Circulation and Spread of Mosquito-Borne Pathogens in Germany and Europe

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Publications and manuscript

This dissertation is based on the following two publications and one manuscript. My contribution to each is states below.

Publications

I) Ute Ziegler, Pauline Dianne Santos, Martin H. Groschup, **Carolin Hattendorf**, Martin Eiden, Dirk Höper, Philip Eisermann, Markus Keller, Friederike Michel, Robert Klopfleisch, Kerstin Müller, Doreen Werner, Helge Kampen, Martin Beer, Christina Frank, Raskit Lachmann, Birke Andrea Tews, Claudia Wylezich, Monika Rinder, Lars Lachmann, Thomas Grünewald, Claudia A. Szentiks, Michael Sieg, Jonas Schmidt-Chanasit, Daniel Cadar, and Renke Lühken: **West Nile Epidemic in Germany Triggered by Epizootic Emergence, 2019**. *Viruses* 2020, 12, 448.

-I participated in the investigation, particularly the analysis of bird samples.

II) **Carolin Hattendorf**, Dániel Cadar, Stefan Bosch, Norbert Becker, Lars Lachmann, Jonas Schmidt-Chanasit, Anna Heitmann, Renke Lühken. **Weak association of Usutu virus and haemosporidian infection in birds collected in Germany**. *One Health* 2024, 19, 100868.

-I participated in the investigation, particularly the dissection of bird samples, carried out the majority of the formal analysis and wrote the first draft of the manuscript. Birds collected in 2018 had already been dissected and analysed by me for my master's thesis.

Manuscript

III) **Carolin Hattendorf**, Renke Lühken. **Spread of *Dirofilaria* spp. In Europe during the 20th and 21st century**.

-I took part in the investigation by extracting the information from the included publications. I performed the majority of the formal analysis. I wrote the first draft of the manuscript.

The manuscript has been submitted to the Journal *Infectious Diseases of Poverty* but has not been published yet. Therefore, it is attached in the form of a preprint.

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Abstract

Over the last two decades, pathogens transmitted by mosquitoes have become a growing health concern in Germany and Europe. The most important limiting factor for mosquito-borne pathogens is temperature, because for one, mosquitoes need relatively warm temperatures to be active and reproduce, and without vectors, the pathogens cannot maintain their transmission cycle. Secondly, mosquitoes are poikilotherm, therefore the outside temperature directly influences the temperature within the mosquito. Because most mosquito-borne pathogens need relatively warm temperatures to replicate in high enough quantities so they can be transmitted again, many pathogens were not able to establish in Central and North Europe, since the temperatures were too low. However, with the warming climate, Northern territories allow for circulation and establishment of temperature-sensitive mosquito-borne pathogens. Two such examples are West Nile virus (WNV) and Usutu virus (USUV), both of which originated in Africa, but have spread to other parts of the world over time, including Europe.

WNV primarily infects birds but can also cause disease in horses and humans. It has been circulating in some areas in Europe for several decades but has displayed a drastic spread involving numerous outbreak events in the last few years. However, Germany has apparently remained free of local WNV transmission, although migratory birds carrying WNV-specific antibodies have been reported. This changed in 2018, when the first WNV infections in local birds and horses were detected. At this point, it was unclear, if this was a one-time event because of the extremely hot temperatures of the summer of 2018 or if WNV would now be established in Germany.

Like WNV, USUV uses birds as its main amplification host, but infections in humans have also been observed. USUV has first been detect in Europe in 2001 in Austria, although retrospective analysis revealed the first outbreak to have occurred as early as 1996 in Italy. Since then, it has continuously spread throughout Europe, reaching Germany in 2010, when it was first isolated from local mosquitoes. Following this, it caused several local outbreaks and then a big, country-wide outbreak in 2018. Additionally, several countries reported a high co-infection rate with avian-infecting haemosporidians. These parasites are also transmitted by arthropods, but unlike WNV and USUV, they have been circulating in Europe for centuries. However, they are understudied and their impact on local bird populations is not well understood. The discovery of co-infections raised concerns about a possible interaction between USUV and haemosporidians, which might increase the burden on local bird populations.

An increased circulation over the last two decades has also been observed for the nematodes *Dirofilaria immitis* and *D. repens* in Europe. They are transmitted by mosquitoes with dogs as their primary hosts, but can also infect humans and other mammals, such as cats and red foxes. They have been traditionally circulating in South Europe but have been found in increasingly Northern regions as of late. This led to growing concerns for animal and human health across Europe.

The aim of this dissertation is to investigate the circulation patterns of WNV, USUV, avian-infecting haemosporidians in Europe, and *D immitis,* and *D. repens* in Europe. For this, the first epidemy of WNV in Germany is described using surveillance data of birds, horses, mosquitoes, and humans from 2018 and 2019. Furthermore, circulation dynamics of USUV and haemosporidians in Germany are explored using data of deceased birds from 2016 to 2021, with particular emphasis on a possible association of the two pathogens. Finally, the distribution of *D. immitis* and *D. repens* throughout Europe is analysed by compiling published cases during the last 70 years, to identify changes between the 20th and 21st centuries.

Zusammenfassung

In den letzten zwei Jahrzehnten sind von Stechmücken übertragene Pathogene in Deutschland und Europa zu einem wachsenden Gesundheitsproblem geworden. Der wichtigste limitierende Faktor für durch Stechmücken übertragene Krankheitserreger ist Temperatur, denn zum einen benötigen Stechmücken relativ warme Temperaturen, um aktiv zu sein und sich zu vermehren und ohne Vektoren können die Pathogene nicht weiterverbreitet werden. Zweitens sind Stechmücken poikilotherm, das heißt, die Außentemperatur hat einen direkten Einfluss auf die Temperatur im Inneren der Mücke. Da die meisten durch Stechmücken übertragenen Pathogene relativ warme Temperaturen benötigen, um sich in ausreichender Menge zu vermehren, damit sie wieder übertragen werden können, konnten sich viele Erreger in Mittel- und Nordeuropa nicht etablieren, weil die Temperaturen zu niedrig waren. Die Klimaerwärmung ermöglicht jedoch in nördlichen Gebieten die Zirkulation und Etablierung von diesen temperaturempfindlichen Pathogenen. Zwei solcher Beispiele sind das West-Nil-Virus (WNV) und das Usutu-Virus (USUV), die beide ihren Ursprung in Afrika haben, sich aber im Laufe der Zeit in andere Teile der Welt ausgebreitet haben.

WNV infiziert in erster Linie Vögel, kann aber auch bei Pferden und Menschen Krankheiten hervorrufen. In einigen Gebieten Europas zirkuliert es bereits seit mehreren Jahrzehnten, hat sich aber in den letzten Jahren mit zahlreichen Ausbrüchen drastisch ausgebreitet. Deutschland blieb scheinbar lange frei von lokalen WNV-Übertragungen, obwohl Zugvögel, die WNV-spezifische Antikörper trugen, identifiziert wurden. Dies änderte sich 2018, als die ersten WNV-Infektionen in lokalen Vögeln und Pferden gemeldet wurden. Zu diesem Zeitpunkt war unklar, ob es sich um ein einmaliges Ereignis aufgrund der extrem heißen Temperaturen im Sommer 2018 handelte, oder ob sich WNV nun in Deutschland etablieren würde.

Wie WNV nutzt auch USUV Vögel als Hauptwirt für die Vermehrung, aber es wurden auch Infektionen beim Menschen beobachtet. USUV wurde in Europa erstmals 2001 in Österreich nachgewiesen, allerdings ergab eine retrospektive Analyse, dass der erste Ausbruch bereits 1996 in Italien stattgefunden hatte. Seitdem hat sich das Virus kontinuierlich in ganz Europa ausgebreitet und erreichte 2010 auch Deutschland, als es erstmals aus lokalen Stechmücken isoliert wurde. In der Folge kam es zu mehreren lokalen Ausbrüchen und schließlich 2018 zu einem großen, landesweiten Ausbruch. Darüber hinaus meldeten mehrere Länder eine hohe Koinfektionsrate mit vogelinfizierenden Haemosporidien. Diese Parasiten werden ebenfalls durch Arthropoden übertragen, zirkulieren aber im Gegensatz zu WNV und USUV seit Jahrhunderten in Europa. Sie sind jedoch wenig erforscht und ihre Auswirkungen auf lokale

Vogelpopulationen sind kaum bekannt. Die Entdeckung von Koinfektionen gab Anlass zur Sorge über eine mögliche Wechselwirkung zwischen USUV und Haemosporidien, die den Druck auf die Vogelpopulationen erhöhen könnte.

In den letzten zwei Jahrzehnten wurde auch eine zunehmende Verbreitung der Nematoden *Dirofilaria immitis* und *D. repens* in Europa beobachtet. Sie werden durch Stechmücken übertragen mit Hunden als Hauptwirte, können aber auch Menschen und andere Säugetiere wie Katzen und Rotfüchse infizieren. Sie sind traditionell in Südeuropa verbreitet, wurden aber in letzter Zeit zunehmend in nördlicheren Regionen gefunden. Dies führt zunehmend zur Sorge um die Gesundheit von Mensch und Tier in ganz Europa.

Ziel dieser Dissertation ist es, die Zirkulationsmuster von WNV, USUV, vogelinfizierenden Haemosporidien in Deutschland und *D. immitis* und *D. repens* in Europa zu untersuchen. Dazu wird die erste Epidemie von WNV in Deutschland anhand von Überwachungs-Daten von Vögeln, Pferden, Stechmücken und Menschen aus den Jahren 2018 und 2019 beschrieben. Darüber hinaus wird die Zirkulationsdynamik von USUV und Haemosporidien in Deutschland anhand von Daten verstorbener Vögel aus den Jahren 2016 bis 2021 untersucht, wobei ein besonderer Schwerpunkt auf einer möglichen Assoziation der beiden Erreger liegt. Schließlich wird die Verbreitung von *D. immitis* und *D. repens* in ganz Europa analysiert, indem veröffentlichte Publikationen von Infektionen während der letzten 70 Jahre zusammengetragen werden, um die Veränderungen zwischen dem 20. und 21 Jahrhundert zu untersuchen.

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List of abbreviations

Abbreviation	Full name
Ae.	*Aedes*
An.	*Anopheles*
CEC	Central and Eastern European clade
Cs.	*Culiseta*
Cq.	*Coquillettidia*
Cyt *b*	cytochrome *b*
Cx.	*Culex*
D.	*Dirofilaria*
mf	microfilariae
NGS	next Generation Sequencing
NUTS	Nomenclature of Territorial Units for Statistics
PCR	polymerase chain reaction
RNA	ribonucleic acid
s.l.	sensu lato
spp.	species pluralis
USUV	Usutu virus
WNV	West Nile virus

1. Introduction

For a long time, the most important mosquito-borne disease in Germany and Europe was human malaria, which had been plaguing Europeans for centuries (1). However, during the 20th century, autochthonous human malaria cases almost completely disappeared in Europe because of mass chemotherapy and prophylaxes, drastic vector control, urbanisation, and socioeconomic improvement (2). Consequently, there was a major decline of interest in mosquito-borne diseases in Europe as they posed no urgent threat anymore. However, in recent years, the interest in pathogens transmitted by mosquitoes has been renewed, as several pathogens have been introduced into Europe and other, already established, pathogens have expanded into new, previously free areas because of global warming and globalisation. Two of the most important (re-)emerging viruses are Usutu virus (USUV) and West Nile virus (WNV). Both have birds as their primary hosts but can also infect other animals such as humans and horses. USUV outbreaks have further drawn additional attention to avian-infecting haemosporidians, which, unlike their human infecting counterparts, have been widely circulating in Europe for centuries. Furthermore, two closely related parasites, *Dirofilaria immitis*, the causative agent of heartworm disease in dogs, and *D. repens* have drastically increased their prevalence and distribution area and are now endemic in countries that have been considered *Dirofilaria*-free only a few decades ago.

1.1 West Nile virus in Europe

West Nile virus (WNV) is a single-stranded *Flavivirus*, belonging to the Japanese Encephalitis Antigenic Complex, which also encompasses viruses such as Murray Valley encephalitis virus and Saint Louis Encephalitis virus (3). It has been isolated from a wide range of birds, but some birds seem to be particularly vulnerable to the virus, such as birds of prey, owls, and passerines, which regularly succumb to an infection (4,5). Because many birds are migratory, WNV can often be carried over long distances, which may at least in part explain its wide spread (6). In Europe, its most important vectors are *Culex pipiens* bioform *pipiens* (7) and bioform *molestus* (8), and *Cx. torrentium* (7,9,10), but mosquitoes such as the invasive mosquito *Aedes albopictus* or *Ae. detritus* have also been experimentally shown to be competent vectors (11,12). The mosquitoes take up the virus through a blood meal from an infected host (13). Inside the mosquito, WNV replicates and migrates to the salivary glands. When the mosquito bites another host, the infective saliva is excreted and the virus transmitted to the new host (figure 1) (13).

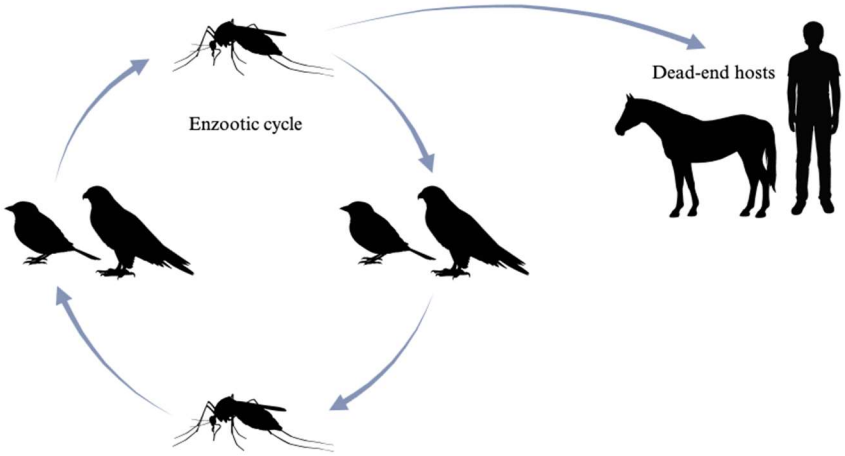

Figure 1: Transmission cycle of West Nile virus. Created with BioRender.com

Because these vectors do not exclusively feed on birds, transmission to mammals occurs regularly (14–16). Particularly vulnerable to WNV infections are horses, where WNV is known to cause disease in about 8 % of the cases (17). The first clinical signs often include fever, depression, anorexia, colic and lameness (18), which can be followed by neurological symptoms, predominantly encephalomyelitis with ataxia (19). It is estimated that the fatality rate of diseased horses ranges from 22 % to 44 % (17). Furthermore, transmission to humans via infected mosquitoes frequently occur (20,21). Most human infections are asymptomatic, but around 20 % of infections are thought to lead to West Nile fever, which includes flu-like symptoms such as fever, headache, and myalgia. About 1 % of infected humans develop West

Nile neuro-invasive disease, which involves the central nervous system and can lead to meningitis, encephalitis, and poliomyelitis (22). Particularly older and immunocompromised individuals are at risk of developing severe neuro-invasive disease (23).

Humans, horses and other mammals are considered dead-end hosts, because WNV cannot replicate in great enough quantities in these hosts to be taken up and transmitted further by mosquitoes. However, there is a risk of human-to-human transmissions via blood-transfusions (24). It is therefore important to detect asymptomatic WNV infections to prevent transmission though blood donation (25). According to EU regulations, potential blood donors are to be temporarily deferred if they have been in an area with active WNV transmission within the last 28 days, unless an individual nucleic acid test is negative (26).

It is mandatory to report a human WNV infection on the EU/EEA level to the ECDC (27) and to report cases of equine and avian WNV infections within the EU/EEA area to the Animal Disease Information System of the EU (28). The initial screening for a suspected WNV infection is usually performed via the detection of WNV-specific IgM and/or IgG (29). However, there is a possibility of cross-reactivity between antibodies specific to related viruses such as USUV, dengue virus, or yellow fever virus (30). Thus, a positive serological test should be confirmed through virus neutralising tests (31). Additionally, WNV genome amplification can be used (32). Virus isolation is not recommended for diagnosis by the ECDC, because it takes up to 5 days and requires biosafety 3 facilities (33). Once a diagnosis is made, the treatment of humans and horses is symptomatic, as there is no specific treatment available for either species (33,34). There are three vaccines for horses approved by the European Medicine Agency to date, but no vaccines for humans are available (35). Prevention measures recommended by the ECDC focus on vector control by reducing mosquito breeding sites like water collections in buckets and barrels and personal mosquito protection via mosquito nets, mosquito repellents and clothing that covers most of the body (33).

1.1.1 Circulation of West Nile virus outside of Europe

WNV was first isolated from a human living in the West Nile district of Uganda in 1937 (36). During the next decades, more identifications of WNV in various countries in Africa, Asia, and Europe followed, illustrating how widespread the virus already was (37,38). Additionally, Kunjin virus, which has been detected in Australia since the 1960s, was recognised as a

subclade of WNV at the beginning of the 21st century (39,40), expanding its area of circulation to the Australian continent. WNV is now recognised as the most widespread *Flavivirus* (37,38).

Besides sporadic cases, there have been a few outbreaks reported since its first detection. The first recorded outbreak occurred in Israel in 1951 with 123 human infections (41), followed by another outbreak in the same country in 1957, during which manifestations of meningitis and encephalitis were observed for the first time (42). Israel experienced further outbreaks in 2000 with 417 human infections (43), and in 2015 with 149 cases (44), which were both linked to preceding heatwaves, which might have enhanced mosquito population numbers (44,45). On the African continent, there have been occasional epidemics involving neurological complications or even mortality in humans and horses. The countries reporting the most cases are South Africa (outbreak in 1974) (46), Algeria (outbreak in 1994) (47), Morocco (outbreaks in 1996, 2003, 2010) (48) and Tunisia (outbreaks in 1997, 2003, 2012) (49). The areas of high WNV circulation in Northern Africa have been linked to a proximity to wetlands (48) and the outbreaks of Morocco are associated with higher precipitation and higher temperatures, which might have facilitated mosquito breeding, thus increasing WNV transmission (50).

On the American continent, no WNV cases were detected until WNV was introduced into New York, USA, in 1999, where it caused an outbreak of human encephalitis and mortality in horses and birds, especially in crows (*Corvus* spp.) (51–53). In total, 62 human infections were reported, of which 59 presented with neuro-invasive symptoms (54). Considering that a large proportion of human infections are asymptomatic, it was estimated that around 8,200 human infections occurred that year (55). Additionally, the population of the American Crow (*Corvus brachyrhynchos*) declined by 45 % during the following six years (56). Phylogenetic analysis revealed a close relationship to WNV isolates from Israel in 1998, identifying this country as a possible origin for the introduction into the USA (57,58). It is possible the virus was carried by migrating birds into New York (6,59), however, this is not definitively proven. An introduction through an infected human returning from an endemic country cannot be excluded either (60,61). WNV overwintered in the USA (62,63) and rapidly spread into other states (64), causing yearly outbreaks (65). The spread was mainly attributed to birds migrating or dispersing along North and South America (66) and to the fact that the virus could overwinter and be vertically transmitted in local mosquito populations (67). To combat the spread, mosquito control measures were implemented as well as surveillance of bird die-off events (62). Nonetheless, WNV further spread into Canada (68), the Caribbean (69,70), Central and South America (65,70) within less than a decade, where it caused infections in humans, horses and

birds, although the case numbers were not as high as in the USA (70). The WNV genotype introduced to New York in 1999 (NY99) was soon replaced by a newly emerged genotype (WN02), a genetic variant of the original NY99 that has evolved on the American continent (71,72). WN02 was shown to be more efficiently transmitted by *Culex* spp. mosquitoes (73). This replacement occurred extraordinarily fast. WN02 was identified as the predominant genotype by 2004, only two years after its first detection in the USA in 2002 (71,74). WNV continues to circulate on the American continent until this day, where WNV is now considered to be endemic in many countries (75). In the USA alone, it has caused mortality in over 300 bird species (76), lead to over 28,000 infections in horses (77) and has caused nearly 60,000 human infections of which almost 3,000 were fatal (78).

1.1.2 First detection and outbreaks of West Nile virus in Europe

The first indication for WNV circulation in Europe was found in 1958, when WNV-neutralising antibodies were detected in two humans from Albania (77). The first isolation of WNV was conducted in 1963 from humans and *Cx. modestus* mosquitoes in France (78). During the following decades, sporadic cases of West Nile fever were reported in Belarus (35), France (79), Portugal (80), Romania (81), Russia (35), and Ukraine (35). Around 1996, WNV caused a major outbreak in and around Bucharest, Romania (82,83). This outbreak included the first reports in Europe of neurological disease because of WNV infection, with a total of 17 fatalities (82). One important driver of the outbreak is suggested to have been a high abundance of *Cx. pipiens* bioform *pipiens* in that area and specifically within housing blocks that emerged after a relatively dry spring, as well as a naïve human population (85). The next year, WNV caused outbreaks in Czech Republic with five confirmed human cases (86) and in 1999, WNV caused an outbreak in Russia with a dramatically high mortality rate: out of 186 confirmed infections, 40 were fatal (87). The latter was again associated with a relatively high population of *Cx. pipiens* following a dry spring (87) as well as high temperatures during the summer (88).

During the following years, equine WNV cases were reported in Italy in 1998 (89), and in France in 2000 (90), 2003 (91), 2004 (92), and 2006 (93), indicating an established WNV circulation. Additionally, WNV seroprevalence among French blood-donors was demonstrated in 2000 (94) and a French patient with meningoencephalitis was found to be infected with WNV in 2003 (91). Italy experienced its first WNV outbreak with human disease in 2008, when eight patients were diagnosed with West Nile neuro-invasive disease and the virus recurred the following summers (95). In Spain, WNV-specific antibodies were detected in 1998 among a

surprisingly high number of residents of the Ebro Delta region in Spain (96), and between 2003 and 2005, seroprevalence was demonstrated in migratory and residential birds, suggesting an ongoing WNV circulation in that country. In Portugal, WNV was isolated from mosquitoes (97) and two Irish tourists were infected during their stay in the Algarve in 2004 (98). In 2003, Hungary experienced its first WNV outbreak associated with clinical symptoms in a flock of geese, but this WNV strain was revealed to be most closely related not to the WNV strains already circulating in Europe, but to the strain circulating in Israel (99), highlighting the ongoing introduction of WNV into Europe. This strain caused repeated outbreaks in humans in 2003 to 2008 (100). Therefore, it became obvious that the outbreak in Rumania in 1996 was not a single exception, but that WNV was re-emerging in Southern Europe, causing numerous and widespread outbreaks.

1.1.3 Emergence of West Nile virus lineage 2 in Europe

Until 2004, almost all European WNV cases were caused by WNV lineage 1. In Czech Republic, WNV lineage 3, originally named Rabensburg virus, was isolated from mosquitoes in 1997, 1999, and 2006, but this lineage was not associated with disease in humans or animals (101,102). Interestingly, in 2023 a patient in the USA was diagnosed with an infection of WNV lineage 1 and lineage 3, which was the first report of WNV lineage 3 on the American continent (103). The patient had encephalitis and multiple organ system failure, but because it was a co-infection of two lineages, the effect of lineage 3 remains unclear.

In 2004, WNV lineage 2 was isolated in Hungary, the first detection outside the African continent (99). It was isolated from a goshawk, which is resident, indicating that WNV lineage 2 was transmitted by local mosquitoes. In the following years, WNV lineage 2 increased its circulation area, causing yearly infections in birds and horses (104). In 2008 and 2009 a rapid spread was detected, with WNV lineage 2 now also circulating in Austria and infecting humans (104). In 2010, WNV lineage 2 was first detected in Greece (105–107) and Serbia (108) and in 2012, it was found in Italy (109), where it quickly replaced the already circulating WNV lineage 1 (93,110,111). Therefore, WNV lineage 2 of the Central/Southern European clade had become the dominant lineage in Europe, leading to regular outbreaks and causing human disease (110–113).

WNV lineage 2 also was also detected in Romania for the first time in 2010 during an outbreak with 54 cases of human West Nile neuro-invasive disease (114) (114) and it continued to

circulate the following years (115,116). It did not belong to the Central/Southern European clade, however, but was more closely related to the strain that had caused an outbreak in Volgograd, Russia in 2007 (88). But in 2015, WNV lineage 2 belonging to the Central/Southern European clade was detected for the first time co-circulating with the established strain (116). By the next year, the Central/Southern European clade had replaced the original strain, causing outbreaks in 2016 and 2017 (116,117). This replacement again indicated that the Central/Southern European clade has evolved to be well adapted to transmission in Europe, enabling it to quickly replace less adapted strains (118,119). It has further been associated with a higher rate of disease and mortality in humans (120).

Greece, Italy, Romania, and Serbia are the countries most affected by WNV in Europe (111). Outbreaks occur regularly and most human cases are reported there. Nonetheless, WNV lineage 2 has spread to many other European countries since its emergence in 2004. By 2018, human cases have been reported in Albania (121), Austria (122), Bulgaria (123), Croatia (124), Cyprus (125), Czech Republic (86), France (91), Greece (110–113), Hungary (100), Italy (93,110,111), Kosovo (126), Montenegro (127), Portugal (98), Romania (84,114,116), Serbia (108), Slovakia (128), and Spain (129,130). Due to the warming climate, WNV is projected to spread further Northward, and outbreaks are expected to occur more often (131) and this trend is already being seeing, with more countries reporting their first WNV occurrences each year (111). 2018 marked a big WNV outbreak and spreading event in Europe with a 7.2-fold increase in human cases compared to 2017 (132). This sharp increase was linked to a wet spring followed by an early and extraordinarily hot summer (132).

In Germany, the presence of WNV-neutralising antibodies in migrating birds was first detected in a study carried out in 2000 and 2002–2005 (133). Subsequent studies further confirmed the presence of seroprevalence among wild, migrating birds across Germany between 2005 and 2017 (134–138). In 2018, WNV-specific antibodies were detected for the first time in resident and short-distance birds (138). Additionally, WNV RNA was isolated from birds and horses (139), both of which indicated that WNV was now transmitted in Germany. Moreover, a veterinarian acquired an infection with WNV, most likely while dissecting a WNV-positive bird (140). Phylogenetic analysis revealed that the virus was presumably introduced from Czech Republic before 2018 and belonged to the lineage 2 Central/Southern European clade, and more specifically to the putative Central European subclade II, which had been found in Czech Republic and Austria (139). Again, the extraordinarily hot summer was linked to the further expansion of the WNV circulation area (138).

1.2 Usutu virus in Europe

USUV is a *Flavivirus* closely related to WNV, thus also part of the Japanese Encephalitis Antigenic Complex (3). Like WNV, its amplification hosts are birds and while it appears to primarily infect Passeriformes or Strigiformes (figure 2), it has been found in 58 bird species of 13 orders and 26 families (141). Its main vectors in Europe are *Cx. pipiens* s.l. mosquitoes. Additionally, vector competence studies have identified *Ae. japonicus* and *Ae. albopictus* as potential vectors, although their role in the wild remains unclear (142–145).

