A survey of the German mosquito fauna (Diptera: Culicidae) and identification of associated pathogens

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››Das Glück hat seine entomologischen Launen:
Man läuft ihm hinterher und erreicht es nie; man
vergisst es, und es klopft an die Tür.‹‹
(Jean-Henri Fabre: Erinnerungen eines Insektenforschers)

››I know, I know the sun is hot
Mosquitos come suck your blood
Leave you there all alone
Just skin and bone
When you walk among the trees
Listening to the leaves
The further I go the less I know
The less I know‹‹
(Queens of the Stone Age: Mosquito song)

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(In chronological order)

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1. GENERAL INTRODUCTION

Mosquitoes are insects of the order Diptera, family Culicidae. This family is a most diverse and abundant group of species which occur in almost all ecozones, except the Antarctic. Most of the species are considered pest animals, not only because of their serious biting nuisance, but also owing to the fact that they are vectors of a wide range of debilitating bacterial, viral, parasitic and protozoal agents of disease affecting both humans and animals. Many of the diseases, such as chikungunya or West Nile fever, are regarded as "emerging vector-borne diseases" which have gained high attention in Europe recently (WEISSENBÖCK et al. 2010).

Emerging diseases are defined as infections which were previously unrecognized or have reappeared, are rapidly increasing in incidence or geographic range or are supposed to become a threat in the near future (GRATZ 1999). The term 'vector' is used in a broad sense, but can be characterized as either biological or mechanical. In general, it refers to any organism that acquires a disease agent from a living host and transmits it to another (SPICKLER et al. 2010).

Multiple factors are in discussion to account for the emergence of vector-borne diseases. Changes in international trade and travel, anthropogenic activities, climate conditions and even pathogen genetics can facilitate the emergence of vector-borne diseases (RANDOLPH & ROGERS 2010). Direct consequences are changes in the temporal and spatial distribution of vectors and pathogens. The movement of travellers and the trade of goods and animals may lead to the introduction and spread of animals and pathogens that are not native to an area. Climate change may promote favourable environmental conditions and thus allow pathogens, their hosts and vectors to invade new areas and to successfully establish.

As mosquitoes are poikilothermal organisms, their internal temperature directly depends on the ambient temperature. Therefore, the environmental temperature and the availability of breeding sites and blood hosts determine the distribution, reproduction rate, biting behaviour and longevity of mosquitoes. With rising temperatures as an effect of climate change, mosquito activity may become seasonally extended and population densities may increase. Furthermore, the extrinsic incubation periods of pathogens, i.e. the time period they need for their development inside their vectors, are temperature-dependent and tend to become shorter at warmer temperatures. Human behaviour, e.g. outdoor recreation, is also strongly influenced by temperature, so climate change will alter our interaction with vectors and the pathogens they carry. Changes in pathogen genetics

or introduction of new pathogenic strains may increase the infection prevalence in vertebrate hosts or affect vector specificity and efficiency, thus enhancing the host range and/or amplification potential of the pathogen (SCHUFFENECKER et al. 2006, WEAVER & REISEN 2010). Basic and applied research is needed to understand the complex biological and ecological interactions that exist between pathogens, vectors, hosts and their environments and to estimate the potential of vector-borne diseases to emerge in the future, conquer new geographic areas and become important public and veterinary health problems (MORENS et al. 2004, LORD et al. 2014).

The scientific field of medical entomology has long been neglected in Germany. The comprehensive works of Erich Martini, Fritz Weyer, Fritz Peus and Werner Mohrig, produced decades ago, are still the basis of present-day research on the ecology of mosquitoes in Germany. With few exceptions, systematic field studies on culicids have not been done for decades. Thus, up-to-date data on the mosquito fauna, including species composition, are basically missing.

According to the "Checkliste der Dipteren Deutschlands", a total of 46 mosquito species have been described for Germany (DAHL et. al. 1999). A number of species commonly regarded indigenous have not been recorded for many years, and it is a moot point whether they are still endemic (KAMPEN et al. 2013a). On the other hand, there are various exotic mosquito species such as *Aedes aegypti, Ae. albopictus, Ochlerotatus atropalpus, Oc. japonicus japonicus*, *Oc. koreicus* and *Oc. triseriatus* that have recently invaded Europe (MEDLOCK et al. 2012). The Asian tiger mosquito *Ae. albopictus* has repeatedly been detected in Germany, both as adult and as immature developmental stages, but it is not clear whether this species has already established or was introduced several times (PLUSKOTA et al. 2008, WERNER et al. 2012, BECKER et al. 2013, KAMPEN et al. 2013b). Unlike *Ae. albopictus*, the Asian rock pool mosquito *Oc. j. japonicus* has become widely established in southern Germany (BECKER et al. 2011, SCHNEIDER 2011, HUBER et al. 2012, WERNER et al. 2012). Ongoing studies also demonstrate that the species has infested large areas in western and northern Germany in the federal states of North Rhine-Westphalia, Rhineland-Palatinate and Lower Saxony (KAMPEN et al. 2012b, WERNER & KAMPEN 2013, KAMPEN & WERNER 2014).

These invasive exotic mosquitoes have been demonstrated to be competent vectors of viruses and filarial nematodes, infecting humans and animals (Figure 1), and may also have an impact on the biodiversity of the invaded area by displacing indigenous species (ANGELINI et al. 2007, ANDREADIS & WOLFE 2010, GOULD et al. 2010, MEDLOCK et al. 2012).

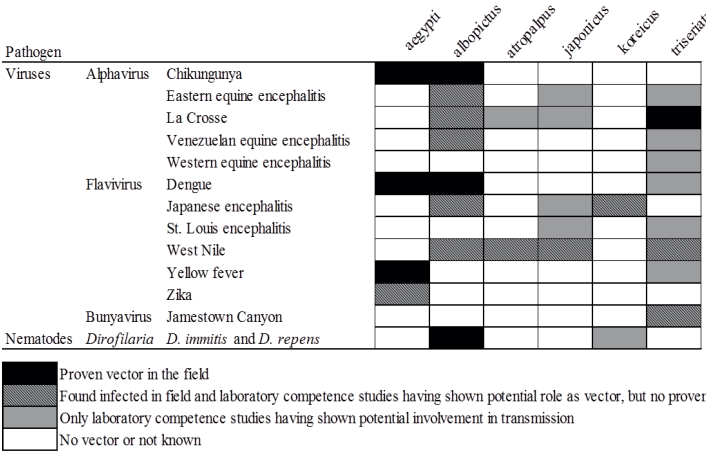

Figure 1 Overview of the vector status of the exotic aedine mosquito species intercepted or established in Europe (figure source: MEDLOCK et al. 2012).

2. LITERATURE REVIEW

2.1 TAXONOMY AND MORPHOLOGY OF MOSQUITOES

At the turn of the nineteenth century, the discovery of the role of mosquitoes in the transmission of pathogens (MANSON 1878) triggered the interest in the description and classification of these insects and entailed the constantly increasing number of known species. Since the introduction of molecular biology technologies in taxonomic research, the classification of the Culicidae has been revised several times. However, the discussion about the relationships of taxa, especially within the tribe Aedini, is still going on. Major generic changes were recently published by REINERT (2000) and REINERT et al. (2004, 2006, 2008, 2009), resulting in two or more names being simultaneously used in the scientific literature for a single taxon. In this thesis, traditional names and the elevation of the subgenus *Ochlerotatus* to generic rank according to REINERT (2000) are used in agreement with the database "Fauna Europaea" (SNOW & RAMSDALE 2013). According to the classification by REINERT (2000) and REINERT et al. (2004, 2006, 2008, 2009), the family Culicidae presently includes 3,537 described species classified in two subfamilies, Anophelinae and Culicinae, and 112 genera (HARBACH 2014).

Conforming to the literature and the results of the present study, the number of mosquito species considered indigenous to Germany has increased to 49, assuming that the cryptic *Anopheles daciae* of the Maculipennis Subgroup is a true species (Table 1). The two forms of *Culex pipiens*, *Culex pipiens* biotype *pipiens* and *Culex pipiens* biotype *molestus*, will be treated accordingly within this elaboration and not as true species. Thus, the German mosquito inventory comprises one species of the genera *Coquillettidia* (*Cq.*) and *Uranotaenia* (*Ur.*), each, four species of the genus *Aedes* (*Ae.*), six of the genus *Culex* (*Cx.*), seven of the genus *Anopheles* (*An.*), eight of the genus *Culiseta* (*Cs.*) and 22 of the genus *Ochlerotatus* (*Oc.*), respectively. These include two newly established species, *Oc. j. japonicus* and *Cs. longiareolata*, as well as *An. daciae*, which was recently recognized as a new sibling species of the Maculipennis Group (BECKER & HOFFMANN 2011, WEITZEL et al. 2012, chapters 4 & 5). The Asian tiger mosquito, *Ae. albopictus*, is not listed as it is still considered not to reproduce in Germany and therefore not to belong to the German mosquito fauna.

Table 1 List of culicid species recorded in Germany. Parentheses around authors' names and dates indicate that a species is currently placed in a genus other than the one in which the author(s) originally placed it. Nomenclature of taxa according to REINERT (2000).

Genus	Species/ biotype	Author & date of description
Aedes	*cinereus*	Meigen, 1818
	rossicus	Dolbeskin, Gorickaja & Mitrofanova, 1930
	geminus	Peus, 1970
	vexans	(Meigen, 1830)
Anopheles	*algeriensis*	Theobald, 1903
	atroparvus	van Thiel, 1927
	claviger	(Meigen, 1804)
	daciae	Linton, Nicolescu & Harbach, 2004
	maculipennis	Meigen, 1818
	messeae	Falleroni, 1926
	plumbeus	Stephens, 1828
Coquillettidia	*richiardii*	(Ficalbi, 1889)
Culiseta	*alaskaensis*	(Ludlow, 1906)
	annulata	(Schrank, 1776)
	fumipennis	(Stephens, 1825)
	glaphyroptera	(Schiner, 1864)
	morsitans	(Theobald, 1901)
	ochroptera	(Peus, 1935)
	subochrea	(Edwards, 1921)
	longiareolata	(Macquart, 1838)
Culex	*hortensis*	Ficalbi, 1889
	martinii	Medschid, 1930
	modestus	Ficalbi, 1890
	pipiens	
	biotype *pipiens*	Linnaeus, 1758
	biotype *molestus*	Forskål, 1775
	territans	Walker, 1856
	torrentium	Martini, 1925
Ochlerotatus	*annulipes*	(Meigen, 1830)
	caspius	(Pallas, 1771)
	communis	(de Geer, 1776)
	cataphylla	(Dyar, 1916)
	cantans	(Meigen, 1818)
	cyprius	(Ludlow, 1920)
	detritus	(Haliday, 1833)
	diantaeus	(Howard, Dyar & Knab, 1913)
	dorsalis	(Meigen, 1830)
	excrucians	(Walker, 1856)
	flavescens	(Müller, 1764)
	geniculatus	(Olivier, 1791)
	intrudens	(Dyar, 1919)
	japonicus	(Theobald, 1901)
	leucomelas	(Meigen, 1804)
	nigrinus	(Eckstein, 1918)
	punctor	(Kirby, 1837)
	pullatus	(Coquillett, 1904)
	refiki	(Medschid, 1928)
	riparius	(Dyar & Knab, 1907)
	rusticus	(Rossi, 1790)
	sticticus	(Meigen, 1838)
Uranotaenia	*unguiculata*	Edwards, 1913

Species of the family Culicidae differ morphologically from each other by their size, colouration and pattern of scales, setae and bristles. Principal characters distinguishing the subfamilies Anophelinae and Culicinae can be found in all developmental stages. Anopheline eggs are laid singly and possess characteristic floats, larvae lack air tubes (siphons) and lie parallel to the water surface, and adults have elongated palps in both sexes and rest with their body forming an angle to the surface. Typical for the subfamily Culicinae, eggs are laid singly or are glued into a raft and never bear floats, larvae have air tubes and hang down in an angle from the water surface, and females have short palps and rest with their body being more or less parallel to the surface.
However, there are groups of rather closely related and often isomorphic so-called "sibling species", which can all the same differ in their biological characteristics such as feeding pattern, breeding preference and vector competence. These sibling species are arranged in informal sections, series, groups, subgroups and complexes, which may not obligatorily indicate natural relationships. From a medical point of view, two such groups are particularly important in Germany, the Pipiens Group and the Maculipennis Subgroup.

Worldwide, the Pipiens Group consists of several species and is believed to be represented in Germany by two distinct biotypes, *Cx. pipiens* biotype *pipiens* and *Cx. pipiens* biotype *molestus*, as well as hybrids of these (RUDOLF et al. 2013). In addition, *Cx. torrentium*, another *Culex* species of temperate regions, is sometimes also being referred to as a member of the Pipiens Group because of similar morphology and sympatric occurrence (SMITH & FONSECA 2004). In Europe and other temperate regions, members of the Pipiens Group serve as principal vectors of various human and animal disease agents, including viruses, protozoans and filarial worms. *Culex pipiens* biotype *pipiens* is considered to be mainly ornithophilic, contrary to *Cx. pipiens* biotype *molestus* which is mainly mammalophilic, whereas hybrids display an indiscriminative biting behaviour and readily feed on both avian and mammalian hosts, including humans. The lacking feeding preference of the hybrids is considered to have important implications for the transmission of multi-host zoonotic vector-borne pathogens like the West Nile virus (KILPATRICK et al. 2007).
The Maculipennis Subgroup has been the subject of many hundreds of scientific publications, reflecting the medical importance of this species group. It consists of at least eleven Palaearctic species and is believed to be represented in Germany by four species: *An. atroparvus*, *An. daciae*, *An. maculipennis* and *An. messeae* (WEITZEL et al. 2012, chapters 4 & 5). The members of this

Subgroup are known to be responsible for most of the malaria cases in historic Europe, but the various species are not equally efficient as vectors of malaria parasites and other pathogens (JETTEN & TAKKEN 1994). With respect to the different vector potential for malaria parasites of geographically distinct mosquito populations, this phenomenon has become famous as "anophelism without malaria" (FANTINI 1994). Based on morphological and physiological studies, BATES (1940) proposed their classification as separate species instead of variable taxonomic sub-categories (e.g. races, varieties or biotypes). Prior to the development of DNA based diagnostic methods, the comparative examination of the egg patterns and structures and later cytotaxonomy (WHITE 1982) were the most common methods of separating Maculipennis Subgroup species.

The occurrence, distribution and ecology of the sibling species of the Maculipennis Subgroup in Germany are elaborately described in the work of WEYER (e.g. 1938, 1948, 1951), which has essentially contributed to our present knowledge. A revised inspection of the Maculipennis Subgroup in Germany is described in chapters 4 and 5.

For the future it can be expected that the use of modern techniques in species differentiation will lead to the detection of additional culicid species, which are possibly not recognized by now. The different ecologies and behaviours of these sibling species and biotypes may have an impact on their epidemiological significance and are important for the understanding of disease outcomes (KAMPEN et al. 2012a).

2.2 BIOLOGY AND DEVELOPMENT OF MOSQUITOES

The development of mosquitoes from egg to imago mostly depends on temperature and is completed in two different environments: an aquatic one and a terrestrial one. In temperate regions, the larval and pupal development of e.g. *Cx. pipiens* takes at least two weeks at 20 °C, but can be prolonged to more than three months under unfavourable conditions (BECKER et al. 2010).

Mosquito eggs are laid in different manners, on the water surface, attached to underwater vegetation or on humid substrates above the water line that will later get flooded. In the latter case, eggs do not hatch immediately after oviposition and the embryo first enters a period of dormancy or diapause. Eggs of these mosquitoes (e.g. *Aedes* and *Ochlerotatus* species) can withstand desiccation and low temperatures up to several months or even years. In temperate

regions, these mosquito species usually overwinter as diapausing eggs. The diapause ends after the eggs have submerged and specific environmental stimuli such as changes in the oxygen level, temperature and day length occur.

Mosquitoes whose larvae hatch soon after embryogenesis usually have several generations per year (multivoltinism) (e.g. *Culex*, *Coquillettidia, Culiseta*), whereas the larvae of univoltine species only hatch after the winter.

There are four mobile larval instars which feed on microorganisms as well as on decaying plant and animal material (filter-feeders). Depending on the species, the breeding habitats vary from temporary surface water (e.g. flood plains, brackish water), permanent water bodies (ponds and lakes) to diverse natural and artificial water storage reservoirs and containers (e.g. tree holes, rain barrels).

Mosquito larval breathing is performed through a pair of spiracles which are located dorsally on the ninth abdominal segment. The breathing apparatus is well developed as an elongated siphon in the subfamily Culicinae and poorly developed in the subfamily Anophelinae. Thus, the anopheline larvae rest in parallel to the water surface. Some species of the genera *Mansonia* and *Coquillettidia* have a specialized siphon to pierce roots, stems or submerged leaves of aquatic plants, enabling them to utilize oxygen from the aerenchyma (SÉRANDOUR et al. 2006). Larvae of species which overwinter in the larval stage (e.g. *Oc. geniculatus, Oc. rusticus*) can survive in water close to freezing or even coated with ice (BECKER et al. 2010). Overall, there are various physiological and phenological adaptations to the environment in the aquatic stages of different mosquito species and populations.

The pupae do not feed and, as a consequence, spend most of their time at the water surface for breathing. When metamorphosis is finished, the adult emerges from the pupal case by ingesting air, causing the cephalothorax to split.

Within the first days of the adult life both mosquito sexes ingest carbohydrates, e.g. in the form of nectar or honeydew, to become sexually mature and mate. Plant sugars are the main energy source during their life in both sexes, mostly spent on flight activity. In most species, females enter a swarm of flying males for mating (eurygamy). Once a male catches a female, the copulation takes place outside the swarm. Whilst male mosquitoes can mate many times, female mosquitoes retain the sperms in their spermathecae (receptacula siminis) to fertilize future egg batches without further copulation. After insemination, females of most mosquito species require a blood meal for each gonotrophic cycle (anautogeny). In contrast, some species/biotypes (e.g.

Cx. pipiens biotype *molestus*) are able to develop the first batch of eggs without a blood meal (facultative autogeny), while other species (e.g. genus *Toxorhynchites*) subsist exclusively on plant sugar (obligatory autogeny).

Females searching for blood hosts are attracted by heat convection, visual stimuli and a specific odour composed of volatile chemicals such as carbon dioxide, ammonia and lactic and fatty acids. There are various patterns of activity as a function of adaptation to the preferred host. Many mosquitoes are primarily active during dusk or dawn (crepuscular), whereas others are active during the night (nocturnal) or during daytime (diurnal). Endophagic mosquitoes enter houses to feed on their host, while others feed exclusively outdoors (exophagy). Host specificity and host preference can vary widely and may also change in certain areas and during the seasons. There are mosquitoes that feed primarily on humans (anthropophily), on animals (zoophily) or on birds (ornithophily), while others are considered generalists and feed on variable hosts (indiscriminative biters). After imbibing a blood meal, mosquitoes search for resting places either indoors (endophily) or outdoors (exophily), where they digest the blood meal and maturation of the eggs takes place. Overall, the behaviour of female mosquitoes is highly diverse and may not only vary from species to species but also from population to population within the same species. These variations can be of fundamental relevance on disease epidemiology when it comes to vector species (SERVICE 2008).

2.3 MEDICAL IMPORTANCE OF MOSQUITOES

Mosquitoes are by far the most important blood-sucking arthropods worldwide, causing considerable nuisance due to their aggressive biting behaviour. As in the case of other blood-feeding arthropods, the saliva proteins, which are injected during feeding, may lead to allergic reactions. Massive floodings entail ideal conditions for mass reproductions of mosquitoes (e.g. the flood-water species *Ae. vexans*), resulting in increased biting rates, as happened in the summer of 2013 along Germany's Elbe and Danube rivers. In some areas of high risk of mass development (e.g. the Upper Rhine Valley), the mosquitoes have therefore been controlled for decades using mosquitocidal bacterial toxins such as *Bacillus thuringiensis* var. *israelensis* toxin or *Lysinibacillus* (formerly *Bacillus*) *sphaericus* toxin (BECKER 1997).

The role of culicid mosquitoes as vectors of disease agents has been investigated very well since the discovery of the transmission cycles of *Wuchereria bancrofti* and malaria parasites at the end of the 19th century (COOK 1994).

In general, pathogens can be transmitted either biologically or mechanically (SPICKLER et al. 2010). A mechanical vector (e.g. a fly or a cockroach) is a vector that simply carries a pathogen from one locale to another by contaminated mouthparts or other body parts. In some cases, the pathogen may even pass the intestines of the mechanical vector. However, during this type of transmission no multiplication or developmental modification of the pathogen takes place.

Biological vectors, such as haematophagous arthropods, transmit pathogens that obligatorily undergo a developmental cycle and/or multiplication in the body of the arthropod. The extrinsic incubation period, i.e. the time required for the development and/or multiplication in the vector, depends on the pathogen species and, particularly, on the ambient temperature. In this type of transmission, the pathogen can be transmitted to a new host via the saliva during a blood meal (e.g. viruses and malaria parasites by mosquitoes), via the faeces (e.g. *Trypanosoma cruzi* by reduviid bugs) or through regurgitation (e.g. *Yersinia pestis* by fleas) (CLEMENTS 2012).

Vector competence is genetically controlled by factors (e.g. immune response of the vector, cell receptor furnishing) determining the ability of a mosquito to acquire, enable the development and transmit a pathogen. However, vector competent mosquitoes are not necessarily vectors in the field. It has been shown for a number of mosquito species that they are vectors in the laboratory but not in their natural distribution area. Environmental and behavioural factors affect the association between a vector, the pathogen transmitted and the vertebrate host, thus defining the vectorial capacity (BEERNTSEN et al. 2000).

2.3.1 MOSQUITO-BORNE DISEASES IN EUROPE

In contrast to many tropical and subtropical regions of the world, the role of mosquitoes as vectors of disease agents has generally been of minor importance in Europe for the last decades. However, this situation has changed as a result of global alterations, and Europe has recently faced a series of mosquito-borne diseases affecting humans and animals. Some of them resurged and have broken out periodically (e.g. West Nile fever) while others are novel (e.g. chikungunya). Further disease entities (e.g. Rift Valley fever) are feared to arrive in the near future (TAKKEN & KNOLS 2007).

With over 200 million estimated cases per year and a high level of morbidity and mortality, malaria is one of the most important infectious diseases worldwide (WHO 2013). Until the middle of the 20th century, high incidences of autochthonous malaria were also reported from Europe, where members of the Maculipennis Group, especially *An. sacharovi*, *An. labranchiae* and *An. atroparvus*, were the principal vectors (JETTEN & TAKKEN 1994).

