 2010) which anneal within conserved gene regions. Each forward and reverse oligonucleotide contained the Fluidigm–specific common sequence tag CS1 (5′–ACACTGACGACATGGTTCTACA–[TS–For]–3′) or CS2 (5′–TACGGTAGCAGAGACTTGGTCT–[TS–Rev]–3′) to enable subsequent barcoding of the generated PCR products (Fluidigm, San Francisco, California, USA). PCRs and metabarcoding of *Sarcocystis*–positive sample pools (roe deer: $n_{WT} = 21$, $n_{CA} = 10$; red deer: $n_{WT} = 10$, $n_{CA} = 4$; wild boar: $n_{WT} = 20$, $n_{CA} = 10$) were conducted as described in Lesniak et al. (2017) ('unpublished data').

Wolf intestinal contents were extracted and processed using the amplicon sequencing approach described in Lesniak *et al*., 2017.

Bioinformatics

Ungulate *Sarcocystis* sequences were sorted into operational taxonomic units (OTUs) using USEARCH (Edgar, 2010; Edgar, 2013), which then were assigned to *Sarcocystis* species as described in Lesniak et al. (2017) ('unpublished data'). Briefly, OTUs were assigned to *Sarcocystis* species sequences from a custom database ('unpublished data') using BLAST® (blastn, Altschul *et al*., 1990) with an identity threshold of 98%. Only hits with a biunique best bit score for one species were collected in a table including the respective *Sarcocystis* species, OTU, amplicon, and sample name. Due to technical limitations, the

proti15_proti440_R1 and proti15_proti440_R2 datasets were split by ungulate species. When describing and discussion our results, the term 'species' instead of 'OTUs' will be used for simplicity, although we are aware of the technical limitations of our approach to determine species, as previously discussed (Lesniak et al., 2017).

The wolf metabarcoding dataset was analysed as previously described (Lesniak et al., 2017).

Statistical analyses

The data on *Sarcocystis* presence in ungulate tissues collected using light microscopy were used to test the prediction that ungulate *Sarcocystis* prevalence was higher in areas affected by wolf recolonization. Generalized linear models (GLMs) were fitted separately for each ungulate species with binomially distributed errors, in which the response variable was the record of '*Sarcocystis* spp. infection' (binary: infected or not infected). All models included the predictors 'wolf presence' (binary: absent or present) and ungulate age (categorical: juveniles, subadults, adults).

In order to interpret the goodness of fit of each model in comparison to the null model, overall likelihood ratio tests were performed with the R package *lmtest* v0.9-34 (Zeileis, 2002), and model predictors were tested for collinearity using the R package *car* v2.6-26 (Fox, 2011).

In order to test whether some parasites occurred more frequently in wolves than expected, considering the frequencies of these parasites in the prey species, we first estimated expected frequencies in wolves (f_{exp}), taking into account that prey species are not necessarily consumed in equal proportions. The expected frequencies were then compared to observed parasite frequencies (f_{obs}) from wolves collected in this study (Table 1) to (1) test whether a 'mismatch' could be detected in terms of a significant difference in both distributions, and (2) identify which *Sarcocystis* spp., if any, were overrepresented in wolves.

Two approaches (A & B) were used to estimate expected parasite frequencies f_{exp} in wolves. In approach A, the conventional approach, f_{exp} was estimated based on the published information on wolf diet (Wagner et al., 2012) to derive the proportion of each prey species in the diet (feeding proportion d_j) and information on relative parasite infection frequencies $p_{i,j}$ in ungulates obtained in this study. The observed relative frequencies $p_{i,j}$ of each *Sarcocystis* species i in ungulate species j in our sample are listed in Table 2. Based on published information on wolf diet, we used the following feeding proportions d_j: red deer: 0.22, roe

deer: 0.59 and wild boar: 0.19. Expected frequencies of *Sarcocystis* species *i* in wolves were then calculated as: $f_{exp,i} = \Sigma_j \, p_{i,j} \times d_j$ (eqn 1).

The conventional approach has the drawback that identified mismatches between observed and expected parasite infections in wolves could be an artefact of erroneously estimated feeding proportions d_j. Published information on the average wolf diet are not necessarily an accurate representation of individuals in the current study, and therefore d_j estimated in this way might be a poor representation of the diet of the wolves we analysed.

To account for this potential problem we also used a conservative approach (B) that aimed to minimize the chance of obtaining an erroneously elevated mismatch between observed and expected parasite frequencies in wolves. For this purpose, we indirectly estimated d_j from the observed parasite frequencies in wolves and their prey. Using an optimisation approach (*optim* function in R), the estimated d_j were those that generate expected parasite infection frequencies in eqn (1) that maximize the match between expected and observed parasite frequencies (which should minimize the risk of obtaining an erroneously elevated mismatch). Using the results from the optimisation approach, the match between estimated and observed parasite infection frequencies was compared with the χ^2 value of a Chi–squared test. As a result of this estimation we obtained the following feeding proportions d_j: 0.09 red deer, 0.87 roe deer, and 0.04 wild boar. These estimates were then used to calculate expected parasite frequencies using eqn (1), with results listed in Table 1.

The expected wolf *Sarcocystis* infection frequencies obtained from both approaches were used in two separate Chi–squared tests in order to check whether observed *Sarcocystis* spp. infection frequencies in wolves differed from expected probabilities. Subsequent post–hoc binomial tests were used to identify *Sarcocystis* species that were overrepresented or underrepresented in wolves and their p-values adjusted by applying the Benjamini–Hochberg procedure with a false discovery rate of 5% (Benjamini and Hochberg, 1995). Overrepresented *Sarcocystis* species were considered candidate species for ‘wolf specialists’, whereas all other species were termed ‘non–wolf specialists’. Using a Mann–Whitney–U test, we tested for each ungulate prey species whether ‘wolf–specialized’ sarcocysts had a higher increase in prevalence ($\delta_{prevalence}$) in wolf areas relative to control areas in comparison to other detected *Sarcocystis* spp.

Table 1: **Relative observed (f_{obs}) and expected (f_{exp}) *Sarcocystis* spp. frequencies in wolves (n = 43).** Expected infection probabilities (f_{exp}) are based on relative *Sarcocystis* spp. occurrence in ungulates (see Table 2) and the average proportion of ungulates in the wolf diet in Central Europe from the literature (approach A) and on estimated prey proportions in the wolf diet in this study (approach B), respectively. Significant p–values are highlighted in bold.

			Approach A		**Approach B**		**Interpretation**
***Sarcocystis* spp.**	**n_{obs}**	**f_{obs}**	**f_{exp}**	**p–value**	**f_{exp}**	**p–value**	
S. bovini	0	0.000	0.053	**0.036**	0.047	**0.036**	underrepresented (A+B)
S. capreolicanis	27	0.194	0.109	0.055	0.148	0.055	
S. elongata	0	0.000	0.014	0.073	0.005	0.127	
S. gracilis	28	0.201	0.116	0.055	0.175	0.109	
S. grueneri	25	0.179	0.086	**0.018**	0.097	**0.036**	overrepresented (A+B)
S. hjorti	3	0.022	0.037	0.091	0.012	0.073	
S. miescheriana	17	0.123	0.246	**0.018**	0.098	0.091	underrepresented (A)
S. silva	0	0.000	0.142	**0.018**	0.194	**0.018**	underrepresented (A+B)
S. taeniata	39	0.281	0.109	**0.018**	0.144	**0.018**	overrepresented (A+B)
S. tarandi	0	0.000	0.005	0.109	0.002	0.127	
S. truncata	0	0.000	0.083	**0.018**	0.081	**0.018**	underrepresented (A+B)

Table 2: **Relative *Sarcocystis* spp. frequencies (f_{inf}) in ungulates identified by metabarcoding of microscopically positive samples** (red deer: $n_{WT} = 10$, $n_{CA} = 4$; roe deer: $n_{WT} = 21$, $n_{CA} = 10$; wild boar: $n_{WT} = 20$, $n_{CA} = 10$).

***Sarcocystis* spp.**	**f_{inf} red deer**	**f_{inf} roe deer**	**f_{inf} wild boar**
S. bovini	0.128	0.042	0.000
S. capreolicanis	0.064	0.161	0.000
S. elongata	0.064	0.000	0.000
S. gracilis	0.000	0.196	0.000
S. grueneri	0.128	0.098	0.000
S. hjorti	0.170	0.000	0.000
S. miescheriana	0.085	0.063	1.000
S. silva	0.085	0.210	0.000
S. taeniata	0.085	0.154	0.000
S. tarandi	0.021	0.000	0.000
S. truncata	0.170	0.077	0.000

The wolf metabarcoding dataset was used to test the prediction that young wolves had a higher *Sarcocystis* species richness than adults, and that 'wolf–specialized' *Sarcocystis* species persist in the adult age class, whereas 'non–wolf specialists' fade out. Using a GLM with Poisson distributed errors, we investigated whether wolf *Sarcocystis* species richness (number of species, range 0 – 10) decreases with wolf age (categorical: pup, yearling, adult), while controlling for wolf population size using the number of wolf packs (range 3 – 31)

during the sampling period over eight years. Post–hoc tests between age categories were performed using the R package *multcomp* v1.4 – 5 (Hothorn et al., 2008). In a next step, we tested whether potential 'wolf–specialized' *Sarcocystis* are more likely to persist with increasing wolf age than 'non–wolf specialists' that should be cleared by immune response. To avoid multiple testing, we only applied this test to those wolf age categories identified as age categories showing a significant decrease in *Sarcocystis* species richness. Firstly, we calculated an expected value for the average prevalence change of 'wolf specialist' *Sarcocystis*. Secondly, we used a one–sample t–test to investigate whether this expected value deviated from the prevalence change of other species. We restricted this test to the three most common 'non–specialist' parasites that reached a minimum prevalence of 20% in wolves and that were detected in both wolves and wild ungulates.

Statistical analyses were performed in the statistical software R version 3.2.1 (R–Development–Core–Team, 2008).

Results

Ungulate Sarcocystis spp. infection status and prevalence

Microscopic examination revealed that *Sarcocystis* sp. prevalence in ungulates was consistently higher in wolf areas than in control areas (Figure 1, Table 2). The increase in *Sarcocystis* sp. prevalence in red deer was significant ($GLM_{red\ deer}$: $p < 0.001$; overall likelihood ratio test: $\chi^2 = 59.94$, $df = 4$, $n = 93$, $p < 0.001$). For roe deer and wild boar there was a non–significant trend of an increase in prevalence in ungulates in the wolf inhabited areas ($GLM_{roe\ deer}$: $p = 0.075$; overall likelihood ratio test: $\chi^2 = 19.903$, $df = 4$, $n = 171$, $p < 0.001$; $GLM_{wild\ boar}$: $p = 0.097$; overall likelihood ratio test: $\chi^2 = 9.224$, $df = 4$, $n = 120$, $p = 0.024$).

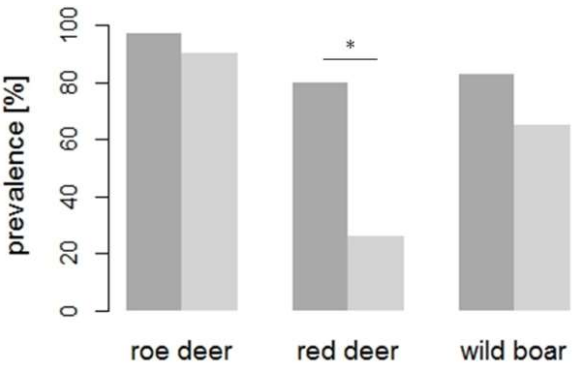


Figure 1: Observed *Sarcocystis* spp. prevalence in three ungulate prey species in relation to wolf presence in their habitat. *Sarcocystis* sp. prevalence was significantly higher in wolf areas (dark grey) than in control areas (light grey) in red deer ($n_{WT} = 75$, $n_{CA} = 18$, $p < 0.001$). A non–significant trend was found for roe deer ($n_{WT} = 99$, $n_{CA} = 72$, $p = 0.075$) and wild boar ($n_{WT} = 83$, $n_{CA} = 37$, $p = 0.097$).