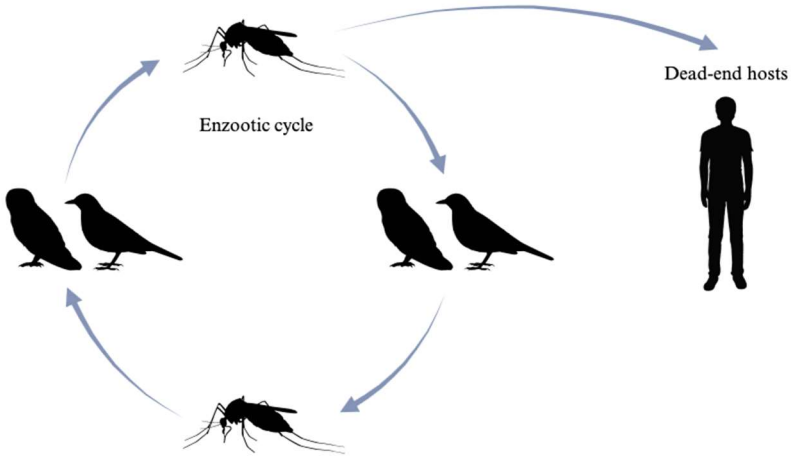

Figure 2: Transmission cycle of Usutu virus. Created with BioRender.com

USUV was first isolated from *Cx. neavei* in 1959 in South Africa, near the river Usutu in Eswatini (146). Phylogenetic analysis estimated that USUV emerged in Africa at least 500 years ago (147). It was subsequently found in various mosquito and bird species in Senegal, Central African Republic, Burkina Faso, Cote d'Ivoire, Nigeria, and Uganda (148–150). However, in these countries, no USUV-related deaths were recorded in birds and only two cases involving other animals were detected. One involved an African soft-furred rat and the other a human who displayed symptoms such as fever and rash, which were linked to USUV (151).

1.2.1 First detection and outbreaks of Usutu virus in Europe

In 2001, USUV was reported for the first time outside Africa, when it was identified as the causative agent of a die-off of common blackbirds (*Turdus merula*) in Vienna, Austria (152). This study furthermore detected USUV in barn swallows (*Hirundo rustica*), which had died at a barn 200 km away from Vienna. Retrospective analysis of deceased common blackbirds from 2000 in Vienna revealed USUV infections as well (152). This indicated that USUV had

overwintered, was transmitted by local mosquitoes and had already spread a significant distance. Indeed, a retrospective study later detected USUV in deceased common blackbirds from a die-off event in the Tuscany region, Italy, in 1996 (153), proving that USUV had been introduced into Europe several years before its first detection. A phylogenetic study furthermore suggested that USUV had been introduced into Europe regularly within the past 50 years (147). The primary way of introduction is most likely through migrating birds, as the major migratory bird flyways could predict the dispersal patterns of USUV (147). However, an introduction through an infected mosquito being brought into Europe through cargo or luggage would theoretically be possible as well (61).

After the outbreak in 2001 in Vienna, USUV caused outbreaks in 2002 and 2003 around the Vienna area as well (154,155). It was estimated that the common blackbird population in Vienna declined by up to 90 % as a result (156). 2003 had an extraordinary long and hot summer, which was linked to a continuous spread of USUV into the Eastern part of the country (155). In 2004 and 2005, however, only a few dead birds were detected (155,157). In 2006, active monitoring was formally discontinued and no USUV infection were reported for 10 years (158). It was thought that the bird populations developed herd immunity, which was supported by findings of lower viremia (155) and high seroprevalence of USUV-specific antibodies in birds (159).

In 2005 and 2006, evidence for USUV circulation was detected in local birds in Hungary (160), but in contrast to Austria, USUV transmission was not linked to increased mortality among common blackbirds. Similarly, USUV was detected in Great Grey Owls (*Strix nebulosa*) in Milan, Italy, in 2006, but no die-offs in birds were seen (161). Continuous USUV circulation in Italy was demonstrated the following years (161–163). On the other hand, an USUV outbreak was observed in Switzerland in 2006, when wild and captive Passeriformes and Strigiformes died in and around the Zurich Zoo (164). Unlike the Austrian outbreak, the most affected birds were house sparrows (*Passer domesticus*). USUV activity was detected during the following summers, although mortality significantly decreased from 2008 onward. Nonetheless, a continuous expansion in circulation area was observed (164).

Interestingly, USUV RNA was isolated in 2006 in Spain from *Cx. pipiens* s.l. mosquitoes, but this strain was more closely related to isolates from South Africa than the ones circulating in Central Europe, implying a separate introduction (165). The same strain was detected again in 2009, indicating an established circulation, however, this strain was never linked to die-off events in birds (166).

The next big USUV outbreak occurred in Northern Italy in 2009, which lead to at least 1,000 dead common blackbirds (163). Notably, half of the USUV-positive birds were co-infected with haemosporidian parasites (163). USUV RNA was further isolated from *Cx. pipiens* s.l. mosquitoes, emphasising their relevance as a vector (163,167,168). Additionally, the first two human cases in Europe were reported during this outbreak in Italy (169,170). Both patients had pre-existing conditions and showed symptoms like fever, headache and skin rash. The second patient also developed neuro-invasive disease (170). Following these two initial cases, retrospective analyses were undertaken to determine if USUV was the causative agent of neuro-invasive diseases with thus far unknown cause. One study from Modena, Italy, diagnosed USUV in seven patients from 2008 and three patients from 2009 (171). It was known for four of the ten patients that they had underlying diseases. Another study detected USUV in an additional three patients with suspected meningoencephalitis, which had been negative for WNV in Italy between 2008 and 2009 (172). Increased effort was also made to detect asymptomatic USUV infections in healthy blood donors. USUV-specific antibodies were found in Italian blood donors in 2008–2012 (171,173,174) and 2014–2015 (175), and USUV RNA was isolated from four blood donors in 2017 and 2018, indicating an acute infection (176). These findings suggested that USUV is more widespread among humans than initially thought and that an infection can lead to serious disease. While patients with pre-existing conditions are particularly at risk, completely healthy individuals can develop severe illness as well.

After the outbreak in Northern Italy in 2009, USUV spread into more European countries, but not always caused outbreaks. In 2011, USUV was identified in one deceased common blackbird in Czech Republic, which was the first USUV isolation in that country, but no increased mortality was reported (177). The next year, two USUV-infected birds were identified in Belgium, which marked the first USUV detection for that country (178). In contrast, in Spain a die-off in birds was observed (179). This was not caused by the already established USUV strain, which was not known to cause disease in birds, but by a variant closely related to the ones circulating in Central Europe. This was another indication that the USUV variants circulating in Central Europe were linked to a higher virulence than other USUV isolates. Croatia reported three more human USUV infections with neuro-invasive disease in 2013 (180). Interestingly, only two patients were known to have underlying disease, suggesting that in some cases, USUV is able to cause disease in seemingly healthy humans.

In 2016, USUV caused an outbreak in Hungary and USUV-positive birds were found in Austria for the first time since 2006 (157). That same year, Belgium and the Netherlands reported their

first USUV outbreak (181). Interestingly, in both countries a high co-infection rate of USUV-positive birds with haemosporidians was reported (182,183). Additionally, USUV-infected birds were found in France close to the border of Southwestern Germany (184). In the same area, a human case was reported with idiopathic facial paralysis, which was an unusual result of an USUV infection (184).The next year, an USUV outbreak among birds was observed in Austria and USUV RNA was isolated from four blood donors (185), while in Hungary, the infected bird numbers decreased (157). USUV continued to circulate in Belgium (186) and the Netherlands (187,188), where USUV was identified in humans for the first time (189), and in Slovakia, where USUV was found to be more prevalent among local mosquitoes than WNV (128).

Seroprevalence studies were also carried out in birds in several countries which had not reported USUV outbreaks yet, in order to estimate the risk of USUV introduction. In the United Kingdom, neutralising antibodies against USUV were already demonstrated in resident, non-migrating birds in 2001 – 2003 (190,191), although no outbreak has been reported there so far. Between 2004 and 2006, USUV-specific antibodies were detected in a migrating bird in Czech Republic (192) and in 2006, USUV-neutralising antibodies were demonstrated in migrating birds in Poland (193). In 2010, USUV-specific antibodies were revealed in birds in Greece (194). Additionally, USUV-specific seroconversion was shown in horses in Croatia in 2011 (195).

1.2.2 Emergence of Usutu virus in Germany

Seroconversion in migrating birds has been shown in Germany as early as 2000, and 2002 – 2005 (133). However, due to the migrative nature of these birds, it was not clear where the birds might have acquired the USUV infection. The first definite proof for USUV circulation in Germany was found in 2010, when USUV RNA was isolated from *Cx. pipiens* bioform *pipiens* in the Upper-Rhine valley in Southwestern Germany (196). The next year, 2011, Germany experienced its first USUV outbreak in the same area (197). In 2012, Germany experienced a second USUV outbreak in the same location, with the circulation area expanding (136). Additionally, USUV-neutralising antibodies were detected in a healthy blood donor from Southwestern Germany (198).

USUV circulation has been detected in Southwestern Germany in all following years (136,199,200). Additionally, a second independent introduction into Bonn, Western Germany

was reported in 2014 (199) and a third introduction into Berlin, Northeastern Germany in 2015 (200). It was becoming clear that as a result of regular introduction from Africa, presumably via migrating birds, and *in situ* evolution in Europe, several distinct lineages of USUV were establishing and circulating in Europe (181).

In 2016, an USUV outbreak was reported in the tristate area of Germany, Belgium, and the Netherlands (181). USUV continued to circulate in 2017 (201), but in 2018, the so far biggest outbreak in Germany occurred (201). Unlike during the previous years, USUV was now present in almost all parts of the country, causing a massive die-off of birds, particularly common blackbirds (201).

Within less than a decade, USUV had established and spread into almost all parts of Germany, leading to a high mortality especially in the common blackbird. Therefore, it is important to continuously monitor USUV activity. It is possible, that the German bird populations will adapt and develop herd immunity, as seen in Austria (155,159). Additionally, the reports of high co-infection rates with haemosporidian from Italy (163), Belgium (183) and the Netherlands (182) are of concern, because co-infections could lead to a higher mortality and increase the pressure on the German bird populations. Thus, this warrants further investigation.

1.3 Avian-infecting haemosporidians in Europe

Haemosporidians are probably best known because the order Haemosporidia encompasses the human malaria causing *Plasmodium* species (202), however, haemosporidians can also infect a range of other animals, including birds (203). Bird infecting haemosporidians were first discovered in 1885 (204) and were used to demonstrate for the first time that haemosporidians (i.e. *Plasmodium* spp.) can be transmitted by mosquitoes in 1898 (205,206). Bird-infecting haemosporidians do not infect humans but they were used as a tool to study human malaria, until rodent-infecting haemosporidians were identified for the first time in 1948 (207), which were henceforth used as a model for human malaria (208). This has led to a steep decline of interest in research of avian-infecting haemosporidians (208).

Since the 1940s, it is recognised that while only parasites of the genus *Plasmodium* can infect humans, birds can be infected by parasites of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* (203,208). For a long time, differentiation between the genera and their respective species relied on morphological differences (203). In 2000, the first molecular approach was developed based on the cytochrome *b* (cyt *b*) gene (209), which is still used today to distinguish avian haemosporidian parasites (210–215). An online library called MalAvi was created, where cyt *b* sequences can be shared and compared (216). There are currently over 3,600 unique genetic lineages cited in MalAvi, although it is often not clear how they relate to the recognised species and whether they represent new, so far undescribed species (217). The vast diversity in bird-infecting haemosporidians is also reflected in the high number of bird species that have been found infected, which are currently over 800 from 25 different orders (208).

1.3.1 Life cycle of avian-infecting haemosporidians

It is thought that the contemporary haemosporidians infecting various vertebrates have emerged from a common ancestor between 20 and 40 million years ago (218,219). Therefore, haemosporidians are well established and widespread. Bird-infecting haemosporidians have been found in all regions of the world except Antarctica, where no vectors are present (220). The dipteran vectors transmitting *Plasmodium* spp. are mosquitoes (Culicidae), while *Haemoproteus* spp. are transmitted by biting midges (Ceratopogonidae) and louse flies (Hippoboscidae), and *Leucocytozoon* spp. are transmitted by blackflies (Simuliidae) (203). As

a result, haemosporidian transmission dynamics are linked to the life cycle and abundance of their vectors, and transmission occurs mainly during the summer (221).

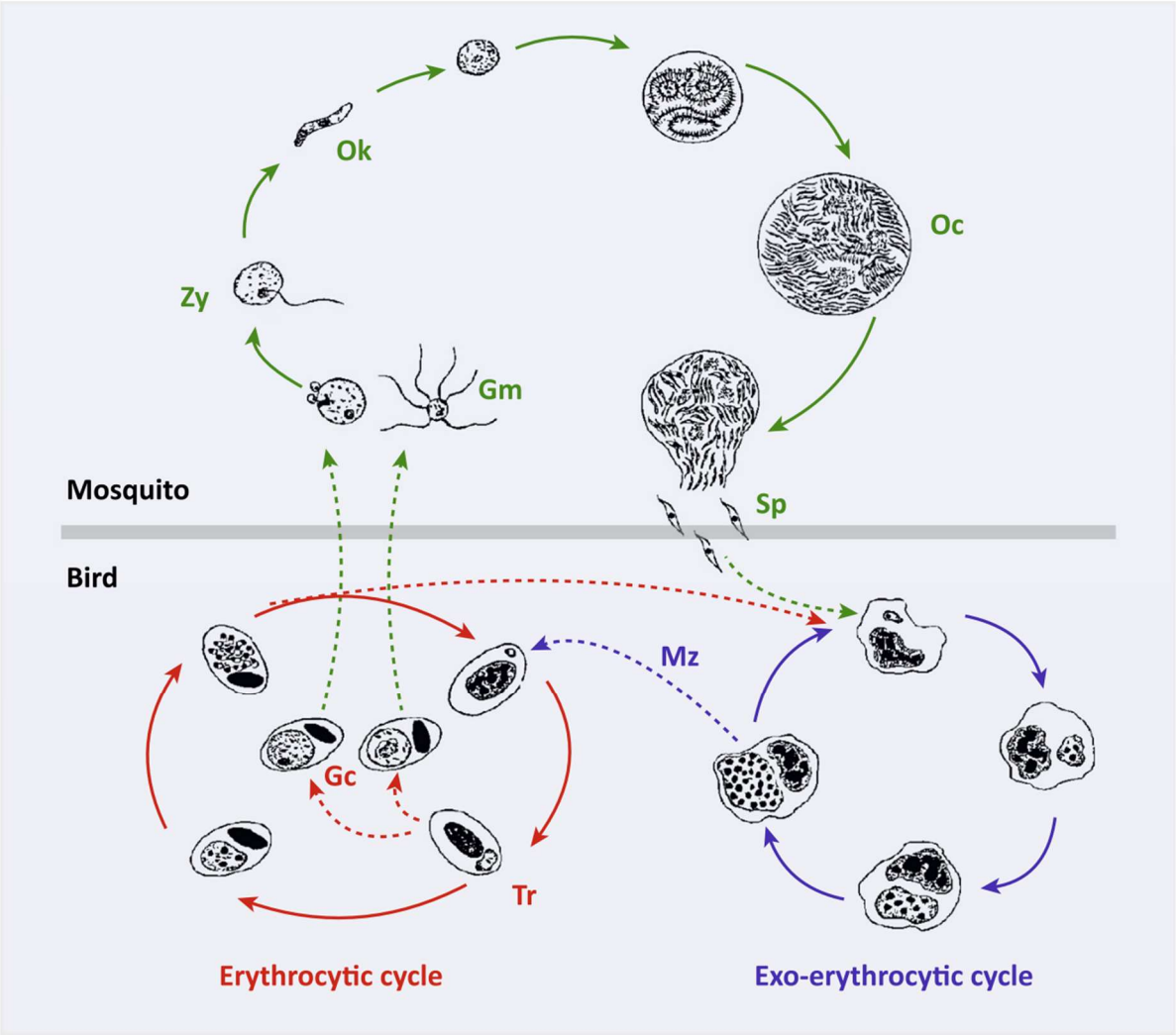

Figure 3: Life cycle of avian-infecting haemosporidians. The upper panel shows the life stages inside the mosquito vector, the lower panel shows the life stages inside the bird hosts, either in endothelial cells of capillaries and reticular cells (exo-erythrocytic cycle) or in erythrocytes (erythrocytic cycle). Gm = gamete, Zy = zygote, Ok = ookinete, Oc = oocyst, Sp = sporozoite, Mz = merozoite, Tr = trophozoite, Gc = gametocyte. Credit: Rivero and Gandon, Trends in Biology, 2018 (208).

Haemosporidians are single-cell parasites with a complex biology and life cycle (203). They are taken up from the host by the vector during a blood meal (figure 3). The parasites are in the form of male or female gametocytes, which then evolve into gametes and sexually reproduce inside the vector, a process called oogamy. The zygote forms an ookinete, which attaches itself to the vector's midgut, where it develops into an oocyst. Inside the oocyst, sporozoites are formed, which are eventually released and migrate into the salivary gland to be transmitted to a new host during the next blood meal. Within the host, haemosporidians amplify asexually in the form of merozoites, trophozoites and schizonts. This occurs primarily inside erythrocytes

and causes an acute infection. The parasites further form gametocytes to be taken up by a vector again and continue the transmission cycle. Additionally, in birds, the parasites invade endothelial cells of capillaries and reticular cells in various organs, leading to a chronic infection. This exo-erythrocytic cycle is not observed for human-infecting haemosporidians on that scale and is the main cause for disease in birds, which occurs past the peak of parasitaemia in the blood (203).

1.3.2 Impact of haemosporidians on birds in Europe

There was a long-standing belief that birds are generally well adapted and haemosporidian infection would be mostly asymptomatic (203), because the late onset of symptoms was not linked to the haemosporidian infection. This assumption has been revised in the past two decades, as studies revealed a significant health impact on bids because of haemosporidian infection. An infection can cause mortality (222–224) reduce reproductive success (225–227), shorten lifespans (228,229) or reduce the body condition, such as decreased body mass or infestation by exo-parasites (225). It has further been shown that chronic infections can relapse into acute infections due to stress or the rising of the corticosterone level at the beginning of the breeding season (230–232). More research is needed to understand the impact of haemosporidian infection on bird populations.

The extent of haemosporidian prevalence and the resulting burden on bird populations in Europe, and Germany in particular, is not clear. This is in part because the relationship of dipteran vectors, birds and haemosporidians is highly complex. There are presumably many *Plasmodium*, *Haemoproteus* and *Leucocytozoon* species circulating in Germany and these are not clearly differentiated and described (220,233). Furthermore, it has been shown that different haemosporidian species display different life traits. For instance, many *Plasmodium* species are attributed to be generalists, infecting a wide range of bird species, while other haemosporidians are believed to be specialised to individual or closely related bird taxa (208). On the other side, different bird species display varying susceptibility to different haemosporidian species and varying prevalence for the three genera overall (203,208,234). Finally, as mentioned, haemosporidians are transmitted by different dipteran vectors, most of which are very little researched in Germany. Due to their relevance for human health, mosquitoes are comparatively well studied, but there are very few studies concerning biting midges, louse flies or blackflies (235). The population dynamics of vector populations can directly impact transmission (e.g. low vector population can lead to decreased transmission), thus it is important to better

understand the ecology of the vectors in order to explain haemosporidian circulation patterns (221,236). For example, one German study showed that forest management decreased dipterian abundance, but at the same time increased haemosporidian prevalence in the vectors (236). Another study suggested that the distance to the nearest water source and human traffic influences haemosporidian transmission (237).

Overall, there are many unknowns about haemosporidians in Germany, including their biology, distribution and prevalence in various bird species, but also regarding their interactions with other pathogens, such as USUV, with which a high co-infection rate has been demonstrated several times (163,182,183). More studies are urgently needed.

1.4 *Dirofilaria immitis* and *D. repens* in Europe

Two species of *Dirofilaria* are found in Europe: *D. immitis* and *D. repens* (238). Both are transmitted by mosquitoes, with the most important species in Europe being *Cx. pipiens* s.l. and *Ae. albopictus* (239–241). Their primary amplifying hosts are dogs (*Canis lupus familiaris*), although they have been found in a range of other animals, particularly cats (*Felis catus*), red foxes (*Vulpes vulpes*), and grey wolves (*Canis lupus*) (242). Moreover, humans can acquire infections through the bite of an infected mosquito, however, they are considered to be dead-end hosts and as such do not contribute to the transmission cycle (243).

1.4.1 Life cycle of *Dirofilaria* spp.

Dirofilaria spp. are nematodes with a complex life cycle that develop from transmissible microfilariae over several larvae stages into adults, that can produce new microfilariae (figure 4) (238). Mosquitoes transmit the parasites in the larvae 3 (L3) stage (243). Within the canine host, the larvae moult into the L4 stage and then into preadult worms within 50–70 days. In the case of *D. immitis*, the preadult worms travel into the pulmonary artery and right ventricle of the heart, which led to the name 'heartworm', and in the case of *D. repens*, they travel to the subcutaneous tissues, and sometimes to the abdominal cavity or connective muscular fasciae (244). The worms reach sexual maturity after 6–9 months post infection and begin to release microfilariae into the bloodstream. Adult worms can survive for more than seven years and microfilariae for up to two years inside the host (243). The microfilariae can be taken up again by a mosquito taking a blood meal. There, they moult into the L2 and then L3 stage, and then migrate to the feeding tube of the mosquito, ready to infect a new host during the next blood meal. The duration of the development of the larvae inside the mosquito vector is strongly temperature dependent. It has been shown that development does not occur if the temperatures are below 14°C (241,245) and that the extrinsic incubation period shortens with rising temperatures (246).

A *D. immitis* infection can lead to a range of symptoms in the dog, from mild persistent coughing, exercise intolerance, and loss of appetite to a blocking of the pulmonary arteries by the worms causing right-sided heart enlargement ultimately resulting in heart failure (247). Less common is a blocking of the blood flow through the lungs due to an accumulation of worms, which can lead to the migration of the worms into surrounding areas and ultimately leading to heart failure as well. *D. repens* infections are often less severe in dogs than *D. immitis* infections

due to their subcutaneous location, causing mainly swelling and local irritation (248,249), although severe cases involving allergic reactions have been reported (250,251). Many *D. repens* infections in dogs are completely asymptomatic, however, leading to an underdiagnosis of this infection (252). As a consequence, there is a much greater awareness among veterinarians for *D. immitis* infections than for *D. repens* infections (253). Additionally, rapid and reliable tests are only available for *D. immitis,* and dogs are only routinely screened for *D. immitis* and not for *D. repens* (254). Current preventative and curative treatment has also been primarily designed for *D. immitis* (255).

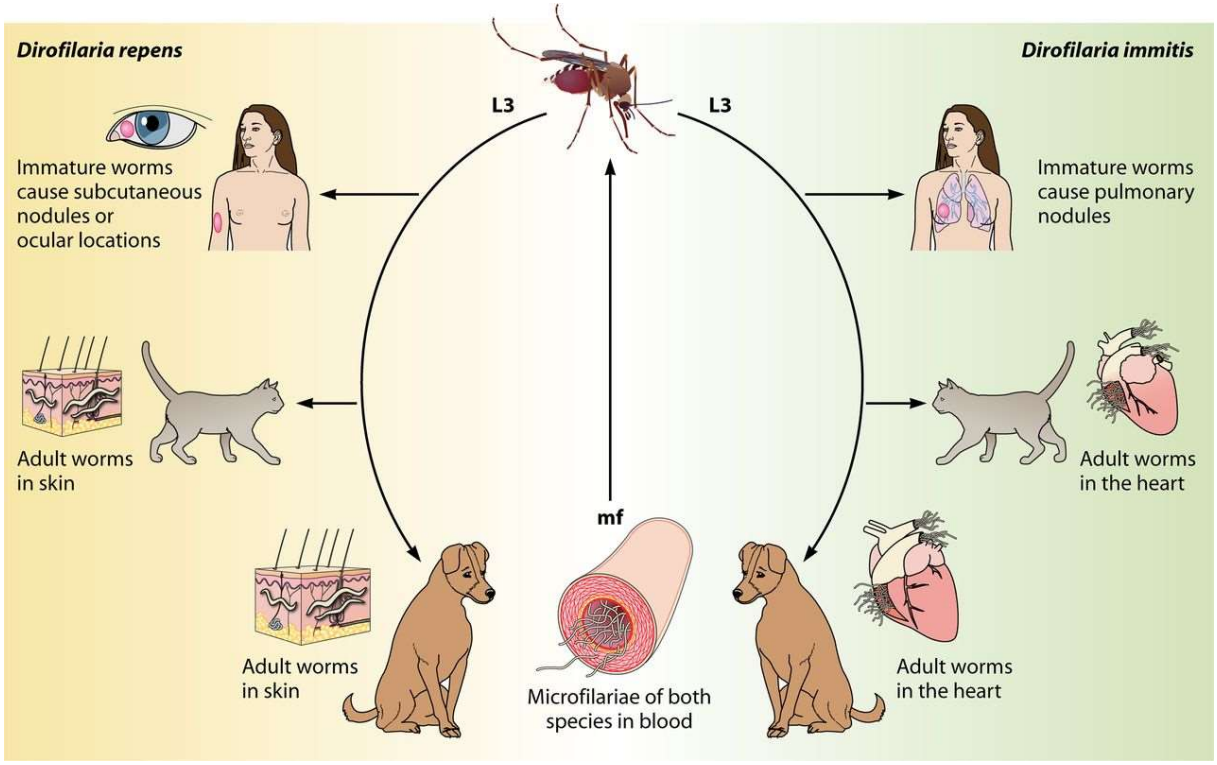

Figure 4: Life cycle of *D. immitis* and *D. repens*. mf = microfilariae. Credit: Simòn *et al*., Clinical Microbiology Reviews, 2012. (243)

Another species in which *Dirofilaria* spp. infections are regularly reported are cats. However, both *Dirofilaria* species are rarely able to produce viable microfilariae, so their relevance as reservoirs is presumed to be small (256). Nonetheless, due to their domestication, an infection in cats is more likely to be diagnosed than for canids other than dogs, whose role in the transmission cycle is not entirely clear (257,258). Additionally, infection of only a few dead or immature worms can lead to symptoms. The most common severe result of an infection is the development of heartworm associated respiratory disease, which can cause a variety of unspecific symptoms such as chronic coughing, laboured breathing and vomiting, and can further induce sudden death (259). Due to the lack of specificity and sometimes complete

absence of symptoms before the cat succumbs, it is important to regularly screen the animal in endemic areas and administer preventative treatment in highly affected regions (254).