Most infections were probably attributed to *Plasmodium vivax* (tertian malaria) and *P. malariae* (quartan malaria), but rare epidemics with high mortality rates were also caused by *P. falciparum* (BRUCE-CHWATT & DE ZULUETA 1980). In historic Germany, malaria was widespread where swamps and meadows concentrated alongside the rivers in certain regions, in particular in the northern lowlands (GROBER 1903). While *Anopheles* mosquitoes were recognized as vectors of malaria parasites only at the beginning of the 20th century, the characteristic remittent fever, called 'ague', had long before been linked with swamps and marshlands. The disease's name has been derived from the Italian "mal'aria", meaning "bad air", and it was commonly believed that fumes from the meadows produced the illness. Without really knowing the aetiology of the disease, this knowledge led to a variety of attempts to avoid or remove the source of the "bad air", such as settling beyond the fatal marshland or by drainage of swamps, ponds, dead stream channels and other stagnant water bodies (DOBSON 1994).

Quinine, as a component of the bark of the cinchona tree which effectively suppresses the clinical symptoms and may contribute to a milder nature of malaria, became a readily available and popular malaria treatment in the 19th century (DOBSON 1994). Changes in agriculture, such as intensification of crop and lifestock farming and introduction of new root crops in consequence to the drainage of the landscape further contributed to the reduction of *Anopheles* breeding places. The significant decline of mosquito population densities in turn led to a steady reduction of malaria which finally disappeared from Europe in the second half of the 20th century (BRUCE-CHWATT & DE ZULUETA 1980).

DOBSON (1994) also speculated that the decrease and regional isolation of malaria led to a reduced genetic diversity of the malarial parasites and a selective loss of virulent variants. The discovery of the existence of *An. maculipennis* sibling species and the realization of differences in their efficiency as vectors, due to their different vector competences and ecologies, brought the breakthrough in the fight against malaria (FANTINI 1994). Furthermore, a number of changes in human behaviour contributed to the decrease of malaria. With the possibility to heat and light

rooms and to regulate indoor humidity, the housing situation improved substantially and conditions became less suitable for mosquitoes.

Other interventions were related to changes in livestock husbandry. The cultivation and storage of agricultural products enabled the people to feed their cattle over the winter months, providing an alternative source of blood for the mosquitoes. At the same time, the cattle sheds were built away from human habitation, making it easier to avoid unnecessary close contact to the vector (vector deviation). While generally on the decrease, malaria cases increased again in Germany and other European countries in the context of the First and Second World Wars and post-war periods in the first half of the 20th century, owing to poor sanitary conditions, massive refugee and population movements and production of numerous *Anopheles* breeding sites in the vicinity of human dwellings (WEYER 1951). In East Frisia alone, the number of autochthonous malaria cases increased from 108 cases in 1914 to more than 4000 cases in the year 1918 (EICHENLAUB 1979). In the following decades, malaria once again diminished continuously as a consequence of the increased application of insecticides, such as DDT, and synthetic drugs, the introduction of epidemiological surveillance, improved social, economic and sanitary conditions, and the further reduction of *Anopheles* breeding sites (DE ZULUETA 1994, MAIER 2004).

While malaria was declared eradicated from geographical Europe in the early 1970s by the WHO, the disease remained endemic in some member states of the WHO European Region, namely Azerbaijan, Georgia, Kyrgyzstan, Tajikistan, Turkey and Uzbekistan (GORDEEV et al. 2008). In western European countries, sporadic cases of autochthonous malaria kept occurring, mostly as the result of the bites of *Anopheles* mosquitoes that became infected by feeding on parasitemic people returning from endemic areas or by introduced *Anopheles* females infected with plasmodia (MAJORI et al. 1999). In the last decades individual locally acquired cases occurred in Italy (SARTORI et al. 1989, BALDARI et al. 1998), Germany (PRAETORIUS et al. 1999, KRÜGER et al. 2001, ZOLLER et al. 2009), Spain (CUADROS et al. 2002, SANTA-OLALLA PERALTA et al. 2010), France (ARMENGAUD et al. 2006, DOUDIER et al. 2007) and Greece (MALTEZOS et al. 1995, KAMPEN et al. 2002, 2003). In 1995, a small outbreak of autochthonous malaria occurred in Bulgaria (NIKOLAEVA 1996) and since 2009, several outbreaks due to *P. vivax* have been reported from Greece, where migrant workers from malaria endemic countries are discussed as potential sources of infection (DANIS et al. 2011a).

Recent studies on the receptivity of the European *Anopheles* species for tropical *Plasmodium* strains were contradictory. *Anopheles labranchiae* and *An. atroparvus* were completely refractory

to strains of *P. falciparum* from India as well as from East and West Africa due to missing coadaptation (SHUTE 1940, ZULUETA et al. 1975, RAMSDALE & COLUZZI 1975, MARCHANT et al. 1998, TAKKEN et al. 2007). Experiments on the susceptibility of *An. atroparvus, An. messeae* and *An. sacharovi* mosquitoes from the former USSR to *P. falciparum* also demonstrated refractoriness to imported strains from nine different countries of tropical Africa (DASHKOVA 1977). On the other hand, some indigenous *Anopheles* are probably fully susceptible to infection with African *P. vivax* and *P. ovale* strains, as shown by the recent autochthonous malaria cases in Europe.

The susceptibility of European *Anopheles* species to infection with tropical *Plasmodium* strains and their potential role as vectors of malaria parasites should be revised and experimentally re-investigated with respect to a changing environment and climate. Recent laboratory and field studies with *An. plumbeus*, for example, suggested both vector competence for *P. falciparum* and a behavioural change in that breeding sites in close vicinity to humans are readily accepted, such as manure pits allowing for mass development (KRÜGER et al. 2001, ELING et al. 2003, DEKONINCK et al. 2011, SCHAFFNER et al. 2012).

Despite the occasional import of *Plasmodium*-infected *Anopheles* specimens and parasitemic humans that may infect vector-competent indigenous *Anopheles* species and cause autochthonous transmission, the risk of large-scale outbreaks or the re-emergence of malaria to an endemic level in Europe is considered rather unlikely. This is in essence due to the high health standard and excellent medical care service in most European countries. As the incubation period for malaria parasites is normally quite short and efficient diagnostic techniques and therapeutic drugs are available, the disease could not spread without being noticed (ALTEN et al. 2007).

In contrast, mosquito-borne viruses are considered to represent a serious threat for public and animal health in Europe. Most clinical symptoms caused by infection with mosquito-borne viruses are unspecific and resemble a common flu, often running under the name of ‘summer flu’. Such disease cases are usually not followed up etiologically, so that in the case of mosquito-borne viruses the causative agents may remain undetected after introduction and spread without any surveillance. Apart from unspecific flu-like symptoms, however, mosquito-borne viruses such as West Nile virus or dengue virus may lead to meningitis, encephalitis and further life-threatening syndromes such as high fever, bleeding disorders, a low blood pressure and haemorrhages that can end up in the death of the patient if untreated.

It is also assumed that vertical transmission of certain viruses within the mosquito population allows the virus to persist through unfavourable periods such as the winter months in temperate regions (DEFOLIART et al. 1987).

Vaccines or chemotherapeutic agents are not yet available against the majority of mosquito-borne viruses. Furthermore, the transmission cycle of most mosquito-borne viruses involves numerous wild animals serving as reservoir and transportation host, which allow the virus to propagate and spread undetected.

West Nile fever, for example, is a viral mosquito-borne disease, whose natural transmission cycle involves birds and mosquitoes, particularly *Culex* spp. and *Aedes* spp., in which the virus is able to overwinter (NASCI et al. 2001) and probably to be transmitted vertically, as shown for *Ae. albopictus* and *Cx. pipiens* (BAQAR et al. 1993, GODDARD et al. 2003). Especially migrating birds play an important role as reservoir hosts and for the dispersal and establishment of new endemic foci along the migratory routes, as illustrated by the introduction of West Nile virus (WNV) into the New World and the subsequent rapid spread (RAPPOLE et al. 2000, REED et al. 2003). A first epidemic in Europe occurred in France, affecting humans and horses during the summer of 1962 in the Camargue region (DEL GIUDICE et al. 2004).

Mammals are usually incidental dead-end hosts, where the disease can trigger a range of symptoms from mild, flu-like ones to encephalitis and even fatal outcome. During the past 20 years outbreaks in birds, horses and also humans tend to occur increasingly often and more regularly (SAMBRI et al. 2013). Epidemics of WNV were reported from Romania (TSAI et al. 1998, CALISTRI et al. 2010), France (MURGUE et al. 2001), Italy (AUTORINO et al. 2002, MACINI et al. 2008, BARZON et al. 2009, RIZZO et al. 2009, ANGELINI et al. 2010) and Greece (DANIS et al. 2011b, 2011c), while travel-associated cases were reported from Belgium, Sweden and Switzerland (EFSA & ECDC 2014). Moreover, WNV was detected in various mosquito species from several European countries, e.g. Austria (STIASNY et al. 2013), Italy (ENGLER et al. 2013), Greece (PAPA et al. 2011) and Spain (VÁZQUEZ et al. 2011a).

As data of WNV infections in animals have been submitted at the EU level since 2012, almost all member states (except Germany and Poland) reported infections of animals, mainly horses. In the light of the reported findings in animals, the overwintering of infected mosquitoes and the permanent presence of susceptible birds, it can be expected that WNV will expand its geographic range and establish in further Europe regions in the next few years causing increasing numbers of outbreaks associated with human morbidity and mortality.

Recently, a special focus was placed on the introduction and establishment of exotic mosquito species such as *Ae. aegypti, Ae. albopictus, Oc. atropalpus, Oc. japonicus, Oc. koreicus* and *Oc. triseriatus,* which have been demonstrated to be competent vectors of numerous viruses infecting humans and animals. Among them, the greatest attention deserves the Asian tiger mosquito *Ae. albopictus*, which is considered the most invasive mosquito species in the world, as illustrated by records from more than 20 European countries (MEDLOCK et al. 2012).

The anticipated threat of a possible disease outbreak mediated by these invasive species in Europe has already become reality: *Aedes albopictus* was the primary vector in the outbreak of chikungunya in Italy in 2007 (ANGELINI et al. 2007) and probably in two cases of autochthonous chikungunya virus transmission in France in 2010 (GOULD et al. 2010, GRANDADAM et al. 2011). Not enough, it was also accountable as a dengue virus vector in France and Croatia in 2010 and 2013 (LA RUCHE et al. 2010, GJENERO-MARGAN et al. 2011, MARCHAND et al. 2013).

While autochthonous transmission of chikungunya virus within continental Europe was reported for the first time, dengue and yellow fever affected Europe already in the eighteenth and nineteenth centuries, with the yellow fever mosquito *Ae. aegypti* being responsible for severe epidemics (FONTENILLE et al. 1997, REITER 2010), such as in Spain (ERITJA et al. 2005) and in Greece in 1927-1928 (ROSEN 1986). It is most likely that *Ae. aegypti* was eradicated from Europe in the course of the malaria control programmes in the early 20th century, but it has recently re-established in Europe, e.g. in Madeira (Portugal) (ALMEIDA et al. 2007) and on the eastern Black Sea coast in southern Russia and Georgia (YUNICHEVA et al. 2008).

In October 2012, *Ae. aegypti* mediated an outbreak of dengue fever on Madeira resulting in approximately 2,000 human cases (SOUSA et al. 2012). Unlike *Ae. albopictus*, *Ae. aegypti* is not adapted to the temperate climates of more northern latitudes and could therefore be quickly eradicated from the Netherlands where it had been introduced in 2010 (SCHOLTE et al. 2010).

Aedes albopictus and *Ae. aegypti* are particularly synanthropic mosquito species and prefer breeding in urban surroundings in small artificial water containers, which highlights the increased potential for contact with humans of these mosquito species (MEDLOCK et al. 2012).

Among mosquito-borne viruses infecting both humans and animals, Rift Valley fever virus (RVFV) is expected to arrive in Europe in the near future. Rift Valley fever is widespread in Africa and recently spread to the Middle East, where it caused high mortality rates in newborn ruminants, especially sheep and goats (BHARDWAJ 2013). Infections in humans are mainly caused by direct or indirect contact with the blood and organs of infected animals, e.g. during

slaughtering. The disease is typically associated with self-limiting febrile illnesses, but among severely affected persons who are hospitalized, the case fatality rate is approximately 10-20 % (MADANI et al. 2003). In contrast, bites by infected *Aedes* and some *Culex* species are the main transmission route in ruminants (CHEVALIER et al. 2010).

The probability of natural introduction and spread of RVFV in Europe is low, but illegal importation of infected ruminants or products, its recent geographic expansion to Egypt and the Arabian Peninsula, combined with the presence of competent vectors and the variety of infection routes constitute a risk to certain areas in Europe (CHEVALIER et al. 2010).

In the light of the increasing risk of introduction, establishment and spread of human- and animal-pathogenic mosquito-borne viruses and the absence of vaccines (except for yellow fever), surveillance of the local mosquito population and the pathogens they carry is of paramount importance in order to assess the risk of autochthonous transmission of mosquito-borne pathogens.

Table 2 offers an overview of the pathogens demonstrated in indigenous mosquito species or transmitted by mosquitoes in Europe.

Table 2 Pathogens demonstrated in indigenous mosquito species or transmitted by mosquitoes in Europe (LUNDSTRÖM 1999, HUBÁLEK 2008, AGÜERO et al. 2011, PUTKURI et al. 2014); pathogens recently demonstrated in Germany (vector or vertebrate host) are highlighted in bold.

Family	Pathogen	Distribution	Vertebrate host	Important vectors
Togaviridae (*Alphavirus*)	**Sindbis virus**	Mostly northern Europe	Wild passeriform birds	Largely ornithophilic mosquitoes (*Culex* spp., *Culiseta* spp.)
	Chikungunya virus	Sporadic outbreaks in Italy and France	Humans	Anthropophilic *Aedes* spp.
Flaviviridae (*Flavivirus*)	West Nile virus	Southern and central Europe	Wild birds, occasional mammals, e.g. humans, cattle, camel, horse	Largely ornithophilic mosquitoes (*Culex* spp.)
	Usutu virus	Austria, Hungary, Italy, Czech Republic, Poland and Germany	Wild passeriform birds and raptors	Largely ornithophilic mosquitoes (*Culex* spp.)
	Dengue virus	Greece, Spain, Portugal, France	Humans	Anthropophilic *Aedes* spp.
	Bagaza virus	Spain	Wild (game) birds	Largely ornithophilic mosquitoes (*Culex* spp.)
Bunyaviridae (*Orthobunyavirus*)	**Batai virus** (Bunyamwera serogroup)	Mostly eastern Europe	Domestic pig, horse, and ruminants, several bird species	Zoophilic mosquitoes, e.g. *An. maculipennis* s.l., *An. claviger*, *Cq. richiardii*
	Tahyna virus (California serogroup)	Throughout Europe	Lagomorphs, hedgehogs and rodents	Several flood-water mosquitoes (*Aedes* spp., *Ochlerotatus* spp.)
	Snowshoe hare virus (California serogroup)	Eastern Europe	Lagomorphs and rodents	*Ae. cinereus, Ae. vexans, Oc. communis, Oc. punctor, Oc. cataphylla, Cs. inornata, Cs. impatiens*
	Inkoo virus (California serogroup)	Northern Europe, including Russia	Mountain hare (*Lepus timidus*)	*Oc. communis, Oc. punctor, Oc. hexodontus*
	Lednice virus (Turlock serogroup)	Czech Republic	Birds, largely of the order Anseriformes	*Cx. modestus*
	Chatanga virus (California serogroup)	Finland	unknown	*Ochlerotatus* spp., *Aedes* spp. (suggested)
Malaria	*Plasmodium vivax*	Recently in Greece, in the past throughout Europe	Humans	*Anopheles* spp.
Filariasis	*Dirofilaria immitis*	Southern Europe	Carnivores	*Cx. pipiens, An. maculipennis* s.l., *Ae. albopictus*
	Dirofilaria repens	Southern and eastern Europe	Carnivores	*Cx. pipiens, An. maculipennis* s.l., *Ae. albopictus*

2.3.2 MOSQUITO-BORNE VIRUSES

During the last 60-year period, twelve mosquito-borne viruses, belonging to the families Togaviridae (Sindbis, Chikungunya), Flaviviridae (West Nile, Usutu, Dengue, Bagaza) and Bunyaviridae (Batai, Tahyna, Snowshoe hare, Inkoo, Lednice, Chatanga) have been isolated in Europe (LUNDSTRÖM 1999, HUBÁLEK 2008, AGÜERO et al. 2011, PUTKURI et al. 2014). Along with these viruses, which may cause diseases in humans and animals, numerous novel „insect-specific viruses" have been discovered in natural mosquito populations (e.g. ROIZ et al. 2012, COOK et al. 2013).

Among these mosquito-borne viruses, Batai, Sindbis, Tahyna and Usutu viruses were isolated either from the mosquitoes or vertebrate hosts in Germany so far (ACKERMANN et al. 1970, PILASKI & MACKENSTEIN 1985, KNUTH et al. 1990, JÖST et al. 2010, 2011a, 2011b, BECKER et al. 2012). In the following sections a short overview of mosquito-borne viruses recognized in Germany is given.

2.3.2.1 BATAI VIRUS

Batai virus (BATV, syn. Calovo or Chittoor virus), a member of the Bunyamwera serogroup in the family Bunyaviridae, is a single-stranded RNA virus that is widely distributed in Southeast Asia, Siberia, Uganda and Europe, but has never been documented in the New World. Since it was first recovered from *An. maculipennis* s.l. mosquitoes in South Slovakia in 1960 (HUBÁLEK 2008), the virus has been isolated either from the vector or the vertebrate host in Austria, the Czech Republic, Italy, Moldova, Norway, Serbia, Sweden and Ukraine. Moreover, serological surveys of humans and wild mammals indicate BATV activity in Bosnia, Finland, Hungary and Portugal (HUBÁLEK 2008, CALZOLARI et al. 2010).

The natural virus transmission cycle includes wild and domestic animals such as cattle, pig and deer, in which the infection causes a mild, febrile illness. In Europe, the virus is transmitted by zoophilic *An. maculipennis* s.l., *An. claviger* and, less often, *Ochlerotatus* spp. mosquitoes (LUNDSTRÖM 1999). It is likely that the virus hibernates in the field in overwintering Maculipennis Group species as demonstrated in laboratory experiments (BELETSKAYA & ALEKSEEV 1988). In the Czech Republic, BATV infections were shown to be associated with mild clinical signs in humans (LUNDSTRÖM 1999).

In southern Germany, GÄRTNER (1973) examined agricultural workers for neutralizing serum antibodies against BATV and found a very low prevalence (0.01 %). Although human cases have not been noticed yet in Germany (LUNDSTRÖM 1999), BATV was isolated from a pool of Maculipennis Group species in the federal state of Baden-Wurttemberg in 2009 (JÖST et al. 2011a). Among 195 serum samples from cattle around the infested area investigated for BATV-specific-IgG antibodies, two samples were tested positive, demonstrating past BATV infections (ENGLER et al. 2013). Overall, the BATV infection prevalence in mammals and in the mosquito population in Germany seem to be low and therefore appear to be of limited public health importance at present.

2.3.2.2 SINDBIS VIRUS

Sindbis virus (SINV) is a single-stranded RNA virus of the genus *Alphavirus* in the family Togaviridae that circulates in Africa, Asia, Australia and Europe.

SINV virus strains may slightly differ genetically depending on their geographical distribution and correlating with major bird migration patterns (LUNDSTRÖM & PFEFFER 2010). SINV has been identified genetically or serologically in domestic and wild mammals in numerous European countries, e.g. Austria, Czech Republic and Poland (ECDC 2014). The natural virus transmission cycle mainly includes wild birds, in which the infection causes sporadic illness (KURKELA et al. 2008). Ornithophilic mosquitoes of the species *Cx. torrentium*, *Cx. pipiens*, and *Cs. morsitans* mostly serve as vectors of SINV in Europe, but *Ochlerotatus* spp. and *Aedes* spp. are also included (HUBÁLEK 2008).

SINV has also been demonstrated in mites feeding on chickens in India (SHAH et al. 1960) and has been isolated from ticks in Italy (GRESIKOVÁ et al. 1978), but it is not proven whether arthropods other than mosquitoes are able to transmit the virus. Clinical disease in humans is primarily reported from northern Europe (locally known as “Pogosta” and “Ockelbo” disease) and is associated with rash, arthritis and respiratory symptoms (SANE et al. 2011).

In Germany, SINV was isolated from *Cx. torrentium, Cx. pipiens* and *An. maculipennis* s.l., captured in late summer of 2009 in the Upper Rhine Valley (JÖST et al. 2010). Phylogenetic analysis of the virus revealed a close relationship to a strain circulating in Sweden, suggesting that migratory birds served as transport hosts for the virus. Although the virus was found in several pools of mosquitoes, it has not been associated with human infection in Germany. Quite

recently, EIDEN et al. (2014) were able to detect a viable SINV in a Hooded Crow (*Corvus corone cornix*) found injured in the city center of Berlin in 2010. The isolated strain clusters within SINV genotype I, but were more closely related to a Finnish strain detected in a mosquito in 2005 than strains isolated from mosquitoes in southwestern Germany in 2009, which suggests the circulation of different SINV strains in Germany and their possible introduction by additional avian species.

2.3.2.3 TAHYNA VIRUS

Tahyna virus (TAHV), family Bunyaviridae, is a human pathogen of the California serogroup and is endemic to Europe, Asia and Africa. TAHV has frequently been demonstrated serologically or has been isolated in several European countries, e.g. Austria, Czech Republic, France, Italy, Great Britain and Poland (HUBÁLEK 2008). Considerable research on the ecology of TAHV has been done in Austria and the Czech and Slovak republics, where the virus is enzootic and circulates between natural hosts and haematophagous insects.

Mosquitoes of the genera *Aedes*, *Culex*, *Culiseta* and *Anopheles* are well documented vectors of TAHV. In addition, HALOUZKA et al. (1991) isolated the virus from biting midges of the genus *Culicoides,* but an involvement of these insects as vectors of TAHV is doubted. As California serogroup viruses are transmitted transovarially, mosquitoes play a major role in the overwintering of the virus (LEDUC 1979). Principal vertebrate hosts are lagomorphs, hedgehogs and rodents, which serve as reservoirs but do not develop signs of illness (ASPÖCK & KUNZ 1970, BÁRDOŠ 1975, RÖDL et al. 1979). TAHV infections in humans mostly occur from summer to early autumn and may predominantly lead to a disease also known as Valtice fever in children. This can present with influenza-like symptoms and, occasionally, have adverse effects on the central nervous system (GRATZ 2004, HUBÁLEK 2008).