Ungulate and wolf Sarcocystis communities

A metabarcoding approach was used to determine the *Sarcocystis* species spectrum in red deer, roe deer, and wild boar. For these ungulate hosts, 148 OTUs were assigned (1 – 66 OTUs/*Sarcocystis* species, mean = 14) which shared the highest sequence similarity with 11 *Sarcocystis* species, and 26 OTUs were identified which were considered to be undetermined *Sarcocystis* sp. (Suppl. Table S1). Based on GenBank entries, the OTUs were assigned to *S. bovini*, *S. capreolicanis*, *S. grueneri*, *S. miescheriana*, *S. silva*, *S. taeniata*, and *S. truncata* in red deer and roe deer, *S. elongata*, *S. hjorti* and *S. tarandi* were exclusively detected in red deer, and *S. gracilis* only occurred in roe deer (Suppl. Table S1). Wild boars were only infected with *S. miescheriana.*

In order to test which of the detected *Sarcocystis* spp. were 'wolf specialists', two approaches were used to calculate expected *Sarcocystis* frequencies in wolves. Under the conventional, more lenient approach A, the observed *Sarcocystis* spp. infection frequencies in wolves differed from the expected probabilities for several *Sarcocystis* species ($\chi^2 = 120.47$, $df = 10$, $p < 0.0001$). Post–hoc tests showed that *S. grueneri* and *S. taeniata* occurred significantly more often in wolves than expected (representing potential 'wolf specialists') whereas *S. bovini*, *S. miescheriana*, *S. silva,* and *S. truncata* occurred less often than expected (Figure 2, Table 1). Under the more conservative approach B, the observed *Sarcocystis* spp. infection frequencies in wolves also differed from expected probabilities ($\chi^2 = 78.067$, $df = 10$, $p < 0.0001$). Post–hoc tests showed that the same species, *S. grueneri* and *S. taeniata*, were overrepresented in wolves, suggesting they were 'wolf specialists', whereas *S. bovini*, *S. silva* and *S. truncata* occurred significantly less often than expected (Figure 3, Table 1).

We also tested in ungulates whether the increase in prevalence from control areas to wolf inhabited areas of 'wolf specialist' *Sarcocystis* was higher than the increase of the other *Sarcocystis* species. Consistent with the idea of *S. grueneri* and *S. taeniata* being 'wolf specialists' identified by approach A and B, their increase in prevalence from control area to wolf inhabited area was significantly higher than the increase of the other *Sarcocystis* species in both red deer and roe deer (red deer: U = 18, p = 0.044; roe deer: U = 18, p = 0.043).

Suppl. Table S1: **Number of OTUs per ungulate *Sarcocystis* species** (mean = 14 OTUs/species) – based on 18S rRNA sequences from 10 different amplicons analysed with USEARCH and number of all and unique 18S rRNA GenBank entries for each species.

species assignment	**n_{OTUs}**	**n 18S rRNA GenBank entries**	**n unique 18S rRNA GenBank entries**
S. bovini	8	26	26
S. capreolicanis	9	12	12
S. elongata	2	20	18
S. gracilis	5	14	7
S. grueneri	3	2	2
S. hjorti	5	6	4
S. miescheriana	66	49	26
S. silva	18	9	8
Sarcocystis sp.	26	NA	NA
S. taeniata	27	27	26
S. tarandi	1	22	22
S. truncata	4	20	20

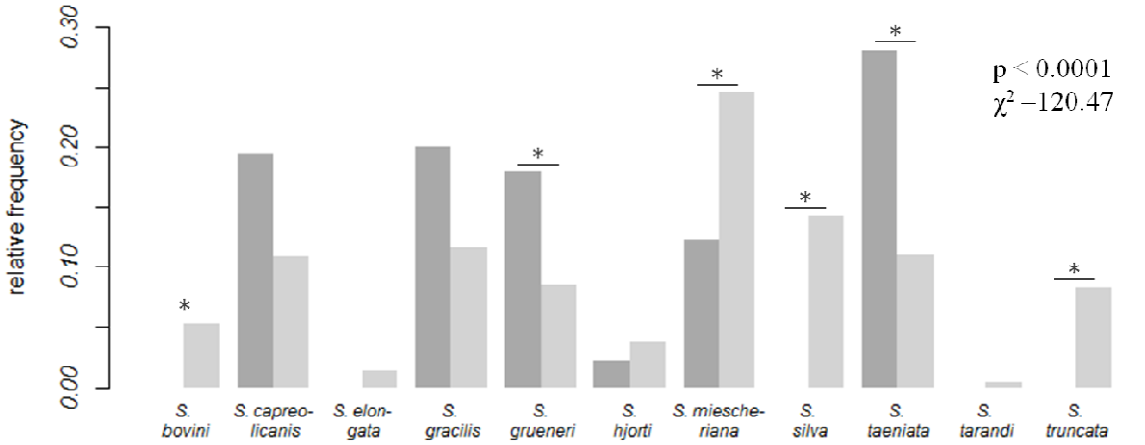


Figure 2: Relative observed (dark grey) *versus* relative expected (light grey) *Sarcocystis* spp. infection frequencies in wolves based on approach A. Expected values have been generated by counting genetically determined *Sarcocystis* spp. occurrences in red deer, roe deer and wild boar and by accounting for relative, normalized ungulate feeding proportions by wolves extracted from literature (Wagner et al., 2012). The general distribution of infection probabilities was significantly different between observed and expected values (Chi–squared test for given probabilities, $\chi^2 = 120.47$, df = 10, $p < 0.0001$). Binomial post–hoc tests showed that *S. grueneri* ($p = 0.018$) and *S. taeniata* ($p = 0.018$) were overrepresented in wolves, whereas *S. bovini* ($p = 0.036$), *S. miescheriana* ($p = 0.018$), *S. silva* ($p = 0.018$) and *S. truncata* ($p = 0.018$) were underrepresented.

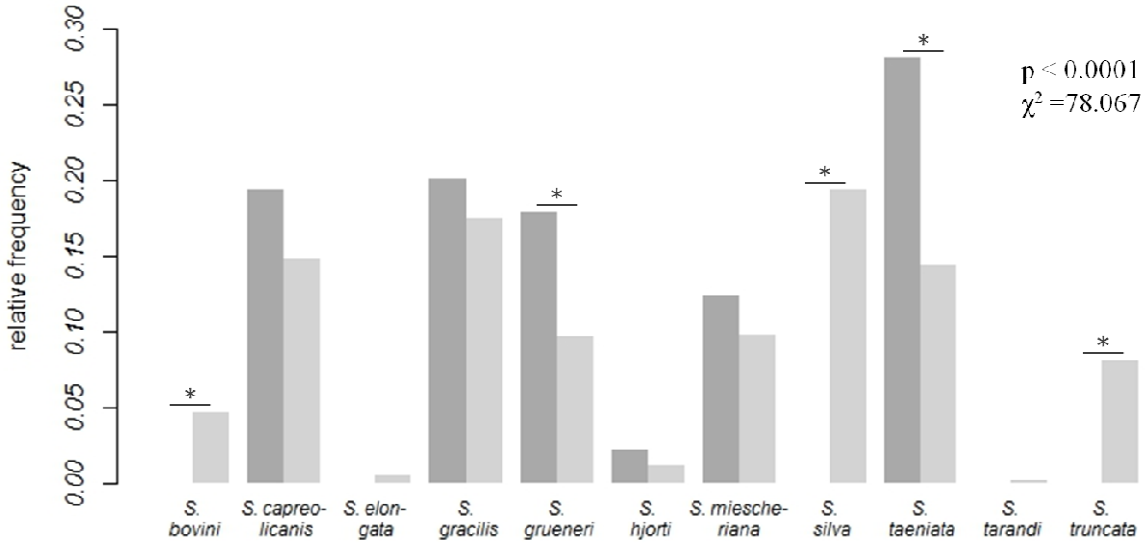
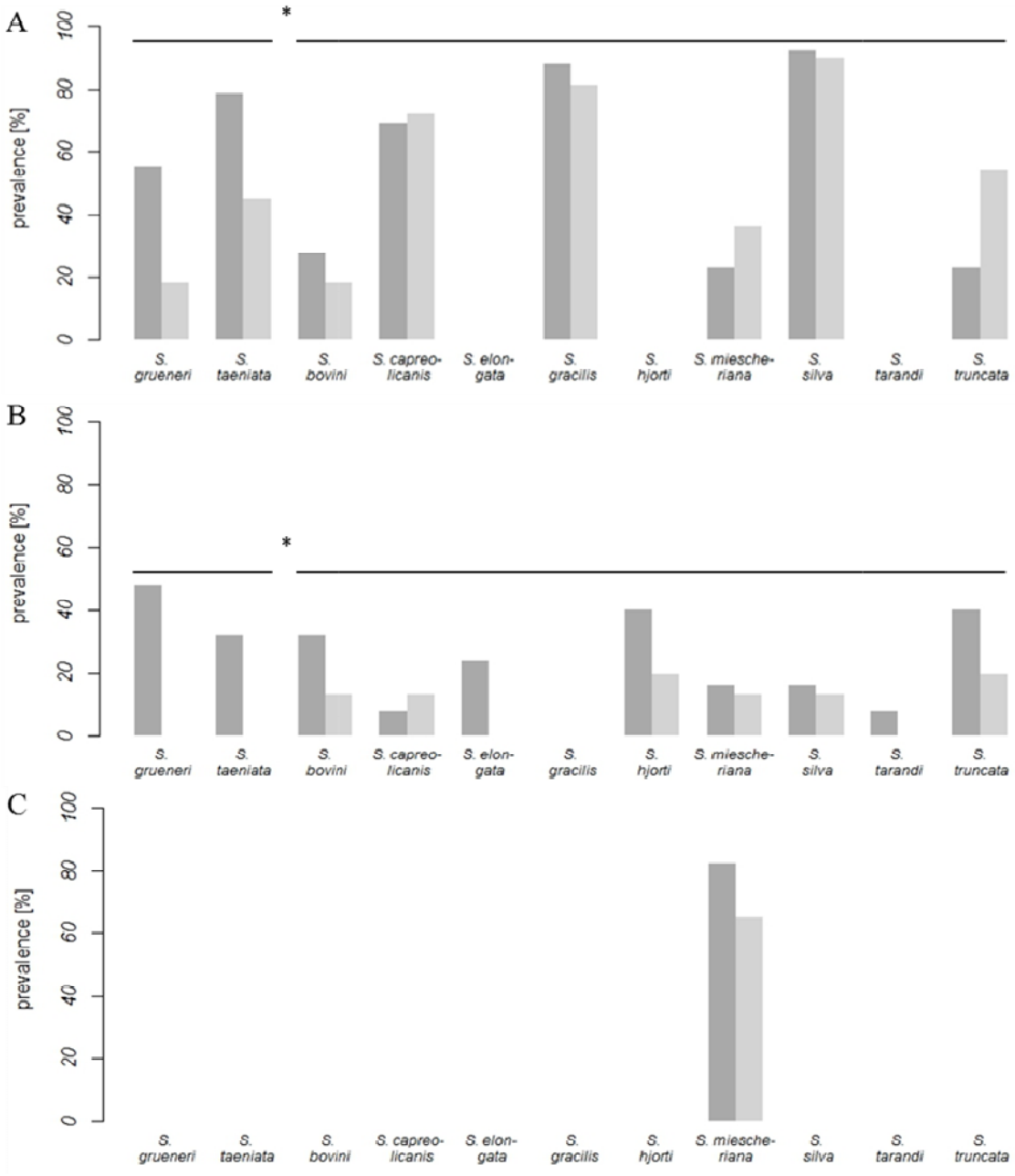


Figure 3: Relative observed (dark grey) *versus* relative expected (light grey) *Sarcocystis* spp. infection frequencies in wolves based on approach B. Expected values have been generated by counting genetically determined *Sarcocystis* spp. occurrences in red deer, roe deer, and wild boar and by accounting for estimated relative ungulate feeding frequency by wolves. The general distribution of infection probabilities is significantly different between observed and expected values (Chi–squared test for given probabilities, $\chi^2 = 79.067$, df = 10, $p < 0.0001$). Binomial post–hoc tests showed that *S. grueneri* ($p = 0.036$) and *S. taeniata* ($p = 0.018$) were overrepresented in wolves, whereas *S. bovini* ($p = 0.036$), *S. silva* ($p = 0.018$) and *S. truncata* ($p = 0.018$) were underrepresented.