Dirofilaria spp. infections have also been detected in wild animals, particularly red foxes, grey wolves, and golden jackals (*Canis aureus*) (260). However, due to a lack of studies, it is unclear if they contribute significantly to the transmission cycle or if they suffer from disease following infection (243). There are only few prevalence studies in wildlife across Europe with varying results. For example, one study found no *D. immitis* infections in red foxes in Serbia in 2013 (261), while another study detected a prevalence as high as 22.2 % in 2015 and 2016 in Serbia (262).

There are a growing number of human *Dirofilaria* spp. infections (263). Although both species are present in Europe, the overwhelming majority of human cases are caused by *D. repens*. Nonetheless, *D. immitis* cases do occur, although they are in most cases asymptomatic (243). The worms usually from pulmonary nodules, which are most often detected through radiography and initially mistaken for cancer. *Dirofilaria repens* mostly causes subcutaneous nodules or localises in the ocular region (264,265). As mentioned, humans are considered to be dead-end hosts, meaning that microfilariae are not produced within them. However, there are a few reported cases where viable microfilariae of *D. repens* were found in the blood stream, although these cases are considered rare exceptions (266–270).

1.4.2 *Dirofilaria* spp. circulation in Europe

Reporting of *Dirofilaria* spp. infections date back as far as the 16th century, with the first *D. repens* infection diagnosed most likely in 1566 in a child from Portugal, while the first *D. immitis* case was described in 1626 in a dog in Italy. For a long time, both species were predominantly circulating in Southern and Eastern Europe, while most Central and Northern European countries were considered *Dirofilaria*-free (263). However, over the last decades, more and more European countries reported their first *D. immitis* and/or *D. repens* cases and it became clear, that while some cases were imported from known endemic countries, others had been transmitted locally. Today, there is a stable *Dirofilaria* spp. transmission in many regions of Central and Northern Europe and previously *Dirofilaria*-free countries are now considered endemic. For example, during the 1990s, there were reports in Germany of imported *D. immitis* and *D. repens* infections in dogs, but local transmission was not observed (271,272). In 2004, the first autochthonous *D. repens* case was diagnosed in a dog from Southwestern Germany

(273). In the following years, more autochthonous infections with *D. repens* were identified in dogs (274,275), as well as infections of local mosquitoes (276–278). In 2012, *D. immitis* DNA was detected for the first time in German mosquitoes (278), although this is not definite prove for local circulation, because the mosquitoes could have fed on a host that had travelled to an endemic country. Nonetheless, there are a growing number of incidents of autochthonous cases, indicating that Germany's climate allows for *Dirofilaria* spp. development and transmission. Similar trends can be seen in many other European countries as well.

Since adult worms can be easily spotted by the naked eye and identified morphologically, a diagnosis can be much more easily made than for viruses such as USUV and WNV, or parasites of microscopic size like haemosporidians. Therefore, there is a record of reported cases dating back more than a century available today. This data is extremely helpful to track the spread of *Dirofilaria* spp. in Europe and to help understand the factors that increase prevalence and distribution.

2. Scope of the thesis

2.1 Monitoring of West Nile virus activity in Germany

In light of the number of neighbouring countries with WNV circulation, it was considered likely that WNV would eventually arrive in Germany. Therefore, regular screening of birds was conducted since 2000 (133). This screening programme was continued to monitor WNV activity in 2019. Additionally, surveillance of infections in horses and humans was continued. Whenever possible, organ or serum samples were used for diagnosis and RNA sequencing. Finally, mosquitoes were collected in areas of WNV circulation in 2019. The aim was to investigate the areas and the extent of WNV circulation in Germany, to explore if temperature data can explain its circulation patterns and to use phylogenetic analysis to better understand how the German WNV strains relate to each other and to other strains circulating in Europe.

2.2 Surveillance of Usutu virus and haemosporidian circulation and their interaction

There have been several reports of high co-infection rates of USUV and haemosporidians in birds, indicating a link between the two. Therefore, data from a dead bird surveillance programme, where citizens are asked to submit dead birds found in the wild, was used to investigate the prevalence of USUV and haemosporidians between 2016 and 2021. Furthermore, the rate of co-infections was explored, and it was tested for a statistically significant association of the two pathogens.

2.3 Meta-analysis of *D. immitis* and *D. repens* reports in Europe

In order to investigate the recent spread of both, *D. immitis* and *D. repens*, published cases of *Dirofilaria* spp. infections in Europe dating back as far as 70 years were complied. Cases were divided into the 20th and 21st century and the respective circulation patterns for *D. immitis* and *D. repens* were compared to better understand the extent of their spread throughout Europe. Furthermore, the impact on human, dogs and other mammalian hosts was investigated, as well as potential mosquito-vectors.

3. Discussion

3.1 Monitoring of West Nile virus activity in Germany

3.1.1 Establishment of West Nile virus in Germany

It was expected that WNV might eventually arrive in Germany (134,135). There has been evidence for WNV circulation in migrating birds since 2000, indicating that long-distance introductions due to bird migration are possible (133,136,201). Additionally, WNV had already established in numerous neighbouring countries, such as France (279), Austria (122), and Czech Republic (280,281), making a short-distance introduction possible as well. Furthermore, all factors needed for WNV transmission and establishment are present in Germany: There is an abundance of hosts, as shown by the detection of WNV-specific antibodies in a range of birds (133,136,201). Secondly, mosquitoes competent for WNV transmission are present in Germany. Some of the most abundant mosquitoes in Germany are *Cx. pipiens* s.l. and *Cx. torrentium* (282,283), which have been shown to be competent vectors for WNV (7,9,284). The included publication I confirmed the importance of *Cx. pipiens* s.l. mosquitoes as vectors for WNV. Thirdly, the climate is increasingly suitable for WNV transmission. Higher temperatures are associated with increased transmission rates (9,284) and with a shorter extrinsic incubation period, i.e. the time it takes the virus to replicate and migrate to the salivary glands within the mosquito vector, so it can be transmitted further (285–287). Because of climate change, temperatures are rising in Germany, with increasingly hot and prolonged summers (288). The year 2018, when the first WNV cases were detected in Germany (139), had an extraordinarily hot summer (289) and the temperatures of the summer of 2019 were above the average as well (290). The included publication I revealed that as a result, several areas of Germany were at high risk for WNV transmission due to short extrinsic incubation periods. Especially Eastern Germany was predicted to be suitable for WNV transmission and this is indeed where most WNV activity was detected in both years. The temperatures in Germany are projected to increase in the future due to global warming, making the climate in Germany ideal for WNV transmission (131,291).

Given the suitable conditions for WNV transmission in Germany, it was expected that WNV might establish circulation after its first detection in 2018, as it had done previously in other European countries (104). The included publication I confirms this expectation, reporting Germany's first WNV epidemy in 2019, with infections in birds, horses, mosquitoes and humans. The outbreak occurred in the same region as in 2018 and the WNV isolates were

genetically highly similar, indicating that WNV had overwintered, and the outbreak was not due to a new introduction. It has been shown for other countries that WNV is able to overwinter in Europe (104,292). There are two main pathways discussed for overwintering. Firstly, there are indications that WNV might overwinter in mosquitoes. This has first been shown for *Cx. pipiens* s.l. mosquitoes in New York (63) and WNV has since been isolated from hibernating *Cx. pipiens* s.l. in Czech Republic (293). Additionally, WNV might overwinter in birds. WNV has been shown to be able to cause persistent infections in birds (294–297) and recent findings of WNV-positive birds in Italy support the hypothesis of WNV overwintering in birds (298,299).

3.1.2 Impact of West Nile virus on human and animal health in Germany

As shown in the included publication I, considerably more WNV cases were identified in 2019 compared to 2018 with a total of 88 bird, 38 horse and the first five presumably autochthonous human cases. Additionally, seven *Cx. pipiens* s.l. pools tested positive for WNV, emphasising this species as an important vector. All infections occurred in Eastern Germany (Berlin, Brandenburg, Saxony, Saxony-Anhalt, Thuringia) with the exception of one positive bird found in Hamburg, indicating that the endemic area remains mostly restricted to the Eastern Germany area (figure 5). While only five human cases were diagnosed, it is likely that many more cases went undiscovered. Given that three of the cases involved West Nile neuro-invasive disease, which only occurs in around 1 % of infected humans (22), it can be assumed that hundreds, if not thousands of human cases occurred during the outbreak. This is of concern not only because of the widespread threat of direct infections of humans, but also because of the danger of transmitting a WNV infection via blood transfusion (25). A study from Hungary revealed a WNV seroprevalence as high as 4.3 % among blood donors in 2019, highlighting the amount of undiagnosed, asymptomatic WNV infections (300). Because of this, blood donations are now screened if the donor has spent time in the affected regions of Germany (301).

In 2020, 22 infections in horses and 65 infected birds were detected in Eastern Germany (table 1) (302). One study investigating the seroprevalence of horses in Eastern Germany found a prevalence of 5.8 % (303), and another study measured a prevalence of 13.8 % (304). Additionally, a seroprevalence of 2.6 % in horses was detected in Western Germany, indicating that while most acute infections are identified in Eastern Germany, WNV had indeed spread to other parts of Germany as well (304). In humans, 20 symptomatic and two asymptomatic autochthonous infections were reported, including the first fatal case (305). Additionally, eight

infections were noticed during blood donation screening, bringing the total infection number up to 30 (306). The infections all occurred in Eastern Germany (figure 5), indicating a stable transmission there (305). Notably, a cluster of five confirmed West Nile neuro-invasive disease, two suspected West Nile neuro-invasive disease and two suspected West Nile fever cases were detected in Leipzig within four weeks (307). This marked the biggest human outbreak reported in Germany so far, especially considering that most human infections are asymptomatic and go undiagnosed, indicating a high number of missed infections in that area. A potential trigger for the outbreak is thought to have been the hot and dry summer which preceded the cluster of cases (307). As mentioned, higher temperatures shorten the extrinsic incubation period of WNV, leading to a more efficient transmission (8). Moreover, dry conditions, i.e. low precipitation over a prolonged period of time, appear to be a stronger driver of WNV circulation than precipitation (308). A possible explanation for this is that decreasing water sources mean that reservoir birds gather at the same water sources more often, which in turn increases their contact with each other and with mosquito vectors, which are also dependent on water for reproduction (308).

Table 1: Confirmed autochthonous WNV infections in Germany as reported by the Robert Koch Institute (316) and the Friedrich Loeffler Institute (317).

Confirmed WNV infections	2019	2020	2021	2022	2023
Humans	5	30	5	17	7
Horses	38	22	19	17	18
Birds	88	65	34	54	25

The following year, five human autochthonous infections were reported, one of which was identified solely through blood donation screening (309). Additionally, 19 infections in horses and 34 infections in birds were reported (302). All cases were located in Eastern Germany (figure 5), Seroprevalence studies among horses in Eastern Germany revealed a prevalence of 3.3 % (310). Overall, the case numbers and seroprevalence were lower than in the previous year and this was associated with a comparatively cold summer (309). Nonetheless, stable WNV transmission was observed throughout the summer and WNV RNA was identified in two *Cx. pipiens* s.l. mosquitoes in Berlin (311) and in an overwintering *Cx. pipiens* bioform *pipiens*

specimen in Saxony-Anhalt (312), illustrating that WNV was transmitted by and overwintering in local mosquitoes.

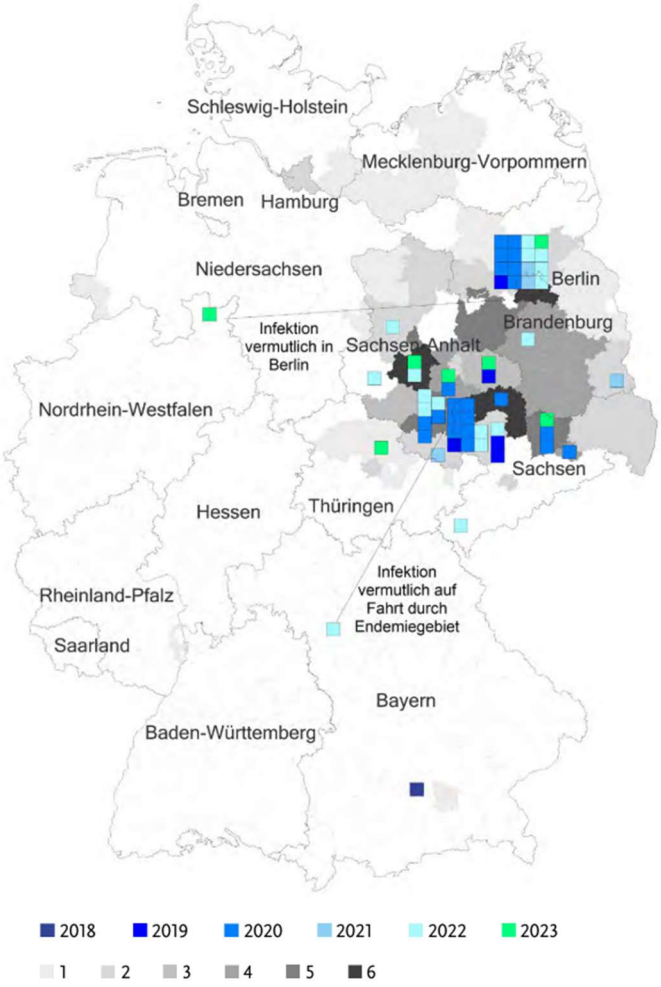

Figure 5: Reported autochthonous WNV infections in Germany between 2018 and 2023. Human cases are coloured by year, the number of WNV infections in horses or birds per county are coloured in shades of grey. Credit: Robert Koch Institute (316).

In 2022, 13 symptomatic and four asymptomatic autochthonous human infections were reported (313). Again, the circulation area was constrained to Eastern Germany, although it seemed to be slowly expanding towards Central Germany (figure 5) (313). One infection was reported in Bavaria, however, it is thought that the infection occurred during travel to an endemic area in Eastern Germany (313). WNV RNA was found in five *Cx. pipiens* s.l. mosquitoes in the same location in Berlin as the previous year (311). A total of 17 horse cases and 54 bird infections were reported (302). Additionally, WNV-specific neutralising antibodies were found in a juvenile bird in Northern Germany, indicating its introduction there and implying a further spread of WNV through the country (314). As with previous years, the

increase in infection numbers, especially in humans, might again be linked to the warm and dry climate during the summer (315).

The next year, seven human autochthonous infections were reported, five of which were detected during blood donation screenings (316). One infected person resided in North Rhine Westphalia but is believed to have acquired the infection in Berlin (316). All other cases occurred in Eastern Germany as well (figure 5). Furthermore, 18 infections in horses and 25 infections in birds were confirmed (302). While the temperatures during the summer of 2023 were comparatively high as well, precipitation was also high (318) and as mentioned before, dry conditions appear to be a stronger driver for WNV transmission than rainy periods (308), which might explain why the numbers were lower than in the previous year.

It is clear that WNV has established in Eastern Germany, where it has caused autochthonous infections every year since its first detection (316,317). It is therefore important to closely monitor WNV activity and its potential spread into other parts of Germany. This is in part achieved through mandatory notification of WNV infections to national registries (316,317). It is complemented by active surveillance systems like screening of human blood donations (306), seroprevalence studies (302), dead bird screening (137), and mosquito trapping (311). Additionally, control measures are crucial to reduce sickness and death in animals and humans. One strategy focuses on reduction of transmitting mosquitoes. This includes reducing water sources for breeding and the application of larvicides and adulticides (319). For horses residing in an endemic area, a vaccination is recommended (320). To combat severe disease or even death in humans, it is crucial to identify an infection as early as possible (321). Routine screening of blood donation has been shown to be an effective tool to detect WNV infections that otherwise might have gone unnoticed (306). Additionally, physicians seeing patients with symptoms such as fever, headache, or myalgia should be aware of the possibility of a WNV infection, if the patient resides in or has visited the endemic area in Eastern Germany (322,323). Furthermore, individuals residing in or visiting the endemic area should use personal protection against mosquito bites to prevent transmission, like the application of mosquito repellents or covering up skin with clothes (323). Especially individuals at higher risk of developing severe disease, like immunocompromised or elderly patients, should apply personal protective measures (324).

3.1.3 Evolution and spread of West Nile virus in Germany

Phylogenetic analysis presented in publication I indicate that WNV diversity in Europe is primarily driven by *in situ* evolution and not by continuous introduction events, although at least six different introduction events were predicted. It appears that WNV lineage 2 strains are adapting to local bird and mosquito species. Phylogenetic analysis of WNV positive birds in Berlin between 2021 and 2022 demonstrate that the virus continues to evolve and form local sub-clades (311). An example of the potential effects of continuous adaptation of WNV has been observed in the USA, when WN02 replaced the original WN99 strain in 2004 (71). This strain was associated with a more efficient transmission by *Culex* spp. mosquitoes, which explains why it was able to replace WN99 (73). More experiments are necessary to understand the consequences of the observed mutations in the dominant Eastern German clade, however, it is reasonable to assume that they increased its transmission efficiency in some way, making it the most successful and thus dominant strain in Germany. There is always a danger of the emergence of a highly virulent variant, which might lead to a massive die-off, as seen in the USA (71) or in Europe with USUV (181,201). Therefore, it is crucial to maintain the monitoring of WNV activity and evolution to detect new variances as early as possible and implement control strategies, as mentioned earlier.

Despite the supposed local adaptation of WNV, however, WNV has remained mostly restricted to the Eastern part of the country. While seroprevalence studies have demonstrated some WNV activity in Western Germany (304), as mentioned earlier, acute cases primarily occur in Eastern Germany (316). Something similar could be observed after WNV lineage 2 was first detected in Hungary in 2004 (104). Between 2004 and 2007 only sporadic infections were observed. However, in 2008, WNV suddenly spread throughout the whole country and into Austria (104). Similarly, local WNV transmission in Serbia was first demonstrated in 2010 (108), but the first human cases occurred only two years later (93). By 2013, WNV had spread throughout the entire country (325). The distribution pattern in Serbia could be linked to climate conditions (325). Therefore, because WNV transmission is climate-dependent, it cannot be ruled out that a sudden spread of WNV into other parts of Germany will occur if the climate is suitable. As mentioned earlier, this includes a prolonged period of high temperatures and potentially low precipitation (308).

Nonetheless, it is striking that WNV has apparently not established circulation along the Upper-Rhine valley, even though in the included publication I it was identified as an area at high risk for WNV transmission. Interestingly, this was the area in which the first USUV outbreak was

reported (136,196,197). By the time the first WNV cases were detected, however, USUV had spread throughout the country (201), making it unlikely that the USUV circulation is the reason for the WNV absence in the Upper-Rhine valley. Nonetheless, the dynamic of WNV and USUV co-circulation is mostly unknown. Co-infections have been reported in a range of birds (168,326,327) and both pathogens were detected in several mosquito pools (128,167). First occurrences of co-infections in birds in Germany have been reported as well (328). Moreover, their envelope proteins are predicted to be highly similar based on their amino acid sequence, which is why cross-reaction in serological tests is often observed (329). Co-circulation of members of the genus *Flavivirus* has led to displacements in the past. Not only have more competent WNV variants replaced other WNV strains (71,116), but WNV is also attributed to have displaced the Saint Louis encephalitis virus in California, after WNV arrived there at the beginning of the century (330). Another scenario is an increase in severity of a *Flavivirus* infection if a previous infection had occurred. This is observed for the four dengue virus serotypes (331), but also for a dengue virus infection after an infection with the Japanese encephalitis virus (332). Neither of those scenarios has been observed in Europe so far, however, it is important to monitor the co-circulation dynamics as new virus variants emerge and their circulation is expanding.

3.2 Surveillance of Usutu virus and haemosporidian circulation and their interaction

3.2.1 Circulation and spread of Usutu Virus in Germany

After USUV was first detected in the region of the Upper-Rhine valley in 2010, it has expanded its circulation area rather slowly in Germany during the first few years. During 2011 and 2012, some mortality in birds was observed, but the virus remained restricted to that area (134,180,181). Phylogenetic analysis has even suggested that the virus had been introduced three years prior to its first detection, extending the time period in which USUV displayed limited spread (419). Indeed, at that time it was thought that USUV might have slower spreading tendencies than WNV (420).

However, in 2016, a large outbreak in Central Europe occurred (181) and a notable expansion of the circulation area in Germany was observed (335). High temperatures in the affected areas were identified as a cause for the outbreak (335). In 2017, a gradual northward spread could be detected, but in 2018, the so-far largest USUV outbreak occurred in Germany (201). The sudden increase in USUV circulation in 2018 is reflected in the data of the included publication II as well. The number of submitted birds as well as the USUV prevalence sharply increased in 2018 compared to the previous years. As seen in other studies, USUV activity was not only found in the known hotspots but was detected throughout the county (201). This sudden and drastic spread was partly attributed to the outstandingly hot summer of 2018 (201). The average temperature for the summer months of June to August was 19.3° C, which was 3° C above the reference period of 1961–1999 (289). It was the second hottest summer ever recorded, exceeded only by the summer of 2003, when USUV presumably had not yet established in Germany (333). Therefore, the hottest summer since USUV established circulation is linked to the largest USUV outbreak and spreading event in Germany, highlighting major the impact of temperature on USUV transmission. Interestingly, the hot and dry climate, i.e. little precipitation, of the summer 2018 are not ideal conditions for mosquitoes, as they require water sources for their larvae stages and relative humidity as adults (336). This indicates that temperature might be a more important driver of USUV transmission than a high vector abundance.

During the three years following the big USUV outbreak of 2018, USUV prevalence continuously declined as seen in the presented publication II. While the summer of 2019 was still relatively hot, the summers of 2020 and 2021 were significantly cooler than 2018. This might be an explanation for the lower prevalence of USUV. An alternative, not mutually exclusive, explanation could be that local birds are developing herd immunity. This has been demonstrated for the bird populations in and around Vienna, where the seroprevalence of

USUV-specific antibodies was reported to be as high as 54 % in 2005 and 2006, only a few years after the initial outbreak in 2001 (159). However, a recent study on birds in Germany revealed that seroprevalence in 2019 and 2020 was even lower than in 2017 and 2018, suggesting that herd immunity had not established (302). Indeed, while USUV circulation was comparatively low in 2022 and 2023, a new outbreak, primarily located in Lower Saxony, was reported in 2024 (337). Interestingly, the average temperature for the summer in 2024 was 18.5° C (338), which was lower than the average of 18.6° C in 2023 (318), and 19.2 ° C in 2022 (315). It is thought that high precipitation during the summer of 2024 might have also contributed to the outbreak (337).

It is important to note that there might be additional reasons why common blackbirds are the most commonly included bird species in dead bird monitoring programmes. Common blackbirds often live in close proximity to humans, thus dying in urban settings where they are more likely to be found by a human than in the wild (339). Many of the other bird species that have been diagnosed with USUV-infections have been living in captivity. (e.g. (164,178,197)). For instance, the great grey owl (*Strix nebulosa*) is also associated with high mortality as a result of an USUV-infection, and infections are reported in captive individuals (152,182,200). However, this species rarely occurs naturally in Germany (340) and other birds of prey usually reside in forests and tend to avoid humans, thus drastically decreasing the chances of being discovered by a human once deceased. This is one of the big drawbacks of employing a passive monitoring of dead birds in Germany (341). The submission locations are heavily biased toward areas with a high human population density. The more humans live in any given area, the higher the chances are that someone who is aware of the call for submissions discovers a dead bird and sends it in. This makes spatial analysis more difficult, because at first glance it appears that USUV is mostly circulating in areas with a high human population density, however, it is unlikely that this is an important factor for USUV, as it uses birds and not humans as the amplification host. Active surveillance efforts are needed to determine the prevalence of USUV in rural and forest areas of Germany. Nonetheless, employing the help of citizens can be a great asset in detecting USUV activity, as it drastically increases the number of people conducting surveillance throughout Germany. Indeed, this monitoring system also helped uncover WNV cases as well (139), showcasing that it is a useful tool for the detection of various bird-affecting pathogens.

3.2.2 Circulation of haemosporidians in Germany

Haemosporidians are well established in Germany and no major outbreak events in birds have ever been recorded as they have for WNV and USUV (203). Nonetheless, it is believed that haemosporidians exert a selective pressure on bird populations, as long-term studies have linked an infection with bird survival, behaviour, mate selection, and reproductive success, although the direction of the association is not clear, i.e. whether the haemosporidian infection is the cause or the consequence (227,342). Although more research is clearly needed, it is becoming increasingly clear that haemosporidians shape the bird population dynamics in Europe and Germany (343–345).

There is no clear picture of haemosporidian prevalence among the different bird species present in Germany. Because the study in the included publication II was executed in the context of an USUV monitoring programme, the vast majority of birds tested were common blackbirds, because as mentioned above, they are most likely to succumb to an infection and subsequently be found by participating citizens. There were only two other studies which investigated the prevalence of haemosporidians in common blackbirds in Germany and the results differ vastly from each other (table 2).

Table 2: **Summary of studies investigating the prevalence of haemosporidians in common blackbirds in Germany**. Columns indicate the prevalence of any haemosporidian infection, *Plasmodium* spp., *Haemoproteus* spp., and *Leucocytozoon* spp. infection.

Year	Overall in %	*Plasmodium* spp. in %	*Haemoproteus* spp. in %	*Leucocytozoon* spp. in %	Source
1965 – 1975	15.2	2.3	13.0	2.3	Haberkorn (346)
2011, 2013	76.7	75*	75*	17.8	Santiago-Alarcon *et al.* (237)

*The study screened for *Plasmodium* spp. and *Haemoproteus* spp. at the same time and did not differentiate between them.

It is important to note that the first study by Haberkorn (346) was done before the development of a molecular screening method, which is why they might have missed some infections.

However, the results of Santiago-Alarcon *et al.* (237) also vastly differ from the results of the included publication II, which detected an overall prevalence of only 38.1 %. This difference might be explained by local variations, because Haberkorn (346) and Santiago-Alarcon *et al.* (237) only tested birds from one specific region in Germany, while this study included birds from all over the country. Additionally, the studies were carried out in different years and annual variations are expected, as shown by the prevalence data per year in the presented publication II. Finally, Haberkorn (346) morphologically diagnosed infections in histological sections and blood smears, while Santiago-Alarcon *et al.* (237) tested the blood of live birds via PCR and the study in the included publication II screened organ samples from dead birds via PCR. Indeed, it has been shown that study design is the best explainer for prevalence variation, which makes comparisons between studies difficult (344).

The overall prevalence of *Haemoproteus* spp. and *Leucocytozoon* spp. was comparatively low in the included publication II with 1.2 % and 3.5 % respectively. It is possible that this is partly due to the study design: In the presented publication II, a PCR protocol was used, which was only able to amplify the most abundant *Plasmodium* spp. or *Haemoproteus* spp. infection present (211). Thus, *Haemoproteus* spp. co-infections with *Plasmodium* spp. might be missed. Furthermore, the PCR protocol used for *Leucocytozoon* spp. also amplified *Plasmodium* spp., so again, co-infections might have been missed. Additionally, the dead birds tested had often been left out at room temperature for several days before the organ samples were collected and it is unknown if this might impact the accuracy of the PCR protocol.