SPIECKERMANN & ACKERMANN (1972) provided the first indication to TAHV circulating in Germany. The authors isolated 54 virus strains from 30,400 mosquitoes, collected in the area of the upper course of the river Main in northern Bavaria. At the same time, ACKERMANN et al. (1970) examined people living in rural areas for neutralizing serum antibodies against the virus and could show that 6 % of the population had had contact with the virus. Interestingly, the highest seroprevalences were found in people living along the banks of the rivers Rhine and Main. The authors thereupon isolated the virus from mosquitoes collected in southern Germany,

which mostly belonged to the floodwater species *Ae. vexans*. In the early 1980s, PILASKI & MACKENSTEIN (1985) also reported the isolation of TAHV from *Ae. vexans* collected in the Rheinaue wetlands in southern Germany.

Although clinical cases of TAHV infection in humans were demonstrated in other European countries (e.g. Czech Republic, France) and the virus had repeatedly been isolated in Germany, no associated clinical infections in humans could be demonstrated in Germany.

2.3.2.4 USUTU VIRUS

Usutu virus (USUV) is a single-stranded RNA virus in the family Flaviviridae of the genus *Flavivirus* that circulates in Africa and Europe. The virus was first isolated from *Cx. univittatus* in South Africa in 1959 (NIKOLAY et al. 2011). Due to the fact that there was no indication of pathogenicity in animals or humans in Africa, the scientific interest in the virus was moderate and information on the virus remained scanty (WEISSENBÖCK et al. 2007). In late summer 2001, a series of deaths in several bird species, particularly the common blackbird (*Turdus merula*), was registered in eastern Austria. At first, a West Nile virus outbreak was assumed, but later USUV was determined as the causative agent, which had never been observed outside Africa before and had also never been associated with fatal disease in animals or humans (WEISSENBÖCK et al. 2002). Interestingly, a retrospective study of archived tissue samples from dead birds collected 1996 in Italy demonstrated USUV. Further analyses confirmed identity with the Austrian strain and thus provided evidence that USUV had emerged in a pathogenic form much earlier in Europe (WEISSENBÖCK et al. 2013).

The disease in the USUV-affected birds in Austria was characterized by encephalitis, myocardial degeneration and necrosis in liver and spleen (CHVALA et al. 2004). In the subsequent year, USUV was repeatedly diagnosed in blackbirds, accompanied by a dramatic population decrease (MEISTER et al. 2008). The virus was also present in mosquito samples containing the predominantly ornithophilic species *Cx. pipiens*, *Cx. hortensis*, *Cx. territans*, *Cs. annulata*, *Ae. vexans* and *Ae. rossicus* (WEISSENBÖCK et al. 2007). Moreover, USUV was demonstrated in mosquito collections from 2004-2005 in overwintering *Culex* spp., which possibly enabled the overwintering and establishment of endemic virus cycles in consecutive years (WEISSENBÖCK et al. 2007). In the following years, a decline of USUV-associated bird mortality and an increasing

number of seropositive birds were observed, suggesting a developing herd immunity in the local resident bird population (CHVALA et al. 2007, MEISTER et al. 2008).

Since its first emergence in Austria, USUV has spread to other European countries, including Hungary (BAKONYI et al. 2007), Italy (MANAROLLA et al. 2010), Spain (BUSQUETS et al. 2008) and Switzerland (STEINMETZ et al. 2011) and could also be demonstrated serologically in wild birds in England (2001–2002), the Czech Republic (2005) and Poland (2006) (VÁZQUEZ et al. 2011b). USUV infections in humans were reported from Italy in two immunocompromised patients causing severe neurological impairment (CAVRINI et al. 2009, PECORARI et al. 2009). Sequence analysis revealed a close relationship between a strain isolated from one of the patients from Italy and the European avian USUV strains but at the same time identified significant amino acid substitutions, which might play a role in the pathogenicity of the virus for humans as observed by other flaviviruses under experimental conditions (GAIBANI et al. 2013). In addition, CAVRINI et al. (2011) detected USUV in the cerebrospinal fluids of three patients, who suffered from acute meningoencephalitis.

LINKE et al. (2007) provided the first indication of a potential emergence of USUV in wild birds in Germany. The authors examined blood samples from over 3,000 migratory and resident birds captured throughout Germany and found that at least in three samples of migrating bird species (*Ciconia ciconia*, *Phoenicurus phoenicurus* and *Pandion haliaetus*) neutralizing antibodies against USUV were present. Furthermore, JÖST et al. (2011b) isolated USUV in 2010 from a pool of *Cx. pipiens* mosquitoes trapped in Southwest Germany that was closely related to a USUV strain isolated from a dead blackbird from Austria. The apprehended scenario finally started in June 2011: A mass mortality in birds caused by USUV occurred in the Upper Rhine Valley in Southwest Germany, which equally affected migratory and resident birds, as well as captive canary (*Serinus canaria domestica*) and the great grey owl (*Strix nebulosa*) from zoological gardens (BECKER et al. 2012, BOSCH et al. 2012). The outbreak persisted to the following year 2012 and affected thousands of birds (ProMED-mail 2012).

Although neutralizing antibodies against USUV were detected in a healthy blood donor from Southwest Germany (ALLERING et al. 2012) and the virus had repeatedly been isolated from birds, no human clinical infections associated with USUV have been demonstrated in Germany so far.

2.3.3 FILARIOID NEMATODES TRANSMITTED BY MOSQUITOES

Filariae are parasitic nematodes (roundworms) belonging to the superfamily Filarioidea, which are characterised by their filamentous habitus. They are transmitted by arthropod vectors, such as ticks (Acari), black flies (Simuliidae), biting midges (Ceratopogonidae), horse flies (Tabanidae) and mosquitoes (Culicidae). The adult worms infect specific tissues or the circulatory system of the vertebrate host where they mate and the females start producing microfilariae. Microfilariae are ingested during a blood meal by competent intermediate arthropod vectors in which they mature to metacyclic infective larvae. These are transmitted to the definite or accidental host during the bite of the arthropod vector.

Infection with filarioids induces subcutaneous nodules, cause severe condition through involvement of the lymphatic or cardio-pulmonary system or even remain asymptomatic.

There are various species of filariae which cause common infections in birds, reptiles, amphibians and mammals. Filariae which are natural parasites of mammals have been recognized as causing zoonotic infections in humans (ORIHEL & EBERHARD 1998).

Filarial diseases associated with transmission of the worms by mosquitoes are a major health problem, especially in tropical and subtropical areas. However, mosquito-borne nematodes also exist in Europe, affecting both humans and animals.

2.3.3.1 INFECTIONS WITH *DIROFILARIA* SPECIES

Dirofilariosis caused by *Dirofilaria immitis* and *Dirofilaria repens* (Spirurida, Onchocercidae) is regarded as an emergent disease of animals and humans in several European regions (OTRANTO et al. 2009, 2013a). Natural vertebrate hosts of both species are domestic and wild canines, felines and other carnivores (SIMÓN et al. 2012). While *D. immitis* induces cardiovascular symptoms known as heartworm disease, *D. repens* causes subcutaneous nodules and allergic dermatitis (MCCALL et al. 2008, ROCCONI et al. 2012).

Dirofilaria species are of worldwide zoonotic concern. Whilst cases of human dirofilariasis in North and South America are caused by *D. immitis*, *D. tenuis* and *D. ursi*, European human cases are mainly attributed to *D. repens* (PAMPIGLIONE et al. 2009, SIMÓN et al. 2012). Infections in humans are usually characterized by subcutaneous, subconjunctival, cardiovascular and/or ocular

lesions (MCCALL et al. 2008, SIMÓN et al. 2012). Also, rare cases of unusual organ infection and meningoencephalitis have been described (POPPERT et al. 2009, HARIZANOV et al. 2014).

Dirofilariae are transmitted by culicid mosquitoes of various species, such as *Cx. pipiens*, *An. maculipennis* s.l. and *Ae. albopictus*, which are probably the most important vectors in the Mediterranean, where *Dirofilaria* species naturally occur (CANCRINI et al. 2003, 2007). In the recent past, several autochthonous cases of canine and human dirofilariosis were reported in more northern countries, including Austria (AUER & SUSANI 2008, DUSCHER et al. 2009), the Czech Republic (SVOBODOVÁ et al. 2006), Hungary (JACSO et al. 2009), the Netherlands (OVERGAAUW & VAN DIJK 2009), Poland (CIELECKA et al. 2012, ŚWIĄTALSKA & DEMIASZKIEWICZ 2012), Romania (MIRCEAN et al. 2012), Slovakia (IGLÓDYOVÁ et al. 2012, BOCKOVÁ et al. 2013, VÍCHOVÁ et al. 2014) and Ukraine (SAŁAMATIN et al. 2013).

Until recently, cases of dirofilariosis caused by *D. repens* were only recorded in dogs in Germany (HERMOSILLA et al. 2006, PANTCHEV et al. 2009, SASSNAU et al. 2009, 2013). Following these detections in the German federal states of Baden-Wurttemberg and Brandenburg, *D. repens* was detected in the same geographic regions in mosquito pools, consisting of *Cs. annulata*, *An. maculipennis* s.l. and *Ae. vexans* (CZAJKA et al. 2014). Taking into account the extrinsic incubation period of *Dirofilaria* spp. and the maximum life expectancy of about 30 days of a mosquito vector, GENCHI et al. (2011) confirmed the possibility of a continuous *Dirofilaria* transmission cycle during summertime within these states and assigned them a risk of stable endemicity (SASSNAU et al. 2013). Moreover, recent interviews of veterinarians on their experience with *D. immitis* revealed that over 20 % (23/101) of the German practitioners diagnosed more than one case of canine heartworm over the last 12 months, presumably acquired in endemic areas (GENCHI et al. 2014). This finding mirrors the permanent threat of infected dogs with a travel history to act as donors of microfilariae to local mosquito species facilitating the spread of the worm to native canines.

Shortly before this thesis was prepared, in March 2014, a first autochthonous infection with *D. repens* was diagnosed in a German citizen in the federal state of Saxony-Anhalt (TAPPE et al. 2014). The patient, who had developed an itching subcutaneous nodule containing a female worm, resides in an area affected by the floods of the nearby Elbe River in summer 2013. As the patient also recalled multiple mosquito bites during the fishing season in September 2013 and the incubation period takes four to eight months, an infection in late summer 2013 is most likely. An infection associated with travelling into endemic areas could be excluded.

2.3.3.2 INFECTIONS WITH OTHER FILARIOID NEMATODES

In addition to well-known filarioid nematodes such as *Dirofilaria* spp. which have been identified in domestic animals, there are other filarioid species in European livestock and wildlife, whose biology and ecology is not yet sufficiently known. These include potential zoonotic species of the genera *Acanthocheilonema*, *Cercopithifilaria*, *Onchocerca, Parafilaria* and *Setaria*, which are transmitted by fleas, ticks, biting midges, dipteran flies and mosquitoes, respectively (CZAJKA et al. 2012, OTRANTO et al. 2013a, 2013b, MASNY et al. 2013).

Nematodes of the genus *Setaria* are common parasites of the peritoneal cavity of wild and domestic ruminants, mainly transmitted by *Ochlerotatus* mosquito species (LAAKSONEN et al. 2009). Infections with *Setaria* nematodes are usually asymptomatic and not considered pathogenic, but occasionally may cause severe cases of peritonitis and perihepatitis (SERVICE 2001, LAAKSONEN et al. 2007).

In Germany, *Setaria tundra* has exclusively been found in roe deer (*Capreolus capreolus*) (BÜTTNER 1978, REHBEIN et al. 2000). Elsewhere, it has been identified in other cervids such as the Finnish forest reindeer (*Rangifer tarandus fennicus*) in Scandinavia, the moose (*Alces alces*) in Lapland and the fallow deer (*Dama dama*) in Austria (LAAKSONEN et al. 2009, REHBEIN et al. 2014). The zoonotic importance of *Setaria* spp. is uncertain, although rare cases of human infections have been reported (PANAITESCU et al. 1999, ŢĂLU et al. 2012).

Due to recent isolations from mosquitoes, there is evidence for further filarial nematode species circulating in Germany (CZAJKA et al. 2012). The identity and biology of these have not sufficiently been clarified yet.

3. AIM AND STRUCTURE OF THE THESIS

During the last decade, viral agents of mosquito-borne diseases such as Sindbis virus and Batai virus have been repeatedly isolated from the indigenous mosquito fauna (JÖST et al. 2010, 2011a), while autochthonous transmission of Usutu virus, *Dirofilaria repens* and *Plasmodium falciparum* were occasionally demonstrated (KRÜGER et al. 2001, HERMOSILLA et al. 2006, PANTCHEV et al. 2009, SASSNAU et al. 2009, 2013, ZOLLER et al. 2009, JÖST et al. 2011b, CZAJKA et al. 2014).

Since the introduction or re-emergence of these mosquito-borne pathogens, sporadic studies investigating their prevalence in both vertebrate hosts and the mosquito fauna have been carried out but were limited to a few regions in southern Germany. Nation-wide longitudinal studies, however, have been lacking in Germany.

Since the middle of the last century the research on the culicid mosquito fauna in Germany has increasingly become neglected. As biological systems are subject to permanent changes, and globalisation and climate change are supposed to have significantly influenced the indigenous arthropod fauna during the last decades, our knowledge about the mosquito fauna in Germany must be considered outdated.

The objectives of the present work were to contribute to investigating the diversity of the mosquito fauna in Germany and to find out whether mosquito-borne pathogens circulate. Therefore, the following studies were conducted:

(i) Entomological monitoring of the occurrence, seasonal and geographic distribution of culicid mosquito species in Germany with emphasis on the analysis of the composition and diversity of the Maculipennis Subgroup (chapter 4).

(ii) Design and establishment of a reliable molecular tool to differentiate the presently known members of the European Maculipennis Subgroup (chapter 5).

(iii) Large-scale molecular cross-sectional examination of mosquitoes to analyse the occurrence and geographical distribution of filarial nematodes of the superfamily Filarioidea (chapter 6).

(iv) Large-scale virological cross-sectional examination of mosquitoes to analyse the occurrence and geographical distribution of viruses of the family Bunyaviridae, Flaviviridae and Togaviridae (chapter 7).

4. MOLECULAR CONFIRMATION OF THE OCCURRENCE IN GERMANY OF *ANOPHELES DACIAE* (DIPTERA, CULICIDAE)

Abstract

Anopheles daciae, a newly described member of the Maculipennis Group of the culicid genus *Anopheles* was recently reported from western, southern and eastern Europe. Before its recognition, it had commonly been listed under the name of *An. messeae*, due to utmost morphological and genetic similarities. As the sibling species of the Maculipennis Group are known to differ in their vector competences for malaria parasites and other pathogens, the occurrence of *An. daciae* in a given region might have an impact on the epidemiology of mosquito-borne diseases. Mosquito collections from Germany were therefore screened for *An. daciae*. Adult and immature Maculipennis Group mosquitoes were collected between May 2011 and June 2012 at 23 different sites in eight federal states of Germany. A standard PCR assay was used to differentiate the previously known sibling species while the ITS2 rDNA of specimens preliminarily identified as *An. messeae*/*daciae* was sequenced and analysed for species-specific nucleotide differences. Four hundred and seventy-seven *Anopheles* specimens were successively identified to Maculipennis Group level by morphology and to species level by DNA-based methods. Four species of the Maculipennis Group were registered: *An. messeae* (n=384), *An. maculipennis* (n=82), *An. daciae* (n=10) and *An. atroparvus* (n=1). *An. daciae* occurred at four sites in three federal states of Germany, three of the sites being located in north-eastern Germany (Brandenburg, Saxony) while one collection site was situated in the northern Upper Rhine Valley in the federal state of Hesse, south-western Germany. The detection of *An. daciae* represents the first of this species in Germany where it was found to occur in sympatry with *An. messeae* and *An. maculipennis*. Given collection sites in both north-eastern and south-western parts of Germany, the species is probably even more distributed in Germany than demonstrated, albeit apparently with low population densities. Research is needed that confirms the species status of *An. daciae* and elucidates its vector competences as compared to *An. messeae* and the other species of the Maculipennis Group in order to optimize management of possible future outbreaks of diseases caused by pathogen transmission through Maculipennis Group mosquitoes.

4.1 INTRODUCTION

The recognition of sibling species within the Maculipennis Group of the culicid genus *Anopheles* in the early 20th century and of their different roles as vectors of malaria parasites was a historical milestone in malaria research (HACKETT & MISSIROLI 1935, JETTEN & TAKKEN 1994). It triggered in-depth research on the biology and ecology of the various geographical "*Anopheles maculipennis* races" and renewed taxonomic revisions of the genus *Anopheles*. Based on nucleotide sequence analysis of the nuclear ribosomal DNA (rDNA) second internal transcribed spacer (ITS2), HARBACH (2004) confirmed the monophyly of the Maculipennis Group species in 2004 and divided them into three hierarchical systems of informal taxonomic subgroups (Maculipennis Subgroup, Quadrimaculatus Subgroup, Freeborni Subgroup). According to this system, and under consideration of *An. artemievi*, a mosquito species described in 2005 (GORDEEV et al. 2005), the Palaearctic members of the Maculipennis Group, including *An. atroparvus, An. labranchiae, An. maculipennis, An. melanoon, An. messeae, An. sacharovi, An. artemievi, An. martinius* and *An. persiensis*, form the Maculipennis Subgroup. The six first-mentioned species plus *An. beklemishevi* (Quadrimaculatus Subgroup of the Maculipennis Group) are distributed throughout Europe. While egg morphology, larval and pupal chaetotaxy, ecological studies, hybridization experiments, zymotaxonomy and cytotaxonomy were mostly applied to identify sibling species in earlier culicid research, recent discoveries of cryptic species are often the results of DNA analyses (LINTON et al. 2003, GORDEEV et al. 2005). Thus, NICOLESCU et al. (2004) described *An. daciae* as an additional previously unrecognized member of the Maculipennis Group on the Black Sea coast in southern Romania by means of differences in the rDNA ITS2 sequence as compared to *An. messeae*, supported by mitochondrial DNA (mtDNA) cytochrome c oxidase subunit I (COI) sequence data and morphological peculiarities of the egg ornamentation. The larvae, pupae and adult stages of both species are indistinguishable, and both species have been found to be sympatric (NICOLESCU et al. 2004, LINTON et al. 2005). Prior to the description of *An. daciae*, a polymerase chain reaction (PCR) assay developed by PROFT et al. 1999 provided a reliable tool for the identification of the then known European Maculipennis Group sibling species. Using that PCR assay, however, *An. daciae* is erroneously identified as *An. messeae* and remains unrecognized.

In the same year that NICOLESCU et al. (2004) described *An. daciae*, DI LUCA et al. (2004) published a comprehensive study on intraspecific polymorphisms in the ITS2 region of

populations of *An. messeae* from Italy, The Netherlands, former Yugoslavia, Kazakhstan and England. The authors came up with five haplotypes each of which corresponded to a distinct geographical area. An additional investigation of an "*An. messeae*" population in southwest England (LINTON et al. 2005) revealed that its ITS2 sequences were identical both to the England haplotype described by DI LUCA et al. (2004) and to the *An. daciae* type series from Romania (NICOLESCU et al. 2004). A comparative analysis of partial mitochondrial COI gene sequences of mosquitoes collected by DI LUCA et al. (2004) in Kazakhstan and Italy with those of specimens of the *An. daciae* type series from Romania collected by NICOLESCU et al. (2004) suggests the occurrence of *An. daciae* in England and Romania as well (LINTON et al. 2005).

While there are now eight species of the Maculipennis Group known to occur in Europe, three of them have been described for Germany: *An. maculipennis, An. atroparvus* and *An. messeae* (WEYER 1948). However, the recent findings of new members of the Maculipennis Group in Europe, in particular of *An. daciae* in eastern, southern and western Europe (Romania, Italy, England), suggested that *An. daciae* might also be present in other European countries such as Germany. Specimens of the Maculipennis Group from a German national mosquito monitoring programme identified as *An. messeae* by the PCR assay according to PROFT et al. (1999) were therefore analysed with regard to their ITS2 DNA sequences.

4.2 METHODS

Mosquito specimens of the Maculipennis Group were collected between May 2011 and June 2012 at 23 sites in eight federal states of Germany within the framework of mosquito monitoring activities (Figure 2, Table 3). Adult *Anopheles* specimens were caught by trapping and netting, as well as by hand collections from resting places in overwintering shelters and in animal stables during summer. Larvae and pupae were removed from their breeding sites and reared to adults for easier morphological identification which was done using the keys by SCHAFFNER et al. (2001) and BECKER et al (2010). Mosquitoes belonging to the Maculipennis Group were further identified by a species-specific PCR assay (PROFT et al. 1999) performed on DNA extracted from whole single specimens using the DNeasy Blood & Tissue Kit (Qiagen, Germany) and the NucleoSpin RNA Virus Kit (Macherey-Nagel, Germany) according to the instruction manuals. PCR products were fractionated on 1.5 % agarose gels containing 0.5 µg/ml ethidium bromide and visualized under UV light. The ITS2 rDNA of specimens preliminarily identified as *An.*

messeae was subsequently amplified using 5.8S and 28S primers published by COLLINS & PASKEWITZ (1996) to generate DNA fragments of 435 bp each. For DNA sequencing, PCR products were cycled using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Germany). They were cleaned by means of SigmaSpin Sequencing Reaction Clean-Up columns (Sigma-Aldrich, Germany) and sequenced on a 3130 Genetic Analyzer (Applied Biosystems). Sequences were edited and aligned with published ITS2 sequences of *An. messeae* and *An. daciae* available in GenBank using CodonCode Aligner (CodonCode Corporation).

4.3 RESULTS AND DISCUSSION

Four hundred and seventy-seven collected *Anopheles* specimens were assigned to the Maculipennis Group according to morphological characters. Of these, ITS2 rDNA species-specific PCR according to PROFT et al. (1999) generated 394 *An. messeae*, 82 *An. maculipennis* and 1 *An. atroparvus*. While *An. messeae* and *An. maculipennis* have previously been shown to have a widespread distribution in Germany, the salt-tolerant species *An. atroparvus* mainly occurs in coastal marsh regions but has also been found in inland areas, although at much lower frequencies (MOHRIG 1969). In total, "*An. messeae*" accounted for 80 % of our *Anopheles* PCR identifications.