Suppl. Figure 1: Normalized prevalences of genetically detected *Sarcocystis* species in ungulates from wolf areas (dark grey) and control areas without wolves (light grey). (A) Roe deer (n_{WT}= 21, n_{CA} = 10) were infected with eight distinct *Sarcocystis* species, all of them occurring in both study sites. The prevalence increase of the 'wolf–specialized' species *S. grueneri* and *S. taeniata* was significantly higher than that of all other species (p = 0.043). (B) Red deer (n_{WT} = 10, n_{CA} = 4) were infected with 10 distinct *Sarcocystis* species, four of them exclusively isolated from samples harvested in wolf areas. The prevalence increase of the 'wolf–specialized' species *S. grueneri* and *S. taeniata* was significantly higher than that of all other species (p = 0.044). (C) Wild boar (n_{WT} = 20, n_{CA} = 10) originating from both study sites were infected with a single *Sarcocystis* species.

Sarcocystis spp. species richness in wolves and prevalence of 'wolf specialists'

Eleven known *Sarcocystis* species were detected from wolf intestinal samples of which six were also detected in wild ungulates. Species richness per individual wolf ranged between zero and 10 species. It significantly decreased with age (GLM, overall likelihood ratio test: $\chi^2 = 7.840$, df = 3, p = 0.049, n = 43), and was significantly lower in adults than in pups (Tukey post–hoc test, p = 0.021,Figure 4).

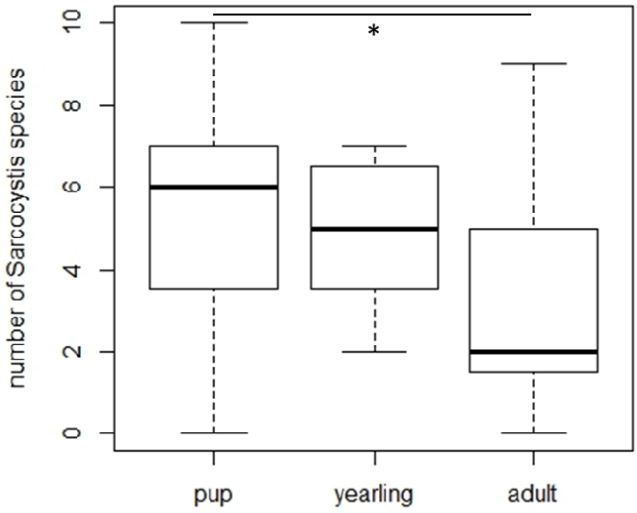


Figure 4: *Sarcocystis* species richness decreases with wolf age (n_{wolves} = 43). Pups had a significantly higher *Sarcocystis* species richness than adults (p = 0.021).

The mean value for the prevalence change of the 'wolf specialists' *S. grueneri* and *S. taeniata* between wolf pups and adults was 19.5%. This expected value differed significantly from the prevalence difference of the three common non–specialist species *S. capreolicanis*, *S. gracilis* and *S. miescheriana* (one–sample t–test, t = 5.885, C.L. 6.587 – 42.413, p = 0.028, Figure 5).

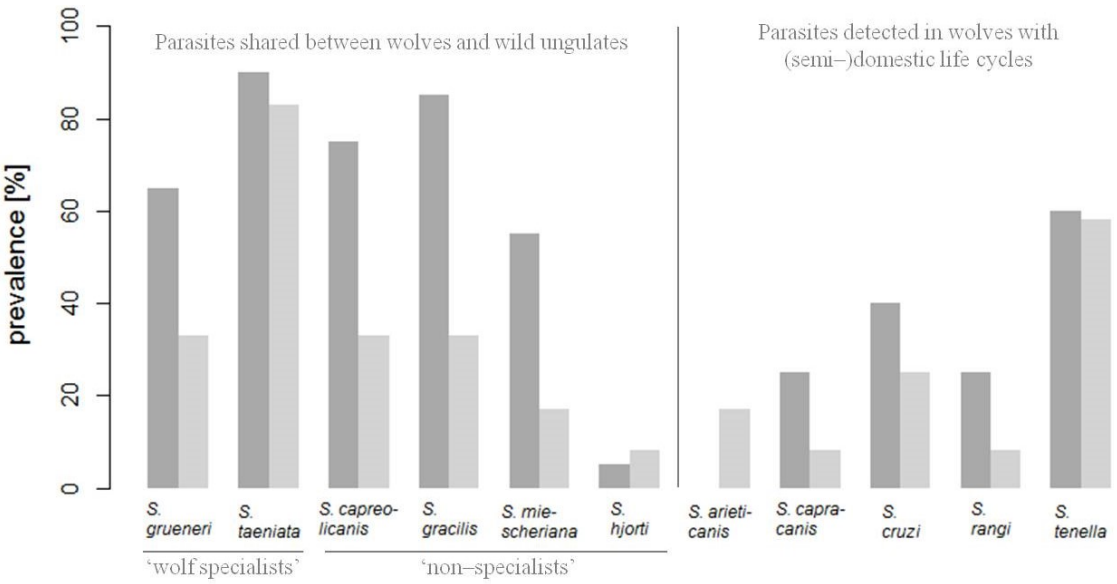


Figure 5: *Sarcocystis* spp. prevalence in wolf pups (n = 20, dark grey) and adults (n = 12, light grey). Eleven *Sarcocystis* species were detected in wolf intestinal samples, of which six species are shared with their wild ungulate prey species, including the 'wolf specialists' *S. taeniata* and *S. grueneri*, whereas the other parasite species are known to have a (semi–)domestic life cycle and did not occur in the investigated wild ungulates in this study. 'Wolf specialists' had a lesser decrease in prevalence from pups to adults relative to 'non–specialist' *Sarcocystis* spp. (one–sample t–test, t = 5.885, C.L. 6.587 – 42.413, p = 0.028).

Discussion

In this study we investigated whether the return of an apex predator affected parasite transmission dynamics in its ungulate prey, as measured by the prevalence of the apicomplexan genus *Sarcocystis*. For most species, sarcocysts reside in muscles in the prey and are only transferred to the definitive host if the intermediate host is eaten by a susceptible definitive host. Apicomplexans such as *Sarcocystis* with a two host life cycle are rarely studied in their definitive hosts due to methodological challenges (Lesniak et al., 2017; Moré et al., 2016; Xiang et al., 2009). In intermediate hosts, morphological cyst characteristics have frequently been used to microscopically identify *Sarcocystis* species (Malakauskas and Grikienienė, 2002; Odening et al., 1995). However, oocysts or sporocysts isolated from definitive host intestinal samples do permit morphological discrimination of species (Khan and Evans, 2006; Stronen et al., 2011). To overcome such limitations, we used a combination of classical microscopy and metabarcoding to investigate *Sarcocystis* fauna, distribution and transmission dynamics between wolves and their ungulate prey. We documented that prevalence was higher in ungulate prey in wolf inhabited areas than in control areas, identified *S. grueneri* and *S. taeniata* as 'wolf specialists', and showed that *Sarcocystis* species richness in wolves declined with age whereas well adapted species persisted in adults.

Sarcocystis infection in ungulates

The presence of wolves in our study site was associated with a general increase in *Sarcocystis* sp. prevalence in their prey. Specifically, red deer had a much higher *Sarcocystis* sp. prevalence when sharing their habitat with wolves than animals from the control area. A similar, albeit statistically insignificant, trend was observed in roe deer and wild boar. A study from the Baltic states, where wolves have been continuously present (Chapron et al., 2014), reported similarly high prevalences of between 84.2% and 89.1% in three ungulate species, including red deer (Malakauskas and Grikienienė, 2002). The wolf–associated prevalence differences measured in red deer in this study and the comparison to wolf range states show, that wolves should be considered a more frequent and important apex predator and consumer of red deer and its sarcocysts than smaller carnivores such as red foxes (*Vulpes vulpes*) or other mesopredators. As a result, red deer–associated *Sarcocystis* spp. cycles have increased in amplitude (prevalence and individual parasite burden) after wolf recolonization, consistent with the 'fading out' hypothesis. Nevertheless, there are *Sarcocystis* spp. which can also be spread by mesopredators as definitive hosts (Moré et al., 2016), as indicated by a high sarcocyst prevalence in wild boar and roe deer in the absence of wolves. During the late 1970s, a study of roe deer in Germany described a *Sarcocystis* prevalence of 71.8%, even though no wolves were present in Central Europe at that time, which is consistent with the idea that mesopredators maintain *Sarcocystis* life cycles, as of species that infect roe deer (Entzeroth, 1981).

Another explanation for the effect of wolves on parasites in red deer and the apparent absence of a similarly strong effect in roe deer could be that both cervids differ in their regional distribution. Recolonizing wolves excreting *Sarcocystis* oocysts with their faeces could now be bridging a rather patchy distribution of roe and red deer, with little overlap in terms of co–occurrence in the same habitat in eastern Germany. There is currently no data on the distribution patterns of these ungulates, so it is unclear whether this is actually the case. Personal observations on hunting bags show that usually one cervid species dominated the hunting bag when samples were collected in a particular area. A recent study by Wu and colleagues showed that suitable habitats for red and roe deer do not necessarily overlap (Wu et al., 2016), even though it is generally accepted that they are sympatric species.

Comparison of Sarcocystis communities between intermediate (ungulate) and definitive (wolf) hosts

In *Sarcocystis* life cycles several intermediate and definitive hosts can be involved which may be linked with each other through the food web. In this study two species, *S. grueneri* and *S. taeniata*, appear to be well adapted to wolves as definitive hosts. Both species occurred in wolves more often than expected on the basis of parasite distribution in prey species. For both red deer and roe deer they showed the strongest increases in prevalence in wolf areas compared to other *Sarcocystis* species. Only red deer from wolf areas were infected with these two types of *Sarcocystis* spp., though some roe deer from the control site also hosted *S. grueneri* and *S. taeniata*. These findings suggest, that (1) *S. grueneri* and *S. taeniata* spread in wolf areas are 'wolf specialists', and that (2) other potential canid definitive hosts such as domestic dogs (*Canis lupus familiaris*), red foxes or raccoon dogs (*Nyctereutes procyonoides*) spread other strains of *S. grueneri* and *S. taeniata* in the absence of wolves. *Sarcocystis* screening of other definitive hosts using metabarcoding techniques will be necessary to clarify the epidemiological relationships amongst intermediate and additional definitive hosts of this protozoan.

Age–related Sarcocystis infection in wolves

Experimental approaches in *Toxoplasma* serve as a model to understand the immunological response of hosts in their interaction with parasites in protozoan infections (Leng et al., 2009). These studies focus on the intermediate hosts, as these are more severely affected by the disease than the definitive hosts which usually suffer little mortality and diarrhoea at worst (Di Genova and Tonelli, 2016; Liang et al., 1998). Studies on immunological defence mechanisms in definitive hosts are the prerogative of human medical research and therefore focus on mouse models (Di Genova and Tonelli, 2016).