It has been demonstrated that higher temperatures lead to increased prevalence of *Plasmodium* spp. (347) and it is projected that it will spread into more Northern regions and increase its prevalence overall (347,348). Whether this applies to *Haemoproteus* spp. and *Leucocytozoon* spp. remains to be seen. However, just as the increasing temperatures in Germany have facilitated the establishment of WNV and USUV, they could enhance the circulation of haemosporidians in German bird populations in the future.

3.2.3 Co-circulation and association of Usutu virus and haemosporidian

There have been numerous reports of a co-infection rate of USUV-positive birds with haemosporidians of 50 % or higher (163,182,183,298), but there also has been one study from Austria indicating no particularly high co-infection rate and no association (349). The included publication II showed a co-infection rate of 41 % and revealed a positive association between

the two pathogens, i.e. USUV-positive birds were more likely to have a co-infection with haemosporidians than be haemosporidian-free.

The vast majority of the investigated birds in the included publication II were common blackbirds. It is well documented that common blackbirds are particularly vulnerable to succumb to an USUV infection, although the reasons behind this are not fully understood (350–352). There are some studies that have associated USUV infection in common blackbirds with hepatomegaly and splenomegaly (154,159,353), as well as coagulative necrosis, lymphoplasmacytic inflammation and vasculitis (187). Moreover, a study in Zurich suggested that birds might suffer from severe disease for several days before succumbing to it, because they found malnourished, USUV-infected dead birds in a zoo, where food was easily accessible and unlimited (164). Citizens who submitted dead USUV-infected birds and observed them shortly before their death in the presented publication II and elsewhere (161,353) have described immobility, apathy, and ruffled feathers, although these observations need to be confirmed by experts. The reduced fitness through an USUV infection could make the birds more accessible to the vectors transmitting haemosporidians (354) and their weakened immune system might make them more likely to develop severe illness following a co-infection as well (355). Vice versa, a pre-existing haemosporidian infection might make a bird more susceptible to developing severe disease following an additional USUV-infection, as it has been shown that chronic haemosporidian infection can significantly reduce a bird's fitness (225,228,229).

Notably, the association was not statistically significant for most individual years, indicating that other factors might have a bigger influence, which led to a masking of the association. This might also explain why the co-infection rate in the publication II was lower than in other studies (163,182,183,298), but also higher than in one Austrian study (349), even when comparing the same years. Different local conditions such as climate or habitat composition might explain this discrepancy.

As mentioned, temperature appears to be one of the most important factors driving USUV transmission (284). It has further been shown that higher temperatures generally increase haemosporidian prevalence (347). However, a study also demonstrated that too high temperatures reduce transmission of haemosporidians in mosquitoes, which could be an explanation for the relatively low prevalence in the extraordinarily hot years of 2018 and 2019 (356). In contrast, USUV appears to be thriving under these hot conditions, but displays lower circulation during comparatively colder summers, when haemosporidian prevalence is in turn

higher. This could be a potential explanation for the seemingly inverse prevalence pattern shown in publication II.

It is further possible that USUV and haemosporidians influence each other's circulation patterns indirectly. Many of the citizens who submitted birds reported the disappearance of their local common blackbird populations during the outbreak of 2018 and a slow return in the following years. The impact on common blackbird populations has been investigated before. For example, during the outbreak in and around Vienna at the beginning of the century, a decline in the local common blackbird population of around 90 % was estimated (156). Several studies analysed the first German USUV outbreak in 2011–2012 in the region of the Upper-Rhine valley and concluded that between 40,000–420,000 common blackbirds died (357–359). A study investigating the same area in 2016 estimated that the common blackbird populations declined by around 15 % in USUV-affected areas compared to USUV-free areas (335). The extremely high number of submissions of dead common blackbirds in 2018 compared to previous and following years in the included publication II indicates that the mortality during that year was even higher than during the previous outbreaks. As host abundance has been shown to influence haemosporidian prevalence (360), the decline in common blackbird populations due to USUV circulation could be an explanation for the lower haemosporidian prevalence during the years that USUV prevalence was high.

While it seems that there is an association between haemosporidians and USUV, it is also becoming clear that there are many differences in their circulation patterns and many more factors influencing their prevalence. More studies are necessary to understand the relationship of these pathogens. Nonetheless, it is clear that co-circulation increases the burden on local bird population and if the warming climate indeed leads to an increase in USUV and haemosporidian prevalence, this impact is likely to increase in the future.

3.3 Meta-analysis of *D. immitis* and *D. repens* reports in Europe

3.3.1 *Dirofilaria* spp. infections in dogs

Dogs are presumed to be the main amplification host of *Dirofilaria* spp. and most infections were indeed shown to be diagnosed in dogs in the included manuscript III. Interestingly, *D. immitis* infections were detected twice as often as *D. repens* infections. Besides differing circulation pattern, an additional explanation for this could be that *D. repens* is harder to diagnose and treat than *D. immitis* for several reasons. Firstly, *D. repens* infections in dogs are less likely to be noticed, because contrary to *D. immitis* infections, they often cause no or mild symptoms (232). Secondly, a *D. repens* diagnosis is significantly more difficult to achieve than a diagnosis for *D. immitis*, as there are no commercially available testing kits for *D. repens*, which is why *D. repens* is usually excluded in routine screenings of dogs (498). Instead, a diagnosis relies on morphological or molecular identification (362). Thirdly, the available treatments in dogs were developed for *D. immitis* infections and there is growing concern over their applicability for *D. repens* infections (363).

During the 20th century, infected dogs were mostly located in Southern Europe, predominantly Spain, Italy and Greece as shown in the included manuscript III. The Po River Valley area in Northern Italy was for a long time considered to be the area in Europe most affected by *D. immitis* (364), with over 50 % of dogs infected during the 1990s (365,366). In other areas, *D. repens* was more prevalent than *D. immitis,* for example in Southern Spain with a *D. repens* prevalence in dog kennels of up to 84.6 % in 2000 (367). During the past two decades, both *Dirofilaria* species have displayed an unprecedented spread into Central and Northern Europe, leading to an overall increase in *Dirofilaria* spp. cases, as shown in the included manuscript III. Despite the overall increase, however, there is evidence that during the last 20 years the prevalence of *D. immitis* has decreased in previously hyperendemic areas (263), which is attributed to increased awareness as well as improved diagnostic and treatment methods. For example, in Italy, *D. immitis* is now less prevalent in the previous hyperendemic North but is now detected in previously *D. immitis*-free areas with an overall infestation rate in dogs of up to 8.5 %, leading to a general increase in *D. immitis* circulation in the country (368). Similarly, *D. immitis* infections are now reported not only in the South of France, but in its centre as well, with a prevalence of up to 35.2 % (369–371). There is growing evidence that several countries that were considered *Dirofilaria*-free during the 20th century must now be viewed as endemic, including Germany (372), Austria (373–375), Czech Republic (376), Hungary (377), and Slovakia (376).

Interestingly, there are indications that *D. repens* is spreading faster than *D. immitis* and that in areas, where both parasites circulate, *D. repens* displays a higher prevalence (244,378,379). It has been shown that *D. repens* can disrupt the transmission of *D. immitis* in Southern Italy (380), although this observation warrants further investigation. Another reason for the fast spread might be that *D. repens* is harder to control due to the lack of rapid and regular testing as well as effective preventative and curative treatment, as discussed above. Therefore, more research on *D. repens* diagnosis and treatment is urgently needed. It would be particularly important to develop a commercially available rapid diagnostic tool in order to identify infections as early as possible.

3.3.2 *Dirofilaria* spp. infections in humans

The mammal, in which *D. repens* was by far the most diagnosed *Dirofilaria* species was humans, as shown in the presented meta-analysis. During the 20th century, there were only a few human *D. immitis* cases, but human *D. repens* infections were found throughout Southern Europe. This is in contrast to *Dirofilaria* spp. infections in dogs, which appeared to have been limited to the far South and coastal regions, as shown in the included manuscript III. A potential explanation is that human *D. repens* infections are relatively easily identified, because the worms usually form subcutaneous nodules and in about 35 % of the cases they are localised in the ocular region (244,264,265). This makes them easily accessible to extract and identify.

In the 21st century, human *D. immitis* cases were still relatively few, but could nonetheless be detected as North as the United Kingdom, indicating a northward spread, as displayed in the presented manuscript III. Similarly, human *D. repens* infections were now found throughout Central Europe and as far North as Finland. *Dirofilaria repens* remains by far the dominant species in humans. Conversely, human *D. immitis* infections are much more common in the American continent, although it has to be noted that *D. repens* does not circulate there. It is not completely understood why the frequency of human *D. immitis* is much lower in Europe and in areas of co-circulation with *D. repens.* The hypothesis, that European *D. immitis* variants might be genetically different to the ones circulating on the American continent and are less adapted to survive inside humans as a consequence has been disproven (381,382). Another potential explanation is that *D. repens* impedes the circulation of *D. immitis* in humans, as it might do in dogs (380). More studies on the interspecies dynamics of the nematodes are warranted to shed some light onto this. Regardless, it is undeniable that *D. repens* infections are an increasing risk to humans. Although *D. repens* infections rarely develop into severe disease, the worms can

survive for up to one and a half years inside a nodule, causing irritation, erythema, and pruritus (264,265,383). More severe cases occur in immunosuppressed patients and can include systemic reactions such as fever, mild eosinophilia, or lymphadenopathy. Therefore, it is important to minimise the transmission of *D. repens* to humans as much as possible.

3.3.3 *Dirofilaria* spp. infections in other mammals

Besides dogs, cats are also often affected by *Dirofilaria* spp. infection, mostly by *D. immitis*, as shown in the presented meta-analysis. However, their role in transmission is thought to be small, because the nematodes rarely produce microfilariae (256) and the prevalence of *D. immitis* in cats is estimated to be 5- to 20-fold lower than it is in dogs (259,384). Nonetheless, infections can lead to severe disease and death in cats (256). Due to their domestication, a cat fallen sick due to a *Dirofilaria* spp. infection is likely to be brought to a veterinarian, where a diagnosis can be made, which might explain why there are so many reports of cat cases (258). This number of infections in cats reported shows that even though cats may not be that important for *Dirofilaria* spp. transmission, the circulation of the worms still poses a health risk for cats.

While dogs are considered to be the main amplification host, infections of wild canids are regularly reported, indicating that they may play a crucial role in transmission as well. As seen in the meta-analysis, the species in which *Dirofilaria* spp. was detected most often were red foxes (*Vulpes Vulpes*), golden jackals (*Canis aureus*) and grey wolves (*Canis lupus*). While human activity such as urbanisation and agricultural intensification has led to a reduction of many carnivore populations during the 20th century, increased conservation efforts have enabled recolonization and growing numbers in the last few decades (385). A study from Italy showed that the prevalence of *D. immitis* in a recolonised grey wolf population was relatively high, implying that they might act as important reservoirs for the parasite (386). Similarly, several studies indicated red foxes as reservoir hosts based on frequently detected *D. immitis* infections (262,387,388). Additionally, golden jackals have recently expanded their distribution range and are now found throughout South, East and Central Europe with occasional reports from West and North Europe (389,390). This movement is attributed at least in part to the warming climate (377). Their increased presence throughout Europe might have impacted the spread of *D. immitis*, by providing a growing number of reservoir hosts (391). Furthermore, human activity has led to a decrease in wildlife habitat, increasing the proximity of humans and dogs to wildlife, thus increasing the chance of transmission of pathogens between the populations (392). More

studies are needed to better understand the role of potential wild reservoir hosts and control strategies have to be adjusted accordingly.

There have been a number of *Dirofilaria* spp. infections in non-canid mammals, as shown in the included manuscript III. Many of these animals resided in zoos or were held as pets and their close proximity to humans likely lead to a diagnosis (393–396). As no viable microfilariae were detected in these animals, it is thought that they were accidental hosts and did not contribute to the transmission cycle (393–396). Nonetheless, it is striking that these animals were almost exclusively infected by *D. immitis.* One explanation could be that a *D. immitis* infection more often leads to symptoms, as it does with dogs (238) and that rapid testing is only available for *D. immitis,* as discussed above (498). Additionally, *D. immitis* might have a broader host range than *D. repens* and is able to survive and cause disease in more species. However, more research is needed to investigate this hypothesis.

3.3.4 *Dirofilaria* spp. infections in mosquitoes

Both *Dirofilaria* species have been found in a range of mosquitoes, as presented in the included meta-analysis. However, a detection alone is not a proof that the mosquito could actually transmit the parasites, as it could have just fed on an infected host. In order for transmission to occur, the worms need to develop inside the mosquito and migrate to its feeding tubes. To differentiate between mosquitoes that have merely fed on an infected host and mosquitoes that are able to transmit the *Dirofilaria* species, some studies have separated the abdomen and the thorax before testing for the presence of *Dirofilaria* spp. Mosquitoes with infections in their thorax are implicated as vectors. As shown in the included manuscript III, there are a number of mosquito species that are implicated as vectors for both, *D. immitis* and *D. repens* (*Ae. albopictus, Ae. caspius, Ae. vexans, An. maculipennis* s.l., *Cx. pipiens* s.l., and *Cx. theileri*) and two mosquito species that are implicated only for *D. immitis* transmission (*Ae. detritus* s.l. and *Cq. richardii*). It has to be noted, however, that there are only a few studies that conducted experimental transmission tests to definitely prove the transmission capacity of the mosquitoes. So far, this has been done for *Ae. vexans* (397)*, Cx. pipiens* bioform *molestus* (398)*,* and *Ae. albopictus* (241). Nonetheless, the fact that most of the implicated vectors are repeatedly found infected with *Dirofilaria* spp., as shown in the included manuscript III, strongly indicates that they are indeed capable vectors. This is of great concern, as most of these mosquito species are distributed throughout the entire continent (399).

One vector believed to be very important for *Dirofilaria* spp. transmission is *Ae. albopictus* (263,392,400). Interestingly, while there were a number of reports of an infection with *D. immitis,* there were only two studies detecting *Ae. albopictus* mosquitoes infected with *D. repens*. This could mean that *Ae. albopictus* is of lesser importance to *D. repens* than previously thought, although this warrants further investigation. Similarly, whether *Ae. detritus* s.l. and *Cq. richardii* are truly vectors only for *D. immitis* or if they can transmit *D. repens* as well, remains to be seen. Since there are very few experimental studies to determine the transmission efficiency of all the implicated vectors, it is difficult to know which mosquito species might be the most relevant for *Dirofilaria* spp. transmission.

Dirofilaria repens was found in a wider range of mosquito species overall, although most of them are not believed to be vectors. Nonetheless, this implicates that *D. repens* is, at least in some areas, more prevalent than *D. immitis.* This is in line with observations of infected mammals, which suggest that *D. repens* might be spreading faster than *D. immitis* (244,378,379) and is harder to control due to the lack of regular testing and effective treatment (361,363), as discussed above.

3.3.5 Causes for the spread of *D. immitis* and *D. repens* in Europe

There are several reasons attributed to the drastic spread over the past few decades. The main reason for the drastic expansion of *Dirofilaria* spp. is thought to be the warming climate. The development of *Dirofilaria* spp. inside the mosquito is strongly temperature-dependent and the extrinsic incubation period shortens with rising temperatures (246,401). As a result, warming climate leads to increased transmission. Additionally, it has been established that the parasites do not develop inside the mosquito below temperatures of 14°C (241,245,402), and with rising temperatures due to climate change, areas that were previously too cold for development are now exceeding that threshold regularly and long enough to enable stable transmission (403). Besides the direct impact on the parasites themselves, the warming climate further allows *Ae. albopictus* to survive in increasingly Northern parts of Europe (404). The introduction and spread of the invasive *Ae. albopictus* is attributed to have increased *Dirofilaria* spp. cases (263,392,400). It was first introduced into Europe during the end of the 20th century and has since continuously spread northward, due to the increasingly suitable climate for its development (405). *Ae. albopictus* has been shown to be a highly competent vector for both *Dirofilaria* species (241) and infected mosquitoes have been detected in Europe several times (239,370,406–408), as mentioned above. This mosquito is furthermore known for its aggressive

daytime biting and is indicated to often feed on mammals, including dogs and humans (409–411). These attributes make it an ideal vector for transmission between dogs, but also as a bridge-vector between dogs and humans.

Finally, the EU passed the Pet Travel Scheme in 2000. This legislation allowed the travel of dogs, cats, or ferrets within the EU or from a non-EU country into the EU if the animal had been microchipped or tattooed, been vaccinated against rabies, had a valid European pet passport or EU animal health certificate, and had been treated against the tapeworm *Echinococcus multilocularis* if travelling into an *E. multilocularis*-free country (412). This made pet travel significantly easier in the EU. However, the requirements included neither preventive treatment against nor testing for *Dirofilaria* spp. infections. The Pet Travel Scheme has been associated with a significant spread of *Dirofilaria* spp. in Europe as a consequence (413). Besides travelling with their owners, dogs are also often moved between countries as part of rehoming programmes. For example, dogs adopted by German owners often originate from South European countries such as Italy, Spain, and Greece, where *Dirofilaria* spp. is well established and as a result, dogs regularly carry an infection when they arrive in Germany (414,415). These dogs often used to be stray dogs and the high number of stray dogs in some European countries, such as Italy, Romania, or Bulgaria have been shown to be important reservoirs for *Dirofilaria* spp., because they are not subjected to regular treatment or testing (416–419).

4. Conclusion and outlook

Pathogens transmitted by mosquitoes and other arthropods are a growing concern in Europe and Germany. Various pathogens have been introduced and established circulation, such as WNV and USUV, posing a threat for native animals, including birds and horses, as well as causing disease in humans. Interactions with already established pathogens like haemosporidians put an additional burden on European bird populations. In addition to the emergence of pathogens, pathogens that have existed in Europe for centuries, such as *Dirofilaria* spp. have notably increased their circulation areas, affecting more animals and humans than ever. Reasons for this drastic increase in mosquito-borne diseases are globalisation with increased travel and the warming climate (420,421).

There are several factors needed for stable transmission of mosquito-borne diseases. Firstly, a sufficient number of reservoir hosts need to be available. The pathogens need to be able to amplify within the host, but also not cause lethal disease, because this would eliminate the host from the transmission cycle (422–424). Secondly, competent vectors need to be present to transmit the pathogens from one host to another. When pathogens are taken up via blood meal by a mosquito, they are located inside the midgut, so they need to migrate into the salivary gland, where they can be deposited into the next host (422,423,425). This requires a high level of adaptation to the mosquito for the pathogen to avoid being eliminated by the mosquito's immune system (425–427). Therefore, pathogens can often only be transmitted by specific mosquito species. Both, host and vector need to be present in sufficient abundance so the chance that a competent mosquito feeds on first an infected and subsequently an uninfected host is high enough to sustain stable transmission (428). The final important factor is a suitable climate. The development of the pathogens inside the mosquito (extrinsic incubation period) is highly dependent on temperature (429,430). Below certain temperatures, that vary per pathogen, development does not occur at all and usually the higher the temperature, the shorter the extrinsic incubation period, increasing transmission. Higher temperatures are also associated with faster development of mosquito larvae into adulthood and mosquito abundance is usually higher in warmer climate (431,432). Due to the climate-dependency, mosquitoes enter dormancy during the winter months in Europe, limiting the mosquito and consequently the transmission season to April to October (433).

It has become clear that the continuously increasing temperatures in Germany are a main driver for the introduction and spread of various pathogens. The warming climate has enabled the establishment of WNV, USUV and *Dirofilaria* spp. While there are some indications that higher

temperatures might increase haemosporidian circulation as well, this needs further investigations before a conclusion can be drawn. The effect of co-circulation of various pathogens that use the same vector and bird species, such as WNV, USUV and haemosporidians or *D. repens* and *D. immitis* needs to be closely monitored. Such a co-circulation is unprecedented in Germany and Central Europe and the consequences are unclear. Several studies, among them the included manuscript III, have indicated that the pathogens influence each other and these relationships need to be further studied.

While it remains to be seen how the circulation of the here discussed pathogens will develop in the future, it is safe to say that they will significantly shape the ecology of the German fauna and will remain of importance for human and animal health in the foreseeable future.

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List of figures and tables

Appendix

viruses

MDPI

Article

West Nile Virus Epidemic in Germany Triggered by Epizootic Emergence, 2019

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Abstract: One year after the first autochthonous transmission of West Nile virus (WNV) to birds and horses in Germany, an epizootic emergence of WNV was again observed in 2019. The number of infected birds and horses was considerably higher compared to 2018 (12 birds, two horses), resulting in the observation of the first WNV epidemy in Germany: 76 cases in birds, 36 in horses and five confirmed mosquito-borne, autochthonous human cases. We demonstrated that Germany experienced several WNV introduction events and that strains of a distinct group (Eastern German

WNV clade), which was introduced to Germany as a single introduction event, dominated mosquito, birds, horse and human-related virus variants in 2018 and 2019. Virus strains in this clade are characterized by a specific-Lys2114Arg mutation, which might lead to an increase in viral fitness. Extraordinary high temperatures in 2018/2019 allowed a low extrinsic incubation period (EIP), which drove the epizootic emergence and, in the end, most likely triggered the 2019 epidemic. Spatiotemporal EIP values correlated with the geographical WNV incidence. This study highlights the risk of a further spread in Germany in the next years with additional human WNV infections. Thus, surveillance of birds is essential to provide an early epidemic warning and thus, initiate targeted control measures.

Keywords: West Nile virus; Germany; epizooty; epidemic; human; bird; horses; mosquitoes; transmission risk; zoonoses

1. Introduction

West Nile virus (WNV, family *Flaviviridae*, genus *Flavivirus)* is maintained in a transmission cycle between birds as amplification hosts and mosquito vectors [1]. Spillover events have significant public health and veterinary relevance [2]. A total of 25% of the infected people develop West Nile fever (WNF) and become symptomatic (e.g., headache or muscle pain) [3]. Severe disease progressions manifesting as WNV neuroinvasive disease (WNND) are rare (<1%) [4]. These include syndromes of meningitis, encephalitis, and acute flaccid paralysis/poliomyelitis. Case-fatality rate of WNND is approximately 10% [5]. Age is the most important risk factor for WNND and a fatal disease outcome [2]. Thus, WNV circulation poses considerable risk for transfusion and organ transplantation safety [6].

WNV is distributed in wide areas of Europe. The main focus of WNV circulation is in south-eastern Europe and Italy [7]. However, low WNV activity is also observed in the neighboring countries of Germany (France, Austria, and Czech Republic). Therefore, over the last decade, different monitoring programs were implemented in Germany to screen for WNV RNA and antibodies in birds, horses, mosquitoes and chicken eggs [8–12]. In 2018, an epizootic emergence of WNV was observed in Germany for the first time [13]. All WNV-positive birds and horses were infected with the same WNV lineage 2 strain of the central European subclade II. WNV activity was detected in eastern Germany over a distance of almost 900 km (Munich to Rostock). At the same time, a large WNV outbreak was observed in south-eastern and southern Europe [7]. However, phylogenetic analysis in combination with the wide distribution in Germany indicates that WNV may have been introduced from the Czech Republic to Germany already before 2018 [13]. The emergence of WNV in Germany and the focus in the central part of eastern Germany was correlated with outstandingly high summer temperatures. As demonstrated for other European countries, WNV is probably predominantly transmitted by different native *Culex* species. *Culex pipiens* biotype *pipiens*, *Culex pipiens* biotype *molestus* and *Culex torrentium* from Germany were experimentally proven to be susceptible to WNV infection [14].

In this study, we report a WNV epidemic in Germany, 2019, triggered by an epizootic emergence among birds with spillover to horses and humans. Human and animal cases were located in the same area, showing a high WNV activity also in 2018. In both years, the region was characterized by suitable temperature conditions allowing a short extrinsic incubation period (EIP). Phylogenetic and phylogeographic analysis showed that Germany experienced several WNV introduction events. Several virus variants circulate in the affected German regions with Austria and Czech Republic as possible origins. The majority of the WNV strains involved in the German outbreak clustered together into a distinct and dominating group (Eastern German WNV clade) comprising of mosquito, bird, horse and human-related virus variants.

2. Materials and Methods

2.1. WNV Screening of Birds, Horses and Mosquitoes

Since the first outbreak of Usutu virus (USUV) in Germany (2011/12), a nationwide bird surveillance network (living and dead birds) was set up to monitor for zoonotic arboviruses with a focus on WNV and USUV. In this context, a variety of dead birds and organ samples were submitted to the Bernhard Nocht Institute for Tropical Medicine and the national reference laboratory for WNV at the Friedrich-Loeffler-Institut (FLI) by the regional veterinary laboratories of the federal states of Germany, by the German Mosquito Control Association (KABS), the Nature and Biodiversity Conservation Union (NABU), citizens and independent bird clinics and zoological gardens. WNV infection in birds and horses is a notifiable disease in Germany if a recent infection is detected by a WNV-specific RT-qPCR result and/or a positive result of horses by IgM-ELISA, i.e., the detection of a fresh WNV infection. A previous vaccination of horses must be excluded. A positive IgG or neutralizing antibody detection is not notifiable in Germany.

Requests for the submission of dead birds were made via press releases of involved institutes and subsequent dissemination of the information by different kinds of media, including newspaper articles, television and radio. Total RNA from homogenized tissue samples (brain, liver, lung, or heart) was extracted and analyzed for the presence of flavivirus RNA by using a modified pan-flavivirus reverse transcription PCR [15] or WNV-specific reverse transcription quantitative PCR (RT-qPCR) [16]. Furthermore, all samples were also tested using the USUV-specific RT-qPCR described by Jöst et al. [17] (data not shown).

Organ samples from affected horses were also tested by the RT-qPCR as stated above. In the case of diseased horses, often with neurological symptoms typical for WNV disease, the serum samples were screened by IgM-and/or IgG-ELISA (IDVet, Grabels, France) and positive samples were confirmed by differentiating virus neutralization tests to exclude cross-reacting flaviviruses (USUV, tick-borne encephalitis virus (TBE)) [12].

Following the first confirmed avian WNV case in the Tierpark Berlin (Wildlife Park) in 2019, mosquitoes were collected in that park by EVS (Heavy Duty Encephalitis Vector Survey) traps (BioQuip Products, Rancho Dominguez, CA, USA) equipped with dry ice as an attractant. Traps were continuously operated from mid-September to early October and emptied daily. Captured mosquitoes were morphologically identified to species or complex using the determination key by Becker et al. [18] and pooled with up to ten specimens per pool. Pools were homogenized and subjected to RNA extraction and WNV RT-qPCR as described above [16]. Positive samples were inoculated on C6/36 cells (L 1299, Collection of Cell Lines in Veterinary Medicine (CCLV), Friedrich-Loeffler-Institut, Greifswald – Insel Riems, Germany). Six days after inoculation, the supernatant of infected cultures was tested again with WNV RT-qPCR and the two samples with the lowest Ct-value were used for NGS analysis [19].