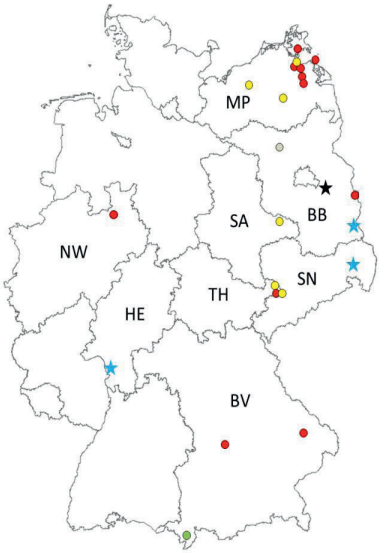

Figure 2 Geographic locations of mosquito sampling sites and distribution of Maculipennis Group species.
Asterisks and dots: sampling sites positive for mosquitoes of the Maculipennis Group. Asterisk = *An. daciae* present. Colours: black = *An. daciae* and *An. maculipennis*; blue = *An. daciae*, *An. maculipennis* and *An. messeae*; red = *An. messeae*; green = *An. maculipennis*; yellow = *An. messeae* and *An. maculipennis*; grey = *An. maculipennis, An. messeae* and *An. atroparvus* present.
BB Brandenburg, BV Bavaria, HE Hesse, MP Mecklenburg-Western Pomerania, NW North Rhine-Westfalia, SA Saxony-Anhalt, SN Saxony, TH Thuringia.

DNA sequence analysis of the ITS2 region of the "*An. messeae*" mosquitoes revealed five single nucleotide polymorphisms in ten specimens, nine females and a male (GenBank accession nos.: JX173885, JX416347-JX416352, JX444557-JX444559), identical to those defining *An. daciae* according to NICOLESCU et al. (2004). Three of the females were hand-collected in August 2011 and June 2012 in a domesticated rabbit stall in Maust, Brandenburg, northeastern Germany, close to the border with Poland. Four *An. daciae* females were sampled in June 2012 in a stable harbouring sheep in Ralbitz-Rosenthal, Saxony, and one male was caught in August 2011 in a rabbit stall in Schoeneiche, Brandenburg. The two remaining females were trapped by a BG-Sentinel mosquito trap (Biogents, Germany) in August and September 2011 in Trebur, Hesse. In all locations, either *An. messeae* or *An. maculipennis* or both were also shown to occur.

Table 3 Origin and species assignment of the Maculipennis Group mosquitoes involved.

Federal state	Locality	No. identified	*An. maculipennis*	*An. atroparvus*	*An. messeae*	*An. daciae*
Mecklenburg-Western Pomerania (MP)	Boltenhagen	1	–	–	1	–
	Dummerstorf	2	1	–	1	–
	Greifswald	2	–	–	2	–
	Gristow	3	1	–	2	–
	Kargow	4	1	–	3	–
	Peendemuende	15	–	–	15	–
	Putbus	4	–	–	4	–
	Spantekow	2	–	–	2	–
	Tutow	13	–	–	13	–
Brandenburg (BB)	Eisenhuettenstadt	2	–	–	2	–
	Maust	72	2	–	67	3
	Schoeneiche	16	15	–	–	1
	Zippelsfoerde	56	8	1	47	–
Saxony-Anhalt (SA)	Kropstaedt	61	36	–	25	–
North Rhine-Westphalia (NW)	Bielefeld	2	–	–	2	–
Saxony (SN)	Haselbach	99	3	–	96	–
	Ralbitz-Rosenthal	96	6	–	86	4
Thuringia (TH)	Windischleuba	4	–	–	4	–
	Zschaschelwitz	7	2	–	5	–
Hesse (HE)	Trebur	5	1	–	2	2
Bavaria (BV)	Deggendorf	1	–	–	1	–
	Agathazell	6	6	–	–	–
	Neuburg	4	–	–	4	–
Total		477	82	1	384	10

This is the first description of *An. daciae* for Germany. Considering known differences in vector competence and/or vectorial capacity for malaria parasites of different Maculipennis Group species in the same geographic region and of the same species in different geographical areas, the status of *An. daciae* as a vector in Germany and elsewhere should be investigated. Such studies, however, should not remain restricted to malaria parasites but should include further pathogens since Maculipennis Group sibling species have been shown to be infected in the field with Tahyna virus in Austria (ASPÖCK 1970), West Nile virus in Portugal (FILIPE 1972), Sindbis and Batai viruses in Germany (JÖST et al. 2010, 2011a), and *Dirofilaria immitis* and *Setaria labiatopapillosa* filaria in Italy (CANCRINI 1997, 2006).

Despite having followed the recent literature and having denominated *An. daciae* a species, the authors do not consider the evidence given for the species status of *An. daciae*, separate from *An. messeae*, as convincing and sufficient. There are three criteria on which the suggested species status of *An. daciae* is based, most importantly ITS2 rDNA sequence polymorphisms, with *An. daciae* being described as an ITS2 variant of *An. messeae* different at five positions out of 435 nucleotides. However, while investigating the intragenomic heterogeneity of the ITS2 region of geographically distinct *An. messeae* populations, BEZZHONOVA & GORYACHEVA (2008) found that the *An. daciae* variant was just one out of various variants in peripheral populations of *An. messeae*, the other variants not being elevated to species status. Admittedly, the *An. daciae* variant was the only one found at more than one, geographically distinct location, which indicates that the genetic divergence is stable. In our ITS2 sequence analyses, the *An. daciae* ITS2 variant was the only one encountered in addition to the *An. messeae* variant.

A second criterion given by NICOLESCU et al. (2004) is the egg structure, which is considered different from that of *An. messeae*. The differences given, however, are minor and have not been shown to be statistically significant, i.e. to be outside the range of natural phenotypic variation within a species. In fact, such variation can be commonly observed in insect specimens of the same species including the Maculipennis Group members (HACKETT et al. 1932).

Most ambiguous is the delimitation of *An. daciae* and *An. messeae* by means of unique polymorphisms in the COI gene, which, although used for species identification by barcoding, displays a certain degree of sequence variability (HEBERT et al. 2003). While some COI sequence haplotypes are said to represent *An. daciae* (NICOLESCU et al. 2004), no data on intraspecific sequence divergence, either for *An. messeae* or for *An. daciae*, in contrast to interspecific divergence, have yet been published.

Phylogenetic tree construction from GenBank COI sequences to check for clustering is not possible since it is not known without the corresponding ITS2 sequences whether sequence entries running under the name of *An. messeae* must actually be assigned to the *An. messeae* or to the *An. daciae* variant. Studies on correlated COI and ITS2 sequence analyses have therefore been initiated. Preliminary analyses of COI sequences of *An. messeae* specimens identified in our lab by ITS2 sequences, as compared to *An. daciae* COI sequences presented by NICOLESCU et al. (2004), have shown an identical haplotype. In support of such studies, the ecological and/or physiological features of *An. daciae* should be studied.

4.4 CONCLUSION

To resolve the species status of *An. daciae*, it is necessary to correlate its genetic variant to well-defined biological characteristics and to carry out crossing experiments. Irrespective of that, vector competences and characteristics different from those of *An. messeae* are conceivable in the *An. daciae* variant that could, for instance, lead to, and explain, differences in the epidemiology of mosquito-borne diseases whose agents are transmitted by species of the Maculipennis Group. Therefore, the exact geographical distribution and the vector status of *An. daciae* should be examined more carefully.

5. PCR IDENTIFICATION AND DISTRIBUTION OF *ANOPHELES DACIAE* (DIPTERA, CULICIDAE) IN GERMANY

Abstract

Based primarily on nucleotide polymorphisms in the internal transcribed spacer 2 (ITS2) of the ribosomal DNA, *Anopheles daciae* was recently described as an additional member of the Maculipennis Group of species, separate from *Anopheles messeae* with which it had previously been confused due to morphological and genetic similarity. Species differentiation between *An. messeae* and *An. daciae* was possible only by ITS2 polymerase chain reaction (PCR) amplification followed by DNA sequencing or RFLP analysis. In addition to its siblings, *Anopheles maculipennis*, *Anopheles atroparvus* and *An. messeae*, *An. daciae* has been shown to occur in Germany, although with limited distribution. We here describe additional collection sites for this species in Germany, showing concentrations in East Germany and the northern Upper Rhine Valley in Southwest Germany. A species-specific multiplex PCR assay is presented that is able to differentiate the four Maculipennis Group sibling species occurring in Germany plus *Anopheles sacharovi*, *Anopheles melanoon* and *Anopheles labranchiae*. The correct identification and detailed knowledge of the biology of *An. daciae* are of relevance since it might be a vector of disease agents, as suggested by the vector potential of its siblings and the recent finding of an *An. daciae* female infected with *Dirofilaria repens* in southern Germany.

5.1 INTRODUCTION

Major inter-population differences in biological traits, such as choice of breeding habitat, blood host preference and vector competence, resulted in the recognition of *Anopheles maculipennis* as a heterogeneous taxon in the 1930s (e.g. HACKETT 1934). Due to the absence of visible morphological differences, the dissimilar populations were first called subspecies, races, biotypes, varieties or forms but were later ranked as separate biological species based on evidence from hybridization and cytogenetic studies (KITZMILLER et al. 1967).

More recent discoveries of cryptic species are often the result of DNA analyses. Thus, NICOLESCU et al. (2004) described *Anopheles daciae* as an additional, previously unrecognized member of the Maculipennis Group on the Black Sea coast in southern Romania, based on differences in the internal transcribed spacer 2 (ITS2) ribosomal DNA (rDNA) sequence as compared with *Anopheles messeae*. Further reports on the existence of *An. daciae* were contributed by LINTON et al. (2005) from Southwest England. The same authors postulated the occurrence of *An. daciae* in Italy and Kazakhstan after analysing mitochondrial DNA cytochrome c oxidase subunit I (COI) gene sequences of mosquitoes studied by DI LUCA et al. (2004).

Prior to the description of *An. daciae*, a polymerase chain reaction (PCR) assay based on ITS2 sequence polymorphisms provided a reliable tool for the identification of the then known European Maculipennis Group sibling species (PROFT et al. 1999, KAMPEN 2005): *Anopheles atroparvus*, *Anopheles beklemishevi*, *Anopheles labranchiae*, *An. maculipennis*, *Anopheles melanoon*, *An. messeae* and *Anopheles sacharovi*. However, using that PCR approach, *An. daciae* is erroneously identified as *An. messeae* due to the high genetic similarity of the two species. In fact, the DNA sequence presented by PROFT et al. (1999) for *An. messeae* is an *An. daciae* sequence.

Taking into account the potential occurrence of *An. daciae* in other European countries and a missed record of occurrence in Germany, studies on the Maculipennis Group sibling species by rDNA ITS2 sequence analysis were initiated that eventually led to the discovery of *An. daciae* in Germany (KRONEFELD et al. 2012, WEITZEL et al. 2012). This article updates the geographical distribution of *An. daciae* and sympatric members of the Maculipennis Group in Germany and presents a PCR assay developed to differentiate seven members of the European Maculipennis Group of species, including *An. daciae*.

5.2 METHODS

Sample origin

Anopheles mosquitoes were collected between April 2011 and November 2013 in 13 federal states of Germany within the framework of a national mosquito monitoring programme. Adult specimens were caught by trapping and netting and by hand-held aspirators from resting places in overwintering shelters and in animal stables during summer. Larvae and pupae were removed from their breeding sites by dipping and were reared to adults for easier morphological identification, which was accomplished using the determination keys by SCHAFFNER et al. (2001) and BECKER et al. (2010). Specimens were stored at −20 °C until DNA extraction.

DNA extraction

Mosquito DNA was extracted by means of the QIAamp DNA Mini Kit (Qiagen, Germany). For tissue homogenization, individual legs were homogenized in 180 µl extraction buffer ATL and 20 µl proteinaseK (20 mg/µl) by shaking for 3 min at 30 Hz with a TissueLyser II (Qiagen) in the presence of stainless steel beads (diameter 3 mm). DNA was then isolated from the supernatant according to the manufacturer's protocol. DNA elution was done in 100 µl AE buffer. The eluates were stored at −20 °C.

PCR amplification

A species-specific ITS2 PCR, modified after PROFT et al. (1999), was performed to differentiate the previously known German species of the Maculipennis Group: *An. maculipennis, An. atroparvus* and *An. messeae*, the latter probably including cryptic *An. daciae*. PCR mixtures contained 5 µl template DNA, 0.6 µM of primers 5.8S-UN and 0.3 µM of primers AMA, AAT and AMS each and 10 µl of 2× QuantiTect Multiplex PCR-Kit (Qiagen). Amplification was carried out in 25 µl total volume in a C1000 Thermal Cycler (BioRad, Germany). The thermoprofile consisted of an initial denaturation step at 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 60 s, and a final 10 min extension step at 72 °C. For amplification of the entire mosquito ITS2 rDNA region (ca. 435 bp), PCR was carried out accordingly, except that 0.4 µM 5.8S-UN and 28S were used as the only forward and reverse primers, respectively (COLLINS & PASKEWITZ 1996). PCR products were visualized on 1.5 % agarose gels run for 1 h at 100 V and stained with ethidium bromide (0.5 mg/ml).

DNA sequencing

For DNA sequencing, amplicons were excised from agarose gels after electrophoresis and recovered by the QIAquick Gel Extraction Kit (Qiagen), according to the user instructions. After cycling using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Germany), PCR products were cleaned by means of SigmaSpin Sequencing Reaction Clean-Up Columns (Sigma-Aldrich, Germany) and sequenced on a 3130 Genetic Analyzer (Applied Biosystems, Germany). Every amplicon was sequenced bidirectionally with the PCR primers. All sequences were edited with Codon Code Aligner (Codon Code Corp., Dedham, MA, USA) and compared to ITS2 sequences of *An. messeae* and *An. daciae* available in the GenBank database. Specifically, nucleotide polymorphisms differentiating *An. daciae* from *An. messeae* were searched (NICOLESCU et al. 2004).

Primer design

Based on consistent sequence differences between *An. messeae* and *An. daciae*, a primer specific for *An. daciae* (ADA) was designed in a way that a species-specific PCR product would be generated in combination with the universal 5.8S-UN primer (Figure 3). The length and the GC content of the primer were adapted to the primers specific for the other Maculipennis Group species according to PROFT et al. (1999) and KAMPEN (2005), and exclusion of intramolecular formation of secondary structures was ensured to enable multiplexing. To achieve the best signal balance in multiplexing, concentrations of PCR reaction compounds and the amplification thermoprofile were adjusted empirically. For this purpose, PCRs were run with various concentrations of *An. messeae* and *An. daciae*-specific primers (AMS and ADA, respectively) ranging from 0.1 to 1 µM, with concentrations of $MgCl_2$ ranging from 0.5 to 2 mM and with an annealing temperature gradient ranging from 50 to 60 °C. The amplification reaction was performed in a 50-µl volume, containing 2 µl template DNA, 0.6 µM of primer 5.8S-UN, 0.3 µM of primers AMA, AAT, ASA, AML and ALA each, 10 µl of 5× Green GoTaq Flexi Buffer, 0.1 mM dNTPs and 1.25 U GoTaq Flexi DNA Polymerase (Promega, Germany), in addition to primers AMS and ADA. The thermoprofile included an initial denaturation step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 50-60 °C for 30 s and 72 °C for 60 s, and a final extension step at 72 °C for 10 min. Primer specificity and cross-reactivity were tested in multiplex PCRs on genetically defined Maculipennis Group control specimens.

The DNA of specimens indicated as *An. daciae* with this assay was PCR-amplified, using an opposite order of verification, for the complete ITS2 region and sequenced for confirmation.

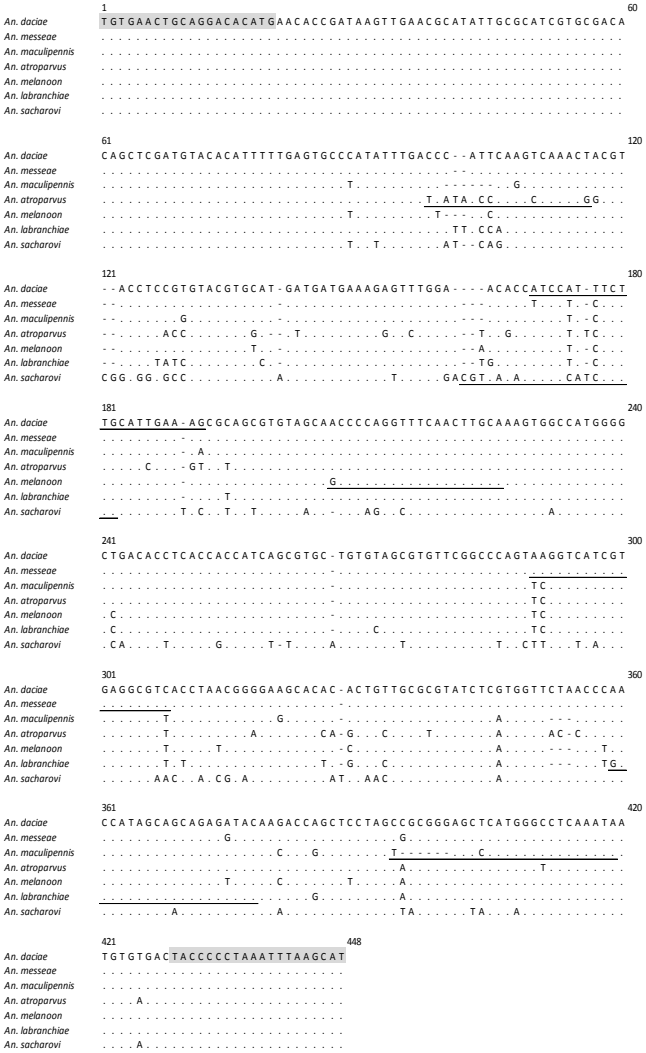

Figure 3 ITS2 sequence alignment of seven European Maculipennis Group members. Dots (.) indicate identity of bases while dashes (−) indicate alignment gaps. ITS2 sequencing primers, including the universal 5.8S-UN primer and 28S primer, are highlighted in grey; species-specific primers for multiplex PCR identification are underlined. Sequences for *An. daciae* and *An. messeae* were produced in this study; sequences for *An. maculipennis*, *An. melanoon*, *An. sacharovi* and *An. labranchiae* are consensus sequences produced from GenBank entries.

5.3 RESULTS

Multiplex PCR for simultaneous detection of European Maculipennis Group species

Sequence analysis of the ITS2 DNA region of *An. messeae* revealed five single nucleotide polymorphisms in 79 specimens (78 females and 1 male) identical to those defining *An. daciae* according to NICOLESCU et al. (2004). Based on three out of these five nucleotide substitutions, a species-specific primer for *An. daciae* named ADA (5'-CTTTCAATGCAAGAAATGGAT-3') was designed (Figure 3). Together with the universal 5.8S-UN primer and the primers specific for *An. maculipennis*, *An. atroparvus*, *An. messeae*, *An. sacharovi*, *An. melanoon* and *An. labranchiae*, a multiplex PCR generated a species-specific amplicon pattern with *An. daciae*, well separated from *An. messeae* and the other Maculipennis Group members (Figure 4).

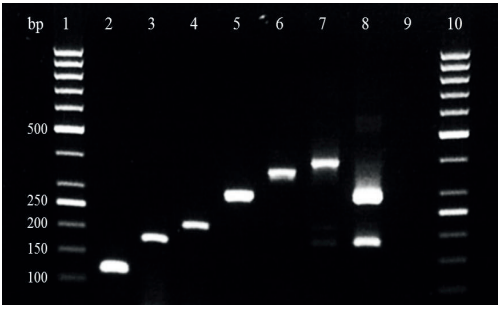

Figure 4 Specific PCR products for seven common European Maculipennis Group species (lanes 1 and 10: 50-bp marker, 2: *An. atroparvus*, 3: *An. sacharovi*, 4: *An. melanoon*, 5: *An. messeae*, 6: *An. labranchiae*, 7: *An. maculipennis*, 8: *An. daciae*, 9: negative control).

The pattern consists of two bands, 184 and 305 bp, the latter alone being specific for *An. messeae*. A weak secondary 184 bp fragment may also occur with *An. maculipennis* in addition to the formerly described species-specific 410-bp fragment. Thus, the sibling species are not generally identified by unique bands in this modified PCR version but in two cases by species-specific patterns of two bands. *Anopheles beklemishevi*, which was included in the original Maculipennis Group PCR according to PROFT et al. (1999) and KAMPEN (2005), was not available for assay evaluation. The optimized PCR mixture contained 0.1 mM dNTPs, 1.5 mM $MgCl_2$, 1.25 U/µl GoTaq Flexi DNA Polymerase, 0.6 µM of primers 5.8S-UN and ADA, 0.3 µM of primers AAT, ASA, AML, AMS, ALA and AMA and 2 µl template DNA. Amplification was carried out with the following thermoprofile: 15 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 58 °C and 1 min at 72 °C, and 10 min at 72 °C. All 79 *An. daciae* specimens and 425 of 793 *An. messeae* included in this study (Table 4) were identified by both DNA sequencing and PCR.

The species status of 12 individuals determined as *An. daciae* by ITS2 sequencing for the purpose of designing a species-specific PCR primer were later confirmed by this PCR. Of 465 additional '*An. messeae*' specimens first processed by the PCR, 67 were *An. daciae* and 398 were *An. messeae*. The identifications of all PCR-identified *An. daciae* and of 30 randomly selected *An. messeae* could be confirmed by subsequent DNA sequencing.

Table 4 Maculipennis Group species analysed in this study.