Even though it is not clear which molecular mechanisms are responsible for *Sarcocystis* defence in wolves, wolf pups hosted more *Sarcocystis* species than older animals, consistent with the predictions from the 'fading out' hypothesis that immunological resistance to *Sarcocystis* is higher in adults. This finding is also consistent with studies of domestic dogs where *Cystoisospora* and *Giardia* infections were most prevalent in pups (Barutzki and Schaper, 2003; Bugg et al., 1999). In wild canids, comparable indications of age–related parasite burden have previously only been reported in helminth etiopathology (Guberti et al., 1993; Lesniak et al., 2017; Veronesi et al., 2014; Webster et al., 2017). In this study, we investigated this phenomenon accepting that co–evolution in a host–parasite–arms race can

drive hosts to counteract parasite invasion by developing immune defence mechanisms. In turn, parasites are expected to evolve strategies to circumvent such barriers (Dawkins and Krebs, 1979; Schmid-Hempel, 2011), which can result in a higher persistence of such parasites despite an increasing immune competence of maturing host individuals. *S. grueneri* and *S. taeniata* appear to be particularly well adapted to wolves as indicated by their lower decrease in prevalence during wolf maturation compared to other *Sarcocystis* spp. If these parasites have adapted to wolves, it may represent a more subtle adaptation than immune escape and might benefit from further investigation.

Ungulate Sarcocystis spp. fauna

The *Sarcocystis* fauna in red deer has been thoroughly studied with 10 species described so far. Of these, seven species were found in our red deer samples: *S. capreolicanis* (Wesemeier and Sedlaczek, 1995b), *S. grueneri* (Prakas, 2012; Wesemeier and Sedlaczek, 1995b), *S. elongata* (Gjerde, 2014b), *S. hjorti* (Dahlgren, 2010; Gjerde, 2013), *S. taeniata* (Reissig et al., 2016), *S. tarandi* (Dahlgren, 2010; Gjerde, 2014b), and *S. truncata* (Gjerde, 2014b) (Suppl. Figure 1 B). *S. hardangeri*, *S. ovalis* and *S. rangiferi* (Dahlgren, 2010) were previously isolated in Norwegian hosts and not found in this study. This is the first study to document *S. silva* – previously only known from roe deer and moose (*Alces alces*) – and the recently characterized species *S. bovini* from German ungulates (Gjerde, 2016) as well as the supposedly suid–specific *S. miescheriana* (Coelho et al., 2015) for the first time in red deer.

Previous molecular studies investigated the *Sarcocystis* species composition of roe deer, yielding four genetically characterized species. Only *S. oviformis* (Gjerde, 2012; Kolenda et al., 2014) was not found in our study whereas *S. capreolicanis* (Gjerde, 2012; Prakas, 2012), *S. gracilis* (Gjerde, 2012; Kolenda et al., 2014) and *S. silva* (Gjerde, 2012; Kolenda et al., 2014) were found. This is also the first record of *S. bovini*, *S. grueneri*, *S. miescheriana, S. taeniata*, and *S. truncata* in this ungulate (Suppl. Figure 1 A). *S. bovini* was previously only described from cattle (Gjerde, 2016). *S. grueneri* was described from reindeer (*Rangifer tarandus*) (Gjerde, 1986), fallow deer (*Dama dama*) (Wesemeier and Sedlaczek, 1995a) and red deer (Prakas, 2012; Wesemeier and Sedlaczek, 1995b). *S. taeniata* was identified in sika deer (*Cervus nippon*) (Prakas et al., 2016), red deer (Reissig et al., 2016) and moose (Gjerde, 2014a). *S. truncata* was previously only described from red deer (Gjerde, 2014b).

In wild boar, one out of two known *Sarcocystis* species was identified. We confirmed *S. miescheriana* (Kia et al., 2011; Prakas, 2012) but not the zoonotic *S. suihominis* (Prakas,

2012) in our sample of wild boar (Suppl. Figure 1 C). The fact that the supposedly suid–specific species *S. miescheriana* was also detected in cervids, and the fact that *S. miescheriana* reads sequenced in this study had a higher intraspecific diversity than all other detected sarcocysts (Suppl. Table S1 C), suggests that *S. miescheriana* sequences deposited in GenBank could potentially derive from more than just one species characterized to date.

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Authors' contributions

This study was designed by HH, IL, and OK; IL and OK collected and dissected the carcasses; IL performed the microscopic analysis; EH and IL performed the molecular analysis; IH and IL did the bioinformatic data analysis; HH, IL and MF performed the statistical analyses; IL wrote the manuscript with contributions of ADG, HH and MF. All authors contributed critically to the drafts and gave final approval for publication.

Data accessibility

NGS data will be deposited on Dryad.

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Chapter III

Surrogate hosts: Hounds and recolonizing grey wolves share their endoparasites

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Surrogate hosts: Hounds and recolonizing grey wolves share their endoparasites

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Key Words

Canis lupus; epidemiology; helminths; hunting dogs; metabarcoding; NGS; protozoa; *Sarcocystis*

Abstract

Understanding to what extent closely related wildlife species and their domesticated counterparts exchange or share parasites, or replace each other regarding functional roles in parasite life cycles, is of great interest to both veterinary and human public health, and wildlife ecology. Grey wolves (*Canis lupus*) host endoparasites which they spread to the environment. These parasites either directly infect canid conspecifics or their prey serving as intermediate hosts of indirectly transmitted species. The wolf recolonization of Central Europe is an excellent opportunity to study the dynamics of parasite transmission between wildlife and domestic species for cases when a definitive host returns after local extinction – a situation equivalent to a 'removal experiment'.

Here we investigate whether the re–appearance of wolves has increased parasite pressure on hounds – a group of companion animals of particular interest as they have a similar diet to wolves and flush wolf habitats when hunting. We compared prevalence and species richness of helminths and the protozoan genus *Sarcocystis* to determine whether they were higher in hounds from wolf areas than a control area without wolves. Of particular interest were *S. grueneri* and *S. taeniata*, known as 'wolf specialists'.

Five helminth and 11 *Sarcocystis* species were identified, of which all helminths and eight *Sarcocystis* species, respectively, were shared between hounds and wolves. Overall prevalence and species richness of helminths and *Sarcocystis* did not differ between wolf and control areas. However, hounds were significantly more likely to be infected with *S. grueneri* in wolf areas. The findings suggest that wolves indirectly increase risk of infection for hounds with *S. grueneri* since cervids are intermediate hosts and cause sarcosporidiosis if fed to dogs. Apart from that, a regular anthelminthic treatment of hounds may be an effective measure to reduce helminth infections even as wolves increase parasite presence in their environment.

Introduction

Many pathogens circulate in multi–host systems and do not depend on one single host species. Recurring outbreaks of avian influenza (Caron et al., 2014; Jones et al., 2014), Ebola (Leroy et al., 2005; Weingartl et al., 2012) or bovine tuberculosis (Nugent, 2011; Renwick et al., 2007) are examples of such pathogens. Understanding the epidemiology of multi–host pathogens is critical to the 'One Health' concept as wildlife, domesticated animals and humans may be affected by such pathogens and share and exchange them (Aguirre, 2002; Haydon et al., 2002; Taylor et al., 2001; Thompson, 2013). Species or populations that maintain a pathogen and are responsible for its spill–over to a target species of interest are generally defined as "reservoirs" (Hatcher and Dunn, 2011; Haydon et al., 2002). In the context of conserving endangered species (Millan et al., 2016; van Kesteren et al., 2015), and recolonization or reintroduction projects (Almberg et al., 2012), the identification of pathogen reservoirs plays an important role for their success. Although spill–over to wildlife species and its effect on endangered or reintroduced species have received increasing attention, the influence of wildlife on closely related domesticated species has rarely been investigated (Thompson, 2013).

The return of an apex predator such as the grey wolf (*Canis lupus*) to a human–dominated landscape, from which it was absent for a century, is the equivalent of an (unintended) 'removal experiment'. Such an event provides an excellent opportunity to study how its close relative, the domestic dog, may be affected by the resurrection of parasite cycles for which returning wolves are definitive hosts. Given their similar biology and close relatedness, domestic dogs and wolves share a long list of helminth species (Otranto et al., 2015a). Both domestic dogs and wolves have been recognized as hosts of the protozoal disease sarcosporidiosis (Barutzki and Schaper, 2011; Stronen et al., 2011). However, it is unclear at present to what extent one canid may act as 'substitute' host for the other and how close their relationship as 'joint' definitive hosts of *Sarcocystis* is (Otranto et al., 2015b). This lack of information is very likely caused by methodological challenges, as there are no morphological techniques to discriminate *Sarcocystis* sporocysts or oocysts shed by definitive hosts (Xiang et al., 2009). With current molecular genetic tools such as metabarcoding, species detection from canid faecal samples has become possible, and recently wolves have been described as hosts for 12 *Sarcocystis* species (Lesniak et al., 2017). Furthermore, epidemiological studies of wolves and their ungulate prey species demonstrated that wolf presence increased the prevalence of sarcocysts in their ungulate prey. Accordingly, the *Sarcocystis* species *S. grueneri* and *S. taeniata*, that were identified as well–adapted to wolves

and therefore termed ‘wolf specialists’, were mostly responsible for this increase (in preparation).

In general, hunting activities have been identified as a risk factor altering parasite infection risk, for instance, by the protozoan *Sarcocystis* (Thompson, 2013). In this context, hounds – domestic dogs trained for hunting ungulates and other game – are of interest for several reasons. They can be considered the most likely source of pathogens or parasites that could be transmitted to wolves, but at the same time are potentially at risk of being exposed to wolf–derived parasites themselves. Transmission could occur when hounds are used for hunting in wolf habitats or when fed with game meat by their owners (ESCCAP, 2010; Otranto et al., 2015b), which usually originates from the same ungulate species that wolves prey on (Wagner et al., 2012). While literature on companion dog parasites is regularly published (Barutzki and Schaper, 2003; Barutzki and Schaper, 2011), little is known about the parasite fauna of hounds, and it is unlikely that they are identical (Al-Sabi et al., 2013; Gómez-Morales et al., 2016). The current wolf recolonization of Central Europe is therefore an ideal system to investigate the potential link between a wild apex predator and its domesticated equivalent, since hounds can be examined in the presence and absence of wolves in comparable habitats.

We hypothesized that wolfs transmit endoparasites to hounds. Such transmission might either occur directly from wolves via the environment to hounds (no intermediate host required) or indirectly via intermediate hosts. In the second case, ungulate prey would therefore serve as a source of infection when fed to hounds. Accordingly, we predicted that (1) the general prevalence and species richness of the protozoan parasite *Sarcocystis* would be higher in hounds from areas affected by wolf recolonization compared to hounds from the control site, and that (2) particularly *Sarcocystis* species recognized as wolf specialists (in preparation) should show a higher prevalence in hounds from the wolf area because of the similar biology of these closely related canids. We also predicted that (3) anthelminthic treatments of hounds can counteract an increased helminth pressure caused by recolonizing wolves, keeping helminth prevalence and species richness of hounds unaffected independent of wolf presence.

Material and methods

Sample collection

Between November 2012 and January 2015, we collected 359 faecal triplicate samples of 78 hounds residing in areas occupied by wolves in the German federal states of Brandenburg and

Saxony (50°10′–53°33′ N and 11°14′–15°2′ E; $n_{dogs} = 49$, $n_{samples} = 230$). Hounds were also sampled in a control area in the German federal state of Schleswig–Holstein (53°20′–54°55′ N and 8°36′–11°7′ E; $n_{dogs} = 29$, $n_{samples} = 129$) where no territorial wolves were recognized during the sampling period. Hound age and information on regular anthelminthic treatments were supplied by their owners.