2.2. Risk of WNV Transmission

The extrinsic incubation period (EIP) gives the time between ingestion of a pathogen via blood meals and the vectors' ability to retransmit the pathogen. In contrast to other indices for transmission risk (e.g., field-measured infection rates of vectors), this approach is a theoretical risk assessment using information on the temperature-dependent EIP from the literature. However, EIP values give an approximation of virus transmission risk through the mosquito vector under local temperature conditions. Therefore, daily EIP values (EIP_d) of WNV were calculated with the formula $-0.132 + 0.0092 \times$ temperature [13,20]. The day-to-day mean E-OBS temperature dataset v20.0e (July 2018 to August 2019) was downloaded from http://www.ecad.eu [21]. Data analysis and visualization was conducted with the program R [22] using the packages lubridate [23] and raster [24]. For the risk assessment, EIP_d values for the subsequent days were summed up until the virus development was completed (=EIP). For each grid cell and year, EIP values were averaged for the period from 15th July to 14th August (=EIP_{ave}).

2.3. Data Sets and Genome Characterization of WNV

A total of 39 WNV genomes from birds, humans, mosquitoes and horses were newly acquired as part of this study (Table 1, Figure 1, Supplementary Table S1). The extracted viral RNA of WNV positive specimens was subjected to a next-generation sequencing (NGS) workflow [25], or to random RT-PCR amplification followed by library preparation by using the QIAseq FX DNA Library Kit (Qiagen, Hilden, Germany). They were normalized, sampled and sequenced using 150-cycle NextSeq550 Reagent Kits v2.5 (Illumina, San Diego, CA, USA) on a NextSeq550 platform (Illumina, San Diego, CA, USA) or the Ion Torrent S5 chemistry (ThermoFisher Scientific, Waltham, MA, USA) on an Ion Torrent S5 XL platform (ThermoFisher Scientific, Waltham, MA, USA). All whole genome sequences of WNV with known sampling time (year) and geographical origin (country) from Europe were retrieved from GenBank (n = 98) and combined with those sequenced in this study. Two data sets have been created: one containing all genomes from Europe incl. Germany, and a second one comprising the "Eastern German clade only." Sequences were aligned using the MAFFT algorithm and then visually inspected in Geneious v2020.0.2 (https://www.geneious.com, Biomatters, Auckland, New Zealand). All sequences were confirmed as non-recombinant by the various methods for recombination detection implemented in RDP4 [26]. The obtained full-length recovered genome sequences of the WNV were submitted to GenBank or the European Nucleotide Archive (accession no. MN794935-MN794939, LR743421-LR743437, and LR743442-LR743458).

2.4. Evolutionary Dynamics and Phylogeography of German WNV

Genomes obtained for the German WNV strains were compared with all European complete or near complete genomes sequences publicly available. For molecular clock phylogenetics, maximum clade credibility (MCC) trees were inferred using the Bayesian Markov chain Monte Carlo (MCMC) approach available in BEAST v1.10 [27]. Analyses were performed under the best fit nucleotide substitution model identified as GTR +Γ for the complete genome data set including "all European" genomes and TN93+Γ for the data set for "Germany only" using jModelTest 2 [28]. To search among maximum likelihood (ML) trees, we employed both nearest neighbor interchange (NNI) and subtree pruning and regrafting (SPR) branch swapping. To assess the robustness of each node, a bootstrap resampling process was performed (1000 replicates) again using the NNI branch-swapping method available in PhyML [29] (data not shown). We have employed the TempEst tool for an interactive regression approach to explore the association between genetic divergence through time and sampling dates [30]. In order to assess the spatiotemporal dynamics of WNV, the time to most recent common ancestor (tMRCA), evolutionary rate and the effective population dynamics of WNV was employed with a relaxed uncorrelated log normal and a strict molecular clock under a flexible demographic model (the coalescent Gaussian Markov Random field (GMRF) Bayesian Skyride) as the best demographic scenario detected. In all cases, each of the MCMC chain lengths was run for 5×10^7 generations (with 10% burn-in) with subsampling every 10^4 iterations to achieve convergence as assessed using Tracer v1.5 [31]. The MCC trees were visualized using FigTree v1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/). To test the hypothesis that WNV was periodically introduced to Germany, a phylogeographic analysis was conducted using a discrete model attributing state characters represented by the detection locality of each strain and the Bayesian stochastic search variable (BSSV) algorithm implemented in BEAST v1.10 [27]. An MCC tree was summarized using TreeAnnotator v1.10. and visualized in FigTree v1.4.3. SpreadD3 v. 0.9.7.1 (https://rega.kuleuven.be/cev/ecv/software/SpreaD3_tutorial) was used to run BSSV analysis and generate Bayes factor (BF) and posterior probability (PP) to test for statistically significant epidemiological links between discrete sampling locations. The potential transmission networks within and between countries for NS5 WNV were inferred in PopART package v1.7.2 using median joining tree method with an epsilon of zero [32].

3. Results

3.1. Spatial Analysis of West Nile Virus Circulation

A total of 88 birds and 38 horses tested positive for WNV in 2018 (diagnosed between 28.8. and 9.10) and 2019 (diagnosed between 8.7. and 21.11) in Germany. In addition, five probably mosquito-borne human WNV cases were diagnosed with no history of travel to WNV-endemic countries within the last month. Except a single specimen (Hamburg, 2019), all WNV-positive animals originated from the eastern part of Germany with a distinct focus for the federal states Saxony-Anhalt, Saxony, Berlin and Brandenburg (Table 1, Figure 1). In addition, the targeted screening in the Tierpark Berlin revealed seven WNV positive *Culex pipiens* complex mosquito pools in 2019.

Low WNV activity was detected for the federal states Bavaria and Mecklenburg-Western Pomerania in 2018, which was not observed in 2019. WNV cases were found in Hamburg and Thuringia in 2019 for the first time. The number of positive birds and horses rose considerably in 2019 (76 birds and 36 horses) compared to 2018 (12 birds and two horses).

Table 1. West Nile virus (WNV)-positive birds, horses and mosquito-borne, autochthonous humans for the federal states of Germany in 2018/2019. Numbers in brackets indicate the number of samples with WNV sequences acquired in this study.

Federal State	Birds (2018)	Horses (2018)	Birds (2019)	Horses (2019)	Humans (2019)	Sum
Bavaria (BY)	2 (2)	0	0	0	0	**2 (2)**
Berlin (BE)	3 (1)	0	33 (6)	0	1 (1)	**37 (8)**
Brandenburg (BB)	0	1	6 (3)	7	0	**14 (3)**
Hamburg (HH)	0	0	1 (1)	0	0	**1 (1)**
Mecklenburg-Western Pomerania (MV)	1	0	0	0	0	**1**
Saxony (SN)	1 (1)	0	21 (8)	9 (1)	3	**34 (10)**
Saxony-Anhalt (ST)	5 (2)	1	15 (10)	19	1 (1)	**41 (13)**
Thuringia (TH)	0	0	0	1	0	**1**
Sum	**12 (6)**	**2**	**76 (28)**	**36 (1)**	**5 (2)**	**131 (37)**

In addition to the 37 WNV sequences, two more genome sequences were obtained from WNV-positive mosquito pools collected in Berlin.

Especially in 2019, a large number of different bird species was affected (Table 2). A total of 52 birds (59.1% of all WNV-positive birds in 2018/2019) were held in captivity. From the total of 88 infected birds only four goshawks in private aviaries survived the infection. Of the 38 infected horses, 29 animals showed typical clinical symptoms, of which five horses died or were euthanized. Most of the other sick horses recovered in a very short time. Another nine horses were asymptomatic and were detected in the framework of additional investigations of holdings in relation to the clinical outbreaks. All 38 infected horses were positive in the IgM-ELISA and were therefore notified.

The area with highest activity of WNV circulation was similar in 2018 and 2019, i.e., central-eastern Germany with most WNV-positive samples (mosquitoes, birds and horses) (Figure 1). In addition, all mosquito-borne, autochthonous human WNV cases were observed in this region. This matches the risk analysis based on the temperature conditions during summer, which indicates short EIP_{ave} (<15 days) for this area. The region along the Upper Rhine Valley (south-western Germany) was also characterized by low EIP_{ave} values, but no WNV circulation was detected in either year. Re-emergence of WNV was not observed for the most northern (Rostock) and southern (Poing) foci of WNV from 2018. This correlates with higher EIP_{ave} values for 2019 (>25 d; Poing: 28.4, Rostock: 26.2) compared to 2018 (<25 d; Poing: 21.6, Rostock: 19.6) for these areas, i.e., lower risk of WNV transmission.

Table 2. Detection of WNV infections in different bird species in 2018 and 2019.

Bird Species	Scientific Name	Housing	Number of WNV-Infected Birds	Affected Federal States *
Eurasian Blackbird	*Turdus merula*	wild	3	ST, MV
Andean Flamingo	*Phoenicoparrus andinus*	captive	1	BE
Great Grey Owl	*Strix nebulosa*	captive	6	SN, ST, BY
Unspecified buzzard	*Buteo* sp.	wild	1	ST
Blue Tit	*Parus caeruleus*	wild	3	SN, ST
Chilean Flamingo	*Phoenicopterus chilensis*	captive	6	BE, SN
Eurasian Jay	*Garrulus glandarius*	wild	1	BB
Coconut Lorikeet	*Trichoglossus haematodus*	captive	1	ST
Scarlet-chested Parrot	*Neophema splendida*	captive	1	SN
Eurasian Golden Plover	*Pluvialis apricaria*	wild	1	SN
Northern Goshawk	*Accipiter gentilis*	wild/captive	19	BB, BE, SN, ST
House Sparrow	*Passer domesticus*	wild	4	SN, ST
Dunnock	*Prunella modularis*	wild	1	HH
Humboldt-Penguin	*Spheniscus humboldti*	captive	1	BB
Inka-Tern	*Larosterna inca*	captive	1	BE
Black-tailed Gull	*Larus crassirostris*	captive	8	BE
Kagu	*Rhynochetos jubatus*	captive	1	BE
Domestic Canary	*Serinus canaria forma domestica*	captive	2	SN
Great Tit	*Parus major*	wild	3	SN
American Flamingo	*Phoenicopterus ruber*	captive	3	BE
Hooded Crow	*Corvus corone cornix*	wild	1	BE
Unspecified pelican	*Pelecanus* sp.	captive	1	ST
Javan Pond Heron	*Ardeola speciosa*	captive	1	BE
Common Wood Pigeon	*Columba palumbus*	wild	1	BE
Snowy Owl	*Bubo scandiacus*	captive	8	BE, ST
Chinese Merganser	*Mergus squamatus*	captive	1	BE
Swift Parrot	*Lathamus discolor*	captive	1	SN
Little Owl	*Athene noctua*	wild	2	BB
European Goldfinch	*Carduelis carduelis*	captive	1	SN
Eurasian Eagle-Owl	*Bubo bubo*	wild	1	SN
Tawny Owl	*Strix aluco*	wild	1	ST
White Eared Pheasant	*Crossoptilon crossoptilon*	captive	2	BE

* abbreviations as in Table 1.

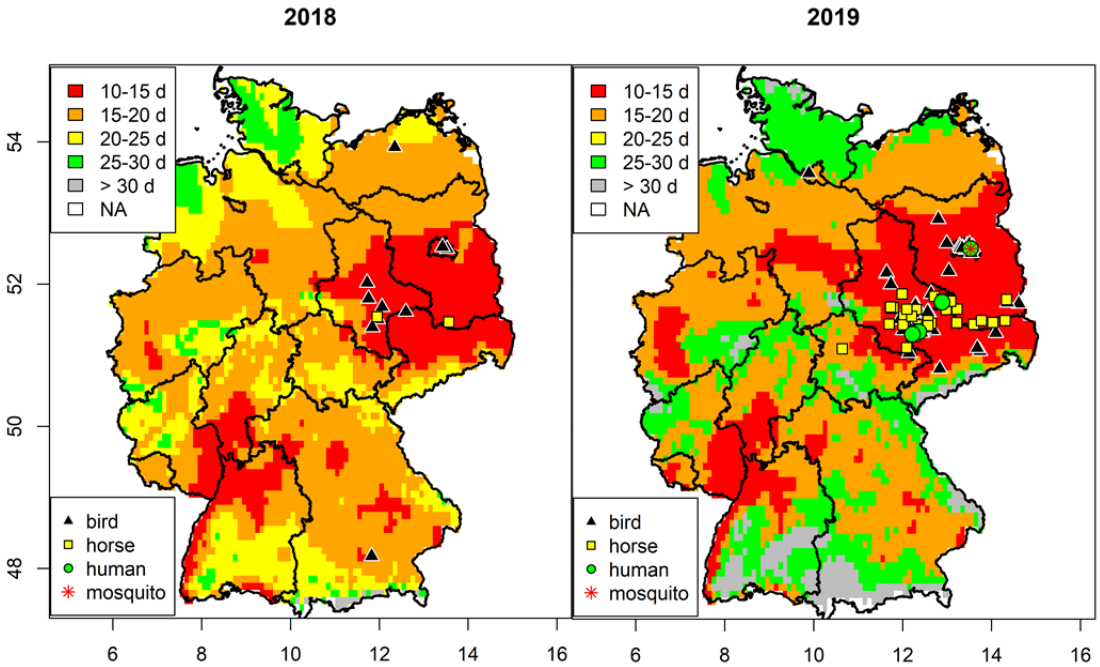

Figure 1. Spatial risk of West Nile virus (WNV) transmission in Germany. Average extrinsic incubation period between 15th July to 14th August 2018/2019 and distribution of WNV-positive birds, horses, humans and mosquitoes.

3.2. Autochthonous Human WNV Cases

In September 2018, a 31-year-old male veterinarian developed flu-like symptoms after necropsy of a WNV-positive owl (https://promedmail.org/promed-post/?id=20181006.6074497). The laboratory confirmation was based on detection of an IgM response against WNV and a cross-reactive IgG response against WNV, which might be also the result of past vaccinations against TBE and yellow fever virus. The first mosquito-borne, autochthonous infection for Germany was confirmed in Leipzig, Federal State of Saxony on 20 September 2019. The 69-year-old male patient presented with WNND, received supportive treatment at the Infectious Diseases (ID) intensive care unit (ICU) between September 3rd and September 20th and was released with restitutio ad integrum. The laboratory confirmation was based on detection of WNV RNA in an early CSF, serum and urine sample and the detection of WNV IgM and IgG in serum samples. A second autochthonous case in Leipzig, an 81-year old male, was admitted to the ICU with presumptive diagnosis of pneumonia, then transferred to the ID-ICU and was found to have WNND confirmed by WNV IgM and IgG in serum samples as well as WNV RNA in CSF samples as early as from 19 September. He also recovered after 12 days of supportive care including mechanical ventilation without neurological sequelae. Both patients reported no history of travel to WNV-endemic countries and routes of non-vector borne transmission were excluded. While one patient had direct contact with the corpse of a Blue Tit (*Parus caeruleus*) five days before onset of fever, the other one reported no obvious contact with animals. Both patients had experienced multiple mosquito bites in the weeks before onset of illness. The third WNV infection was diagnosed on 24 September in a 46-year-old female patient from Berlin, Federal State of Berlin, who presented with West Nile Fever (WNF). The patient did not receive any treatment and recovered within two weeks. The laboratory confirmation was based on detection of WNV RNA in an early serum sample and seroconversion of WNV IgM and IgG in later serum samples. The fourth WNV infection was diagnosed retrospectively based on IgM and IgG detection in a serum sample on 16 October in a 44-year-old female patient from the district Wittenberg Federal State of Saxony-Anhalt. The patient was initially admitted to a local hospital with WNND-like symptoms on 10 September. After receiving supportive care, she was discharged on 17 September with restitutio ad integrum. The serological diagnosis was confirmed on 23 October by the detection of WNV RNA in a serum sample from the acute phase of infection. The fifth WNV infection was diagnosed on 23 October in a 24-year-old female patient from the district Leipzig, Federal State of Saxony, who presented with WNF (onset of symptoms 6 of October) and did not receive any treatment and recovered within one week. The laboratory confirmation was based on detection of WNV-specific IgM and IgG in a serum sample. As of 20 December, no further cases have been reported.

3.3. Genetic Characterization of German WNV

The genetic variations across the viral genome were low and homogenous (0.1%–0.7%) indicating that the analyzed WNV has maintained genetically stable since its first detection in 2018. The identity matrices for the genome and for individual genes were greater than 99.2%. The greatest variation was observed in the nonstructural genes coding for the NS1, NS2A, NS3 and NS5.

3.4. Phylogeny, Phylogeography and Spatiotemporal Dynamics of WNV

In order to investigate the evolutionary relationship and origin of WNV in Germany, a Bayesian MCMC sampling method and ML method were implemented. Similar topologies inferred by ML (not shown) and Bayesian MCC phylogenies of the European WNV lineage 2 data set revealed that all European strains fell into two distinct highly supported groups designated as Southeastern European clade (SEEC) and Central and Eastern European clade (CEC). All WNV strains from Germany fell into the CEC (Figure 2). The detailed analysis of the CEC showed that the German strains clustered in six distinct subclades (Figure 2) of which four consisted of singleton strains (WNV strains ED-I-155_19/ LR743422, ED-I-177_19/ LR743431, ED-I-201_19/ LR743448 and ED-I-205_19/ LR743454) associated with

Austrian relatives (Figure 2). However, the majority of the WNV strains from Germany clustered into a well defined monophyletic group designated as Eastern German clade (EGC). The EGC is also notable for a star-like structure in which several subclades connect viruses sampled from multiple locations and time points (Figures 2a and 3). These and other findings revealed that the genetic diversity of WNV in Europe is shaped primarily by in situ evolution rather than by extensive migration. No specific phylogenetic clustering and differences between the WNV strains from birds, horses, mosquitoes and humans in Germany were observed. The genetic variations of WNV combined with sample collection dates and locations can help to identify the possible source and the evolutionary history of the newly emerging viral variants. In order to assess viral migration and explore the origin of the WNV outbreaks in Germany, a discrete-trait phylogeography analysis was used to reconstruct the WNV movements between European countries and within Germany. Both data sets (European and German strains only, EGC) exhibited strong temporal signals (R^2 = 0.31 for the "European" data set and 0.19 for the "German strains only," $p < 0.001$). The coefficient of rate variation supports the use of a strict clock model for European data set and a relaxed clock for the data set "German strains only" (data not shown). The Bayesian MCC tree showed that although the WNV diversity in the "German strains only" group appears to have emerged in the last four years, the phylogeny of CEC which includes EGC suggests relative long-term circulation and evolution in Central Europe (Figure 2).

In further detail, the phylogeographic analysis suggests at least six distinct introductions of WNV into Germany from neighboring countries. It is predicted that all viral clade evolution events occurred during the last 16 years (Figure 2a). It should be noted that unlike its designation may suggest, the EGC can have developed in the wider southeastern and central European hemisphere and may have been translocated only later to Eastern Germany. Sequencing a larger number of more current WNV strains from e.g., Austria, the Czech Republic, and Poland would help to answer the circumstances of when and what in regard to the development of the East German Clade variants. Overall, the number of recent whole genome sequences is limited and should be markedly increased using NGS-based approaches.

Based on the albeit only limited Central European strain data, the tMRCA of the EGC group indicates a very recent emergence which was most likely introduced into Germany as a single introduction event. The progenitor of this Eastern European clade dates back to 2011, most probably circulating in Czech Republic (95% HPD 2010–2012; posterior probability, pp = 0.88) (Figure 2a). The EGC shares a common ancestor with basal WNV from Germany providing strong support for in situ evolution of WNV in Germany (Figures 3 and 4). Except for the members of the EGC, all other WNV strains found most recently in Germany seem to be descendants of ancestors from Austria (95% HPD for 2000 to 2015; pp = 0.83–0.97). The spatial diffusion pattern of WNV within Germany and between Germany and neighboring countries has been reconstructed using a Bayes Factor (BF) test under Bayesian Stochastic Search Variable Selection analysis (BSSVS). The strongest epidemiological links based on the BF estimates have been detected between Austria–Germany and Czech Republic–Germany, while the links within Germany have been detected between Halle–Berlin, Berlin–Halle, Berlin–Hamburg, Berlin–Dresden and Halle with neighboring localities (Figures 5 and 6). Similar star-like relationships of the WNV as for EGC have been also observed for Italian and Greek strains within both, SEEC and CEC (Figures 2a and 3). These results further provide indication for the in situ evolution of the European lineages.

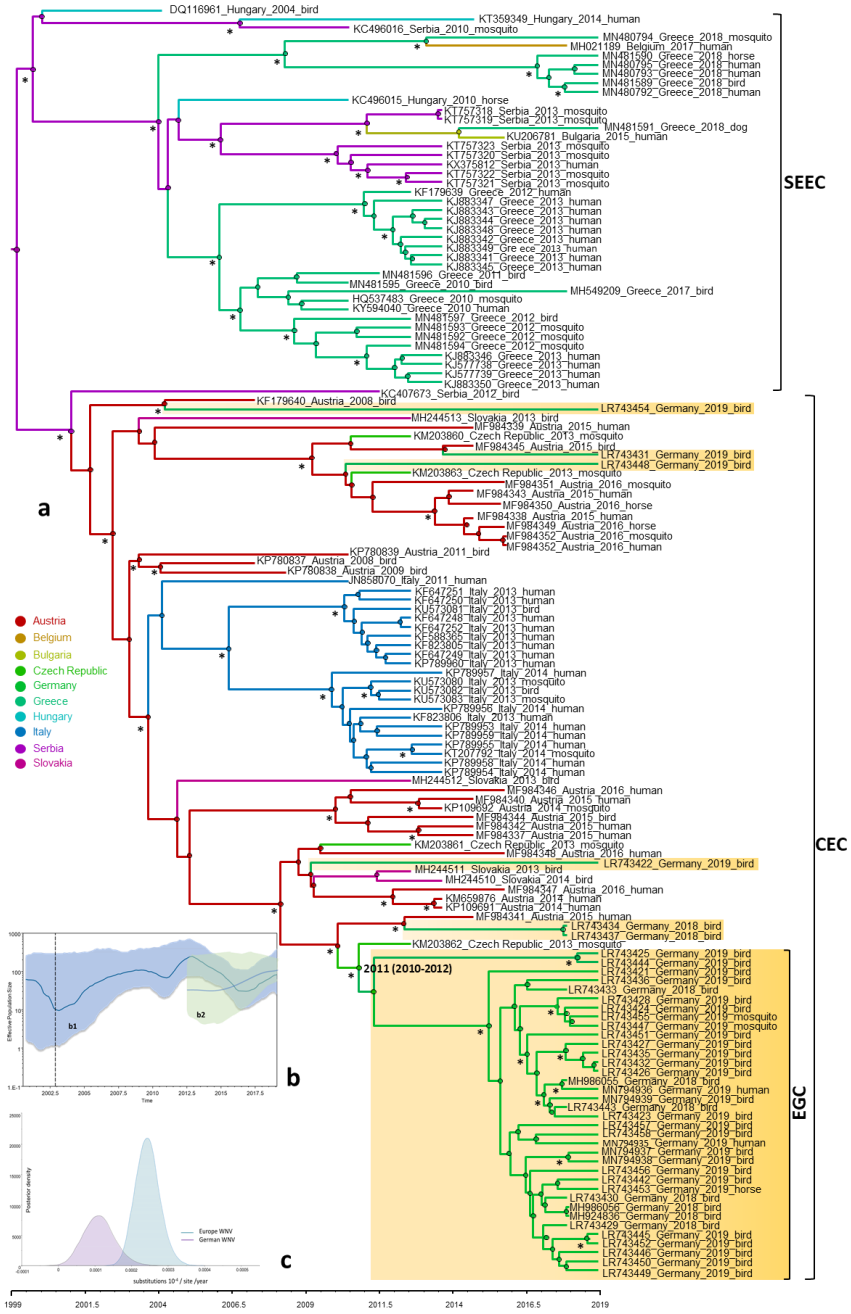

Figure 2. Bayesian maximum clade credibility (MCC) tree; (**a**) representing the time scale phylogeny; (**b**) effective population size; and (**c**) evolutionary rate of the European and German WNV lineage 2. The colored branches of MCC trees represent the most probable geographic location of their descendant nodes (see color codes); (**a**) the main clades are indicated to the right of the tree (SEEC, South Eastern European clade; CEC, Central and Eastern European clade), including the newly proposed German clade (EGC, Eastern German clade). Time is reported in the axis below the tree and represents the year before the last sampling time (2019). The German WNV strains sequenced in this study are highlighted. The estimated tMRCA of German WNV strains of EGC clade is shown with 95% posterior time intervals in parentheses. Bayesian posterior probabilities (≥90%) and 1000 parallel maximum likelihood bootstrap replicates (≥70%) are indicated at the nodes (asterisks); (**b**) temporal variation in the effective population size of the European WNV lineage 2; (**b1**) and EGC; (**b2**) estimated using the coalescent Gaussian Markov Random field (GMRF) Bayesian Skyride model of polyprotein sequences. The Bayesian Skyride plot represents temporal variation in the virus effective population size (Ne) through time. The blue line represents the median Ne estimate and the shaded area corresponds to the 95% high-probability density (HDP) intervals; (**c**) evolutionary rate estimates with 95% credible intervals for the distribution of evolutionary rates observed for the whole European WNV lineage 2 and for WNV from the 2018–2019 German epidemic.

3.5. Population Dynamics, Protein Changes and Analysis of Selection Pressure

The mean rate of evolution estimated for the polyptrotein of the EGC was 1.26×10^{-4} (95% HPD, 1.15×10^{-5}–2.84×10^{-4}) subs site^{-1} year^{-1} two times lower than for the European data set, 2.51×10^{-4} (95% HPD, 2.13×10^{-4}–2.88×10^{-4}) subs site^{-1} year^{-1} (Figure 2c). EGC population dynamics showed a slightly increased growth phase from the beginning of emergence when the virus effective population size (Ne) remained constant until 2015. From that year, a constant increasing tendency for the Ne values was observed, which is in line with the strong population expansion started in 2015–2016 (Figure 2a,b). The monophyletic LysArg mutation located in the C terminus of the NS3 gene appeared only in Eastern German clade strains, while the paraphyletic $Lys_{3056}Arg$ mutation from the NS5 gene was found to be common for EGC strains and some WNV from Austria (MF984341), Czech Republic (KM203862) and Germany (LR743437 and LR743434). There are several non-synonymous mutations in the nonstructural genes, which exhibit geographical structures specific of the members of the CEC (Figure 7). The overall d_N/d_S ratios in the polyprotein of EGC, CEC and SEEC were 0.118, 0.136 and 0.154, respectively, indicating that most sites are subject to strong purifying or negative selection. There was no evidence for positive or episodic diversifying selection in the WNV strains from Germany.