Federal state	No. of sampling sites	No. of mosquitoes identified	*An. maculipennis*	*An. atroparvus*	*An. messeae*	*An. daciae*
BB	45	284	30	2	223	29
TH	16	196	51	0	145	0
SA	20	166	70	0	87	9
SN	17	148	30	0	109	9
MP	22	103	7	1	95	0
BW	3	55	10	0	21	24
LS	20	46	9	0	37	0
SH	8	45	2	0	43	0
BV	19	36	15	0	19	2
HE	6	12	2	0	5	5
NW	4	6	1	0	5	0
HH	2	5	1	0	4	0
RP	3	3	2	0	0	1
Total	185	1,105	230	3	793	79

BB Brandenburg, BV Bavaria, BW Baden-Wurttemberg, HE Hesse, HH Hamburg, LS Lower Saxony, MP Mecklenburg-Western Pomerania, NW North Rhine-Westfalia, RP Rhineland-Palatinate, SA Saxony-Anhalt, SH Schleswig-Holstein, SN Saxony, TH Thuringia

Distribution of Maculipennis Group species in Germany

Based on morphological identification, a total of 1,105 *Anopheles* specimens of the Maculipennis Group were collected at 185 localities across 13 federal states throughout Germany (Table 4). DNA-based differentiation using the newly developed multiplex PCR, plus additional sequencing in the cases of *An. daciae* and *An. messeae*, confirmed all four sibling species known for Germany: *An. maculipennis, An. atroparvus, An. messeae* and *An. daciae*. In general, *An. messeae* was the most abundant sibling species, with 793 specimens (71.8 %) detected at 140 sampling sites, followed by *An. maculipennis* with 230 specimens (20.8 %) at 67 sampling sites and *An. daciae* with 79 specimens (7.1 %) at 24 sampling sites. *Anopheles atroparvus* was the rarest member of the Maculipennis Group, with three individuals (0.3 %) captured at different sampling sites.

Anopheles daciae occurred in sympatry with *An. messeae* and *An. maculipennis*, but not with *An. atroparvus*, in seven federal states of Germany (Table 5). Seventeen of the sites were in eastern Germany (federal states of Brandenburg, Saxony-Anhalt and Saxony) while seven collection sites were in more southern regions (northern Upper Rhine Valley) in the federal states of Hesse, Rhineland-Palatinate, Baden-Wurttemberg and Bavaria (Figure 5).

Table 5 Collection details and composition of Maculipennis Group species from sites where *An. daciae* was found.

Site no.	Federal state	Localitiy	Geographic coordinates (N, E)	Collection date	No. mosquitoes identified	*An. maculipennis*	*An. messeae*	*An. daciae*	Collection method	Reference
1	BB	Biesenthal	52.761230, 13.644762	2012-09-15	1	0	0	1	Aspirator	Unpublished
2		Gramzow	53.210505, 14.008083	2012-09-11	3	0	1	2	Aspirator	Unpublished
			53.210505, 14.008083	2013-08-03	8	0	3	5	Aspirator	Unpublished
3		Havelsee	52.508372, 12.53456	2013-07-30	1	0	0	1	Aspirator	Unpublished
4		Lietzen	52.469867, 14.340849	2012-11-22	1	0	0	1	Aspirator	Unpublished
5		Maust	51.826198, 14.402591	2011-08-22	9	1	7	1	Netting	Kronefeld et al. 2012
			51.826198, 14.402591	2012-06-13	45	1	42	2	Netting	Kronefeld et al. 2012
			51.826198, 14.402591	2012-07-23	1	0	0	1	Trapping	Unpublished
6		Müncheberg	52.515849, 14.112775	2012-05-13	1	0	0	1	Aspirator	Unpublished
			52.515849, 14.112775	2012-07-01	2	0	1	1	Aspirator	Unpublished
			52.515849, 14.112775	2012-09-30	5	0	3	2	Aspirator	Unpublished
7		Peitz	51.859638, 14.413376	2012-07-26	1	0	0	1	Aspirator	Unpublished
8		Schöneiche	52.483313, 13.703679	2011-08-25	13	12	0	1	Netting	Kronefeld et al. 2012
9		Herzberg	51.715942, 13.206618	2012-08-10	1	0	0	1	Trapping	Unpublished
10		Waldsieversdorf	52.542015, 14.077907	2012-07-15	1	0	0	1	Trapping	Unpublished
			52.542015, 14.077907	2012-07-19	1	0	0	1	Trapping	Unpublished
			52.542015, 14.077907	2012-07-21	3	0	2	1	Trapping	Unpublished
			52.542015, 14.077907	2012-07-28	2	0	0	2	Trapping	Unpublished
			52.542015, 14.077907	2012-08-16	3	0	1	2	Trapping	Unpublished
11		Chorin	52.937931, 13.934201	2013-10-25	1	0	0	1	Aspirator	Unpublished
12	BW	Leopoldshafen	49.101904, 8.3828530	2012-08-03	36	0	12	24	Netting	Unpublished
13	BV	Bamberg	49.901620, 10.874501	2012-07-30	1	0	0	1	Trapping	Unpublished
14		Burgheim	48.707198, 11.010808	2013-09-15	1	0	0	1	Aspirator	Unpublished
15	HE	Hochheim/Main	50.014772, 8.3559820	2012-08-22	1	0	0	1	Aspirator	Unpublished

Table 5 (continued)

Site no.	Federal state	Localitiy	Geographic coordinates (N, E)	Collection date	No. mosquitoes identified	*An. maculipennis*	*An. messeae*	*An. daciae*	Collection method	Reference
16	HE	Ludwigsaue	49.901532, 8.3555320	2011-08-23	2	0	1	1	Trapping	Kronefeld et al. 2012
			49.901532, 8.3555320	2011-09-07	2	0	1	1	Trapping	Kronefeld et al. 2012
			49.901532, 8.3555320	2012-06-29	2	0	1	1	Trapping	Kronefeld et al. 2012
17		Riedstadt	49.856696, 8.4387480	2013-07-22	1	0	0	1	Aspirator	Unpublished
18	RP	Wörth	49.034817, 8.2903610	2013-08-20	1	0	0	1	Aspirator	Unpublished
19	SN	Laske	51.302059, 14.229902	2012-06-13	94	6	84	4	Netting	Kronefeld et al. 2012
20		Schönheide	50.480845, 12.530444	2011-10-08	4	0	0	4	Trapping	Unpublished
21		Schkeuditz	51.371489, 12.202139	2013-11-01	1	0	0	1	Aspirator	Unpublished
22	SA	Seegrehna	51.850486, 12.522933	2012-06-12	1	0	0	1	Trapping	Unpublished
			51.850486, 12.522933	2012-06-26	2	0	0	2	Trapping	Unpublished
			51.850486, 12.522933	2012-07-24	2	0	0	2	Trapping	Unpublished
			51.850486, 12.522933	2012-08-14	1	0	0	1	Trapping	Unpublished
23		Magdeburg	52.165358, 11.644832	2013-08-27	20	3	15	2	Netting and dipping	Unpublished
24		Schönebeck/Elbe	52.022791, 11.720284	2013-09-17	1	0	0	1	Aspirator	Unpublished
25	BW	Dettenheim	49.161474, 8.4171150	2007-08-02	6	0	0	6	Trapping	Weitzel et al. 2012
Total					282	23	174	85		

BB Brandenburg, BW Baden-Wurttemberg, BV Bavaria, HE Hesse, RP Rhineland-Palatinate, SN Saxony, SA Saxony-Anhalt

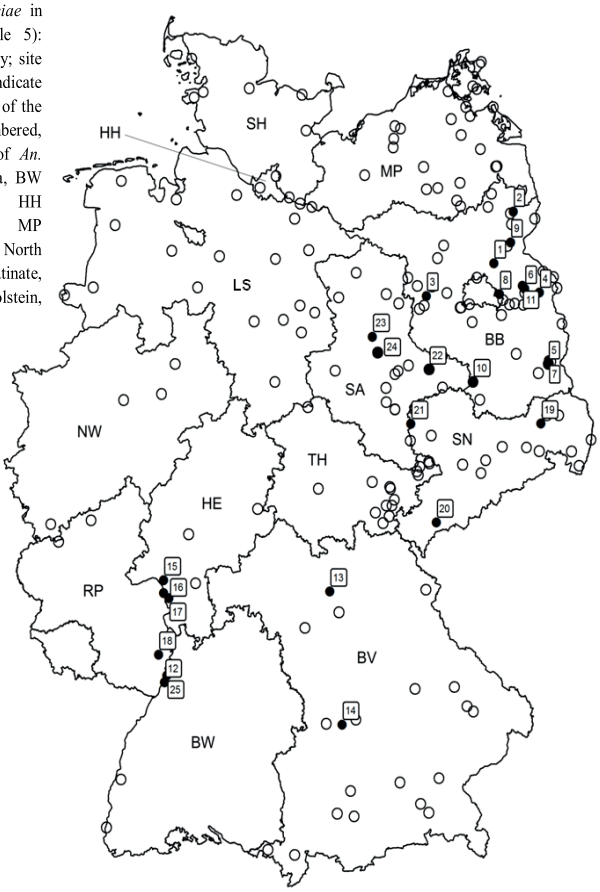

Figure 5 Collection sites of *An. daciae* in Germany (site nos. 1–24 (cf. Table 5): KRONEFELD et al. (2012) and this study; site no. 25: WEITZEL et al. (2012)). Dots indicate sampling sites positive for mosquitoes of the Maculipennis Subgroup, while numbered, black dots indicate collection sites of *An. daciae*. BB Brandenburg, BV Bavaria, BW Baden-Wurttemberg, HE Hesse, HH Hamburg, LS Lower Saxony, MP Mecklenburg-Western Pomerania, NW North Rhine-Westfalia, RP Rhineland-Palatinate, SA Saxony-Anhalt, SH Schleswig-Holstein, SN Saxony, TH Thuringia.

5.4 DISCUSSION

Four *Anopheles* species of the Maculipennis Group were demonstrated to occur in Germany, among them the recently recognized *An. daciae*. The new data on *An. daciae* in Germany supplement previous data by WEITZEL et al. (2012) who found six specimens at a single site in Baden-Wurttemberg (site no. 25 in Figure 5) and by KRONEFELD et al. (2012) who identified specimens at four sites in three German federal states (site nos. 5, 8, 16 and 19 in Figure 5). In the present study, *An. daciae* was collected in several additional federal states of Germany,

suggesting an even wider, probably nationwide, distribution. The results also show a sympatric occurrence with other members of the group, in particular *An. messeae* and *An. maculipennis*.

The scanty occurrence of *An. atroparvus* is remarkable, even though typical breeding sites in the North German coastal areas, as described by WEYER (1938), were sampled. Further studies are required with larger sample sizes, especially along the coastal areas of the North Sea, to determine if *An. atroparvus* has been replaced by *An. messeae*, as observed in the Netherlands (TAKKEN et al. 2002).

A PCR assay was developed and successfully validated for seven European sibling species of the Maculipennis Group including *An. daciae*, suggesting it might become a useful and reliable tool for species differentiation. In particular, the assay will help determine if *An. daciae* is indigenous in other European countries. *Anopheles beklemishevi*, a Maculipennis Group species from northern Scandinavia and Russia (RAMSDALE & SNOW 2000), could not be considered in the multiplex PCR design due to a lack of specimens. Also, non-European Palaearctic members of the Maculipennis Group, such as *Anopheles persiensis*, which is known only from Iran (SEDAGHAT et al. 2009), *Anopheles artemievi* from Kyrgyzstan (GORDEEV et al. 2005) and *Anopheles martinius* from the Caucasus (WHITE 1978) were not included.

Although called a species in this article, the species status of *An. daciae* is not generally accepted (cf. GORDEEV et al. 2008, KRONEFELD et al. 2012). The only reliable differences between *An. messeae* and *An. daciae* known to date are five single nucleotide substitutions in the ITS2 rDNA region (NICOLESCU et al. 2004). Little is known, however, about the biological characteristics of *An. daciae*, including its vector competence, although *D. repens*, the agent of subcutaneous dirofilariosis, was recently detected in an *An. daciae* female from Germany (KRONEFELD et al. 2014). DANABALAN et al. (2013) reported that *An. daciae* feeds on humans and other mammals and on birds, predisposing it to serve as a possible vector, including a bridge vector, of zoonotic disease agents. The characteristics of *An. daciae* may, or may not, differ from those of *An. messeae* and may result in divergent epidemiologies of mosquito-borne diseases, such as malaria, filarial or viral infections, mediated by *Anopheles* species of the Maculipennis Group.

6. MOLECULAR DETECTION OF *DIROFILARIA IMMITIS*, *DIROFILARIA REPENS* AND *SETARIA TUNDRA* IN MOSQUITOES FROM GERMANY

Abstract

As a result of globalization and climate change, *Dirofilaria immitis* and *Dirofilaria repens*, the causative agents of dirofilariosis in Europe, continue to spread from endemic areas in the Mediterranean to northern and northeastern regions of Europe where autochthonous cases of dirofilarial infections have increasingly been observed in dogs and humans. Whilst *D. repens* was recently reported from mosquitoes in putatively non-endemic areas, *D. immitis* has never been demonstrated in mosquitoes from Europe outside the Mediterranean.

From 2011 to 2013, mosquitoes collected within the framework of a German national mosquito monitoring programme were screened for filarial nematodes using a newly designed filarioid-specific real-time PCR assay. Positive samples were further processed by conventional PCR amplification of the cytochrome c oxidase subunit I (COI) gene, amplicons were sequenced and sequences blasted against GenBank.

Approximately 17,000 female mosquitoes were subjected to filarial screening. Out of 955 pools examined, nine tested positive for filariae. Two of the COI sequences indicated *D. immitis*, one *D. repens* and four *Setaria tundra*. Two sequences could not be assigned to a known species due to a lack of similar GenBank entries. Whilst *D. immitis* and the unknown parasites were detected in *Culex pipiens/torrentium*, *D. repens* was found in a single *Anopheles daciae* and all *S. tundra* were demonstrated in *Aedes vexans*. All positive mosquitoes were collected between mid-June and early September.

The finding of dirofilariae in German mosquitoes implies the possibility of a local natural transmission cycle. While the routes of introduction to Germany and the origin of the filariae cannot be determined retrospectively, potential culicid vectors and reservoir hosts must prospectively be identified and awareness among physicians, veterinarians and public health personnel be created. The health impact of *S. tundra* on the indigenous cervid fauna needs further investigation.

6.1 INTRODUCTION

The dirofilarial species *D. immitis* and *D. repens* are the causative agents of cardiopulmonary and subcutaneous dirofilariosis, respectively, in canines, felines and other carnivores in Europe (SIMÓN et al. 2012). In occasional infections of humans, the nematodes may evoke subcutaneous, subconjunctival and cardiovascular lesions with infections of the lung and pulmonary blood vessels. Intra- and retroocular infections as well as infections of deeper locations such as the peritoneal cavity, the omentum and the male sexual organs may occur (MCCALL et al. 2008, SIMÓN et al. 2012). Also, rare cases of meningoencephalitis have been described (POPPERT et al. 2009). Both worms are endemic in southern Europe where numbers of notified human cases of dirofilariasis have substantially increased recently (SIMÓN et al. 2005, GENCHI et al. 2011a). Contrary to previous assumptions, the majority of these had probably been caused by *D. repens* (PAMPIGLIONE et al. 2009). In addition, an ongoing north and eastward spread of both species has been observed, attributed to increased travel and movement of infected animals, the expansion of vector-competent mosquito species, global warming and a change in human activities (GENCHI et al. 2011b, MORCHÓN et al. 2012). Thus, autochthonous cases of *D. repens* infection in dogs were reported from Germany in 2004 (HERMOSILLA et al. 2006) and from the Netherlands in 2008 (OVERGAAUW & VAN DIJK 2009), while several human autochthonous cases have been diagnosed in Poland since 2007 (CIELECKA et al. 2012). In 2007 and 2012, *D. repens* was again diagnosed in dogs in the German federal states of Baden-Wurttemberg and Brandenburg (PANTCHEV et al. 2009, SASSNAU et al. 2009, 2013), suggesting that endemic circulation takes place. Moreover, autochthonous cases of *D. immitis* infection in dogs were reported from Hungary in 2009 (JACSÓ et al. 2009), Slovakia in 2010 (MITERPÁKOVÁ et al. 2010) and Poland in 2012 (ŚWIĄTALSKA & DEMIASZKIEWICZ 2012).

The primarily boreal filarial species *S. tundra* lives in the abdominal cavity of cervids. Setariae are commonly believed to be non-pathogenic in their natural hosts but severe disease outbreaks with associated peritonitis and perihepatitis caused by *S. tundra* have been reported (LAAKSONEN et al. 2007). In Scandinavian countries, reindeer is the main vertebrate host (LAAKSONEN & OKSANEN 2009) whereas in Central Europe only roe and red deer have been found parasitized so far (BÖHM & SUPPERER 1955, KUTZER & HINAIDY 1969, REHBEIN et al. 2000). Human infections have not been described.

Dirofilariae are transmitted by culicid mosquitoes (Diptera, Culicidae) of various species, such as *Cx. pipiens*, *Anopheles maculipennis* s.l. and *Aedes albopictus*, which are probably the most important vectors in the Mediterranean (CANCRINI et al. 2003, 2006, 2007). Some of these, such as *Cx. pipiens* and *An. maculipennis* s.l., are widely distributed over Europe while others, such as *Ae. albopictus*, are expanding northwards from established distribution areas in the Mediterranean (KAMPEN et al. 2013b). The main vectors of *S. tundra* are supposed to belong to the genus *Aedes* (LAAKSONEN et al. 2009).

Within a German national mosquito monitoring programme launched in 2011, mosquito samples were screened for various pathogens such as viruses and filarial nematodes.We describe here the finding of at least four filarial nematode species in mosquitoes collected in Germany, including the first detection of *D. immitis* in Germany.

6.2 METHODS

Adult mosquitoes were collected at numerous sites all over Germany between 2011 and 2013 using BG sentinel traps (Biogents, Germany) equipped with BG Lure™ and CO_2 as attractants, or by hand. The mosquitoes were caught by trained non-specialists who kept them frozen until further processing. Upon transportation to the laboratory, the mosquitoes were identified morphologically (SCHAFFNER et al. 2001, BECKER et al. 2010) or genetically, following RNA/DNA extraction as described below. Specifically, Maculipennis Group species (*An. maculipennis* s.l.) were identified by species-specific PCR (PROFT et al. 1999), whereas mosquitoes neither identifiable morphologically nor by PCR were subjected to COI barcoding (FOLMER et al. 1994).

A total of approximately 17,000 female mosquitoes belonging to six genera (*Aedes, Anopheles, Coquillettidia, Culex, Culiseta* and *Ochlerotatus*) were pooled by species, collection site and date with up to 25 specimens per pool. Mosquitoes identified by COI barcoding or species-specific PCR represented pools consisting of one specimen only. Each pool was homogenized in the presence of stainless steel beads (diameter 3 mm) in a maximum of 750 µl minimum essential medium (MEM) containing 10 µg/ml gentamicin, 0.25 µg/ml amphotericin B, 100 U/ml penicillin and 100 µg/ml streptomycin by a TissueLyserII (Qiagen, Germany) for 3 min at 30 Hz. The homogenate was centrifuged for 1 min at 14,000 g, and the supernatant was used for

simultaneous RNA/DNA extraction by means of the NucleoSpin 96 Virus Core Kit (Macherey-Nagel, Germany) according to the user manual.

For screening the mosquito pools for filarial nematodes (Filarioidea), a filarioid-specific real-time PCR assay was developed targeting a 90-bp fragment of the mitochondrial 16S rRNA gene with the newly designed primers PanFilaF (5'-TGTGCTGCGCTACATCGATG-3') and PanFilaR (5'-AAACCGCTCTGTCTCACGAC-3'). The primers were constructed after alignment of partial and complete mitochondrial genome sequences of nine parasitic filarial nematode species (*D. immitis, D. repens, S. tundra, Setaria digitata, Brugia malayi, Wuchereria bancrofti, Onchocerca flexuosa, Onchocerca volvulus*), which are epidemiologically important in human and animal health, and, additionally, are appropriately represented in GenBank. Sequences were analysed with BioEdit Sequence Alignment Editor (HALL 1999), and conserved DNA regions were identified for primer design taking into account standard rules of designing primers for real-time PCR assays (DORAK 2006). Specificity of the primers was confirmed on *D. immitis, D. repens, O. volvulus* and *W. bancrofti* DNA. A more in-depth testing was not considered necessary as the PCR was meant for sample screening only, thus possibly allowing false negative but not false positive results. The real-time PCR was performed using the CFX96 Touch™ Real-Time PCR Detection System (BioRad, Germany) and ResoLight non-specific detection chemistry, followed by high-resolution melting-analysis. The reaction mixture (25 µl) contained 1 µl ResoLight dye (Roche Diagnostics, Germany), 10 µl of 2× QuantiTect Multiplex PCR Master Mix (Qiagen, Germany), 0.4 µM forward and reverse primer each, and 5 µl of extracted DNA. The thermoprofile consisted of an initial denaturation step at 95°C for 15 min, 35 cycles of 95°C for 45 s, 58°C for 30 s and 72°C for 45 s, and a final extension step at 72°C for 5 min. All amplifications and detections were carried out in Multiplate™ Low-Profile 96-Well PCR Plates with optical Microseal 'B' Film (BioRad). After each annealing cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence of double-stranded DNA-binding ResoLight at 518 nm. After the PCR, a dissociation curve was constructed for steps of 5°C in the range from 60°C to 95°C. All data were analysed using the BioRad CFX-Manager software.

Samples yielding a signal in the real-time PCR were processed by a second conventional PCR amplifying about 650 bp of the filarioid COI gene (CASIRAGHI et al. 2001). After agarose gel electrophoresis, PCR products were excised from the gels and recovered by the QIAquick Gel Extraction Kit (Qiagen, Germany). They were cycled bidirectionally using the BigDye

Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Germany), and sequencing products were purified by SigmaSpin Sequencing Reaction Clean-Up Columns (Sigma Aldrich, Germany) before loading onto a 3130 Genetic Analyser (Applied Biosystems). For species identification, consensus sequences of positive samples were compared with sequences available in GenBank.

6.3 RESULTS

16,878 female mosquitoes representing 15 species or species complexes in six genera were processed, with *Cx. pipiens/torrentium* (73 %), *Anopheles plumbeus* (11 %) and *Aedes vexans* (8 %) being the most frequent mosquito taxa examined (Table 6).

Table 6 Mosquito species and pools examined.