DNA extraction

Dog faeces were collected on three consecutive days and pooled. DNA was extracted using the NucleoSpin® Soil Kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's protocol. For subsequent analyses, DNA from dog faecal pools (n = 359) was again pooled in equimolar ratios per individual (n = 78). Extraction success and DNA concentrations were determined using the NanoDrop® 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

Metabarcoding

Portions of the helminth and apicomplexan 18S rRNA and cox1 genes were amplified using a set of five primer pairs (Table 1). Each forward and reverse oligonucleotide was tagged with the Fluidigm–specific common sequence CS1 (5′–ACACTGACGACATGGTTCTACA–[TS–For]–3′) or CS2 (5′–TACGGTAGCAGAGACTTGGTCT–[TS–Rev]–3′) to enable subsequent barcoding of the generated PCR products (Fluidigm, San Francisco, California, USA). Target–specific PCRs had a total volume of 12.5 µL containing 1 µL DNA template and were run in 40 cycles in an epGradient S thermocycler (Eppendorf, Hamburg, Germany). The reactions contained 1 × FastStart High Fidelity Reaction Buffer without $MgCl_2$, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.2 µM each primer, 5% DMSO, 0.4 µg/µl BSA (only cox1 PCR) and 0.5 U FastStart High Fidelity Enzyme Blend (all components from Roche, Basel, Switzerland). PCR conditions included an initial denaturation step of 95°C (10 min); 40 cycles of 95°C (45 s), 53°C (18S rRNA PCRs) or 55°C (cox1 PCR) (45 s), 72°C (60 s), and a final elongation of 72°C (10 min). PCR products were purified using Agencourt® AMPure® XP beads (Beckman Coulter GmbH, Krefeld, Germany) in a 1:1 ratio to reduce adapter concatemerization during barcoding. Post–PCR quality control steps included amplicon quality and length check using the 2200 TapeStation Instrument with D1000 ScreenTapes and D1000 Reagents (Agilent Technologies, Santa Clara, California, USA). A 10–fold dilution of the purified amplicon pools was used for the subsequent barcoding PCR with the Access Array Barcode Library for Illumina Sequencers – 384 (Single Direction) (Fluidigm, San

Francisco, California, USA) according to the manufacturer's protocol (Access Array™ System for Illumina Sequencing Systems, Chapter 6, pp. 70–72, Fluidigm, San Francisco, California, USA). Barcoded amplicons were pooled and target fragments between 400–800 bp were size–selected using the BluePippin® instrument (Sage Science, Inc., Beverly, Massachusetts, USA) with the 1.5% agarose gel cassettes, 250 bp–1.5 kb (Sage Science, Inc., Beverly, Massachusetts, USA). The purified libraries were sequenced on an Illumina MiSeq at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv) using the MiSeq Reagent Kit v3 (600 cycles) (Illumina, Inc., San Diego, California, USA) and a spike–in of 50% PhiX.

Table 1: Target–specific primers (F: forward direction, R: reverse direction) for the amplification of the variable regions of the helminth and *Sarcocystis* spp. cox1 and 18S rRNA genes.

#	target	primer name	primer sequence 5′–3′	source	~product size
1	cox1	JB3F	TTTTTTGGGCATCCTGAGGTTTAT	(Bowles et al., 1992)	396 bp
		JB4.5R	TAAAGAAAGAACATAATGAAAATG	(Bowles et al., 1992)	
2	18S	18S_965F	GGCGATCAGATACCGCCCTAGTT	(Guardone et al., 2013)	606 bp
		18S_1573R	TACAAAGGGCAGGGACGTAAT	(Guardone et al., 2013)	
3	18S	proti15F	TGCCAGTAGTCATATGCTTGTYT	(Lesniak et al., 2017)	378 bp
		proti440R	CAGGCYCSCTCTCCGGA	(Lesniak et al., 2017)	
4	18S	SarAF	CTGGTTGATCCTGCCAGTAG	(Kutkienė et al., 2010)	530 bp
		SarAR	TTCCCATCATTCCAATCACT	(Kutkienė et al., 2010)	
5	18S	SarBF	GGGAGGTAGTGACAAGAAATAACAA	(Kutkienė et al., 2010)	467 bp
		SarBR	GGCAAATGCTTTCGCAGTAG	(Kutkienė et al., 2010)	

Bioinformatic analysis

As a first step, forward and reverse reads from the Illumina metabarcoding dataset were stratified per sample using bcl2fastq v2.17.1.14 (Illumina, Inc., San Diego, California, USA) with no mismatch tolerance allowed in the barcode. Fastq files were grouped into amplicon sets according to the respective primer used (forward and reverse reads were not merged), and were further processed with USEARCH (Edgar, 2010; Edgar, 2013). Sequences were trimmed using the USEARCH 'fastq_filter' command with the parameter *fastq_maxee* set to two nucleotides per read, and the amplicon–specific optimal trim length (*fastq_trunclen*). Singleton reads were discarded with the USEARCH 'dedup_fulllength' command, applying a *minsize* parameter of two reads. Remaining sequences were clustered using the USEARCH 'cluster_otus' command, setting the parameter *minsize* to two reads per cluster and allowing

one nucleotide substitution as value for the parameter *otu_radius_pct*. OTUs were assigned with a similarity of 99% (parameter *id* set to 0.99) by applying the USEARCH 'usearch_global' command.

A custom database to identify *Sarcocystis* species was constructed from a set of 586,784 sequences for Apicomplexa (Taxonomy ID: 5794) extracted from the NCBI database using their taxonomy browser on 18 Oct 2016 (Sayers et al., 2009). Likewise, a custom database to identify helminth species was constructed from a set of 2,948,076 sequences for the taxon of plathelminthes and 1,959,651 sequences for the taxon of nematodes. Identified OTUs were aligned to the custom databases using BLAST® (blastn, Altschul *et al*. 1990) with an identity threshold of 98%. To assign OTUs to parasite species, only hits with a biunique best bit score for one species were collected in a table including the respective parasite species, OTU, amplicon, and sample name.

Statistical analyses

Statistical analyses were performed in R version 3.2.1 (R–Development–Core–Team, 2008). To test our predictions, we used five different generalized linear models (GLM). While each model contained a different response variable, all models included the same predictor variables: 'wolf presence' (binary: present, absent), 'dog age' (0–14 years) and 'sampling effort' (1–11 samples). To test whether the general prevalence of *Sarcocystis* is higher in wolf areas (prediction 1) we used a binomial model with a binary response variable '*Sarcocystis* spp. infection' (present vs. absent). To test whether *Sarcocystis* species richness is higher in wolf areas (prediction 1) we used a Poisson model with '*Sarcocystis* species richness' (the number of species) as a response. To test prediction 2 that particular *Sarcocystis* species recognized as wolf specialists (in preparation) are more prevalent in hounds from wolf areas, we used two binomial models with the binary response variables: (1) '*S. grueneri* infection' and (2) '*S. taeniata* infection' (present vs. absent). To test whether the prevalence of helminths is different between wolf and control areas (prediction 3), we used a binomial model with the binary response variable 'helminth infection' (present vs. absent). Finally, to test whether helminth species richness is different between wolf and control areas (prediction 3), we used a Poisson model with 'helminth species richness' (the number of species) as a response.

Results

Infections with Sarcocystis spp.

Overall *Sarcocystis* spp. prevalence was 63.3% in hounds from wolf areas and 65.5% in hounds from control areas. In the dataset, 109 operational taxonomic units (OTUs) were detected (Table 2) which shared the highest sequence similarity with 11 *Sarcocystis* species (Figure 1). In 22.5% (wolf area) and 37.9% (control area) of all hounds, OTUs were assigned to unknown *Sarcocystis* sp.

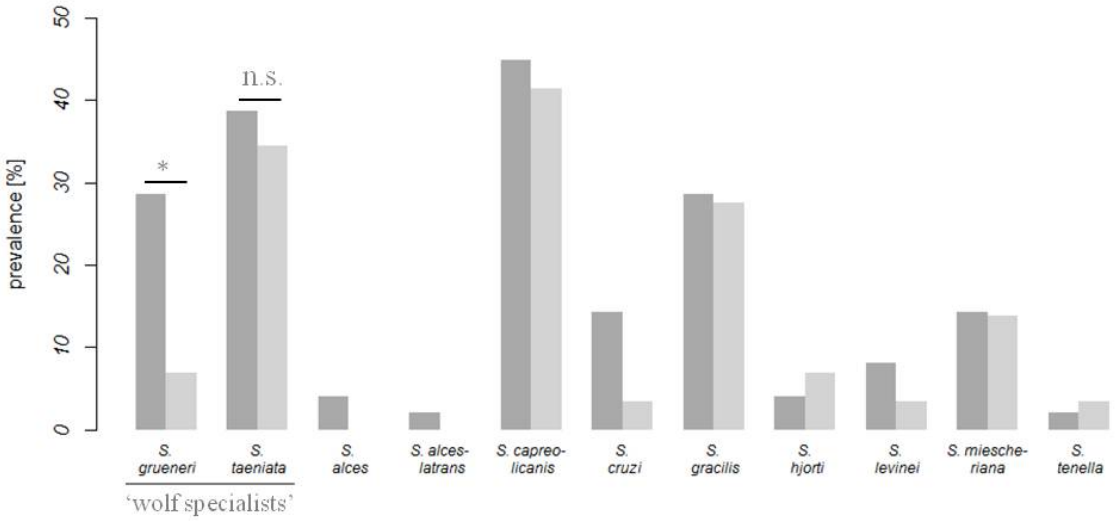

Figure 1: *Sarcocystis* spp. prevalence in hounds from the wolf area (dark grey, n = 49) and control area without wolves (light grey, n = 29). Hounds were infected with 11 distinct *Sarcocystis* species, of which two species only occurred in wolf inhabited areas. They were significantly more likely to be infected with the 'wolf–specialized' parasite *S. grueneri* when sharing their habitat with wolves (p = 0.035). It was not possible to determine a correlation for an infection with the other 'wolf specialist' *S. taeniata* and wolf presence (n.s. = not significant, p = 0.476). P values were extracted from GLMs.

Table 2: Number of detected OTUs per *Sarcocystis* species based on 18S rRNA sequences from six different amplicons analysed with USEARCH.

species assignment	number of OTUs
S. alces	2
S. alceslatrans	1
S. capreolicanis	22
S. cruzi	6
S. gracilis	6
S. grueneri	3
S. hjorti	4
S. levinei	2
S. miescheriana	5
Sarcocystis sp.	9
S. taeniata	57
S. tenella	1

For general *Sarcocystis* prevalence (GLM, overall log likelihood ratio test, $\chi^2 = 14.280$, df = 4, n = 78, p = 0.003) no significant difference was detected between study sites (p = 0.556), although prevalence significantly increased with sampling effort (p = 0.004) and significantly decreased with hound age (p = 0.014). Similarly, for *Sarcocystis* species richness (GLM, overall log likelihood ratio test, $\chi^2 = 17.965$, df = 4, n = 78, p < 0.001) no significant difference was detected between study sites (p = 0.380), although species richness significantly increased with increasing number of analysed samples (p < 0.001) and significantly decreased with hound age (p = 0.013). In contrast, an infection with *S. grueneri* (GLM, overall log likelihood ratio test, $\chi^2 = 8.088$, df = 4, n = 78, p = 0.044, Figure 1) was significantly more likely to occur in hounds sharing their habitat with wolves than in hounds from control areas (p = 0.035), while sampling effort (p = 0.779) and hound age (p = 0.157) had no significant effect. For infections with *S. taeniata* no significant effect for any of the predictors was detected (GLM, overall log likelihood ratio test, $\chi^2 = 2.500$, df = 4, n = 78, p = 0.476).

Infection with helminths

Overall helminth prevalence was 38.5% in hounds from the wolf inhabited area and 24.1% in hounds from the control area. In the dataset, 416 OTUs were detected which shared the highest sequence similarity with one known trematode, three cestode and one nematode species (Figure 2). Sequences were assigned to *Taenia* sp. in 4.1% of hounds from the wolf area. No significant effect for any of the predictors was detected for overall helminth infection risk of hounds (GLM, overall log likelihood ratio test, $\chi^2 = 1.675$, df = 4, n = 78, p = 0.643) or helminth species richness (GLM, overall log likelihood ratio test, $\chi^2 = 2.129$, df = 4, n = 78, p = 0.546).