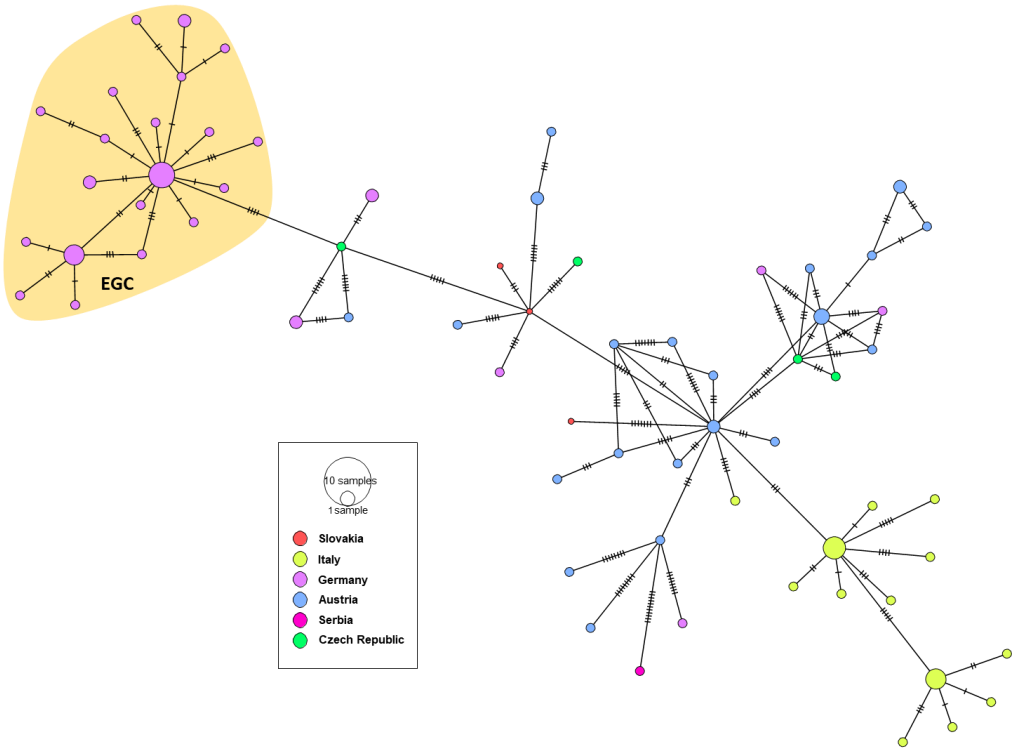

Figure 3. A median-joining haplotype network constructed from complete WNV NS5 gene alignment of the Central European WNV clade (CEC). Each colored vertex represents a sampled viral haplotype, with different colors indicating the different country of origin. The size of each vertex is relative to the number of sampled viral strains and the dashes on branches show the number of mutations between nodes. The Eastern German clade (EGC) is highlighted.

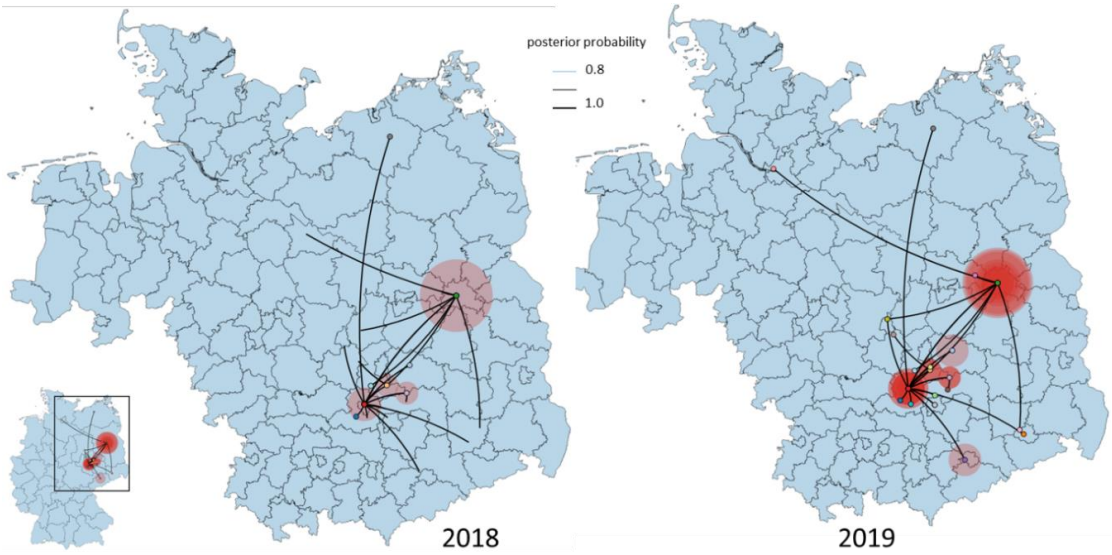

Figure 4. Temporally framed snapshots of the dispersal patterns (2018–2019) among regions in Germany for the Eastern German WNV clade. Lines between locations represent branches in the Bayesian maximum clade credibility (MCC) tree along which the relevant location transition occurs. Circle diameters are proportional to the square root of the number of MCC branches maintaining a particular location state at each time point.

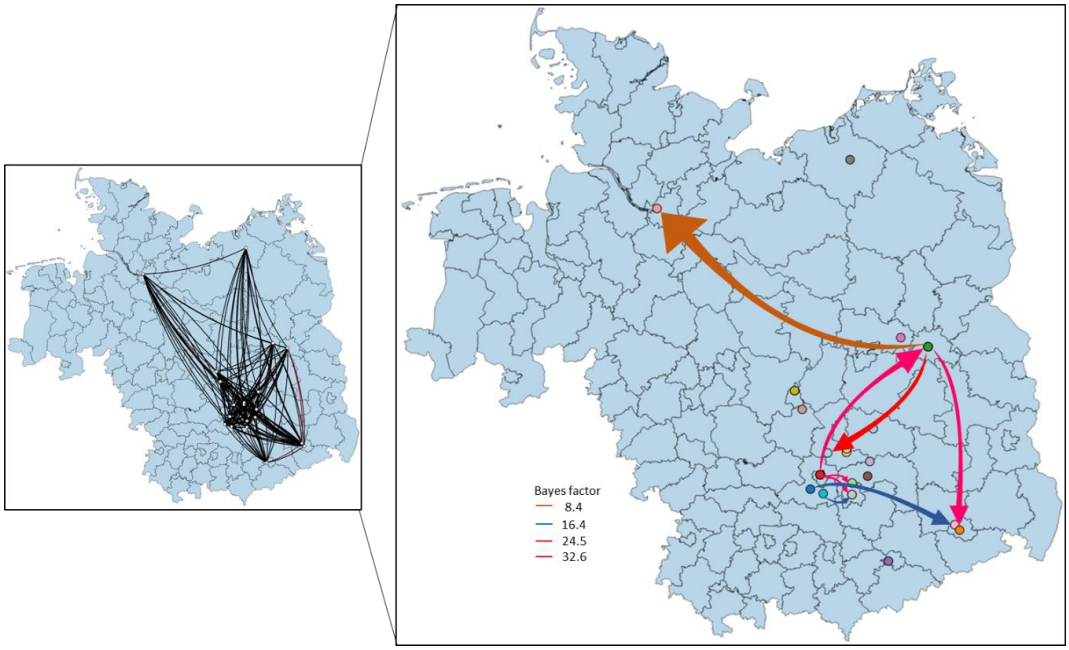

Figure 5. Calculated migration pattern of WNV between German locations based on Bayes factor test for significant non-zero rates. The arrows indicate the origin and the direction of migration between locations, while the colors indicate the strength of the connections.

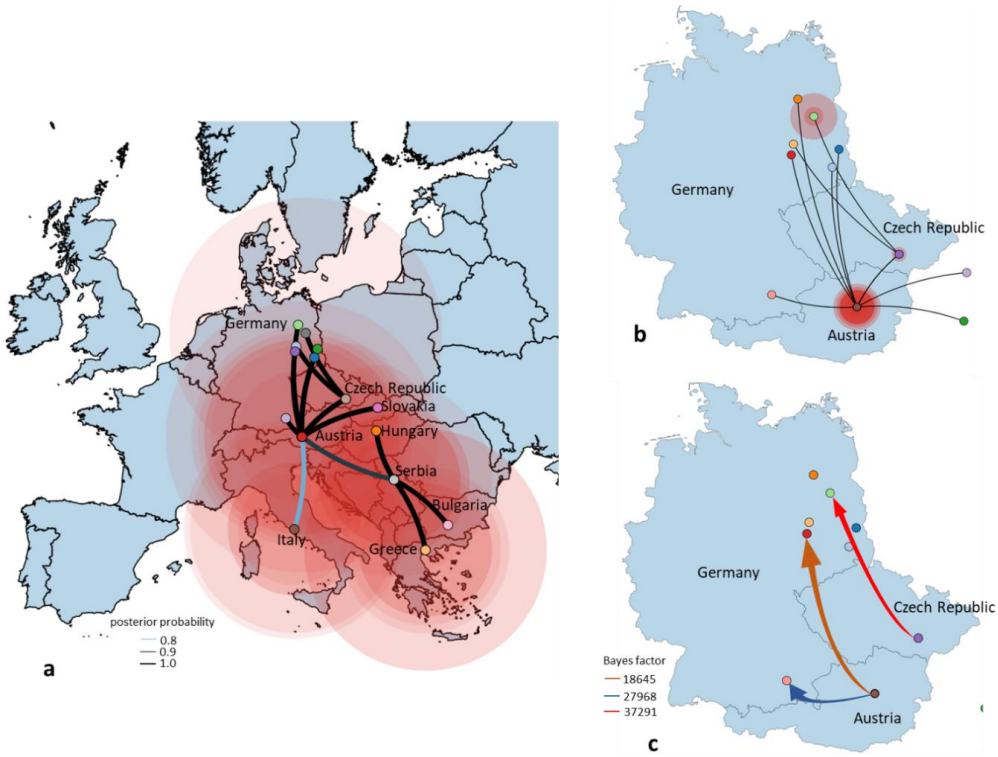

Figure 6. Spatial dynamics of the European clade of WNV lineage 2 including the origin of the German WNV reconstructed from the Bayesian maximum clade credibility (MCC) tree, a flexible demographic prior with location states and a Bayesian Stochastic Search Variable Selection (BSSVS); (**a**) the directed lines between locations connect the sources and target countries. Circles represent discrete geographical locations of viral strains and represent branches in the MCC tree along with where the relevant location transition occurs. All introductions for Germany are shown. Circle diameters of locations are proportional to square root of the number of MCC branches maintaining a particular location state at each time-point. Discrete locations are geographic coordinates for each European country; (**b**) the directed lines between the source of viral strains (Czech Republic and Austria) and target locations in Germany. Location circle diameters are proportional to square root of the number of MCC branches maintaining a particular location state at each time-point; (**c**) migration pattern of WNV between Czech Republic–Germany and Austria–Germany based on Bayes factor (BF) test for significant non-zero rates. Viral migration patterns are indicated between the different regions of Germany and neighboring countries and are proportional to the strength of the transmission rate. The color of the connections indicates the origin and the direction of migration and are proportional with the strength of connections. Only well supported paths between locations are shown.

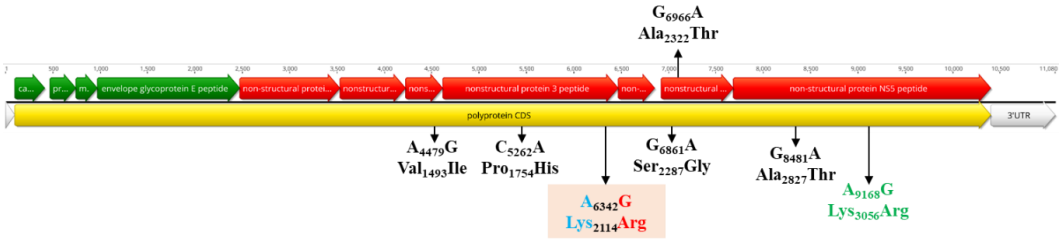

Figure 7. Schematic representation of the WNV genome and the positions of amino acid mutations. The position of the unique amino acid mutation of the Eastern German clade (colored in red/blue) in the NS3 gene is highlighted. The specific non-synonymous amino acid mutations for the CEC are shown in black, while the mutation in the NS5 specific for the subclade including the Eastern German group, one Austrian, one Czech and two German strains is presented in green.

4. Discussion

Globalization and climate change enhance or can lead the migration of exotic pathogens and their hosts to new environments promoting the contacts with naïve and vulnerable hosts. Thus, understanding the local ecological factors and evolutionary processes which navigate the emergence, establishment and spread of newly introduced viral diseases is critical for developing and implementing surveillance strategies for disease control. The present study aimed to elucidate the possible origins, spatiotemporal spread pattern tendencies and eco-epidemiological factors that facilitate WNV becoming an established pathogen in Germany causing neuroinvasive disease in multiple vertebrate species, including humans.

One year after the first observed autochthonous WNV transmission to birds and horses in Germany [9,13], an epizootic emergence of WNV was again observed in 2019. The number of infected birds and horses was considerably higher (76 birds and 36 horses) compared to 2018 (12 birds and two horses). In contrast to the USA, WNV-associated mass mortality in birds had not been observed in Europe before [33]. Previous hypotheses for this difference have been refuted by several research studies, e.g., demonstrating that European birds are susceptible to WNV infections and *Culex* mosquitoes in Europe are competent to transmit WNV. An alternative explanation might by that the bird mortality is so low that it is not detected with current European surveillance programs. A comprehensive USUV/WNV monitoring system is in place, but we also only see the tip of the iceberg of WNV infected birds in Germany. In addition, a huge number of positive specimens were obtained from captive animals (e.g., birds kept in zoos), which have a higher probability of detection compared to wild animals. Furthermore, from these birds, a considerable proportion were birds of prey, which must be considered to have a higher susceptibility to WNV infection [34]. This in combination with a widespread enzootic circulation of WNV and large number of equine cases—36 in 2019 in contrast to 2 in 2018—indicates an increased risk of WNV spillover into the human population.

This is reflected in the detection of five laboratory confirmed, mosquito-borne, autochthonous human WNV cases in 2019. It has to be kept in mind that less than 25% of infected humans develop noticeable symptoms [3]. Even fewer patients (<1%) have a risk of developing WNND [4]. The number of observed WNND cases (three of the five confirmed human WNV) gives rise to the speculation that hundreds of undetected human WNV infections in Germany occurred during the epidemic in 2019.

WNV transmission and spread is significantly influenced by climatic conditions, e.g., shaping phenology and abundance of the vector. Temperature is one of the most important factors directly affecting the EIP in different mosquito vector species [14]. High daily average temperatures (> 20 °C) over several days are required to allow for WNV transmission, which is correlated to the main distribution of WNV in south-eastern Europe. This also matches the spatial pattern of WNV in Germany. The summers in 2018 and 2019 were both characterized by extraordinarily high temperatures allowing low EIP values. The area in central-eastern Germany as the main focus of WNV circulation in both years was characterized by shorter EIP compared to previous years and most other areas in Germany [13]. Furthermore, these areas in Germany are directly neighboring countries reporting several years of WNV circulation (e.g., Czech Republic), leading to a high risk of short distance introductions e.g., by infected birds. The analysis also indicated that the areas along the Upper Rhine Valley in south-west Germany had a high suitability for WNV circulation, but no WNV activity was observed in all previous surveillance programs [8–11]. Most likely, no WNV introduction and circulation occurred yet, which underpins the thesis of the entries over short distances. Future studies are needed to understand if the virus did not yet spread to this area or if there are other factors reducing the risk of virus circulation (e.g., distribution of suitable vector or host species). The phylogenetic analyses indicated that Germany experienced at least six distinct WNV introduction events, with Austria and Czech Republic as possible origin for the progenitors of the German WNV epizootic strain variants. The majority of these strains clustered together into a distinct subclade (EGC).

The ongoing circulation and dominance of the EGC detected in 2019 indicates successful overwintering of WNV in Germany, e.g., through WNV persistence in hibernating mosquitoes

throughout the winter season [35]. The virus variants of the EGC at multiple sites detected in the epidemic in 2019 are descendants of a common ancestor in the wider central European environment which dates back to the time span 2010–2012. Where and when the subsequent virus evolution to the current variants took place and how descendants were eventually transferred to Germany remains elusive. However, such a translocation and subsequent virus amplification may have been fostered by the extremely favorable climatic conditions for mosquitos in Germany in spring/summer 2018, and the short distance transmission with infected birds from neighboring countries.

There has been a comprehensive USUV/WNV monitoring system in place in Germany for over a decade which involves ornithologists, zoological gardens and bird clinics supplying thousands of zoo and wild bird samples for WNV antibody and genome analysis. Moreover, there has been an exhaustive mosquito surveillance in place in Germany since 2009. By both surveillance approaches a variety of viruses were found, such as Sindbis virus, Batai virus and USUV, but not WNV [9–11,13,36,37]. At the same time, different long-distance, partial and short-distance migratory birds showed neutralizing antibodies against WNV before 2018 [9,11]. Although any such monitoring scheme has its predictive limitations due to sampling size constraints, all the negative WNV monitoring results from birds, horses and mosquitos before 2018 and the proximity to a larger region with active WNV circulation supports a recent introduction of multiple WNV descendants e.g., from Czech Republic to Germany. However, sequencing a larger number of more current WNV strains from e.g., Austria or the Czech Republic would help to answer the circumstances of when and what in regard to the development of the East German Clade variants. Overall, the number of recent whole genome sequences is limited and should be markedly increased using NGS-based approaches.

Most of the singleton WNV variants in Germany do not contain the monophyletic Lys2114Arg mutation located in the C terminus of the NS3 gene, even if these strains circulate in the central-eastern part of the country with very high WNV activity and rapid expansion of the EGC. Although these singletons have circulated and evolved under the same ecological conditions as members of EGC, it seems that these variants were not able to perpetuate and establish a stable enzootic cycle leading to a similar epizootic/epidemic scenario as for the EGC group. In case of the EGC, the adaptation to naïve vector and host populations leads to the emergence of local virus variants. The most likely scenario for EGC might be enzootic maintenance similar to that observed for WNV in the United States [38,39]. This hypothesis is supported by the observation that EGC form a star-like structure (population expansion after a single viral introduction) in which the variant viral strains accumulate changes during the rapid adaptation to the local ecological conditions (adaptation of the virus to the host populations and its enzootic maintenance), as observed for Usutu virus [40].

We found evidence that the phylogenetic structure of EGC and virus genetic population growth is shaped by the geographic location and average extrinsic incubation period, which likely facilitated rapid short-distance virus dispersal in 2018/2019. This demonstrates that local ecological factors (e.g., average temperature profile during the vector season) could predict the local and regional dispersal patterns of WNV in our data sets.

The purifying and negative selections observed for WNV in Germany were expected given the transmission and infection modes of arboviruses, leading to the accumulation of synonymous mutations [41]. Mutation observed at amino acid position Lys2114Arg has been found to be involved in the formation of EGC, while Val1493Ile (NS2b), Pro1754His (NS3), Ser2287Gly and Ala2322Thr (NS4b), Ala2827Thr and Lys3056Arg (NS5) are specific for the CEC (convergent evolution). Similar patterns of parallel or convergent evolution have been observed for WNV. This suggests that a limited number of residue changes are permitted due to functional constraints [42]. Viral adaptation in vector and vertebrate hosts by local overwintering or reintroduction of the virus and local ecological conditions (e.g., high average EIP) could be considered key determinants in the spatial dispersal and establishment of WNV. It is interesting to note that the Lys2114Arg mutation is specific for the newly described EGC. The impact of this mutation is unclear; a similar change in the WNV NS3 helicase (Thr1754Pro)

generated a highly virulent phenotype to American crows [43]. In vitro and in vivo experiments with strains from the EGC might show the role of fitness and pathogenicity in the future.

5. Conclusions

This study provides a first comprehensive summary and phylogeographic analysis on the WNV epidemic in Germany in 2018 and 2019 and highlights the risk of human WNV infections causing considerable risks for transfusion and organ transplantation safety. Therefore, intensive surveillance of mosquitoes, birds, horses and humans should remain a public health priority, e.g., to monitor the occurrence and subsequent spread of WNV or to develop targeted control mechanisms. Our study also highlights the need for international cooperation in the area of WNV surveillance and monitoring, especially across national borders and as a "one-health" approach for an improved risk analysis. This should also include the generation of higher numbers of whole-genome sequences, allowing for a more precise molecular epidemiology and strain characterization.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/12/4/448/s1, Table S1: Epidemiological data of West Nile virus with full genome sequences (except for 1 human sample), their corresponding accession numbers and sequencing protocol performed.

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Weak association of Usutu virus and haemosporidian infection in birds collected in Germany

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ABSTRACT

The Usutu Virus (USUV) is a mosquito-borne flavivirus originated in Africa. The virus circulates in Germany since 2010. It is primarily transmitted and maintained in the natural cycle by *Culex* mosquitoes and primarily affects birds, particularly Eurasian blackbird (*Turdus merula*), leading to significant mortality. Several studies have reported a high co-infection rate of European birds with both USUV and haemosporidians. Haemosporidians are blood parasites which maintain an enzootic life cycle with birds via different arthropod vectors. This study conducted screenings of birds from Germany received through a citizen's science project for both, USUV and haemosporidians between 2016 and 2021. The prevalence of USUV reached its peak in 2018, when it was first detected throughout most parts of Germany rather than being limited to localised hotspots. Subsequently, USUV prevalence consistently declined. On the other hand, the prevalence of haemosporidians initially declined between 2016 and 2019, but experienced a subsequent increase in the following years, exhibiting a more or less inverse pattern compared to the prevalence of USUV. In 2020, a statistically significant positive association between both pathogens was found, which was also detected across all years combined, indicating if at all a weak relationship between these pathogens.

1. Introduction

Usutu virus (USUV) is a zoonotic flavivirus transmitted by mosquitoes. Birds serve as amplifying hosts [1]. In Europe, the primary vectors are likely *Culex pipiens* s.s. and *Culex torrentium* [2]. The virus originally emerged in Africa over 500 years ago and has been introduced into Europe several times, leading to local establishment and subsequent spread [3,4]. Currently, USUV is considered established in at least 17 European countries [5,6], including Germany [7], where it has caused several outbreaks with massive bird die-offs in recent years [8,9]. Among the affected bird species, the Eurasian blackbird (*Turdus merula*) seems to be particularly vulnerable to the virus. In 2016, areas with USUV circulation in Germany experienced an estimated 15% decline in local Eurasian blackbird populations [10]. While humans can be infected with USUV, they are considered dead-end hosts, and asymptomatic infection are reported frequently [11,12]. However, in immunocompromised individuals more severe cases were observed, including meningoencephalitis or neurological disorders like diopathic facial paralysis [13,14].

Haemosporidians are widely distributed blood parasites [15]. The most well-known representative is *Plasmodium falciparum*, which causes malaria in human and results in over 600.000 fatal cases per year [16]. Birds can not only be infected by haemosporidian species of the genus *Plasmodium*, but also by members of the genera *Haemoproteus* and *Leucocytozoon* [17]. They are transmitted between birds through pathogen genus-specific dipteran families, such as Culicidae (*Plasmodium* spp.), Ceratopogonidae and/or Hippoboscidae (*Haemoproteus* spp.), and Simuliidae (*Leucocytozoon* spp.). Studies conducted in Germany and other European countries indicated a relatively high prevalence of avian malaria infections in wild bird populations often exceeding 50% (e.g.

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[18–20]). While most bird species are considered to be well adapted to haemosporidian infections [17], recent research has challenged this notion by demonstrating significant fitness loss [21] and mortality [22] among native birds regularly exposed to haemosporidians.

Different studies suggest a positive association between USUV and haemosporidian infections in birds [23–25]. During the large-scale USUV outbreak in Central Europe in 2016, two independent studies reported high co-infection rates of USUV and haemosporidians in birds. In the Netherlands, half of the 16 birds infected with USUV were also infected with *Plasmodium* spp. [24]. In Belgium, out of 91 birds diagnosed with USUV, 90 were co-infected with *Plasmodium* spp. or *Haemoproteus* spp. [23]. Additionally, an earlier study from Italy in 2009 reported a co-infection with haemosporidia in 18 out of 35 USUV-positive birds [25].

Co-infections of pathogens with haemosporidians have been shown to reduce fitness and the survival probability of birds. Examples include double haemosporidian infections [26], co-infections with *Plasmodium* spp. and Bagaza virus [27], or *Plasmodium* spp. and chicken anaemia virus [28]. USUV already poses a threat to local bird populations. Therefore, it is crucial to determine whether co-circulating pathogens play a significant role in the spatial transmission risk of USUV. The interaction between avian malaria parasites and the probability of USUV infection in birds, might also help to understand the potential impact of this interaction on the spillover to humans.

As part of a dead bird surveillance programme in Germany, we conducted screenings on dead birds collected from 2016 and 2021 to detect USUV and haemosporidian infections. The results were used to analyse the association between USUV and haemosporidians.

2. Materials & methods

Through press releases and subsequent media coverage, citizens all over Germany were asked to contribute to the dead bird surveillance programme by sending dead birds to the Bernhard Nocht Institute for Tropical Medicine in Hamburg, Germany. They were also requested to provide information regarding the date and location of the bird's discovery, i.e. street, house number and city. Bird carcasses were shipped as post parcel (priority within 24 h or non-priority) and the delay between dead and finding of the bird are generally unclear. This surveillance programme has been in operation since the initial observation of the USUV outbreak in 2011 [7,10,29,30]. Whenever possible, samples of the heart, liver, and brain were collected from each dead bird specimen. The bird species are dominated by the European blackbirds and only data for this species are presented here.

A mix of heart, liver, and brain tissues of each bird specimen were homogenised and subsequently subjected to DNA/RNA extraction using KingFisher™ Flex Magnetic Particle Processor (Thermo Fisher Scientific, Waltham, MA, USA) with the MagMAX™ Pathogen ribonucleic acid/DNA Kit (Thermo Fisher Scientific, Waltham, MA, USA).

USUV screening was conducted with a modified pan-flavivirus reverse transcription PCR [29]. For haemosporidian screening, we used a nested PCR protocols developed by Bell et al. [31]. This included a nested PCR targeting the cytochrome *b* gene of *Plasmodium* spp. and *Haemoproteus* spp., and a nested PCR for the same gene of *Leucocytozoon* spp. All PCR amplicons were sent to LGC Genomics (Berlin, Germany) for Sanger sequencing. The sequences were processed with Geneious 7.1.9 (Biomatters, Auckland, New Zealand) and compared to available sequences in the GenBank [32] using the basic alignment search tool (BLAST) in the GenBank DNA sequence database [33]. Haemosporidian sequences were additionally compared to sequences in the MalAvi database [34].

The data were analysed using R software [35] with the packages raster, sp., zoo, and ggplot2. The association of co-infection cases was tested using a Pearson's χ^2-test with Yates' continuity correction and temporal correlation was analysed via a Pearson's product-moment correlation test.

3. Results

This study includes birds received between 2016 and 2021 (n = 2272). 89 birds were screened in 2016, 136 birds in 2017, 1164 birds in 2018, 515 birds in 2019, 231 birds in 2020, and 137 birds in 2021 (Fig. 1).