Species	No. of mosquitoes tested (%)	No. of pools tested / No. of pools positive
Culex pipiens/torrentium	12,292 (72.83)	554 / 1
Anopheles plumbeus	1,843 (10.92)	93 / 0
Aedes vexans	1,356 (8.03)	111 / 0
Aedes cinereus/geminus	451 (2.67)	22 / 0
Anopheles maculipennis s.l.	336 (1.99)	99 / 0
Culiseta annulata	253 (1.50)	39 / 11
Coquillettidia richiardii	132 (0.78)	10 / 0
Ochlerotatus diantaeus	73 (0.43)	5 / 0
Ochlerotatus cantans/annulipes	64 (0.38)	8 / 0
Anopheles claviger	50 (0.30)	6 / 0
Ochlerotatus leucomelas	12 (0.07)	1 / 0
Ochlerotatus detritus	10 (0.06)	1 / 0
Ochlerotatus caspius	3 (0.02)	3 / 0
Ochlerotatus sticticus	2 (0.01)	2 / 0
Culiseta alaskaensis	1 (0.01)	1 / 0
Total	16,878	955 / 12

In total, 955 pools were screened using the pan-filarioid real-time PCR. Nine pools (0.94 %) testing positive were confirmed to contain filarioid DNA by the COI PCR assay. Of the sequences obtained, two showed 100 % and 99 % identity to *D. immitis* (Table 7) while presenting two nucleotide differences in direct comparison. One sequence displayed 99 % identity to *D. repens* and four sequences exhibited 99 % identity to *S. tundra*, with the *S. tundra* sequences being variable among each other at six positions. Two positive samples, displaying 91 % homology in direct comparison, could not be assigned to a species due to insufficient identities to GenBank entries (89 and 92 % maximum; Table 7). The *D. immitis*-positive pools as well as those with unknown filarioid DNA sequences were composed of *Cx. pipiens/torrentium*

specimens, the *D. repens*-positive pool was equivalent to a single *An. daciae* female, and the *S. tundra*-positive pools contained *Ae. vexans* mosquitoes (Table 7).

All filariae-carrying culicids had been collected between mid-June and early September in 2011, 2012 and 2013 in four German federal states **(**Figure 6, Table 7). The COI DNA sequences of the identified worms found in the mosquitoes have been deposited in GenBank under accession numbers KF692100-KF692106.

Table 7 Collection details of filarioid-positive mosquito pools.

Filarial species	GenBank accession no.	Max. % identity to GenBank entry (accession no.)*	Collection site	Collection date	Mosquito species	Pool size (no. mosquitoes)
D. immitis	KF692100	100 % (e.g. EU159111)	Freiburg (BW)	2012-07-13	*Cx. pipiens/torrentium*	25
D. immitis	KF692101	99 % (e.g. EU163945)	Buschow (BB)	2012-08-23	*Cx. pipiens/torrentium*	5
D. repens	KF692102	99 % (e.g. DQ358814)	Eggenstein-Leopoldshafen (BW)	2012-08-03	*An. daciae*	1
S. tundra	KF692103	99 % (e.g. DQ097309)	Braunschweig (LS)	2012-08-22	*Ae. vexans*	20
S. tundra	KF692104	99 % (e.g. DQ097309)	Radolfzell (BV)	2012-08-02	*Ae. vexans*	21
S. tundra	KF692105	99 % (e.g. DQ097309)	Regensburg (BV)	2011-09-02	*Ae. vexans*	10
S. tundra	KF692106	99 % (e.g. DQ097309)	Regensburg (BV)	2013-07-07	*Ae. vexans*	25
Filarioidea	-	89 % (HQ186250)	Regensburg (BV)	2011-06-17	*Cx. pipiens/torrentium*	9
Filarioidea	-	92 % (JX870433)	Drochtersen (LS)	2012-08-02	*Cx. pipiens/torrentium*	25

BW Baden-Wurttemberg, BB Brandenburg, LS Lower Saxony, BV Bavaria, *as of 07 November 2013

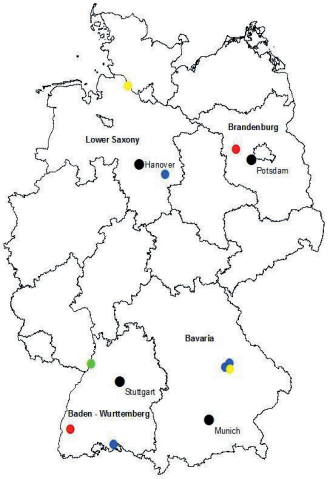

Figure 6 Geographic origin of the mosquitoes tested positive (red dots: *D. immitis*, green dot: *D. repens*, blue dots: *S. tundra*, yellow dots: filariae of unknown species).

6.4 DISCUSSION

As dirofilariosis is a vector-borne disease, its epidemiology is highly susceptible to climatic and environmental conditions. In the recent past, it has become an emerging problem in numerous countries of the world, including many European ones (GENCHI et al. 2011b, SIMÓN et al. 2012). The two causative agents in Europe, *D. immitis* and *D. repens*, have been found outside their traditional distribution ranges in the Mediterranean with increasing frequency. In particular, in Central and eastern European states, such as Austria, Hungary, Slovakia and Poland, autochthonous cases have been diagnosed in dogs and humans (AUER & SUSANI 2008, SZÉNÁSI et al. 2008, DEMIASZKIEWICZ et al. 2009, DUSCHER et al. 2009, MITERPÁKOVÁ et al. 2010, CIELECKA et al. 2012).

With *D. immitis* and *D. repens*, two mosquito-borne zoonotic filarial nematode species endemic to southern Europe were detected in mosquitoes collected in Germany. Hence, this is the first report from Germany of *D. immitis* apparently acquired locally.

Notably, the various filarial species were demonstrated in their putative vectors which included *An. daciae*, a recently recognized member of the Maculipennis Group (KRONEFELD et al. 2012), a complex of several closely related isomorphic *Anopheles* species, in addition to two previously described potential vectors of the filariae, *Ae. vexans* and *Cx. pipiens* (due to the pooling of the mosquitoes, a molecular differentiation between the morphologically indistinguishable females of *Cx. pipiens* and *Cx. torrentium* was not carried out in this study). The detection of the filariae in these mosquito species is not surprising as CANCRINI et al. (2003, 2006) found *D. repens* in *Cx. pipiens* and Maculipennis Group specimens in Italy while BOCKOVÁ et al. (2013) only recently reported *D. repens* in a pool of *Ae. vexans* mosquitoes from Slovakia. *Dirofilaria immitis* has been described from *Cx. pipiens* in Spain (MORCHÓN et al. 2007), and from *Ae. vexans* and *Cx. pipiens* in Turkey (YILDIRIM et al. 2011).

The finding of two unknown filarial species in *Cx. pipiens/torrentium* mosquito pools suggests avian bloodmeal sources of the mosquitoes due to the feeding preferences within this group of culicids, and therefore avian nematode species, one of them possibly being *Cardiofilaria pavlovskyi*, as discussed by CZAJKA et al. (2012).

The route through which the dirofilariae found their way to Germany or the sources of filarial ingestion by the mosquitoes, respectively, must remain speculative. The *D. immitis*-positive mosquito/es from Baden-Wurttemberg was/were collected at the same site where *Ae. albopictus*

had repeatedly been trapped previously (KAMPEN et al. 2013b). This site is characterized by its close proximity to a railway transhipment station where cargo from trucks coming in from southern Europe is transferred to trains. Hence, it is conceivable that, as with *Ae. albopictus*, the filarioid-positive *Cx. pipiens/torrentium* mosquito/es was/were introduced from southern Europe by vehicle transport. By contrast, the finding of *D. immitis* in a pool of mosquitoes from Brandenburg must be attributed most probably to a local uptake by the feeding mosquito/es. A possible source might have been a dog imported from, or with a travel history to, southern Europe. The detection of *D. repens* in *An. daciae* is noteworthy not only because nothing is known about the vector potential of this mosquito species but also because it was collected in the same area where *D. repens* had been isolated from dogs in 2007 (PANTCHEV et al. 2009). Possibly, a local transmission cycle has established in that area.

The third mosquito-borne filarial nematode described, *S. tundra*, seems to be more common in Germany than generally assumed, as it had been detected microscopically or by PCR on several occasions in the past (REHBEIN et al. 2000, CZAJKA et al. 2012). Detailed studies regarding its abundance, distribution and even pathology in areas south of Scandinavia, however, are lacking. As surprising as the dirofilarial findings are, Genchi and colleagues (GENCHI et al. 2011a, SASSNAU et al. 2013) considered both Baden-Wurttemberg and Brandenburg as climatically suitable for dirofilarial development in mosquitoes and assigned to these regions a risk of stable endemicity.

SIMÓN et al. (2012) calculated a transmission period of 3-4 months in Central Europe for both *D. repens* and *D. immitis*, taking into account the extrinsic incubation periods of the worms. At a mean temperature of 18°C, for example, these will last about 29 days, at 22°C still 16–20 days. *Setaria tundra* needs an average of 14-16 days at 21°C to reach the metacyclic infectious L3 stage in the mosquito (LAAKSONEN 2010). Considering the lifespan of a mosquito of a few weeks at its best, these long developmental periods may presently limit the rate of dispersal of the filariae. However, as hot summer periods are predicted to become more frequent and longer as a result of climate change, mosquito-borne filarioses will probably become a growing problem to veterinary and human health in Central and eastern Europe in the future.

6.5 CONCLUSION

With progressing globalization and climate change, the risk of the introduction of zoonotic *D. immitis* and *D. repens* from endemic areas in southern Europe to previously infection-free areas in northern Europe and their subsequent establishment increases. Vector-competent mosquitoes are probably already present there, and the climatic conditions are regionally and seasonally adequate for the filariae to finish their development in infected mosquitoes. The possibility of dirofilarial infections in dogs and other carnivores as well as in humans should therefore be considered with regard to differential diagnosis in unclear cases of appropriate symptomatology. No information exists on a possible spread and an increase in prevalence of *S. tundra* in Central Europe as respective studies are missing. Although this worm does not appear to significantly affect the health of indigenous cervids at present, further research on the epidemiology of setariosis in Central Europe is desirable.

7. SURVEY OF ARTHROPOD-BORNE VIRUSES OF PUBLIC HEALTH SIGNIFICANCE IN GERMAN MOSQUITOES

Abstract

During recent mosquito surveillance programmes, several mosquito-borne viruses were detected for the first time in Europe, including novel insect-specific viruses. In this study, mosquitoes collected from 2011-2013 throughout Germany were screened for the presence of flaviviruses in general and for specific alpha- and orthobunyaviruses (Tahyna, Inkoo, Sindbis, Batai and Chikungunya) by realtime PCR approaches. No zoonotic virus was detected, indicating that the overall prevalence of mosquito-borne viruses in Germany is low. However, approximately 1 % of the mosquito pools examined were positive for flavivirus, further recognised by sequence comparison as related to viral sequences detected in *Aedes vexans* mosquitoes in Spain. Attempts to obtain additional sequence data and to isolate the viruses were not successful.

Note: *The molecular detection of flavivirus RNA and the subsequent attempts to isolate virus by cell culture were performed in cooperation with scientists of the Institute of Novel and Emerging Infectious Diseases (director: Prof. Dr. M. H. Groschup) at the Friedrich-Loeffler-Institut, Greifswald-Insel Riems.*

7.1 INTRODUCTION

Mosquito-borne viruses include several causative agents of diseases affecting both humans and animals in Europe (LUNDSTRÖM 1999, HUBÁLEK 2008). As a result of globalisation and climate change, putatively tropical mosquito-borne diseases are supposed to extend into more northern areas, which cause great concern in Germany, too. Virus surveillance within the native mosquito populations offers an opportunity to detect virus prior to the emergence of disease in the susceptible host population (CALZOLARI et al. 2012a). While past investigations have demonstrated four mosquito-borne viruses exclusively in southwestern Germany (PILASKI & MACKENSTEIN 1985, JÖST et al. 2010, 2011a, 2011b), the occurrence of mosquito-borne viruses in other parts of Germany is mainly unknown.

7.2 METHODS

Trapping and processing of mosquitoes

Within the framework of a large-scale mosquito surveillance programme, adult mosquitoes were collected at numerous sites all over Germany between 2011 and 2013 using BG sentinel traps (Biogents, Germany) equipped with BG Lure™ and CO_2 as attractants, or by hand. The mosquitoes were caught by trained non-specialists who kept them frozen until further processing. Upon transportation to the laboratory, the mosquitoes were identified morphologically (SCHAFFNER et al. 2001, BECKER et al. 2010) or genetically following RNA/DNA extraction as described in chapter 6.

A total of approximately 17,000 female mosquitoes belonging to six genera (*Aedes, Anopheles, Coquillettidia, Culex, Culiseta and Ochlerotatus*) were pooled by species, collection site and date with up to 25 specimens per pool (Table 8). Mosquitoes identified genetically by COI barcoding or species-specific PCR represented pools consisting of one specimen only. Each pool was homogenized in the presence of stainless steel beads (diameter 3 mm) in a maximum of 750 µl minimum essential medium (MEM) containing 10 µg/ml gentamicin, 0.25 µg/ml amphotericin B, 100 U/ml penicillin and 100 µg/ml streptomycin by a TissueLyser II (Qiagen, Germany) for 3 min at 30 Hz. The homogenate was centrifuged for 1 min at 14,000 g, and 150 µl of the supernatant was used for simultaneous RNA/DNA extraction by means of the NucleoSpin 96

Virus Core Kit (Macherey-Nagel, Germany) according to the user manual. The remaining homogenate was stored frozen in two aliquots of 300 µl for further applications.

Table 8 Mosquito species and pools examined.

Species	No. of mosquitoes tested (%)	No. of pools tested / No. of pools positive
Culex pipiens/torrentium	12,292 (72.83)	554 / 1
Anopheles plumbeus	1,843 (10.92)	93 / 0
Aedes vexans	1,356 (8.03)	111 / 0
Aedes cinereus/geminus	451 (2.67)	22 / 0
Anopheles maculipennis s.l.	336 (1.99)	99 / 0
Culiseta annulata	253 (1.50)	39 / 11
Coquillettidia richiardii	132 (0.78)	10 / 0
Ochlerotatus diantaeus	73 (0.43)	5 / 0
Ochlerotatus cantans/annulipes	64 (0.38)	8 / 0
Anopheles claviger	50 (0.30)	6 / 0
Ochlerotatus leucomelas	12 (0.07)	1 / 0
Ochlerotatus detritus	10 (0.06)	1 / 0
Ochlerotatus caspius	3 (0.02)	3 / 0
Ochlerotatus sticticus	2 (0.01)	2 / 0
Culiseta alaskaensis	1 (0.01)	1 / 0
Total	16,878	955 / 12

Viral RNA detection and sequencing

For specific detection of Tahyna, Inkoo, Sindbis, Batai and Chikungunya virus RNA, two multiplex reverse transcriptase-polymerase chain reactions (qRT-PCR) were performed using the primers and probes listed in Table 9. A universal heterologous internal control system according to HOFFMANN et al. (2006) was used to control RNA/DNA extraction and RT-PCR efficiency in each sample or sample pool.

Amplification and detection was performed in the CFX96 Touch™ Real-Time PCR Detection System (BioRad, Germany) using the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Germany) according to the manufacturer's protocol. Each tube contained a reaction mixture of 25 µl consisting of 5 µl of extracted RNA, 12.5 µl of AgPath One-Step RT-PCR Buffer, 1 µl of AgPath-ID One-Step RT-PCR enzyme mix, 0.12 µM of specific probes, and 0.4 µM of each of the forward and reverse primers. The cycling conditions were as follows: an initial reverse transcription at 48 °C for 10 min, followed by reverse transcriptase inactivation and DNA polymerase activation at 95 °C for 10 min, and 40 cycles of amplification at 95 °C for 15 s and 60 °C for 45 s. The reporter dyes were measured after each amplification cycle. For final analysis, threshold-crossing values (Ct) were assigned to each sample in the exponential phase of the amplification plot of each cycle.

Table 9 Oligonucleotides used for the detection of Tahyna, Inkoo, Sindbis, Batai and Chikungunya virus RNA.

	Virus	Primer & probe	Sequence (5' to 3')	Target region	Reference
Multiplex 1	Sindbis	SIND F	CAC WCC AAA TGA CCA TGC	nsP1 gene	JÖST et al. 2010
		SIND R	KGT GCT CGG AAW ACA TTC		
		SIND P-Fam	CAG AGC ATT TTC GCA TCT GGC		
	Chikungunya	ChikSI	TGA TCC CGA CTC AAC CAT CCT	nsP1 gene	PANNING et al. 2006
		ChikAsI	GGC AAA CGC AGT GGT ACT TCC T		
		ChikP-Cy5	TCC GAC ATC ATC CTC CTT GCT GGC		
	Batai	Batai F	GCT GGA AGG TTA CTG TAT TTA ATA C	S-segment	JÖST et al. 2011a
		Batai R	CAA GGA ATC CAC TGA GTC TGT G		
		BataiP-Rox	AAC AGT CCA GTT CCA GAC GAT GGT C		
Multiplex 2	Tahyna	TAH FP	CAA AGC TGC TCT CGC TCG	S-segment	WEIDMANN et al. 2003
		TAH RP	TTC CAG GAA AAT GAT WAT TGA CGA		
		TAH P-Rox	CCG GAG AGG AAG GCT AGT CCT AAA TTT GGA		
	Inkoo	INK FP	CAT TGG AAC AAT GGC CC	S-segment	WEIDMANN et al. 2003
		INK RP	AGG ATC CAT CAT ACC ATG CTT		
		INK P-Fam	TCC CAG GAA CAG AAA TGT TTC TAG AAG TTT TC		
	EGFP	EGFP-10-R	CTT GTA CAG CTC GTC CAT GC	EGFP gene	HOFFMANN et al. 2006
		EGFP-11-F	CAG CCA CAA CGT CTA TAT CAT G		
		EGFP-1-Hex	AGC ACC CAG TCC GCC CTG AGC A		

In addition, extracted RNA was examined by a SYBR green-based qRT-PCR for the presence of flaviviral RNA using generic *Flavivirus*-specific primer targeting a conserved region of the flaviviral NS5 gene and designed across the entire genus *Flavivirus* (RODRIGUEZ et al., unpublished).

Samples yielding a specific signal in the qRT-PCRs were sequenced using the PCR primer and further processed for virus isolation by cell culture. Nucleotide sequences were compared with sequences available in public databases (GenBank).

Virus isolation by cell culture

For virus isolation, the remains of the mosquito homogenates were again centrifuged and the clear supernatants were inoculated into a 96-well plate containing a confluent African green monkey kidney cell (Vero) monolayer. In parallel, this was performed with a confluent *Ae. albopictus* C6/36 cell monolayer. The cultures were incubated at 37 °C in the case of the Vero cells and at 27 °C in the case of the mosquito cells. Five passages of the virus were carried out either solely in Vero or C6/36 cells, or alternately between both cell systems. Cytopathic effects (CPE) in Vero cells were checked for daily, while the supernatants of both culture lines were tested by qRT-PCR for the presence of virus after each passage.

7.3 RESULTS

16,878 female mosquitoes were processed in 955 pools. Although a wide range of mosquito species were tested, no alpha- and orthobunyaviruses could be detected. In contrast, twelve mosquito pools were tested positive for the presence of flaviviruses by the generic qRT-PCR. Eleven positive pools were composed of *Cs. annulata* specimens and one pool contained *Cx. pipiens/torrentium* mosquitoes (Table 10). Out of these twelve pools, nucleotide sequences of about 101 bp could be generated from nine *Cs. annulata* pools. These sequences displayed 99 % homology in direct comparison. The alignment with sequences from the GenBank database suggested the presence of a sequence closely related to flaviviral sequences derived from *Ae. vexans* mosquitoes in Spain (96 % identity to accession nos: JF707859, JF707860) (VÁZQUEZ et al. 2012). Despite considerable efforts of cultivation in vertebrate and insect cells, viruses could neither be isolated from these mosquito pools, nor could positive PCRs be obtained from the supernatants of the exposed cells or CPE be observed after five passages.

Table 10 Collection details of *Flavivirus*-positive mosquito pools.

	Species	Collection site	Collection date	Pool size (no. of mosquitoes)
qRT-PCR positive	*Culiseta annulata*	Gütersloh-Bielefeld	2011-05-31	4
	*Culiseta annulata**	Gütersloh-Bielefeld	2011-08-23	2
	*Culiseta annulata**	Rodleben	2011-09-01	9
	*Culiseta annulata**	Gütersloh-Bielefeld	2011-09-13	2
	*Culiseta annulata**	Tutow Airport	2012-02-10	10
	*Culiseta annulata**	Tutow Airport	2012-02-10	10
	*Culiseta annulata**	Tutow Airport	2012-02-10	10
	*Culiseta annulata**	Spantekow	2012-02-24	10
	*Culiseta annulata**	Spantekow	2012-02-24	10
	*Culiseta annulata**	Spantekow	2012-02-24	10
	Culiseta annulata	Spantekow	2012-02-24	10
	Culex pipiens/torrentium	Jena	2012-08-02	3

*Mosquito pools from which flaviviral sequences could be generated.

7.4 DISCUSSION

Entomological surveys targeting pathogenic arthropod-borne viruses have been done all over the world. Mosquito-borne viruses are of particular interest, as they include pathogenic agents such as West Nile virus or dengue fever virus, which may cause encephalitis and haemorrhagic disease. In the scope of such surveys, flaviviruses without a known vertebrate reservoir and detected only in insects, particularly in mosquitoes, were repeatedly described in recent years (CALZOLARI et al. 2012b, COOK et al. 2013). In this study, flaviviral nucleotide sequences were detected in *Cs. annulata* mosquitoes collected in Germany. However, the short nature of the amplification products that were obtained during the analysis allowed only preliminary characterization. Comparison with sequences from GenBank revealed a close relationship with flaviviral sequences observed in *Ae. vexans* in Spain by VÁZQUEZ et al. (2012). In addition to three novel insect-specific flaviviruses from *Oc. caspius* and *Cx. pipiens,* the authors of that study describe putative genomic integration events of flaviviral sequences into the genome of *Ae. vexans*, *Oc. caspius*, *Oc. detritus*, and *Cs. annulata*.

Interestingly, *Flavivirus* sequences were obtained from *Cs. annulata* mosquitoes from different collection sites in one third of the examined *Culiseta* pools in this study, although other species were also investigated in sufficient quantities. Based on this and the fact that the virus did not propagate in cell culture, occasional viral genome integration resulting in a latent infection is assumed.

It could not be clarified whether our findings were attributed to integrated sequences as described by CROCHU et al. (2004) or a DNA form of the virus as described by COOK et al. (2006).

The data obtained allow the conclusion that zoonotic mosquito-borne viruses in Germany are currently of minor importance. Nevertheless, evidence of zoonotic virus circulation in Baden-Wurttemberg, Bavaria and neighbour countries of Germany (SPIECKERMANN & ACKERMANN 1972, JÖST 2011a, 2011b, ZELLER et al. 2013), even though limited in time and space, indicate the possibility of future epidemics. As the introduction of travel-related cases of mosquito-borne disease and establishment of potential vector mosquitoes such as *Ae. albopictus* lead to unpredictable disease occurrence, continuous surveillance is recommended.