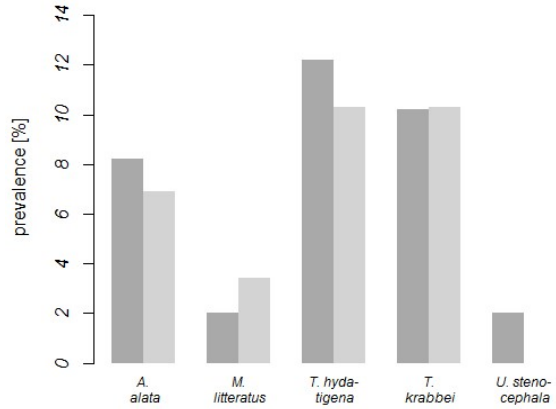

Figure 2: **Helminth prevalence in hounds from the wolf area (dark grey) and control area without wolves (light grey).** Lack of statistical significance was determined using a GLM.

Table 3: Number of detected OTUs per helminth species based on cox1 and 18S rRNA sequences from four different amplicons analysed with USEARCH.

species assignment	number of OTUs
Alaria alata	4
Mesocestoides litteratus	26
Taenia hydatigena	76
T. krabbei	308
Taenia sp.	1
Uncinaria stenocephala	2

Discussion

The recent recolonization of Central Europe by wolves has triggered several scientific studies on their ecology (Andersen et al., 2015; Ansorge et al., 2010; Nowak and Mysłajek, 2016; Nowak et al., 2011; Reinhardt, 2015; Szentiks, 2016; Wagner et al., 2012). Investigating parasite infections in wildlife has a long history that mainly focused on helminth occurrence (Craig and Craig, 2005). Recently, metabarcoding techniques have enabled the high–throughput species identification of protozoan parasites such as *Sarcocystis* in ungulates (in preparation) and wolves (Lesniak et al., 2017), representing the intermediate and definitive hosts, respectively. By applying these techniques, we explored the helminth and *Sarcocystis* fauna of hounds in the context of the ongoing wolf recolonization. We showed that overall prevalence and species richness of *Sarcocystis* and helminths in hounds did not significantly differ between hounds from wolf and control areas. This result indicates that wolves have a minor epidemiological influence on their domesticated conspecifics regarding general parasite burden. The only statistically significant effect detected was an increased prevalence of the wolf specialist parasite *S. grueneri* in hounds from areas affected by wolf recolonization compared to hounds from the control area. This finding underlines the impact of wolves regarding the spread of multi–host pathogens in a predator–prey system, in which prey species are intermediate hosts that serve as source of infection for alternative definitive host such as dogs.

Hound Sarcocystis fauna and impact of wolves

We identified 11 *Sarcocystis* species in hounds, of which eight were shared with wolves (Lesniak et al., 2017). These included the two wolf specialists *S. grueneri* and *S. taeniata* that are known to occur in German roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) (in preparation), and hence serve as the most likely source of infection for hounds. In contrast

to our first prediction, overall *Sarcocystis* prevalence in hounds did not significantly differ between wolf and control areas. A likely explanation is that in another study (1) a wolf–associated substantial increase in overall *Sarcocystis* prevalence was only documented in red deer only, whereas in other ungulates the increase was only slight and limited to a non–significant trend (in preparation), and that (2) this overall increase in *Sarcocystis* prevalence was mainly driven by the two wolf specialist species (in preparation). The current study demonstrates that hound infection with *S. grueneri* – but not with *S. taeniata* – was more likely to occur when wolves were present. Consistent with the above mentioned study, wolves most likely increased the prevalence of *S. grueneri* in their ungulate prey species, thereby being indirectly responsible for an increase in prevalence in hounds because *Sarcocystis* cannot be directly transmitted between canids but require an intermediate host (Figure 3B). Hounds had a lower prevalence of *S. grueneri* in control areas where wolves were absent. Here, as elsewhere, hounds and other unknown mesopredators are the most likely source of infection for grazing ungulates. A study of ungulates from the control area suggested that both *S. grueneri* and *S. taeniata* occur in roe deer but not in red deer (in preparation). In order to explain these findings we propose the following conceptual model of *Sarcocystis* transmission dynamics: parasite strains of species that are spread by hounds in control areas are only well–adapted to roe deer but not to red deer (Figure 3A), whereas strains spread by wolves are well–adapted to both cervids (Figure 3B). Investigation of other definitive hosts, including studies using more variable genome sequences in order to identify parasite strains, would be required to either increase confidence in this model or reject it.

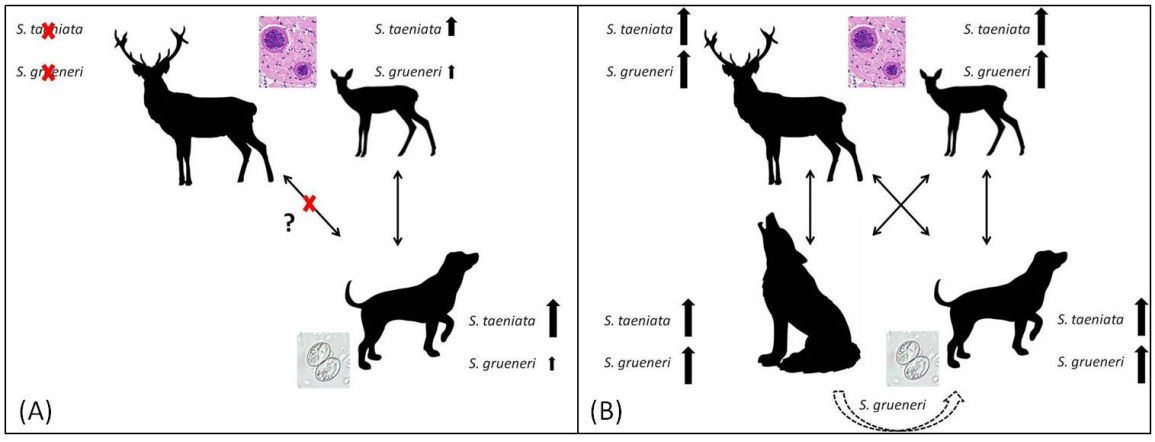

Figure 3: ***S. grueneri* and *S. taeniata* developmental cycles with their intermediate and definitive host in areas without wolves (A) and with wolves (B).** In wolf habitats, wolves increase *S. grueneri* prevalence in their prey, in turn leading to a higher infection rate in hounds. *S. grueneri* and *S. taeniata* strains spread by wolves are well–adapted to both ungulate species, while *S. grueneri* and *S. taeniata* strains spread by hounds from the control area are restricted to roe deer (right ungulate pictogram). The epidemiological influence of wolves regarding the spread of *Sarcocystis* in comparison to hounds has a higher impact on red deer (left ungulate pictogram) than on roe deer. *Sarcocystis* strains in hounds from the wolf area are likely to be a mixture of both hound and wolf strains.

Hounds receive game remains but also commercial meat according to the Biologically Appropriate Raw Food (BARF) method (Schlesinger and Joffe, 2011), both resulting in a high *Sarcocystis* prevalence and diversity detected in hounds. Both types of raw diet may explain the lack of a systematic link between wolf presence and overall *Sarcocystis* prevalence or species richness in hounds. The detection of species such as *S. alces* and *S. alceslatrans* shows that hounds, unlike wolves, have access to ungulates rarely seen in Germany such as moose (*Alces alces*) (Dahlgren and Gjerde, 2008; Gjerde, 2014), most likely because of hunting tourism of their owners. Moreover, this is the first description of domestic dogs being a suitable definitive host for *S. alces*, previously only known from red foxes (*Vulpes vulpes*) and arctic foxes (*Vulpes lagopus*) (Dahlgren, 2010). *S. alceslatrans* has been experimentally shown to reproduce in dogs and coyotes (*Canis latrans*, Dubey, 1980). However, since moose are rare in Germany (Niedzialkowska et al., 2014) the import of these parasite species by domestic dogs can be considered a minor threat to wolves because their developmental cycles cannot be completed without the appropriate intermediate host(s).

The two *Sarcocystis* spp. known from domesticated ungulate intermediate hosts, *S. cruzi* from cattle (*Bos taurus*) and *S. tenella* from sheep (*Ovis* spp.), are known to reproduce in domestic dogs (Dubey, 1976; Erber, 1982). Both parasites were also identified in our sample of hounds, indicating that, besides game, hounds also have access to meat of domesticated ungulates. Here, we demonstrate for the first time that *S. levinei*, native to North Africa (El-Dakhly et al., 2011) and Asia (Claveria and Cruz, 2000; Huong, 1999), also circulates in a domestic cycle in Germany. This species uses water buffaloes (*Bubalus* spp.) as intermediate hosts and can reproduce in domestic dogs as shown by experimental infection (Ghosal et al., 1987). Previously, there were no reports of *S. levinei* from Europe. We assume that this *Sarcocystis* species was either imported via infected domestic dogs that were adopted from the original range countries, or by feeding infected meat to resident domestic dogs. Due to an increasing popularity of water buffaloes for pasturing purposes and meat production, *S. levinei* is able to circulate in Germany (Braun and Preuss, 2008).

To the best of our knowledge, this is the first report of domestic dogs being a possible definitive host for the six *Sarcocystis* species using wild ungulate intermediate hosts – *S. capreolicanis, S. gracilis, S. grueneri, S. hjorti, S. miescheriana,* and *S. taeniata*. Domestic dogs share all of these species with wolves (Lesniak et al., 2017) but only *S. capreolicanis, S. gracilis* and *S. miescheriana* with red foxes and raccoon dogs (*Nyctereutes procyonoides*, Moré et al., 2016), and *S. hjorti* with red and arctic foxes, respectively (Dahlgren, 2010).

Sarcocystis as zoonotic agent

S. silva, one of the most common sarcocysts in roe deer (in preparation), has not been isolated from wolves (Lesniak et al., 2017), other wild canids (Moré et al., 2016) or dogs. An explanation could be that *S. silva* is a zoonotic species using humans as its definitive host. The three currently known zoonotic *Sarcocystis* spp. with a human definitive host are *S. suihominis* from wild and domestic pigs, and *S. hominis* and *S. heydorni* from cattle (Dubey, 2015). Like other meat products, game products such as sausages and ham are cold–smoked, and often undercooked (personal communication by dog owners that took part in this study). Culinary preference can be a potential source of infection as the inactivation of sarcocysts requires cooking over 60°C (Dubey et al., 2002; Gorman et al., 1984). Still, sceptics might argue that oocysts are only shed back into the environment on rare occasions by human defaecation. However, as the zoonotic agent *S. hominis* circulates in Germany (63% cattle, 7.3% human sarcocystosis; Fayer 2004) proves that the human definitive host does contaminate the environment with oocysts. We suggest that illegal wastewater disposal in rural areas or the official installation of irrigation fields could be potential sources of infection for grazing roe deer. Further investigation of human samples will be required to test this hypothesis.

Helminth fauna and impact of wolves

Overall helminth prevalence of hounds was similar between wolf (38.5%) and control areas (24.1%). We were not able to detect any link between wolf presence and helminth infection risk or helminth species richness in hounds, which is consistent with our prediction that regular dog anthelminthic treatments prevent an increase of helminth burden, even if wolf areas might be more contaminated with helminths than control areas. This finding is also consistent with the lack of a link between indirectly transmitted metacestodes (*Taenia* spp.) and wolf presence as shown by a previous study (Lesniak et al., 2017).

A second explanation for similar helminth prevalences in both study sites is that hounds represent equally good hosts for the detected helminths and that during the absence of wolves hounds may have replaced wolves as definitive host, keeping those parasite life cycles alive, even though their ‘main’ host went locally extinct.

Out of five helminth species detected, four have an indirect life cycle involving at least one intermediate host. *Alaria alata* requires snails, then frogs and then wild boars (*Sus scrofa*), *Mesocestoides* sp. requires mites, then small mammals or birds, *Taenia* spp. require ungulates which hounds, unlike companion dogs, have access to. Whereas in our study we

found that helminth prevalence in hounds amounted to 2.0% for *Uncinaria stenocephala*, 3.4% for *Mesocestoides litteratus*, 8.2% for *A. alata* and up to 12.2% for *Taenia* spp., previous studies in companion dogs found that helminth prevalence ranges between 0.0% for *A. alata* and *U. stenocephala*, 0.2% for *Mesocestoides* spp., and 1.2% for Taeniids (Barutzki and Schaper, 2003; Barutzki and Schaper, 2011). The divergent feeding behaviour of companion dogs and hounds probably explains the higher prevalence of these helminths in the latter.