The overall USUV-prevalence rose from 22.5% in 2016 to 41.2% in 2017 (Fig. 2). In 2018, we observed the biggest USUV outbreak so far, with a sharp increase of USUV prevalence to 71.2%. The massive outbreak was also reflected in the total number of birds submitted, which increased more than 8-fold from 2017 to 2018 (Fig. 1). Subsequently, the prevalence of USUV decreased to a level similar to that of 2017 (Fig. 2), but USUV cases were still detected across all regions of Germany. In the subsequent years, the prevalence of USUV continued to decline even further, reaching 19.5% in 2020 and 12.4% in 2021. These cases remained scattered throughout the entire country.

Haemosporidian infected birds were found throughout the country in all years (Fig. 1). The highest prevalence was detected in 2016 with 60.7% (Fig. 2). Afterwards, the prevalence decreased yearly by roughly 10% until it reached its lowest point in 2019 with 28.5%. 2018 and 2019 were the only years in which the haemosporidian prevalence was exceeded by USUV prevalence. The following years, the haemosporidian prevalence rose again to 40.3% in 2020 and 44.5% in 2021. The vast majority of haemosporidian infections were caused by *Plasmodium* spp. We identified only a few cases of *Haemoproteus* spp. and *Leucocytozoon* spp. each year.

In all six investigated years, USUV-positive birds were more often co-infected with at least one haemosporidian species than USUV-negative birds (Fig. 3), although this association was only statistically significant for the year 2020 ($p = 1.103*10^{-6}$). Nevertheless, looking at the whole period of six years, this association was found highly significant ($p = 4.108*10^{-3}$).

Most haemosporidian cases were caused by *Plasmodium* spp. and there was a statistically strong association of USUV and *Plasmodium* spp. in 2020 ($p = 2.09*10^{-7}$) and for all years together ($p = 1.6*10^{-4}$) as well. Additionally, there was a slight association in 2017 ($p = 0.02192$). The other years showed no significant association. There were only 27 *Haemoproteus* spp. cases of all 2272 birds screened and there was no statistically significant association with USUV for any year, except for 2021 ($p = 1.648*10^{-4}$). However, there were only 3 *Haemosporidian* spp. infections in 2021, so the chi-square test might not be reliable. An association of *Leucocytozoon* spp. and USUV was only found for all years

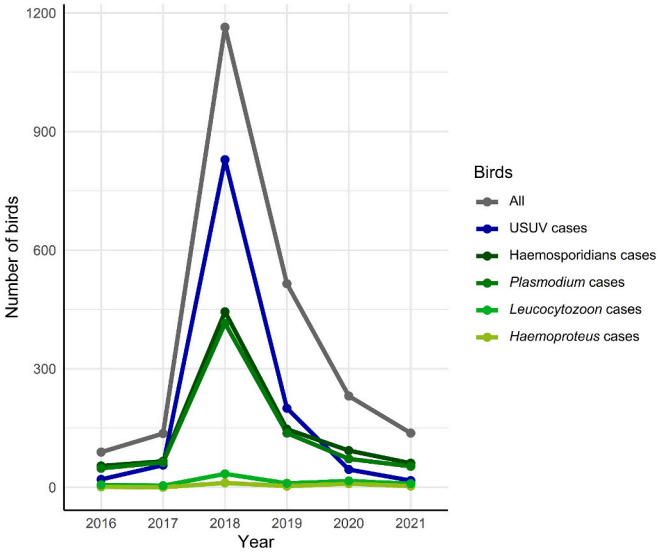

Fig. 1. Number of birds and infections between 2016 and 2021. Haemosporidian cases are shown accumulatively and separated per genera.

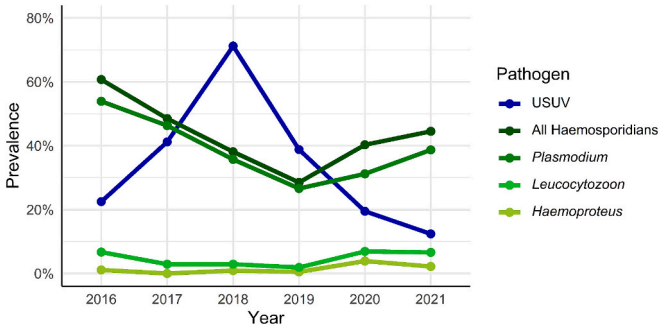

Fig. 2. USUV and haemosporidian prevalence in investigated birds. The prevalence for haemosporidians are shown accumulated and separated for each genus.

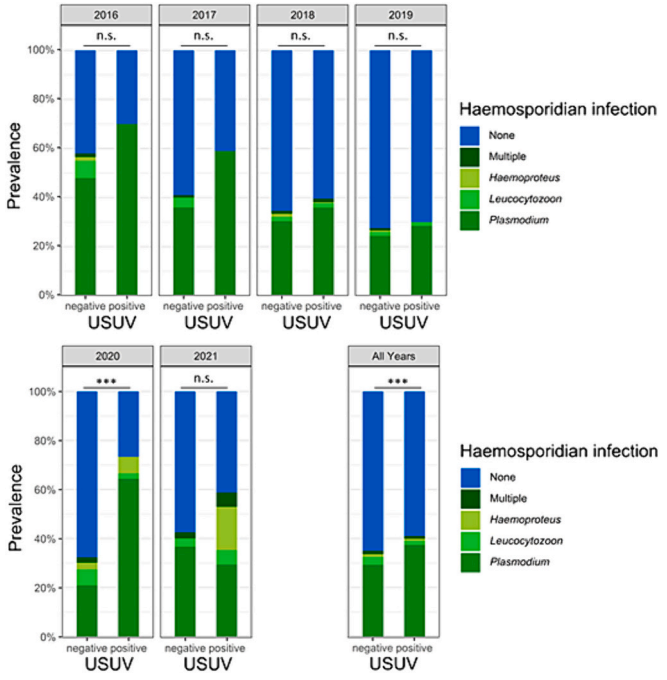

Fig. 3. Dispersion of haemosporidian infected and uninfected birds for USUV positive and negative birds for the years 2016–2021. Single haemosporidian infection are shown by genus, infections of multiple genera in single birds are shown as "Multiple". The total number of USUV-positive/USUV-negative were 20/69 for 2016, 56/80 for 2017, 829/335 in 2018, 200/315 in 2019, 45/186 in 2020, and 17/120 in 2021.

($p = 0.02094$) and not for any individual year.

4. Discussion

Since its initial detection in German in 2010, outbreaks of USUV had been initially limited to South-West Germany, gradually expanding their circulation area towards the North [29,36]. However, in 2018, a rapid and wide emergence of USUV was observed throughout Germany and other western European countries [5,8], resulting in the largest USUV outbreak reported so far. The number of dead birds submitted to our dead bird surveillance programme increased more than 8-fold, suggesting a significant mortality rate among the German bird population due to USUV infections. In the following years, USUV prevalence in the submitted dead birds continuously decreased to only 12.4% in 2021. This suggests that the major outbreak was driven by the expansion of USUV into new regions, where previously unexposed bird populations were highly susceptible to the virus.

In contrast to USUV, haemosporidians are not a re-emerging pathogen among birds, but have been circulating in Germany and Europe for a very long time [17]. Numerous studies consistently demonstrate the presence of haemosporidian infections in birds throughout Europe (e.g. [37,38]). Therefore, it was not surprising to find circulation of haemosporidia across Germany in all years. However, it is noteworthy that the prevalence consistently decreased from 2016 to 2019, only to rise again in 2020 and 2021. This trend appears to be an inversion to the USUV prevalence pattern, although the peak of USUV prevalence was one year prior (2018) to the lowest point of haemosporidian prevalence (2019). This might suggest that the spread of USUV and subsequent decrease in local bird populations [10] may be one of the primary factors influencing the haemosporidian prevalence. There have been some studies indicating that haemosporidian prevalence may be linked to host abundance and density, although this appears to vary for individual haemosporidian species [39,40]. As USUV circulation decreased and the bird populations recovered, the haemosporidian circulation increased once again.

Varying prevalence of the haemosporidian genera are reported regularly. For instance, Lüdtke et al. discovered in 2011 that 99.4% of diagnosed blood parasite infections in German passerines were caused by *Haemoproteus* spp., with an overall prevalence of 39% [41]. Another study by Schumm et al. conducted on German passerines in 2015, 2017, and 2018 identified *Leucocytozoon* spp. as the most prevalent genus, with 71% of tested birds infected, compared to a prevalence of 13% for *Plasmodium* spp. and 31% for *Haemoproteus* spp. [42].

In contrast, our study revealed *Plasmodium* spp. to be the predominant causative agent in the vast majority of haemosporidian infections (91.1%). It is important to consider that the discrepancies in findings among studies can arise from different study design. It is in the nature of such a citizen science project that the birds are predominantly sent from urban areas, which generally show a high density of mosquitoes as vectors of *Plasmodium* spp., but low densities of Ceratopogoniae/ Hippoboscidae or Simuliidae as vectors of *Haemoproteus* spp. and *Leucocytozoon* spp., respectively (REF). In addition, although all studies focused on German passerines, the bird species in focus varied. This study analysed predominantly Eurasian blackbirds, which were tested neither in the studies by Lüdtke et al. [20] nor by Schumm et al. [42]. Furthermore, like most studies, they tested blood samples rather than organ samples, as done in this study. Haemosporidians are considered to be primarily blood-parasites and testing the blood has been shown to be a sensitive method [43]. Nonetheless, it has been shown that haemosporidians can be diagnosed using organ samples as well, because after an initial replication period in the blood stream, haemosporidians often enter latent, exoerythrocytic stages [44]. However, this study used bird carcasses that have been potentially left at room temperature for several days during shipping and the potential effects of this handling procedure on the detection sensitivity are not fully understood. Furthermore, it is worth noting that the nested PCR for the genus *Leucocytozoon* also amplified members of *Plasmodium*. It has been previously observed that these PCR assays tend to amplify the most abundant haemosporidian DNA [45]. This limited our study to detect haemosporidian-double infections and their impact on an additional USUV infection.

USUV-infected birds were more likely to have co-infections with haemosporidians compared to USUV-uninfected birds, although this difference was not statistically significant for most individual years. These findings align with other studies, which reported different results. Studies from Italy (2011) and Netherlands/Belgium (2016) reported a significant positive association between both pathogens [23–25]. More recent studies from the Netherlands confirmed regular co-infection of European blackbirds with USUV and *Plasmodium* spp. (2016–2018, 2016–2020) [46,47]. It was also demonstated that the same organs are affected by both pathogens, but the severity of lesions in multiple organs (liver, spleen, heart, brain, and lungs) is increased in co-infections. In contrast, a study from Austria in 2018 found no association [48]. Environmental factors such as climate or vector density might have a

stronger influence on circulation patterns of both pathogens, thereby masking a potential association in certain years and areas. Birds, particularly the Eurasian blackbird, can experience severe illness as a result of USUV infection [46], which can leave them weakened and less mobile. This reduced mobility might make them more susceptible to haemosporidian-transmitting vectors [49] and their already compromised health may increase the risk of haemosporidian infection. While native birds are generally considered to be well adapted to haemosporidians and often do not develop severe illness [17], recent studies suggest that haemosporidian infection can have significant negative fitness consequences [21,22]. The long-term effects of chronic manifestations, which occur after the peak of parasitaemia have often been overlooked in the past [50]. Therefore, birds with haemosporidian infections may be more susceptible for an additional USUV infection and vice versa. This fits in with the fact that it has recently been shown that co-infections with USUV and *Plasmodium* spp. leads to higher lesion severity in European blackbirds compared to single-infections [47].

5. Conclusion

There appears to be a weak correlation between USUV and haemosporidian infections in birds from Germany, although the exact nature of this relationship remains unclear. We observed that as the prevalence of USUV increased sharply, the prevalence of haemosporidians decreased and vice versa. However, additional studies including the environmental parameters, e.g. land-use or mosquito abundance, driving the infection risk are required to gain a deeper understanding of the causal relationship between USUV and haemosporidians.

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Ethics declarations

The ethcis committee of the lead organization confirmed that ethics approval was not required.

CRediT authorship contribution statement

Carolin Hattendorf: Data curation, Formal analysis, Writing – original draft. **Dániel Cadar:** Formal analysis. **Stefan Bosch:** Data curation. **Norbert Becker:** Data curation. **Lars Lachmann:** Data curation. **Jonas Schmidt-Chanasit:** Conceptualization, Writing – review & editing. **Anna Heitmann:** Conceptualization, Formal analysis. **Renke Lühken:** Conceptualization, Data curation, Formal analysis, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Dirofilaria immitis and *D. repens* in Europe: a systematic literature review on vectors, host range, and the spatial distribution in the 20th and 21st century

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Abstract

Background: *Dirofilaria immitis* and *D. repens* are mosquito-borne nematodes with dogs as primary hosts, but other mammalian species including humans can be also infected. In the last century, circulation of both pathogens was predominantly restricted to Southern Europe. However, different studies indicated a potential establishment in Central, Eastern and Western parts of Europe as an increasing threat to animal and human health.

Methods: We conducted a systematic literature review of publications reporting *D. immitis* and *D. repens* screening in mosquitoes and mammalian vertebrates in Europe. These data were used to analyse the range of vectors and hosts and for a comparison of the spatial distribution between the 20th and 21st century.

Results: Both nematodes appear to have a high overlap of *Aedes*, *Anopheles* and *Culex* vector species, which are abundant in Europe. Most *D. immitis* infections were reported in dogs, while *D. repens* predominated in humans. *Dirofilaria immitis* infections were detected in a wider range of wild and zoo animals. Compared to the last century, many more countries especially in Central Europe were affected by *Dirofilaria* spp. circulation, illustrating a significant spread over the last 20 years.

Conclusion: Our findings suggest that *D. immitis* and *D. repens* are a growing health concern for animals and humans in Europe. Continuous globalisation and climate warming will probably lead to a further spread and increased circulation in the future. All data are made available open access, which will enable further analysis in the future.

Keywords: *Dirofilaria immitis*, *Dirofilaria repens*, spread, globalisation, climate warming

Introduction

Two *Dirofilaria* species are present in Europe: *D. immitis* and *D. repens* (1). Both circulate in an enzootic cycle between mosquitoes and domestic dogs, although other carnivores like Red Foxes and Grey Wolves can also be infected (e.g. (2–5)). Mosquitoes are infected with microfilaria during blood-feeding on an infected host, which then develop to infective larvae in susceptible vectors (6). *Dirofilaria* can be transmitted to other mammals, such as humans and rodents, although these are generally 'dead-end' hosts (6), i.e. no development of microfilaria occurs. *Dirofilaria immitis* localise in the pulmonary arteries of dogs, where they sexually reproduce and release microfilariae into the bloodstream (1,7). Infections can lead to severe disease in dogs and cats with symptoms ranging from chronic cough to heart failure (8,9). In humans, *D. immitis* mostly forms pulmonary nodes, which are generally asymptomatic, but frequently mistaken with lung cancer in radiography (6). However, some humans develop severe symptoms including fever, chest pain, coughing, haemoptysis, wheezing arthralgia or malaise (10). *Dirofilaria repens* infections generally localises subcutaneously (1,6). Approximately 35 % of human *D. repens* infections occur in the ocular region, which can lead to impaired or a complete loss of vision (11). Around 10 % of affected patients suffer permanent complications like retinal detachment or glaucoma (12). Notably, there have been a few reported cases where viable *D. repens* microfilariae have been found in the blood stream of

infected humans (13–17), but these seem to be rare exceptions. The majority of human *Dirofilaria* infections in Europe are caused by *D. repens* (18), while the majority of reported *Dirofilaria* cases in dogs are *D. immitis* (1). However, it has to be noted that *D. immitis* is easier to diagnose in dogs because it more often leads to severe symptoms in dogs and respective tests are available (19).

First cases of human dirofilariosis presumably were diagnosed in 1566 in a Portuguese girl (20) and 1626 in an Italian dog (21) for *D. repens* and *D. immitis*, respectively. In the 20th century, autochthonous circulation of these parasites was predominantly reported from the Southern parts of Europe, but currently there are increasing reports of a spread towards Central, West and East Europe (22). Many previously *Dirofilaria*-free countries are now considered endemic (23). Climate warming is thought to be the main reason, allowing the successful development of the nematodes in the mosquito (24–26). Another important factor is the movement of dogs in Europe, which was made considerably easier with European regulations for traveling with pets (27). To gain a better picture of the vector range and spatial expansion of *D. immitis* and *D. repens* in Europe over the last two centuries, we conducted a systematic literature review of *Dirofilaria* data in mosquitoes and vertebrate hosts, including the collection of different metadata (e.g. sampling time and site).

Methods

All published articles matching the keyword ‘dirofilaria’ in any search field recorded in PubMed (28) were extracted on 24.01.2022. Papers were selected using the following inclusion criteria: 1) article language English or German, 2) a host was diagnosed with an acute infection of *Dirofilaria* spp., i.e. excluding studies only screening antibodies, and 3) the sampling was conducted in Europe. The following information was extracted from each publication: country, date of diagnosis/sampling, sampling location, host species, travel history, screening method,

number of tested and number of positive specimens per *Dirofilaria* species. In addition, for mosquito studies the mosquito trap and pooling information (pool size, body part, etc.) were noted.

If the date of diagnosis was not specified, the date of publication was used and if only a sampling period was given, the total number of cases was split evenly across the sampling years. The accuracy of the sampling locations was classified to decide which level of the Nomenclature of Territorial Units for Statistics (NUTS) classification of the European Union (29) was used for visualisation of parasite distribution in humans, dogs and other vertebrate hosts: 'very high' (coordinates or address, NUTS-3 level), 'high' (town or specific area, NUTS-2 level), 'medium' (hospital or greater area (e.g. county), NUTS-1 level), and 'low' (country, NUTS-0 level). For the spatial analysis of the *Dirofilaria* distribution, we only included reports with unremarkable travel history. However, many studies did not include any information on the travel history. Therefore, we also conducted the spatial visualisation with all unremarkable and unknown travel history for the supplement. Reports with a known travel history were excluded from analysis. Furthermore, we compiled visual summaries of country-specific *Dirofilaria* screening results from mosquitoes and less common vertebrate hosts, excluding humans and dogs. All computational analysis was performed in R (Version: 4.2.2) using the R-Studio IDE (Version:2022.12.0) (30). Additionally, functions from the following packages were used for data preparation, visualization and analysis: terra (31), tidyterra (32), geodata (33) readxl (34), ggpubr (35), plyr (36), dplyr (37), and ggplot2 (38).

Results

A total of 3,847 publications were extracted from PubMed. Of these, 473 (12.3 %) matched our inclusion criteria. We observed an increase in publications reporting *Dirofilaria* from the beginning of the 1990s and another increase in the mid-2000s (Fig. 1).

38 publications (8.0 %) included screenings of mosquitoes for *Dirofilaria* with a total of 1,658,041 specimens tested over 62 mosquito taxa collected in 14 different European countries (Fig. 2). *Dirofilaria immitis* was detected in 17 different mosquito taxa from 12 countries, most frequently in *Culex pipiens* s.l. (11 countries) and *Aedes caspius* (7 countries). *Dirofilaria repens* infections were reported for 31 different mosquito species from 13 countries with *Aedes vexans* (8 countries), *Cx. pipiens* s.l. (6 countries) and *Anopheles maculipennis* s.l. (6 countries) most frequently found positive. A total of 15 mosquito taxa were found positive for both *Dirofilaria* species. *Dirofilaria immitis* was exclusively detected in *Ae. behningi*, while *D. repens* was exclusively found in 16 different taxa of the *Aedes*, *Anopheles*, *Culiseta* and the *Uranotaenia* genus, e.g. *Ae. cantans*, *An. claviger*, *Cs. annulata* or *Ur. unguiculata*. Most studies on *Dirofilaria* prevalence in mosquitoes focused on Southern and Eastern Europe, but some studies also confirmed autochthonous circulation in Central Europe, e.g. Austria or Germany.

198 publications (41.9 % of included publications) reported dog infections with a total of 11,713 cases. Of these, 7,757 (66.2 %) were identified as *D. immitis*, 3,948 (33.7 %) as *D. repens*, and eight (0.1 %) were not further differentiated *Dirofilaria* species. In 199 publications (42.1 %), human *Dirofilaria* spp. infections were described, summing up to 2,555 reported human cases, of which the majority of 2,438 (95.4 %) was *D. repens*, followed by 95 (3.7 %) not further specified *Dirofilaria* spp. and 22 (0.9 %) *D. immitis*. Only 33 publications (7.0 %) reported *Dirofilaria* infections in cats (278 cases): 252 (90.1 %) *D. immitis*, 24 (8.6 %) *D. repens* and two (0.7 %) not further specified *Dirofilaria* species. In addition, 59 publications (12.5 %) described *Dirofilaria* infection in other mammals, the majority of which were caused by *D. immitis* (Fig. 3). These studies predominantly focused on domestic cats (34 publications, 7.2 %) and Red Foxes (15 publications, 3.2 %). In addition, *Dirofilaria* were detected in a wide variety of wild carnivores (e.g. Golden Jackal, Grey Wolf or Eurasian Otter) and zoo animals

(e.g. Lion or California Sea Lion). A wider variety of vertebrate hosts was studied in Slovakia, Serbia and Romania, while studies in other countries focused on few potentially infected species like Red Foxes or only reported single cases.

Only focusing on the studies with unremarkable travel history, the majority of the few *D. immitis* cases in dogs and other mammals until 2001 were recorded in Southern Europe, particularly in Spain, Italy and Portugal (Fig. 2; see supplementary file 1 and supplementary file 2 for visualisation of all cases with unremarkable and unknown travel history). No human cases were reported before 2001. In the 21st century, *D. immitis* infections were found in most countries of South and Central Europe and even in Central Europe, such as Poland and France. A wide distribution in particular was confirmed in dogs and other mammals for wide parts of Eastern Europe and Italy. *Dirofilaria repens* infections, especially looking into human cases, were reported much more widespread than *D. immitis* already during the 20th century in particular for various regions in Italy and France, while dogs were only tested positive in Italy and Spain (Fig. 3). We observed a strong increase of affected countries for both, humans and dogs, including countries in Eastern and Southern Europe (e.g. Ukraine, Slovakia, Greece), but also Central Europe including the Netherlands, Germany or Poland. The most Northern infection was reported in humans from Finland.

Discussion

The number of publications reporting *Dirofilaria* spp. infections have increased in the last two decades compared to the previous century (18,22). This is most likely driven by both, increased research and awareness, but also the spread of the parasites (25,39). *Dirofilaria immitis* and *D. repens* have to be considered endemic in countries that were considered to be *Dirofilaria*-free in the 20th century, e.g. Czech Republic (40,41). The spread of competent vector species probably does not play a major role here. There is a huge overlap between the vector species

for *D. immitis* and *D. repens*, which are widespread in Europe and show host-feeding patterns with a substantial proportion of mammals, e.g. *Cx. pipiens* s.l. or *An. maculipennis* s.l. (42–44). Interestingly, the exotic *Ae. albopictus* was much more often reported to be infected with *D. immitis* than *D. repens*. This mosquito species has been implicated as an important driver of the spread of *Dirofilaria* (22,26,45).

Most infections were reported from dogs as the primary host of *Dirofilaria* (1). The majority of these cases were caused by *D. immitis*, which is well known to cause a more severe disease in dogs compared to *D. repens*, leading to a higher probability of diagnosis (7). Additionally, rapid tests are only available for *D. immitis* and not *D. repens*. Therefore, *D. repens* might be underreported and its actual prevalence among dogs is probably higher (11). In contrast, the overwhelming majority of cases in humans were caused by *D. repens*, confirming previous observations that most human *Dirofilaria* infections in Europe are caused by this species (46,47). The reason for this remains unclear, given that human infections with *D. immitis* are regularly reported, particularly in North America (48). One hypothesis suggested that European *D. immitis* might be genetically distinct from *D. immitis* found in other regions, making it less capable of surviving within humans (11). However, this hypothesis has later been disproven (49,50). Another explanation could be that *D. repens* influences the circulation of *D. immitis*, e.g. it has been shown for Southern Italy that *D. repens* impedes the spread of *D. immitis* in dogs (51). If this plays a general epidemiological role and if this is also true for humans requires further research. Furthermore, it has been proposed that *D. repens* is more difficult to control, because, as mentioned above, rapid tests are only available for *D. immitis* and current preventative and curative treatments are designed for *D. immitis* and are not as effective against *D. repens* (18). Additionally, *D. repens* infections are often asymptomatic in dogs which might lead to a longer time period where a dog is infective, and a mosquito can ingest and transmit the parasite to further hosts (6,52).

Besides dogs and humans, there were also several reports of infections in cats, although it is assumed that cats do not play an important role for *Dirofilaria* transmission (53). Similarly, several other mammalian species diagnosed with an infection were held in zoos or as pets, allowing diagnosis (54–57). Furthermore, there are some wild animals in which *Dirofilaria* infections were identified, predominantly in canids like Red Foxes (4,5,58–69), Golden Jackals (59–62,70,71), and Grey Wolves (2,3,58,59,62,72,73). Zoo and wild animals were almost always infected by *D. immitis*, which again might be because *D. immitis* in comparison to *D. repens* infections more often leads to severe symptoms, corresponding test kits are available or because *D. immitis* has a broader host range.

It is undeniable that both, *D. immitis* and *D. repens*, are spreading in Europe and more humans and animals are at risk of infection. In part this might be also a diagnostic artefact, i.e. imported and travelling dogs are more routinely tested, which leads to more detection (39). Transport of pets has significantly increased during the 21st century as a consequence of the Pet Travel Scheme, which was introduced by the EU in 2000 and made travel of companion animals significantly easier and led to an increase in imported cases (27). Another reason is the continuously high number of stray dogs in some countries, which are not subject to regular treatment and act as reservoirs for the parasites, e.g. countries with many stray dogs, such as Romania or Bulgaria continue to regularly report *Dirofilaria* spp. cases (74–76). However, probably one of the most important factors for the spread of *Dirofilaria* is climate warming. Higher temperatures lead to faster development of *Dirofilaria* larvae inside the mosquito vector (77,78). Prolonged warm periods extend the transmission season (26). There is a significant increase in areas at risk, especially in more Northern countries. This spread has been predicted since the early 2000s (25,52) and with continuous climate warming will further increase in the future. Finally, increasing temperatures in Europe also allowed the widespread establishment of exotic mosquito species such as *Ae. albopictus* (79–81), which is an important vector for *D.*

immitis and *D. repens* and the establishment of the vector species in numerous European regions has been linked to increased *Dirofilaria* circulation (22).

Conclusion

Dirofilaria immitis and *D. repens* are an increasing threat to veterinary and public health in Europe. Both parasites have dramatically expanded their circulation area and are now endemic in areas that were considered *Dirofilaria*-free only one or two decades ago (23). The warming climate and the abundant presence of competent vectors allows the establishment of the parasites in Central Europe, e.g. Germany and Poland. Due to their rising relevance in animal and human health, a Europe-wide unified surveillance system similar to the system in the United States (82) should be implemented in order to better understand the change of circulation patterns and to plan and execute preventative strategies, e.g. dog treatment. All data and code are provided as open access, allowing for future analyses.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article and all codes used for data analysis are available at https://github.com/luehkenecology/dirofilaria_review_europe

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Conceptualization: RL; data collection: CH; data analysis: CH, RL; writing: CH, RL; all authors read and approved the final manuscript.