8. GENERAL DISCUSSION

Autochthonous transmission of chikungunya, dengue and West Nile fever in several countries of Europe indicate that "exotic" mosquito-borne diseases can no longer be exclusively regarded as travel-related. In contrast, autochthonous sporadic and sustained transmission to humans and animals in continental Europe appears to be possible (ANGELINI et al. 2007, SAMBRI et al. 2013, TOMASELLO & SCHLAGENHAUF 2013).

In Germany, the most common vector-borne diseases are associated with ticks and rodents, such as Lyme borreliosis, tick-borne encephalitis and Hantavirus infection (FRANK et al. 2014). However, mosquito-borne diseases such as malaria, chikungunya and dengue fever are of growing importance, as they are frequently acquired abroad and are subject to the imminent threat of introduction and subsequent risk of autochthonous transmission. For example, an annual average of 300 dengue fever cases were imported into Germany mostly from South and Southeast Asia between 2001 and 2009, whereas in the years 2010, 2012 and 2013 the number of imported cases increased to 595, 616 and 879, respectively (RKI 2014). The risk of autochthonous transmission is continuously on the rise, if the number of imported mosquito-borne pathogens keeps increasing.

The question whether exotic viruses can be transmitted in Germany basically depends on the establishment of competent vectors, such as *Ae. albopictus*, and their population densities. Given the repeated introduction of non-native mosquitoes representing efficient vectors of *Dirofilaria* spp., dengue, yellow fever, chikungunya and several other viruses that can cause encephalitides (WERNER et al. 2012, BECKER et al. 2013), and the recent detection of pathogenic viruses transmitted by native mosquitoes (JÖST et al. 2010, 2011a, 2011b), the question about the necessity of a continuous mosquito monitoring throughout Germany becomes more and more relevant.

Up to now, mosquito monitoring and mosquito control operations have only been carried out regionally such as in the Upper Rhine Valley, where the regular mass development of mosquitoes strongly impairs the quality of human and animal life. However, continuous mosquito surveillance throughout Germany would allow the early detection of an introduction of invasive mosquitoes and pathogens prior to their establishment and spread. Furthermore, surveillance measures are a basis for evaluating the potential for transmission of mosquito-borne disease

agents affecting human and animal health and to asses the effect on biodiversity, including impact of nuisance species and possible adverse effects on the native fauna. The data obtained from an integrated surveillance system would enable the responsible authorities to implement preventive measures, appropriate control strategies and to evaluate the efficacy of these control strategies. As a first step in this direction, the German authorities prompted a three-year nationwide mosquito monitoring programme, which aimed to revise the knowledge of the native mosquito fauna and circulating mosquito-borne pathogens that could have a substantial impact on public health.

This study presents results of a comprehensive entomological survey conducted during three consecutive years from 2011 to 2013 throughout Germany. It focussed on the diversity and distribution of the morphologically indistinguishable *Anopheles* species of the Maculipennis Subgroup on the one hand, and on providing preliminary data on the abundance of pathogens circulating in the mosquito fauna.

The obtained results revealed the occurrence and even widespread distribution of *An. daciae*, a previously unrecognized member of the Maculipennis Subgroup in Germany, which so far had been represented by *An. messeae*, *An. maculipennis* and *An. atroparvus*. It is well known that the various species of the genus *Anopheles* formerly used to play different roles in the transmission of human malaria parasites, with *An. atroparvus* considered as the primary malaria vector in Germany. In addition, members of the Maculipennis Subgroup are probably capable of transmitting further mosquito-borne pathogens, as they have been shown elsewhere to be infected in the field with Tahyna virus (ASPÖCK 1970), West Nile virus (FILIPE 1972), *D. immitis* and *S. labiatopapillosa* filariae (CANCRINI et al. 1997, 2006) as well as with Sindbis and Batai viruses in Germany (JÖST et al. 2010, 2011a).

In fact, *An. daciae* collected in the Upper Rhine Valley, was demonstrated in this study to be infected with the zoonotic filarial nematode *D. repens*, the causative agent of subcutaneous dirofilariosis. The Upper Rhine Valley is one of the warmest regions in Germany and is characterized by vast natural and restored meadows and forests regularly covered with water during flooding events. In that region, *An. daciae* was repeatedly recorded and present in great abundance throughout the season from May to November. In historic Germany, the emergence of malaria was strongly correlated with natural floodplains, where environmental conditions enabled a mass development of *Anopheles* mosquitoes (GROBER 1903). It is therefore possible that, at the

time malaria was endemic in Germany, *An. daciae* attributed to malaria transmission along with *An. atroparvus* and *An. messeae* (WEYER 1934).

Unexpectedly, only few specimens of *An. atroparvus* were collected from only a few sampling sites, although historic studies demonstrated a widespread distribution of this species. Similar developments were also observed by TAKKEN et al. (2002) in the Lower Rhine delta plains close to Rotterdam in the Netherlands. Whether these findings display current changes in the mosquito fauna composition, for example as a result of modifications in the waterbody structure or water management, or whether this is an artefact of sampling strategies (e.g. unsuitable sampling technique for certain species) or has another unknown cause should be examined in more detail. A systematic sampling of aquatic stages of mosquitoes would be an alternative, but also a more laborious method in contrast to the trapping technique predominantly applied in this study.

As little is known on the biology and ecology of *An. daciae*, reliable species diagnostics is essential. For this purpose a multiplex-PCR assay was developed, which allows the differentiation of all sibling species of the Maculipennis Subgroup known today in Central Europe, namely *An. messeae*, *An. maculipennis*, *An. atroparvus*, *An. sacharovi, An. melanoon, An. labranchiae* and *An. daciae.* What's more, the assay will enable extensive distribution studies and facilitate a much more accurate risk assessment of competent vector species of the Maculipennis Subgroup and pathogens transmitted by them throughout Europe.

After all, there still remains the question of the species status of *An. daciae*, which has not been sufficiently answered. Further studies focusing on sexual reproduction ability (hybridisation/crossing tests) and morphological and genetic characters distinguishing *An. daciae* from the other siblings of the Maculipennis Group should be conducted.

The screening of mosquitoes from a large-scale cross-sectional monitoring programme with a newly developed filarioid-specific real-time PCR assay demonstrated the presence of filarial DNA from various origins. Among others, *Setaria tundra,* a nematode parasite of cervids, was identified several times in Lower Saxony, Baden-Wurttemberg and Bavaria. Whilst infection with this species in Fennoscandia was documented to be associated with substantial morbidity and mortality in moose and reindeer (LAAKSONEN et al. 2007, 2009), the occurrence of the disease has rarely been addressed in detail in Germany. Here, only few detections in roe deer without any health impairments were reported (BÜTTNER 1978, REHBEIN et al. 2000). Mosquitoes of the genus *Aedes* are considered the most important vectors during epidemics of setariosis in

Scandinavia (LAAKSONEN et al. 2009). *Aedes vexans* was concordantly found to be infected in the field in this study, although the mere finding of worm larvae in mosquitoes does not necessarily equate them with being a vector. This mosquito species usually develops in large numbers in inundated areas and is known to migrate considerably long distances seeking for a blood meal and to invade new habitats (BECKER et al. 2010). It therefore becomes the predominant species during the summer months along river plains and, due to its mammalophilic and day-biting behaviour, the most important nuisance mosquito (BECKER et al. 2010). It should also be noted that, in addition to *Ae. albopictus* and *Cx. pipiens, Ae. vexans* is probably also the most important vector of dirofilariae in Europe (YILDIRIM et al. 2011, LATROFA et al. 2012, BOCKOVÁ et al. 2013). Within this survey, dirofilariae have been demonstrated in *Cx. pipiens/torrentium* and in *An. daciae* mosquitoes.

The findings of *D. immitis* and *D. repens* during the summer of 2012 in Brandenburg and Baden-Wurttemberg could be attributed to the establishment of new mosquito-borne pathogens in Germany. Recent analyses of local temperature data in the surroundings of the sampling sites confirm that the climatic conditions have favourably evolved in the last 30 years, allowing the *Dirofilaria* larvae to complete their development in infected mosquito vectors and thus the establishment of local transmission cycles (SASSNAU et al. 2014). This is consistent with the additional finding of *D. repens* by a second research group in *Ae. vexans*, *Cs. annulata* and *An. maculipennis* s.l., which had also been collected in the federal state of Brandenburg in 2011 and 2012 (CZAJKA et al. 2014).

The assumption that local transmission cycles of *D. repens* have established in Brandenburg and Baden-Wurttemberg is supported by the previous repeated finding of autochthonous infections in dogs (HERMOSILLA et al. 2006, PANTCHEV et al. 2009, SASSNAU et al. 2009, SASSNAU et al. 2013). Dogs imported from, or with a travel history to, south-eastern Europe are possible introduction sources, as Mediterranean countries also reported increased autochthonous transmission of *Dirofilaria* in the recent past (SIMÓN et al. 2012). Furthermore, wild carnivores such as the red fox (*Vulpes vulpes*) and the beech marten (*Martes foina*) which have been found infected in the field (MAGI et al. 2008, LECOVÁ & LETKOVÁ 2009, MITERPÁKOVÁ et al. 2013) may be epidemiologically relevant as reservoir and transportation hosts. The recent report of an autochthonous human *Dirofilaria* infection in a German citizen in the federal state of Saxony-Anhalt and the detection of infected mosquito populations in the same area substantiate the

assumption that *D. repens* has succuessfully established and will continue to spread, resulting in further infections of dogs and humans (TAPPE et al. 2014).

In addition to *S. tundra* and the dirofilarial species, there is evidence of further filarioid nematodes circulating in Germany. Two filarioid DNA sequences were detected in *Culex pipiens/torrentium*, which could not be assigned to a species due to insufficient homologies to GenBank entries. Despite this, the detection demonstrated that the newly developed filarioid-specific real-time PCR assay is well-suited to identify a broad range of filarioid nematodes circulating among wild and domestic animals. This provides an essential advantage in studying nematode infections in the future, particularly of wild animals, in which sufficient sampling is possible only with huge difficulties.

No evidence was found for circulating viruses, which leads to the conclusion that the risk of mosquito-borne viruses is presently low. This is not surprising, as mosquito-borne diseases need a combination of factors to become epidemic, including the presence of competent vectors in sufficient densities overlapping with a susceptible vertebrate host population within an environment (e.g. climate) that is permissive for such interaction.

Previous findings of Batai, Sindbis and Tahyna virus in mosquitoes and the first emergence of Usutu virus in wild and captive birds illustrate that the settings have already been favourable in Germany within certain areas and periods of time. Some of these factors that may cause a mosquito-borne disease becoming epidemic are still unexplored. For example, there are no studies, which assessed the vector competence of indigenous mosquito populations for zoonotic viruses and just a few that examined populations from other Central European countries (e.g. BALENGHIEN et al. 2008). Arising from the interaction between multiple changing factors, such as climate, patterns of travel, urbanisation and land use, it is therefore reasonable to draw significantly more attention in future studies to conditions which could lead to further epidemics as a whole.

In conclusion, the results of this study emphasize the need for regular monitoring of the mosquito fauna and associated pathogens in the context of increasing climate change and globalization.

9. SUMMARY

As a consequence of fundamental environmental, economic and demographic changes worldwide, vector-borne diseases are becoming of increasing public and animal health significance in Europe, owing to their geographical expansion and the growing number of cases. Recently, exotic mosquito species, such as the Asian tiger mosquito (*Aedes albopictus*) and the Asian bush mosquito (*Ochlerotatus j. japonicus*) have repeatedly been found in Germany and mosquito-borne viruses including Sindbis, Batai and Usutu viruses have been documented in native mosquito species. As systematic studies on the occurrence and distribution of mosquitoes and pathogens associated with them have only regionally been realized, focussed on southern Germany, there was an urgent need to update and expand data available on the indigenous mosquito fauna. Meeting this objective, a study on the occurrence and distribution of mosquitoes of the Maculipennis Group in Germany was undertaken and a method for their reliable differeniation was developed within the framework of a nationwide mosquito monitoring programme from 2011 to 2013.

A total of 1,105 *Anopheles* specimens of the Maculipennis Group were collected at 185 localities across 13 federal states throughout Germany. Following a comparative sequence analysis of the internal transcribed spacer 2 (ITS2) ribosomal DNA (rDNA), *Anopheles daciae* was demonstrated as the 49th mosquito species in Germany. Due to the high genetic similarity to *Anopheles messeae*, *An. daciae* has previously been overlooked in Germany using the most common species-specific multiplex PCR assay.

The discovery of *An. daciae* prompted the adjustment of the species-specific multiplex PCR assay to distinguish the presently known members of the European Maculipennis Group including *An. daciae*. Taking into account the described sequence polymorphisms, a primer species-specific for *An. daciae* was designed, allowing the differentiation of the four Maculipennis Group species occurring in Germany plus *Anopheles sacharovi, Anopheles melanoon* and *Anopheles labranchiae*, which are mainly present in southern Europe.

In addition to the formerly known species *Anopheles atroparvus*, *Anopheles maculipennis* and *An. messeae*, the newly described cryptic species *An. daciae* was identified in seven federal states of Germany, indicating a nationwide distribution. The sympatric occurrence together with *An. messeae* and *An. maculipennis* at some sampling sites also indicates that *An. daciae* has similar

ecological requirements, but corresponding investigations have yet to be carried out. Contrary to numerous historical records of *An. atroparvus* along the German coastal areas, this species could only be recorded in low numbers at few sampling sites, possibly as a consequence of recent changes in the water body structures and the water quality. The second part of the thesis focusses on the molecular examination of mosquitoes for viruses of the family Bunyaviridae, Flaviviridae and Togaviridae and for filarial nematodes of human and veterinary importance. Approximately 17,000 identified female mosquitoes were pooled by species, collection site and date with up to 25 specimens per pool. This was followed by a combined isolation of DNA and RNA, allowing the simultaneous screening for various pathogens. For screening the mosquito pools for filarial nematodes of the superfamily Filarioidea, conserved regions were identified in mitochondrial DNA sequences of parasitic nematode species and primers for a filarioid-specific real-time PCR assay were designed. For screening the mosquitoes for viruses, published qRT-PCRs and newly developed in-house assays were applied.

Except for two unknown filarioid species in *Culex pipiens/torrentium*, *Setaria tundra*, a common filarial nematode of cervids, was detected in the flood water mosquito *Aedes vexans* collected in Baden-Wurttemberg, Lower Saxony and Bavaria in three consecutive years. Of particular importance are the findings of two zoonotic filarial nematode species, *Dirofilaria immitis* and *Dirofilaria repens*, in *Cx. pipiens/torrentium* and *An. daciae* from Baden-Wurttemberg and Brandenburg in 2012.

Zoonotic mosquito-borne viruses of the family Bunyaviridae, Flaviviridae and Togaviridae could not be demonstrated in indigenous mosquitoes, suggesting a minor importance at present. However, flaviviral sequences found in the banded house mosquito *Culiseta annulata* revealed a close relationship with insect-specific flaviviruses from *Ae. vexans* in Spain. Similar findings of viruses with no recognized pathogenic role in humans are increasingly reported worldwide from studies targeted at mosquito-borne flaviviruses.

The new insights obtained by the present work provide an important basis for further research on the biological characteristics of *An. daciae* with a focus on its species status and vector potential. The genetic detection of *D. repens* provides a first indication of this species as to a possible vector of zoonotic filarial nematodes. In addition to their primary importance as vectors of malaria parasites, members of the Maculipennis Subgroup have been described to be capable of transmitting Tahyna, West Nile, Sindbis and Batai virus as well as *D. immitis* and *Setaria*

labiatopapillosa filariae. The optimised species-specific multiplex PCR assay to differentiate the European siblings of the Maculipennis Group does not only provide the advantage to find out where *An. daciae* occurs in Europe, but also simplifies to survey its distribution with regard to globalisation and climate change.

Given that other research groups also recently demonstrated *D. repens* in mosquitoes collected in Brandenburg and autochthonous infections had previously been demonstrated in dogs from Baden-Wurttemberg and Brandenburg, the establishment of local transmission cycles of *D. repens* in Brandenburg and Baden-Wurttemberg can be assumed. Only recently, the first case of autochthonous infections with *D. repens* was diagnosed in a German citizen in the federal state of Saxony-Anhalt. Thus, dirofilariosis also poses a risk to public and animal health in regions north of the traditional distribution areas in the Mediterranean.

The results of this study clearly indicate that mosquito-borne diseases should no longer be neglected by physicians, veterinarians, public health personnel and policy makers. After all, the surveillance of mosquitoes and associated pathogens enable the early detection of changes in the mosquito fauna and the introduction of non-indigenous mosquito-borne pathogens.

10. ZUSAMMENFASSUNG

Als Folge fundamentaler globaler, ökonomischer und demographischer Veränderungen ist eine weltweite Zunahme und Ausbreitung Vektor-assoziierter Erkrankungen festzustellen, welche auch in Europa zunehmend an Bedeutung für die Gesundheit von Mensch und Tier gewinnen. In Deutschland wurden in den letzten Jahren wiederholt „exotische“ Stechmücken, wie die Asiatische Tigermücke (*Aedes albopictus*) und die Asiatische Buschmücke (*Ochlerotatus j. japonicus*), sowie Viren, wie das Sindbis-, Batai- und das Usutu-Virus, in einheimischen Stechmücken nachgewiesen. Da systematische Studien zum Vorkommen und zur Verbreitung von Stechmücken und assoziierten Erregern in Deutschland bisher nur regional in Süddeutschland durchgeführt worden waren, bestand ein dringender Bedarf, den gegenwärtigen Kenntnisstand zu aktualisieren und zu ergänzen. Mit dieser Zielsetzung wurden im Rahmen eines bundesweiten Stechmücken-Monitoringprogramms von 2011 bis 2013 im ersten Teil dieser Arbeit das Vorkommen und die Verbreitung von Stechmücken der Maculipennis-Gruppe in Deutschland untersucht sowie eine Methode zu deren molekularbiologischen Differenzierung entwickelt.

Insgesamt wurden bei der Auswertung der Stechmückenfänge 1.105 *Anopheles*-Mücken der Maculipennis-Gruppe von 185 Standorten in 13 Bundesländern erfasst. Nach vergleichender Analyse der DNA-Sequenz der ribosomalen internal transcribed spacer-Region 2 (ITS2) der Stechmücken konnte *Anopheles daciae* als neu beschriebene und damit 49. Stechmückenart in Deutschland identifiziert werden. Aufgrund der hohen genetischen Ähnlichkeit zu *An. messeae* war *An. daciae* mit der bisher gängigen spezies-spezifischen Multiplex-PCR in Deutschland nicht erfasst worden.

Die Entdeckung von *An. daciae* veranlasste eine Modifizierung der artdiagnostischen PCR, die es zukünftig ermöglichen sollte, *An. daciae* unter den in Europa vorkommenden Arten der Maculipennis-Gruppe eindeutig zu identifizieren. Unter Berücksichtigung der fünf beschriebenen Nukleotidunterschiede in der ITS2 Sequenz zwischen *An. daciae* und *An. messeae* wurde ein spezies-spezifischer Primer für *An. daciae* entwickelt, der es erlaubt, alle vier in Deutschland vorkommenden Arten der Maculipennis-Gruppe, zuzüglich der hauptsächlich in Südeuropa vorkommenden Arten *An. sacharovi, An. melanoon* und *An. labranchiae*, zuverlässig zu differenzieren.

Neben den bereits bekannten Vertretern der Maculipennis-Gruppe in Deutschland, *An. atroparvus*, *An. maculipennis* und *An. messeae*, wurde *An. daciae* im Untersuchungszeitraum in insgesamt sieben Bundesländern nachgewiesen, was eine bundesweite Verbreitung vermuten lässt. Das sympatrische Vorkommen mit *An. messeae* und *An. maculipennis* an einigen Untersuchungsstandorten deutet zudem auf ähnliche ökologische Ansprüche dieser Arten hin. Entsprechende vertiefende Untersuchungen stehen jedoch noch aus. Entgegen zahlreicher historischer Belege von *An. atroparvus* an den deutschen Küstengebieten konnte diese Art in der vorliegenden Untersuchung nur mit wenigen Exemplaren nachgewiesen werden, womöglich als Folge jüngerer Veränderungen in der Gewässerstruktur und -qualität.

Der zweite Teil der Arbeit widmete sich der molekularbiologischen Untersuchung der einheimischen Stechmücken auf Viren aus den Familien der Flaviviridae, Togaviridae und Bunyaviridae sowie auf Filarien von human- und veterinärmedizinischer Bedeutung. Dazu wurden knapp 17,000 zuvor bestimmte Stechmücken nach Fundort und –datum mit bis zu 25 Individuen pro Probe gepoolt. Anschließend erfolgte die kombinierte Isolierung von DNA und RNA, was die simultane Untersuchung der Proben auf verschiedene Erreger ermöglichte. Für das Screening der Stechmücken auf parasitäre Nematoden aus der Überfamilie Filarioidea wurden auf Grundlage mitochondrialer DNA-Sequenzen konservierte Regionen identifiziert und Primer für eine Filarioidea-spezifische Real-time PCR konstruiert. Für das Screening der Stechmücken auf Viren wurden bereits publizierte qRT-PCRs und im FLI neu entwickelte Testsysteme eingesetzt.

Neben dem Nachweis von zwei unbekannten Filarien in *Culex pipiens/torrentium* wurde *Setaria tundra*, ein Parasit von Hirschen (Fam. Cervidae), in drei aufeinanderfolgenden Jahren in Exemplaren der Überschwemmungsmücke *Aedes vexans* aus Baden-Württemberg, Niedersachsen und Bayern gefunden. Von besonderer Bedeutung sind die Nachweise der zoonotischen Filarien-Spezies *Dirofilaria repens* und *Dirofilaria immitis* in Stechmücken der Arten *Cx. pipiens/torrentium* und *An. daciae* aus Baden-Württemberg und Brandenburg im Jahr 2012.

Viren aus den Familien Bunyaviridae, Flaviviridae und Togaviridae konnten in einheimischen Stechmücken hingegen nicht nachgewiesen werden, was aus heutiger Sicht auf eine geringe Bedeutung dieser zoonotischen Viren in Deutschland hinweist. Allerdings wurden Flavivirus-Sequenzen in der Ringelmücke *Culiseta annulata* gewonnen, die eine große Übereinstimmung mit Sequenzen von Insekten-spezifischen Flaviviren aus *Ae. vexans* in Spanien zeigten. Über

ähnliche Viren ohne humanpathogene Bedeutung wird weltweit zunehmend berichtet, insbesondere in Studien zur Erfassung von Stechmücken-übertragenen Flaviviren.