Conclusion

Using current molecular genetic tools, we characterized endoparasites from faecal samples of hounds and investigated whether *Sarcocystis* and helminth transmission dynamics change when a large carnivore host returns as an apex predator into its former habitat. The findings support the idea that hounds from the wolf area experience an increase in infection risk by a wolf specialist *Sarcocystis* species. Hounds most likely acquire this parasite because the game that they are fed showed an increase in prevalence of this species when wolves are present (in preparation). Not detected in wolves, hounds, and several mesopredators, it is still unclear which species is the definitive host of the roe deer parasite *S. silva*. For helminths, this study suggests that hounds may have substituted wolves as hosts for specific helminth species during the period of wolf extinction in Central Europe. Our findings support the recommendation to regularly deworm hounds in order to prevent wolves from increasing hound helminth loads.

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General Discussion

General discussion

European grey wolves are currently recolonizing their former habitat in Central Europe. Although wolves are generally known to be a reservoir of a wide range of pathogens and parasites (Craig and Craig, 2005; Watts and Benson, 2016), little is known about the health status of the CEL wolves regarding viral, bacterial and parasitic infections. Endoparasites of canids often develop in multi-host life cycles, involving prey species as intermediate hosts, other canids as alternative definitive hosts, and – in the case of zoonotic parasites – humans as accidental hosts (Otranto et al., 2015a; Otranto et al., 2015b; Schantz, 1991). The ongoing wolf recolonization and range expansion in Germany and Poland are an ideal process to investigate parasite epidemiology among this apex predator population, its ungulate prey populations and domestic dogs. Current molecular genetic methods applied in this thesis helped to clarify (1) the role of this returning apex predator as definitive host for helminths and protozoa in Central Europe, (2) its impact on parasite load in associated hosts, and (3) its significance for the amplification of parasite species that are well adapted to this apex predator.

Parasite etiopathology in canids

Many empirical studies investigating the factors responsible for parasite presence and diversity have either focused on non-mammalian (Hayward et al., 2017; Lo et al., 1998; Takemoto et al., 1996) or rodent populations (Froeschke et al., 2013; Kiffner et al., 2014; Tanaka et al., 2014), whereas others represent meta-analytical approaches comparing determinants across species (Huang et al., 2015; Lindenfors et al., 2007; Morand and Poulin, 1998). Due to their limited availability fewer studies have analysed parasite ecology in apex predator populations; and if so they were commonly conducted in stable or fluctuating host populations (East et al., 2015; Guberti et al., 1993). Thus the effect of predator population expansion after bottleneck events remains poorly understood in the context of parasite ecology (Niskanen et al., 2014). Moreover, such studies preferentially used helminths, since technical limitations have restricted the analysis of protozoan communities and accordingly their determining factors. Owing to their potential correlation with host fitness and pathogen susceptibility, parasite burden is often linked with host genetic constitution (Acevedo-Whitehouse et al., 2006; Niskanen et al., 2014), age (Bagrade et al., 2009; Hamalainen et al., 2015), life-history traits such as reproductive investment (Allander, 1997; East et al., 2015; Sheldon and Verhulst, 1996) or social status (East et al., 2015; Halvorsen, 1986), but also body mass or size (Ezenwa et al., 2006; Lo et al., 1998), sex (Hamalainen et al., 2015; Massey

et al., 2009), population density (Arneberg et al., 1998; Nunn et al., 2003), habitat (Sousa and Grosholz, 1991) and geographical range (Lindenfors et al., 2007; Torres et al., 2006). Particularly in wolves, only few studies have investigated how ecological and biological variables shape parasite etiopathology. Bagrade *et al*. (2009) showed that prevalence of the cestode *T. multiceps* was significantly higher in adult wolves than pups. They did not detect a relationship of age and sex with parasite prevalence. Regarding *T. multiceps* infection, Segovia *et al.* (2001) reported the opposite with a higher prevalence in juveniles than adults. These contradictory findings exemplify the need for more robust investigations in wolf parasite ecology. Such analyses should not be limited to certain parasite species, ideally be applicable to wolf populations not infected with these particular parasites and allow for broader conclusions and deductions about biological and ecological associations. In order to fulfill these needs and based on the knowledge extracted from non-wolf studies, I predicted for CEL wolves that parasite species richness and diversity will be controlled by age, sex, genetic heterozygosity, and geographic origin. Moreover, I accounted for 'growing population size' as wolf carcasses had been collected over an eight years period when the wolf population in Germany increased from three packs in 2007 to 31 packs in 2014 (Reinhardt, 2015). Including this predictor in my analyses represented the rare opportunity to investigate how parasite ecology is altered in a definitive host population during recolonization.

In **chapter I**, firstly I describe the helminth and – for the first time in this predator – protozoan *Sarcocystis* communities in CEL wolves as a basis for the subsequent tests on factors controlling parasite burden. For helminths, I showed in **chapter I** that species richness and diversity significantly drop from pups (wolves between 0 and 12 months of age) to yearlings (wolves in their second year). Comparably, a previous study of Italian wolves reported a significantly increased prevalence and abundance of three helminth species (*T. canis*, *E. granulosus*, *Trichuris vulpis*) in juveniles, even though these findings are limited to single species (Guberti et al., 1993). Measures of alpha diversity such as the Shannon index and species richness are commonly used for inter-specific comparisons (Lindenfors et al., 2007; Nunn et al., 2003) and have not been applied in wolves before. However, the results presented in this thesis are concordant with an age-dependent decrease in parasite burden reported from amphibians (Raffel et al., 2009), baboons (Müller-Graf et al., 2009), horses (Ruegg et al., 2007) to humans (Fulford et al., 2009; Galvani, 2005; Woolhouse, 1998). The decrease of prevalence and/or intensity of the particular parasitoses, after a particular 'peak'

phase in young individuals, have been interpreted as consequences of acquired immunity and seem to be a likely explanation for wolves as well.
Although not significant, I also reported an increase of helminth diversity and species richness from yearlings to adults. Such an increase could likely occur as a consequence of an accumulation of helminths that are potentially well adapted to the wolf as their definitive host and therefore evolved mechanism to cope with host immune defence barriers. Similar observations of an increased helminth diversity and species richness in adult individuals from wild populations are rare but exist, such as in a wood mouse (*Apodemus sylvaticus*) population (Fuentes et al., 2009). On the single species level, evidence of elevated cestode (*T. multiceps*) levels with increasing age exists as well in wolves (Bagrade et al., 2009). Similarly, findings of an increase in prevalence of certain helminth species in older individuals have also been recognized in domestic mammals such as domestic pigs (Roepstorff and Nansen, 1998).

In parts of **chapter II,** I present – in accordance with my findings in helminth etiopathology – that also species richness of the protozoan genus *Sarcocystis* decreases with increasing wolf age. As before, evidence is lacking from other wild populations and most comparable reports have focused on particular protozoan species such as *Giardia* or *Cystoisospora* in domestic dogs (Barutzki and Schaper, 2011; Bugg et al., 1999).

In accordance**,** I also present data of protozoan age-intensity relationships in dogs in **chapter III**. In hounds, prevalence and species richness of *Sarcocystis* decreased with increasing dog age, when controlling for potential age effects in the respective general linear model. Consistent with these findings, a study of sled dog reported highest parasite (helminth and protozoan) species richness in pups (Bajer et al., 2011). However, this thesis represents, to the best of my knowledge, the first comprehensive analysis of the *Sarcocystis* community in wolves and hounds enabling first insights into *Sarcocystis* etiopathology in canids. By developing a metabarcoding approach designed to enrich parts of the *Sarcocystis* 18S rRNA gene, it was possible to overcome previous limitations of *Sarcocystis* species determination using intestinal or faecal samples from definitive hosts. So far, the majority of *Sarcocystis* ecology studies in definitive hosts have been limited to the genus level (Khan and Evans, 2006; Stronen et al., 2011) or utilized conventional (non-deep sequencing) approaches when identifying species (Moré et al., 2016; Prakas et al., 2015). Others have focussed on

experimental feeding and excretion studies to selectively test canid host susceptibility to particular *Sarcocystis* species (Dahlgren, 2010; Erber, 1982).

In **chapter I**, I furthermore provide evidence that helminth diversity and species richness significantly increased with increasing wolf population size, whereas in **chapter II** the data suggest that *Sarcocystis* species richness is not affected. In this thesis, the variable 'wolf population size' has been measured as the number of wolf packs recognized per year. In grey wolves, an increase in population size does not affect population density *per se*. New generations of wolves disperse to occupy their own territories and thus population range expands on a temporal scale during the years of recolonization until, at some point, local saturation will be reached (Fechter and Storch, 2014) and density-dependent mechanisms will become a stronger regulating factor (Packard and Mech, 1980). The strong correlation of the two predictors 'population size' and 'year of sampling' has been reflected by a high degree of multi-collinearity of both predictors leading to the exclusion of one (year of sampling) in the respective linear models.

The positive correlation of wolf helminth burden and population growth and expansion presented in this thesis can most likely be explained by a combination of factors. Previously, literature has merged this phenomenon under the term 'effect of geographical range' when investigating factors controlling parasite species richness or diversity across different species, ranging from fish (Price and Clancy, 1983) to carnivores (Lindenfors et al., 2007; Torres et al., 2006). This idea of a 'species-area relationship' deducts from the conceptual framework of the *habitat diversity hypothesis* which states that "larger islands [areas] provide more habitats, which promotes species diversity" (Strona and Fattorini, 2014; Turner and Tjørve, 2005). Accordingly, applied in a parasite ecological context, host species occupying larger geographical ranges are supposed to encounter and consequently exhibit a richer diversity of parasites, which has also been predicted and evidence consistent with this was found for viral species richness, e.g. in bats (Maganga et al., 2014) and primates (Nunn et al., 2003). The ongoing wolf recolonization in Central Europe is a unique opportunity to (indirectly) study the geographical range effect, not amongst different species as done before, but as an intra-species approach in grey wolves and therefore corresponding to a quasi-experimental setup. For different sampling time points during the study, different geographical ranges had been occupied by CEL wolves, along with an increasing number of wolf packs (predictor 'population size'). Like for the predictor 'year', I suspect a high degree of multi-collinearity

between ‘population size’ and ‘geographical range’; a potential association I could not test for, as population range data have not been available for the conducted analyses.

The causative agents for the documented positive effect of ‘population size’ on helminth diversity and species richness may in parts be explained by (1) the additive parasite species richness as a function of area (according to the *habitat diversity hypothesis*). In the case of the current wolf recolonization this positive effect might furthermore be driven by (2) the accumulation of such parasites in an area over time due to wolf presence, and (3) an increased contact rate and therefore, chance of infection amongst conspecific (e.g. for wolves on dispersal) that all go along with population growth.

However, there was no link between wolf population size and *Sarcocystis* species richness (when controlling for potential effects of population size in the linear model used to investigate age-intensity relationships in *Sarcocystis* etiopathology) in **chapter II**. The most likely explanation for this missing association was that alternative definitive hosts such as dogs or red foxes keep the prevalence of at least some *Sarcocystis* species in the environment high – even in the absence of wolves. The *Sarcocystis* species fauna that the first wolf recolonizers faced two decades ago could have been very similar to the current one, and is therefore independent of the predictor wolf population size. Additional support consistent with this ides is provided and further discussed in an epidemiological context in **chapters II** and **III**.