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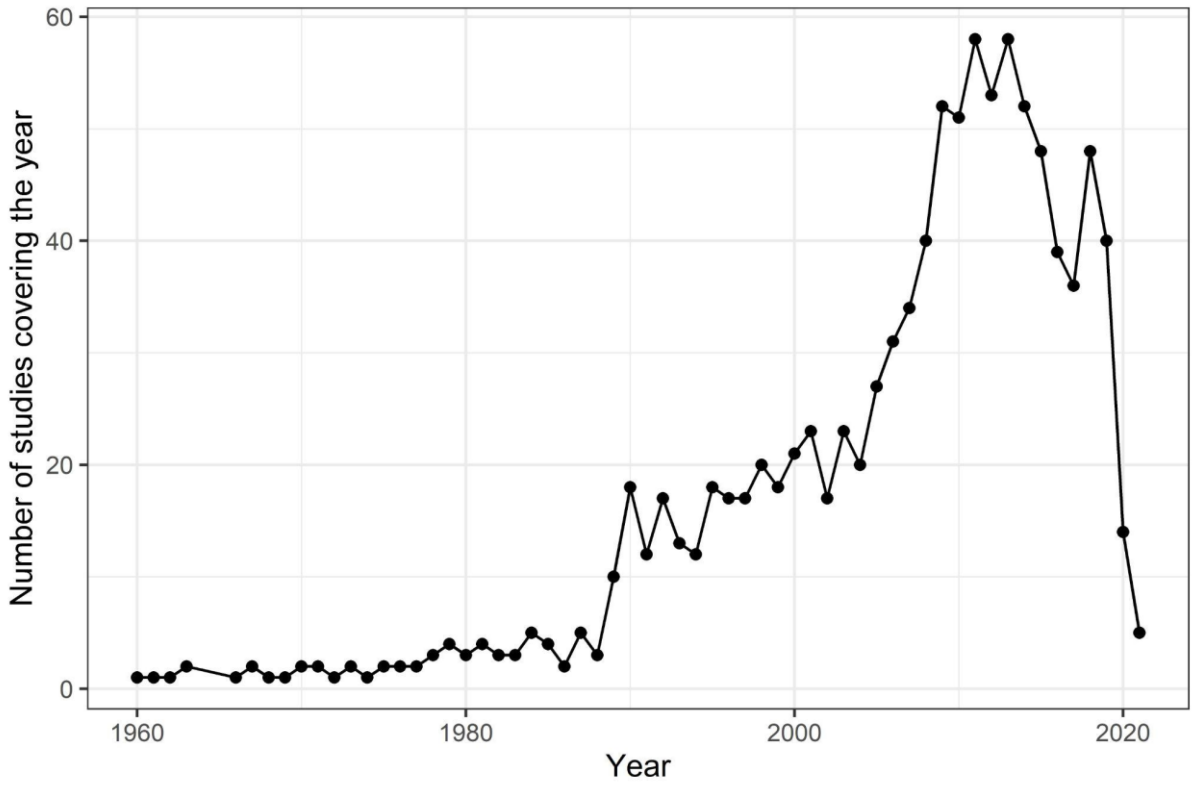

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463 Fig. 1: Number of studies reporting *Dirofilaria immitis* and *D. repens* in Europe

464

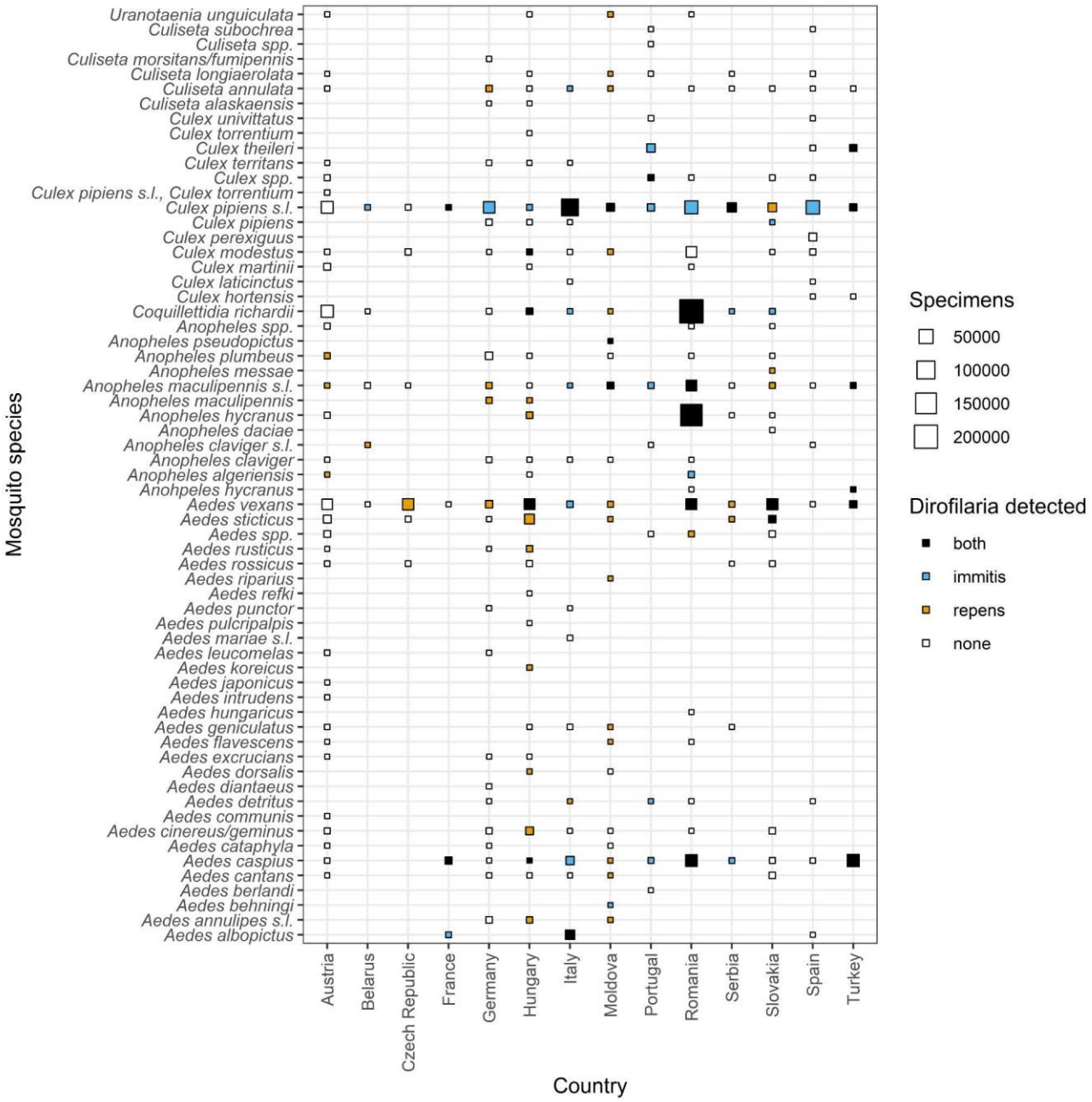


Fig. 2: *Dirofilaria immitis* and *D. repens* reports in mosquitoes for different European countries

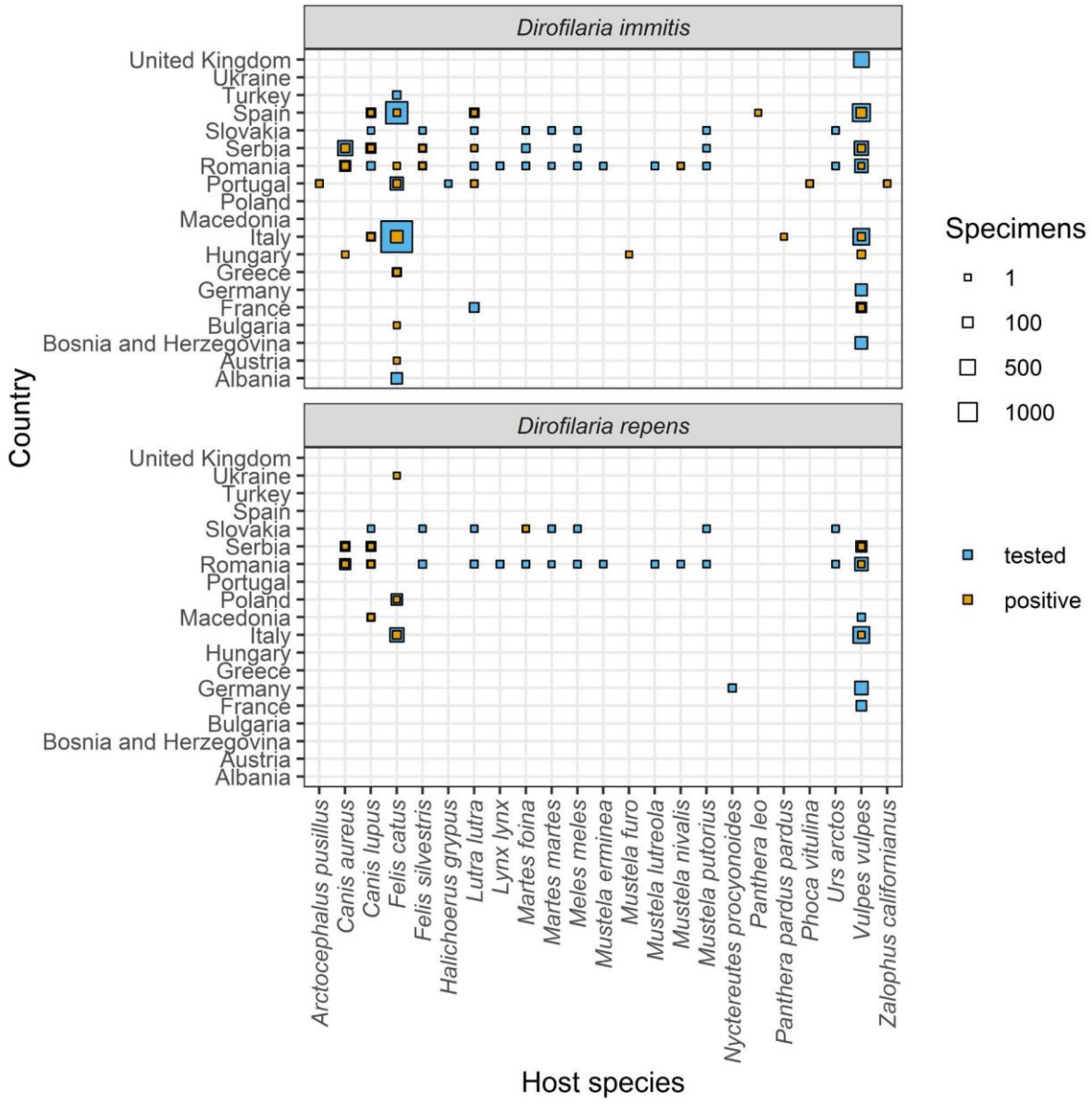


Fig. 3: *Dirofilaria immitis* and *D. repens* reports in vertebrates except humans and dogs for different European countries

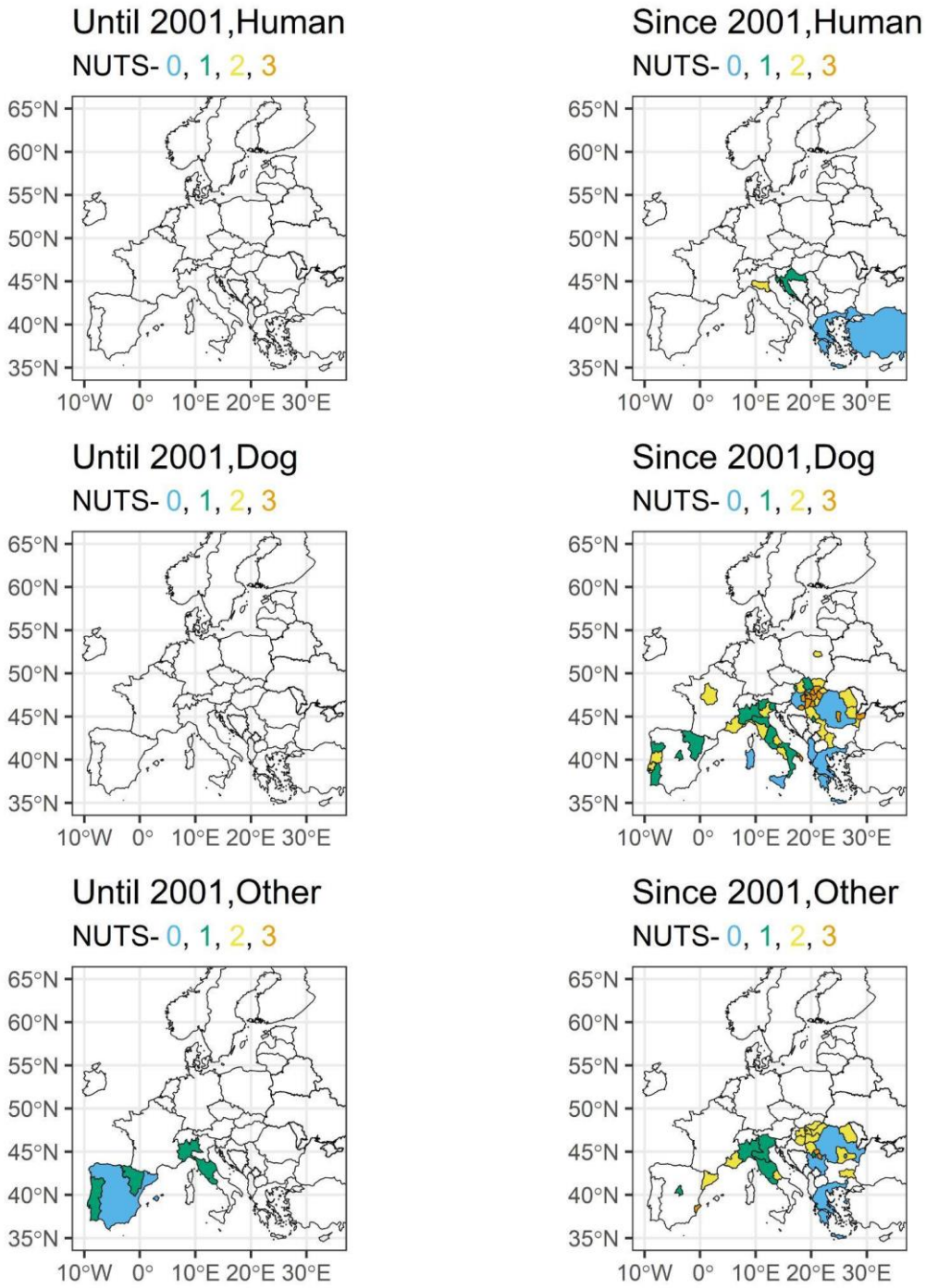
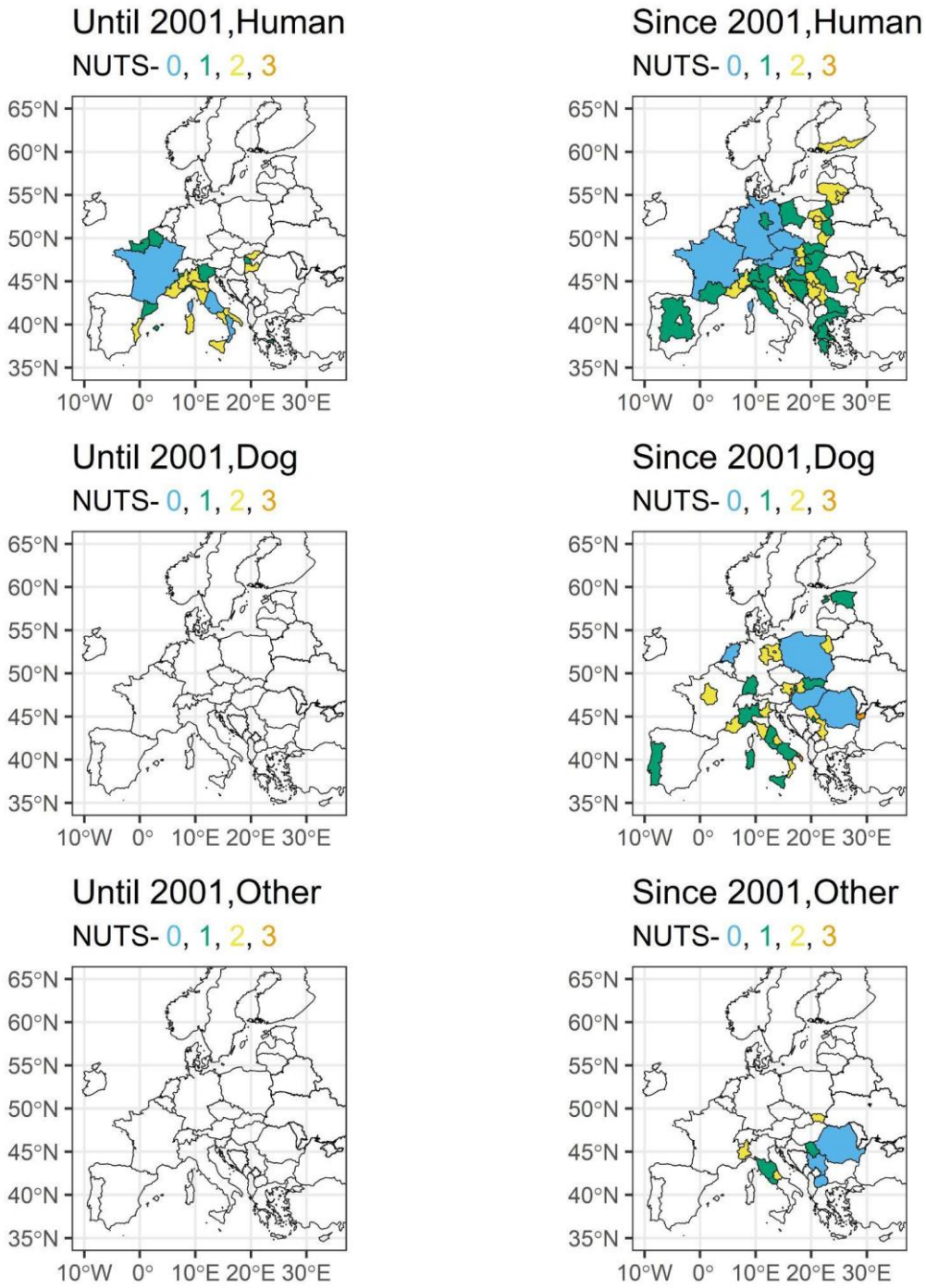


Fig. 4: *Dirofilaria immitis* cases in humans, dogs and other mammals with unremarkable travel history in Europe until and since 2001 at different geographical levels



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477 Fig. 5: *Dirofilaria repens* cases in humans, dogs and other mammals with unremarkable travel

478 history in Europe until and since 2001 at different geographical levels

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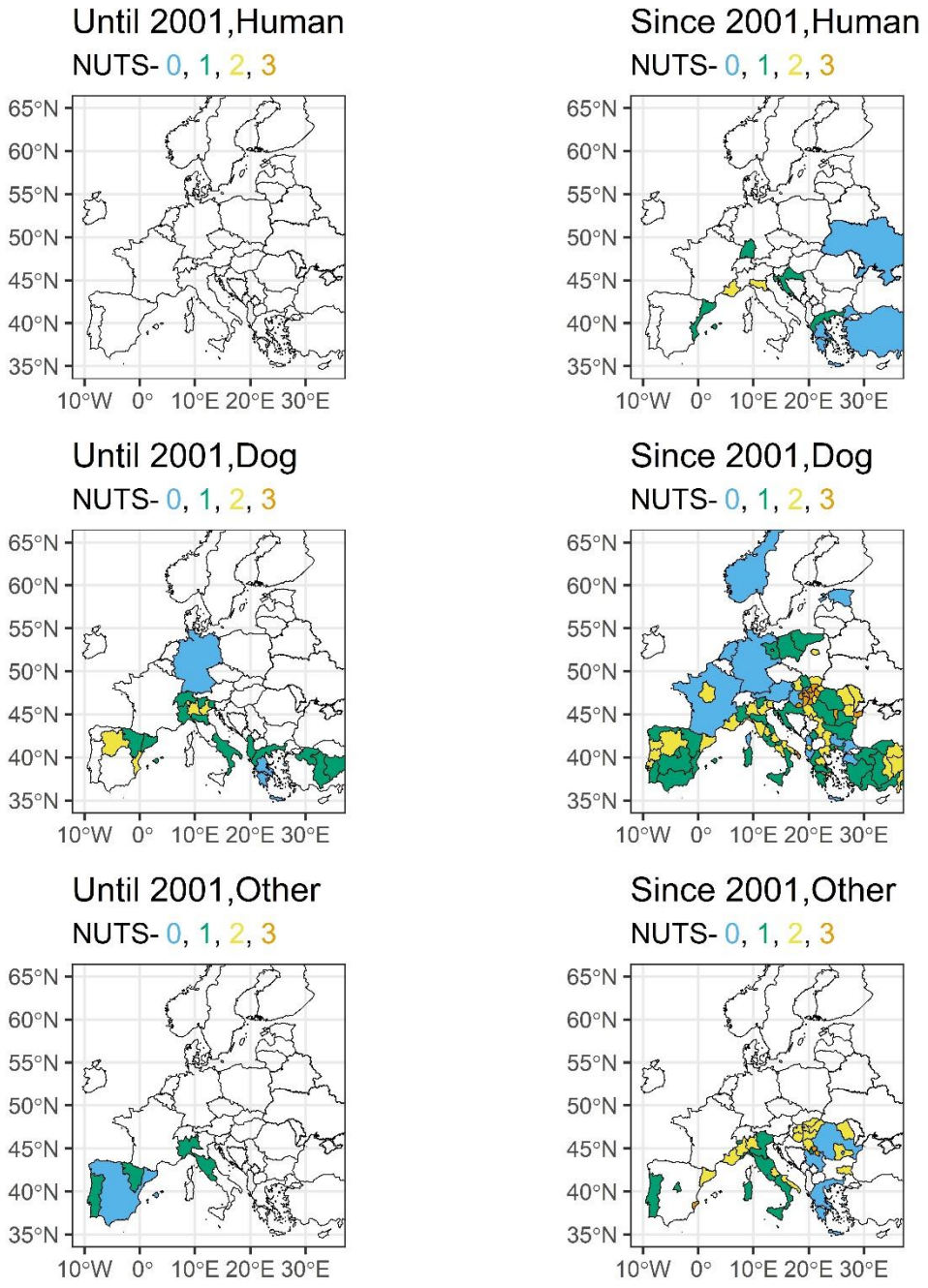

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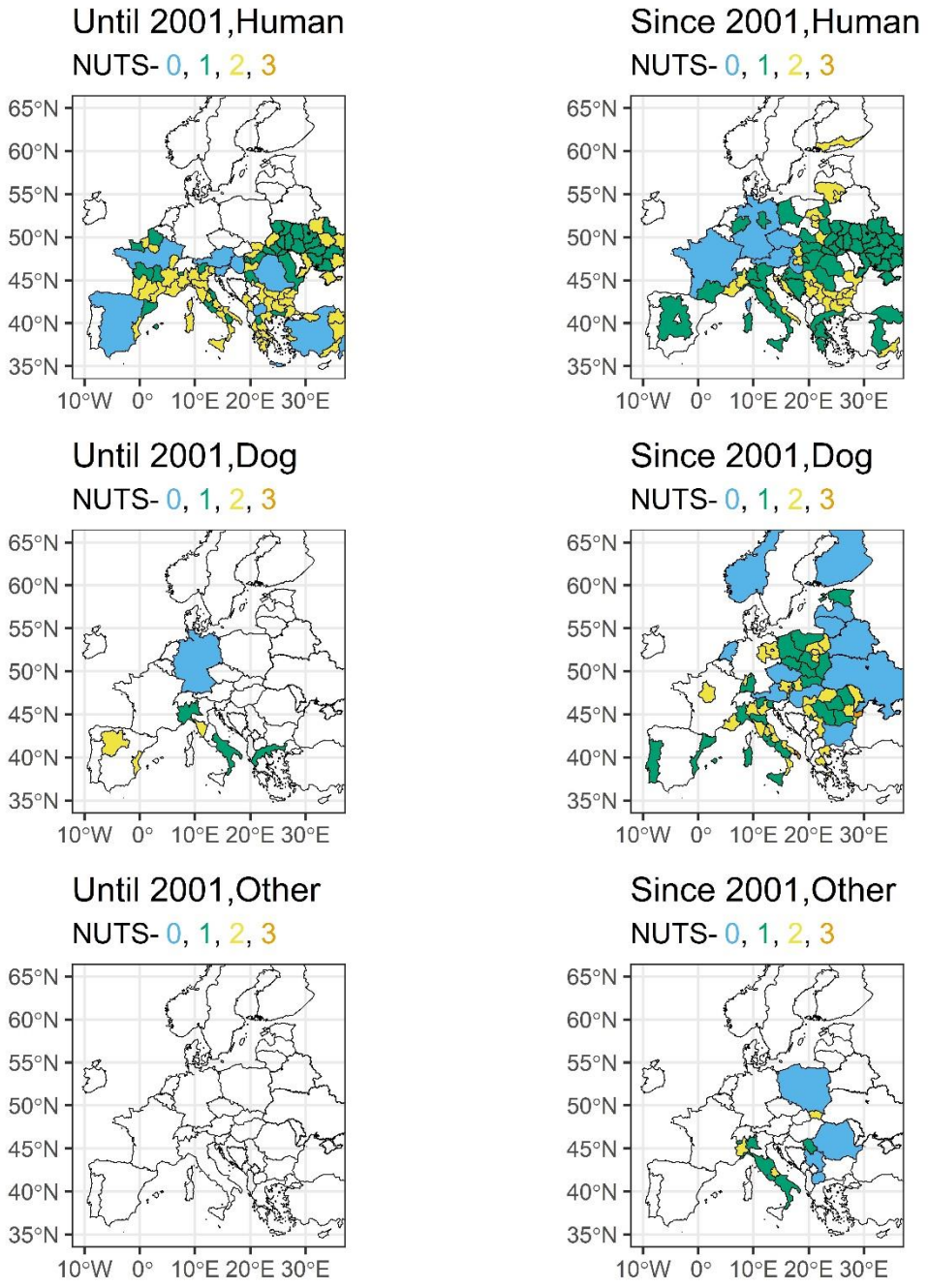
482 Supplementary file 1: *Dirofilaria immitis* cases in humans, dogs and other mammals with
483 unremarkable and unknown travel history in Europe until and since 2001 at different
484 geographical levels



485

486 Supplementary file 2: *Dirofilaria repens* cases in humans, dogs and other mammals with with

487 unremarkable and unknown travel history in Europe until and since 2001 at different

488 geographical levels