Parasite epidemiology among wolves, hounds and their prey

Studying parasite epidemiology in stable environmental settings has significantly contributed to our understanding of ecological mechanisms in predator-prey communities. Infection with trophically transmitted parasites can increase the risk of predation for intermediate hosts (Hoogenboom and Dijkstra, 1987; Lamberton et al., 2008) and consequently shape predator-prey communities (Fenton and Rands, 2006). Underlying mechanisms have been intensely reviewed in the past (Lafferty, 1992) and involve energetic constraints or behavioural changes resulting in an impaired anti-predatory behaviour of intermediate hosts (Afonso et al., 2012). However, the consequences for parasite transmission dynamics and infection patterns are presently not clear, if a locally extinct apex predator and definitive host returns to its former habitat after an absence of almost 100 years.

Both *Sarcocystis* protozoa and *Taenia* cestodes are common canid intestinal parasites with a two host life cycle using ungulates as possible intermediate hosts. According to my predictions, I analysed whether wolf presence increased metacestode and *Sarcocystis* infection risk for ungulates in **chapter I** and **II**, respectively. Likewise, I investigated in **chapter III** whether wolf presence increased hound infection risk for (1) *Sarcocystis* – which hounds would indirectly acquire via infected ungulate meat – and for (2) helminths – which they would either indirectly acquire via infected ungulate meat (metacestodes) or directly via the environment. Regarding *Sarcocystis*, I furthermore investigated in both **chapters II** and **III** whether particular species that are potentially well adapted to wolves would cause the stipulated increase in prevalence.

Infection with metacestodes of the genus *Taenia* was the only metacestode disease in ungulates that could possibly be influenced by wolf presence; as *Taenia* were the only cestodes isolated from CEL wolves that use ungulate intermediate hosts (Murai, 1979; Shimalov and Shimalov, 2003). Correspondingly, *Taenia* metacestodes were the only cysticerci detected in all three ungulate species. Although red deer and wild boar have been reported as suitable hosts of hydatid cysts caused by *E. granulosus* (Murai, 1979; Onac et al., 2013), they were absent in the analysed samples. Hence, like wolves, mesopredators and domestic dogs that are potential hosts of this cestode (Mobedi et al., 2013; Moks et al., 2006) seem to play a minor role in the transmission of this disease within the sylvatic cycle in Germany. Regarding *Taenia* infection, CEL wolves are indeed a primary definitive host responsible for the spread of *T. krabbei* in Central Europe. *T. krabbei* prevalence in wolves (77 %) was significantly higher than in hounds (10 %, Fisher's exact test, $p < 0.001$, 95% C.L. 10.34 – 90.18, $n_{wolves} = 53$, $n_{dogs} = 78$). Thus, wolves seems to be more important than hounds for the transmission of *T. krabbei* to intermediate hosts, even though wolf presence did not significantly impact the general *Taenia* infection risk for ungulates (see **chapter I**). However, this might change with ongoing wolf presence and an accumulation of *Taenia* eggs in the environment. Both canids represent the only (presently known) canid definitive hosts of *T. krabbei* in Central Europe (Loos-Frank and Zeyhle, 1982).

Wolves (15 %) and dogs (12 %) had a similar *T. hydatigena* prevalence (Fisher's exact test, $p = 0.602$, 95% C.L. 0.42 – 4.31, $n_{wolves} = 53$, $n_{dogs} = 78$). Accordingly, the epidemiological influence of wolves on *T. hydatigena* metacestode levels in ungulates can be considered negligible compared to hounds as they occur in lower densities than the latter. However, in

the case of this cestode, alternative definitive hosts such as Eurasian lynx (*Lynx lynx*) (Valdmann et al., 2004) and red foxes (Ballek et al., 1992) might also contribute to the infection risk of ungulates.

In line with the currently negligible effect of wolves on metacestode levels in ungulates shown in **chapter I**, I could not show a wolf-associated effect of helminth prevalence and species richness in hounds in **chapter III**. *T. krabbei* and *T. hydatigena* represent two out of five detected helminth species in hounds, with ungulates as the most likely source of infection. A combination of (1) low ungulate metacestode levels – independent of wolf presence – and the fact that (2) hounds undergo regular anthelmintic treatments, is the most likely explanation for the lack of a systematic link between wolf presence and helminth infection risk for hunting dogs presented in **chapter III**.

Anthelmintics only control helminth infections but not infections with protozoans. Therefore, the missing link regarding wolf presence and overall *Sarcocystis* prevalence and species richness in hounds presented in **chapter III** must exclusively rely on ungulate infection rates (investigated in **chapter II**). The results from this study show that only the overall *Sarcocystis* prevalence of red deer was positively associated with wolf presence. Roe deer and wild boar prevalence were not significantly affected, although the non-significant trend was positive for both ungulates. It is very likely that other carnivores such as mesopredators and domestic dogs themselves keep the *Sarcocystis* sp. prevalence and also species richness in roe deer and wild boar high. If so, wolves can be considered of less significance for *Sarcocystis* etiopathology in these two ungulates. Only in red deer do wolves have a significant amplification effect regarding muscular sarcocysts. Such an effect can most likely be explained by the feeding behaviour of wolves compared to other smaller wild canids.

Returning wolves represent, apart from humans, the main predator of large ungulates such as red deer, leading to the re-amplification of parasites specifically occurring in a red deer-wolf life cycle. Such ‘host-specific’ life cycles are not necessarily restricted to different parasite species using different hosts, but could even be strain-specific within the same parasite species as known for, e.g. *E. granulosus.* Here, ten strains (G1-G10) are known to be host-specific with differing geographical distribution and even variable zoonotic potential (Moro and Schantz, 2009). However, the genetic screenings performed in this thesis were limited to the *Sarcocystis* species level and strains could not be distinguished. Nevertheless, when questioning the existence and occurrence of wolf-specialized *Sarcocystis* species in **chapters**

II and **III**, I found strong indication that *S. grueneri* and *S. taeniata* are well adapted to wolves and that wolf-specific strains existed. Firstly, the latter two species were more prevalent in wolves than one would expect based on the available *Sarcocystis* fauna in ungulate muscular tissues serving as a food source for wolves (**chapter II**). According to our hypothesis, parasite species that were well adapted to the wolf definitive host would evolve mechanisms of persistence in order to circumvent wolf immune barriers. Hence, such species would be detected more frequently than other ingested species that are cleared by an immune response. Secondly, in red deer and roe deer the increase in prevalence of both *S. grueneri* and *S. taeniata* between wolf and control areas was significantly higher than that of other *Sarcocystis* species (**chapter II**). Thirdly, *S. grueneri* and *S. taeniata* were absent in red deer from the wolf-free control area, whereas in the same study site both species occurred in roe deer (**chapter II**). Thus, *S. grueneri* and *S. taeniata* occurring in red deer from the wolf study site could be wolf-specific strains. Accordingly, alternative definitive hosts, other than wolves, exist in the wolf-free control regions and are responsible for the spread of particular strains of both *Sarcocystis* to roe deer. Fourthly, even though the overall *Sarcocystis* prevalence and species richness of hounds was not affected by wolf presence, hunting dogs living in the wolf-area had a significantly higher *S. grueneri* infection risk than hounds from the control area (**chapter III**). This finding indicates that a particular, yet unknown wolf-specific strain of *S. grueneri*, which does not occur in the control area, was transmitted to dogs via infected ungulates. Given their similar biology and physiology, parasite species and strains that are well adapted to wolves have a high chance of also being well adapted to domestic dogs. It presently remains unclear and requires further investigation using strain-specific, genetic markers such as single nucleotide polymorphisms (SNPs) to unravel whether *S. grueneri* and *S. taeniata* strains existed, and if so, whether some are exclusive to the one or other intermediate (red or roe deer) or definitive host (dog, wolf or others), respectively.

Additional findings

Apart from the etiopathological and epidemiological findings described above, this thesis provokes scientific discussion beyond the return of wolves to Central Europe.

In **all chapters**, metabarcoding was applied to intestinal/faecal samples of wolves and hounds, both representing definitive hosts of *Sarcocystis*, whereas in **chapter II** *Sarcocystis* intermediate hosts were additionally investigated. Although it was shown that, with a prevalence of more than 80%, *S. silva* is one of the most common sarcocysts in roe deer,

neither wolves nor hounds were infected with this particular parasite. The absence of *S. silva* in both canids, despite the high infection rate of roe deer, indicates that both canids are not suitable hosts for this protozoan. Hence, *S. silva* uses other, yet unknown, definitive hosts to complete its life cycle. However, it was not detected in another copro-genetic study in red foxes and raccoon dogs (Moré et al., 2016). Therefore, we hypothesize in **chapter III** that *S. silva* could be a zoonotic parasite using humans as its definitive host. To date, there are only three *Sarcocystis* species described as zoonotic and all have been isolated from domesticated intermediate hosts (*S. hominis* and *S. heydorni* from cattle, *S. suihominis* from domestic pigs (Dubey, 2015)). Only the latter *S. suihominis* has recently also been detected in wild boar (Calero-Bernal et al., 2016) representing first evidence that zoonotic *Sarcocystis* species do circulate within sylvatic cycles. Accordingly, a literature screening on human sarcocystosis showed that *S. silva* might have been overlooked so far, as copro-genetic tools have not been used for *Sarcocystis* species identification in human medical studies. Thus, all findings rely on feeding-excretion experiments in which the sarcocyst was morphologically identified from the intermediate host (Dubey et al., 2015; Kimmig et al., 1979) or on results limited to the genus level (Agholi et al., 2016; Khieu et al., 2017; Meloni et al., 1993). Applying the coprological metabarcoding approach presented in this thesis to human faecal samples would help to elucidate *S. silva's* role as a zoonotic parasite.

In **chapter II**, the *Sarcocystis* fauna of three ungulate species including wild boar was presented. With 66 OTUs, the suid-specific parasite *S. miescheriana* had a considerably higher intra-specific diversity than the other 10 detected species ($n_{allOTUS}$=148, $mean_{OTUS}$=14), while the number of available GenBank (Benson et al., 2009) entries for *S. miescheriana* did not differ from that of other species – excluding database bias. One way to explain this finding is the incompleteness of reference databases which could lead to a misassignment of species when applying a 98% identity criterion. An alternative cause could be that *S. miescheriana* sequences deposited in GenBank belong to more than only one species. Phylogenetic studies in *S. miescheriana* reanalysing the available GenBank entries are urgently needed to clarify this issue. To achieve a more adequate reconstruction of phylogenetic relationships amongst taxa, these studies should also include the cox1 gene. Based on cox1 sequences, species previously considered as the same had been described as two different species (Gjerde, 2014).

Concluding remarks

Within the framework of this thesis classical parasitological techniques were applied, but also new methods developed to enable the high-throughput detection of intestinal protozoa from definitive hosts for the first time. This resulted in the description of 12 known *Sarcocystis* species from CEL wolves, previously only described from intermediate hosts. Likewise, seven out of 11 detected *Sarcocystis* species have been described for the first time in domestic dogs. Regarding intermediate hosts, the current work provides evidence of 11 known *Sarcocystis* species isolated from ungulate muscular tissue. In red deer, three out of seven detected species have been reported for the first time, whereas for roe deer five out of eight species are newly described in this thesis.

Based on the genetic identification of helminths and *Sarcocystis*, new insights into relevant factors of parasite etiopathology, including host age and wolf population size, were provided for dog and wolf definitive hosts. Nevertheless, more research is needed to improve our understanding of protozoan etiopathology and epidemiology in the involved host groups, especially regarding alternative definitive hosts. Current methodological improvements, provided in this thesis, overcome previous limitations when identifying protozoans from faecal samples and will assist to extend screenings to further wild and domestic populations or even humans in the future.

Because of the quasi-experimental set-up due to the current wolf recolonization in Central Europe, this thesis has also improved our understanding of *Sarcocystis* epidemiology and infection patterns amongst two closely related definitive hosts and their common ungulate prey. With the identification of two potentially wolf-specialized protozoa, new hypotheses have simultaneously emerged from the gained knowledge, questioning the existence of host-specific *Sarcocystis* strains whose confirmation requires future experiments.

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