Die neu gewonnen Erkenntnisse bilden eine wichtige Grundlage für weitere Untersuchungen von *An. daciae*, insbesondere zu deren Artstatus und ihrer Rolle als Überträger von Krankheitserregern. Der molekularbiologische Nachweis des Hundehautwurms *D. repens* in dieser für Deutschland neu beschriebenen Art lässt eine mögliche Bedeutung als Vektor zoonotischer Filarien vermuten, wobei Vertreter der Maculipennis-Gruppe neben ihrer primären Rolle als Überträger von Malariaparasiten bereits als mögliche Vektoren des Tahyna-, Westnil-, Sindbis- und Batai-Virus als auch von *D. immitis* und *Setaria labiatopapillosa* beschrieben wurden. Die optimierte spezies-spezifische Multiplex-PCR bietet ein geeignetes Hilfsmittel, die europaweite Verbreitung der Arten der Maculipennis-Gruppe zu studieren und im Hinblick auf Globalisierung und Klimawandel weiterhin auch in Deutschland zu überwachen.

Da *D. repens* kürzlich in Brandenburg auch von anderen Arbeitsgruppen wiederholt in Stechmücken nachgewiesen wurde und bereits zuvor in Baden-Württemberg und Brandenburg in Hunden nachgewiesen worden war, kann mittlerweile von einer Etablierung dieses Parasiten in Deutschland ausgegangen werden. Erst kürzlich wurde eine autochthone Infektion mit *D. repens* bei einem Mann aus Sachsen-Anhalt diagnostiziert. Ein Gesundheitsrisiko für Mensch und Tier ist demnach auch deutlich nördlich des traditionellen mediterranen Verbreitungsgebietes gegeben.

Die Ergebnisse der Arbeit unterstreichen deutlich, dass den Stechmücken-assoziierten Krankheiten von Seiten der Human- und Veterinärmedizin, des öffentlichen Gesundheitswesens und der politischen Entscheidungsträger eine größere Beachtung zukommen sollte. Das Monitoring von Stechmücken und die Überwachung der von ihnen übertragenen Krankheitserreger ermöglichen letztendlich eine frühzeitige Erkennung von Veränderungen in der Stechmückenfauna und der Zirkulation eingeschleppter Stechmücken-assoziierter Krankheitserreger.

11. BIBLIOGRAPHY

Ackermann, R., Spithaler, W.I., Profittlich, W. Spieckermann, D. (1970) Über die Verbreitung von Viren der California-Encephalitis-Gruppe in der Bundesrepublik Deutschland. Deutsche Medizinische Wochenschrift 29: 1507-1513.

Agüero, M., Fernández-Pinero, J., Buitrago, D., Sánchez, A., Elizalde, M., San Miguel, E., Villalba, R., Llorente, F., Jiménez-Clavero, M.Á. (2011) Bagaza virus in partridges and pheasants, Spain, 2010. Emerging Infectious Diseases 17: 1498-1501.

Allering, L., Jöst, H., Emmerich, P., Günther, S., Lattwein, E., Schmidt, M., Seifried, E., Sambri, V., Hourfar, K., Schmidt-Chanasit, J. (2012) Detection of Usutu virus infection in a healthy blood donor from south-west Germany, 2012. Eurosurveillance 17: pii=20341.

Almeida, A.P., Gonçalves, Y.M., Novo, M.T., Sousa, C.A., Melim, M., Gracio, A.J. (2007) Vector monitoring of *Aedes aegypti* in the Autonomous Region of Madeira, Portugal. Eurosurveillance 12: pii=3311.

Alten, B., Kampen, H., Fontenille, D. (2007) Malaria in Southern Europe: resurgence from the past? In: Takken, W., Knols, B.J.G. (eds.), Emerging Pests and Vector-Borne Diseases in Europe, Volume 1. Wageningen, The Netherlands: Wageningen Academic Publishers: 500 pp.

Andreadis, T.G., Wolfe, R.J. (2010) Evidence for reduction of native mosquitoes with increased expansion of invasive *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) in the northeastern United States. Journal of Medical Entomology 47: 43-52.

Angelini, R., Finarelli, A.C., Angelini, P., Po, C., Petropulacos, K., Silvi, G., Macini, P., Fortuna, C., Venturi, G., Magurano, F., Fiorentini, C., Marchi, A., Benedetti, E., Bucci, P., Boros, S., Romi, R., Majori, G., Ciufolini, M.G., Nicoletti, L., Rezza, G., Cassone, A. (2007) Chikungunya in north-eastern Italy: a summing up of the outbreak. Eurosurveillance 12: pii=3313.

Angelini, P., Tamba, M., Finarelli, A.C., Bellini, R., Albieri, A., Bonilauri, P., Cavrini, F., Dottori, M., Gaibani, P., Martini, E., Mattivi, A., Pierro, A.M., Rugna, G., Sambri, V., Squintani, G., Macini, P. (2010) West Nile virus circulation in Emilia-Romagna, Italy: the integrated surveillance system 2009. Eurosurveillance 15: pii=19547.

Armengaud, A., Legros, F., Quatresous, I., Barre, H., Valayer, P., Fanton, Y., D'Ortenzio, E., Schaffner, F. (2006) A case of autochthonous *Plasmodium vivax* malaria, Corsica, August 2006. Eurosurveillance 11: pii=3081.

Aspöck, H., Kunz, C. (1970) Felduntersuchungen über die Bedeutung des Igels (*Erinaceus europaeus roumanicus* BARETT-HAMILTON) im Zyklus des Ťahyňa-Virus. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, I. Abteilung, Originale 213: 304-310.

Aspöck, H., Kunz, C., Pretzmann, G. (1970) Phänologie und Abundanz der Stechmücken des östlichen Neusiedlersee-Gebietes (Ost-Österreich) in ihrer Beziehung zum Auftreten der durch Stechmücken übertragenen Arboviren. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, I. Abteilung, Originale 214: 160-173.

Auer, H., Susani, M. (2008) Der erste autochthone Fall einer subkutanen Dirofilariose in Österreich. Wiener Klinische Wochenschrift 120: 104-106.

Autorino, G.L., Battisti, A., Deubel, V., Ferrari, G., Forletta, R., Giovannini, A., Lelli, R., Murri, S., Scicluna, M.T. (2002) West Nile virus epidemic in horses, Tuscany region, Italy. Emerging Infectious Diseases 8: 1372-1378.

Bakonyi, T., Erdélyi, K., Ursu, K., Ferenczi, E., Csörgo, T., Lussy, H., Chvala, S., Bukovsky, C., Meister, T., Weissenböck, Nowotny, N. (2007) Emergence of Usutu virus in Hungary. Journal of Clinical Microbiology 45: 3870-3874.

Baldari, M., Tamburro, A., Sabatinelli, G., Romi, R., Severini, C., Cuccagna, P., Fiorilli, G., Allegri, M.P., Buriani, C., Toti, M. (1998) Malaria in Maremma, Italy. Lancet 351: 1246-1247.

Balenghien, T., Vazeille, M., Grandadam, M., Schaffner, F., Zeller, H., Reiter, P., Sabatier, P., Fouque, F., Bicout, D.J. (2008) Vector competence of some French *Culex* and *Aedes* mosquitoes for West Nile virus. Vector-Borne and Zoonotic Diseases. 8: 589-596.

Baqar, S., Hayes, C.G., Murphy, J.R., Watts, D.M. (1993) Vertical transmission of West Nile virus by *Culex* and *Aedes* species mosquitoes. The American Journal of Tropical Medicine and Hygiene 48: 757-762.

Bárdoš, V. (1975) The role of mammals in the circulation of Ťahyňa virus. Folia Parasitologica 22: 257-264.

Barzon, L., Squarzon, L., Cattai, M., Franchin, E., Pagni, S., Cusinato, R., Palu, G. (2009) West Nile virus infection in Veneto region, Italy, 2008-2009. Eurosurveillance 14: pii=19289.

Bates, M. (1940) The nomenclature and taxonomic status of the mosquitoes of the *Anopheles maculipennis* complex. Annals of the Entomological Society of America 33: 343-356.

Becker, N. (1997) Microbial control of mosquitoes: Management of the Upper Rhine mosquito population as a model programme. Parasitology Today 13: 485-487.

Becker, N., Geier, M., Balczun, C., Bradersen, U., Huber, K., Kiel, E., Krüger, A., Lühken, R., Orendt, C., Plenge-Bönig, A., Rose, A., Schaub, G.A., Tannich, E. (2013) Repeated introduction of *Aedes albopictus* into Germany, July to October 2012. Parasitology Research 112: 1787-1790.

Becker, N., Hoffmann, D. (2011) First record of *Culiseta longiareolata* (Macquart) for Germany. European Mosquito Bulletin 29: 143-150.

Becker, N., Huber, K., Pluskota, B., Kaiser, A. (2011) *Ochlerotatus japonicus japonicus* – a neozoon in Germany and a revised list of the German mosquito fauna. European Mosquito Bulletin 9: 88-102.

Becker, N., Jöst, H., Ziegler, U., Eiden, M., Höper, D., Emmerich, P., Fichet-Calvet, E., Ehichioya, D.U., Czajka, C., Gabriel, M, Hoffmann, B., Beer, M., Tenner-Racz, K., Racz, P., Günther, S., Wink, M., Bosch, S., Konrad, A., Pfeffer, M., Groschup, M.H., Schmidt-Chanasit, J. (2012) Epizootic emergence of Usutu virus in wild and captive birds in Germany. PLoS ONE 7: e32604.

Becker, N., Petrić, D., Zgomba, M., Boase, C., Madon, M., Dahl, C., Kaiser, A. (2010) Mosquitoes and their Control, 2nd Ed. Berlin Heidelberg, Germany: Springer-Verlag: 577 pp.

Beerntsen, B.T., James, A.A., Christensen, B.M. (2000) Genetics of mosquito vector competence. Microbiology and Molecular Biology Reviews 64: 115-137.

Beletskaya, G.V., Alekseev, A.N. (1988) Experimental demonstration of the Batai virus maintenance in hibernating female *Anopheles maculipennis* mosquitoes. Meditsinskaia Parazitologiia i Parazitarnye Bolezni 66: 23-27.

Bezzhonova, O.V., Goryacheva, I.I. (2008) Intragenomic heterogeneity of rDNA internal transcribed spacer 2 in *Anopheles messeae* (Diptera: Culicidae). Journal of Medical Entomology 45: 337-341.

Bhardwaj, N. (2013) Rift valley fever; an emerging viral zoonoses. Advances in Animal and Veterinary Sciences 1: 47-52.

Bocková, E., Rudolf, I., Kočišová, A., Betášová, L., Venclíková, K., Mendel, J., Hubálek, Z. (2013) *Dirofilaria repens* microfilariae in *Aedes vexans* mosquitoes in Slovakia. Parasitology Research 112: 3465-3470.

Böhm, L.K., Supperer, R. (1955) Untersuchungen über Setarien (Nematoda) bei heimischen Wiederkäuern und deren Beziehung zur „epizootischen cerebrospinalen Nematodiasis" (Setariosis). Zeitschrift für Parasitenkunde 17: 165-174.

Bosch, S., Schmidt-Chanasit, J., Fiedler, W. (2012) Usutu virus as cause of mass mortality in blackbirds *Turdus merula* and other bird species in Europe: experiences from five outbreaks between 2001 and 2011. Vogelwarte 50: 109-122.

Bruce-Chwatt, L.J., de Zulueta, J. (1980) The Rise and Fall of Malaria in Europe. A historico-epidemiological Study. Oxford, Great Britain: Oxford University Press: 240 pp.

Busquets, N., Alba, A., Allepuz, A., Aranda, C., Núñez, J.I. (2008) Usutu virus sequences in *Culex pipiens* (Diptera: Culicidae), Spain. Emerging Infectious Diseases 14: 861-863.

Büttner, K. (1978) Untersuchungen zur Parasitierung des Rehwildes bei steigendem Jagddruck. Zeitschrift für Jagdwissenschaft 24: 139-155.

Calistri, P., Giovannini, A., Hubálek, Z., Ionescu, A., Monaco, F., Savini, G., Lelli, R. (2010) Epidemiology of West Nile in Europe and in the Mediterranean basin. Virology Journal 4: 29-37.

Calzolari, M., Bonilauri, P., Bellini, R., Caimi, M., Defilippo, F., Maioli, G., Albieri, A., Medici, A., Veronesi, R., Pilani, R., Gelati, A., Angelini, P., Parco, V., Fabbi, M., Barbieri, I., Lelli, D., Lavazza, A., Cordioli, P., Dottori, M. (2010) Arboviral survey of mosquitoes in two northern Italian regions in 2007 and 2008. Vector-Borne and Zoonotic Diseases 10: 875-884.

Calzolari, M., Gaibani, P., Bellini, R., Defilippo, F., Pierro, A., Alvieri, A., Maioli, G., Luppi, A., Rossini, G., Balzani, A., Tamba, M., Galletti, G., Gelati, A., Carrieri, M., Poglayen, G.,

Cavrini, F., Natalini, S., Dottori, M., Sambri, V., Angelini, P., Bonilauri, P. (2012a) Mosquito, bird and human surveillance of West Nile and Usutu viruses in Emilia-Romagna Region (Italy) in 2010. PLoS ONE 7: e38058.

Calzolari, M., Ze-Ze, L., Ruzek, D., Vazquez, A., Jeffries, C., Defilippo, F., Costa Osorio, H., Kilian, P., Ruiz, S., Fooks, A.R., Maioli, G., Amaro, F., Tlusty, M., Figuerola, J., Medlock, J.M., Bonilauri, P., Alves, M.J., Šebesta, O., Tenorio, A., Vaux, A.G., Bellini, R., Gelbič, I., Sánchez-Seco, M.P., Johnson, N., Dottori, M. (2012b) Detection of mosquito-only flaviviruses in Europe. Journal of General Virology 93: 1215-1225.

Cancrini, G., Frangipane Di Regalbono, A., Ricci, I., Tessarin, C., Gabrielli, S., Pietrobelli, M. (2003) *Aedes albopictus* is a natural vector of *Dirofilaria immitis* in Italy. Veterinary Parasitology 118: 195-202.

Cancrini, G., Magi, H., Gabrielli, S., Arispici, M., Tolari, F., Dell'Omodarme, M., Prati, M.C. (2006) Natural vectors of dirofilariasis in rural and urban areas of the Tuscan region, central Italy. Journal of Medical Entomology 43: 574-579.

Cancrini, G., Pietrobelli, M., Frangipane Di Regalbono, A., Tampieri, M.P. (1997) Mosquitoes as vectors of *Setaria labiatopapillosa*. International Journal for Parasitology 27: 1061-1064.

Cancrini, G., Scaramozzino, P., Gabrielli, S., Di Paolo, M., Toma, L., Romi, R. (2007) *Aedes albopictus* and *Culex pipiens* implicated as natural vectors of *Dirofilaria repens* in central Italy. Journal of Medical Entomology 44: 1064-1066.

Casiraghi, M., Anderson, T.J.C., Bandi, C., Bazzocchi, C., Genchi, C. (2001) A phylogenetic analysis of filarial nematodes: comparison with the phylogeny of *Wolbachia* endosymbionts. Parasitology 122: 93-103.

Cavrini, F., Della Pepa, M.E., Gaibani, P., Pierro, A.M., Rossini, G., Landini, M.P., Sambri, V. (2011) A rapid and specific real-time RT-PCR assay to identify Usutu virus in human plasma, serum, and cerebrospinal fluid. Journal of Clinical Virology 50: 221-223.

Cavrini, F., Gaibani, P., Longo, G., Pierro, A.M., Rossini, G., Bonilauri, P., Gerunda, G.E., Di Benedetto, F., Pasetto, A., Girardis, M., Dottori, M., Landini, M.P., Sambri, V. (2009) Usutu virus infection in a patient who underwent orthotropic liver transplantation, Italy, August-September 2009. Eurosurveillance 14: pii=19448.

Chevalier, V., Pépin, M., Plée, L., Lancelot, R. (2010) Rift Valley fever - a threat for Europe? Eurosurveillance 15: pii=19506.

Chvala, S., Bakonyi, T., Bukovsky, C., Meister, T., Brugger, K., Rubel, F., Nowotny, N., Weissenböck, H. (2007) Monitoring of Usutu virus activity and spread by using dead bird surveillance in Austria, 2003-2005. Veterinary Microbiology 122: 237-245.

Chvala, S., Kolodziejek, J., Nowotny, N., Weissenböck, H. (2004) Pathology and viral distribution in fatal Usutu virus infections of birds from the 2001 and 2002 outbreaks in Austria. Journal of Comparative Pathology 131: 176-185.

Cielecka, D., Żarnowska-Prymek, H., Masny, A., Sałamatin, R.V., Wesołowska, M., Gołąb, E. (2012) Dirofilariosis in Poland: the first autochthonous infections with *Dirofilaria repens*. Annals of Agricultural and Environmental Medicine 19: 445-450.

Clements, A.N. (2012) The Biology of Mosquitoes. Transmission of Viruses and Interaction with Bacteria, Volume 3. Cambridge, USA: CABI: 572 pp.

Collins, F.H., Paskewitz, S.M. (1996) A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. Insect Molecular Biology 5: 1-9.

Cook, G.C. (1994) Manson's demonstration of the malaria parasite 100 years ago: the major stimulus of Ross' discovery? Journal of Infection 28: 333-334.

Cook, S., Bennett, S.N., Holmes, E.C., De Chesse, R., Moureau, G., de Lamballerie, X. (2006) Isolation of a new strain of the flavivirus cell fusing agent virus in a natural mosquito population from Puerto Rico. Journal of General Virology 87: 735-748.

Cook, S., Chung, BY-W., Bass, D., Moureau, G., Tang, S., McAlister, E., Culverwell, C.L., Glücksman, E., Wang, H., Brown, T.D.K., Gould, E.A., Harbach, R.E., de Lamballerie, X., Firth, A.E. (2013) Novel virus discovery and genome reconstruction from field RNA samples reveals highly divergent viruses in dipteran hosts. PLoS ONE 8: e80720.

Crochu, S., Cook, S., Attoui, H., Charrel, R.N., De Chesse, R., Belhouchet, M., Lemasson, J.J., de Micco, P., de Lamballerie, X. (2004) Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of *Aedes* spp. mosquitoes. Journal of General Virology 85: 1971-1980.

Cuadros, J., Calvente, M.J., Benito, A., Arevalo, J., Calero, M.A., Segura, J., Rubio, J.M. (2002) *Plasmodium ovale* malaria acquired in central Spain. Emerging Infectious Diseases 8: 1506-1508.

Czajka, C., Becker, N., Jöst, H., Poppert, S., Schmidt-Chanasit, J., Krüger, A., Tannich, E. (2014) Stable transmission of *Dirofilaria repens* nematodes, northern Germany. Emerging Infectious Diseases 20: 329-331.

Czajka, C., Becker, N., Poppert, S., Jost, H., Schmidt-Chanasit, J., Krüger, A. (2012) Molecular detection of *Setaria tundra* (Nematoda: Filarioidea) and an unidentified filarial species in mosquitoes in Germany. Parasites & Vectors 5: 14.

Dahl, C., Kaiser, A., Becker, N. (1999) Culicidae. In: Schuhmann H, Bährmann R, Stark A (eds.), Entomofauna Germanica. Checkliste der Dipteren Deutschlands. Studia Dipterologica 2.

Danabalan, R., Monaghan, M.T., Ponsonby, D.J., Linton, Y.M. (2013) Occurrence and host preferences of *Anopheles maculipennis* group mosquitoes in England and Wales. Medical and Veterinary Entomology 28: 169-178.

Danis, K., Baka, A., Lenglet, A., Van Bortel, W., Terzaki, I., Tseroni, M., Detsis, M., Papanikolaou, E., Balaska, A., Gewehr, S., Dougas, G., Sideroglou, T., Economopoulou, A., Vakalis, N., Tsiodras, S., Bonovas, S., Kremastinou, J. (2011a) Autochthonous *Plasmodium vivax* malaria in Greece, 2011. Eurosurveillance 16: pii=19993.

Danis, K., Papa, A., Papanikolaou, E., Dougas, G., Terzaki, I., Baka, A., Vrioni, G., Kapsimali, V., Tsakris, A., Kansouzidou, A., Tsiodras, S., Vakalis, N., Bonovas, S., Kremastinou, J. (2011c) Ongoing outbreak of West Nile virus infection in humans, Greece, July to August, 2011. Eurosurveillance 16: pii=19951.

Danis, K., Papa, A., Theocharopoulos, G., Dougas, G., Athanasiou, M., Detsis, M., Baka, A., Lytras, T., Mellou, K., Bonovas, S., Panagiotopoulos, T. (2011b) Outbreak of West Nile virus infection in Greece, 2010. Emerging Infectious Diseases 17: 1868-1872.

Dashkova, N.G. (1977) New data on susceptibility of *Anopheles* mosquitoes of the U.S.S.R. fauna to imported strains of the causative agents of human malaria. Medicinskaja Parazitilogija i Parazitarnye Bolezni 46: 652-657.

DeFoliart, G.R., Grimstad, P.R., Watts, D.M. (1987) Advances in mosquito-borne arbovirus/vector research. Annual Review of Entomology 32: 479-505.

Dekoninck, W., Hendrickx, F., Van Bortel, W., Versteirt, V., Coosemans, M., Damiens, D., Hance, T., De Clercq, E.M., Hendrickx, G., Schaffner, F., Grootaert, P. (2011) Human-Induced expanded distribution of *Anopheles plumbeus*, experimental vector of West Nile virus and a potential vector of human malaria in Belgium. Journal of Medical Entomology 48: 924-928.

Del Giudice, P., Schuffenecker, I., Vandenbos, F., Evelyne, C., Zeller, H. (2004) Human West Nile virus, France. Emerging Infectious Diseases 10: 1885-1886.

Demiaszkiewicz, A.W., Polańczyk, G., Pyziel, A.M., Kuligowska, I., Lachowicz, J. (2009) The first foci of dirofilariosis of dogs evoked by *Dirofilaria repens* Railliet et Henry, 1911 in central Poland. Wiadomości Parazytologiczne 55: 367-370.

De Zulueta, J. (1994) Malaria and ecosystems: from prehistory to posteradication. Parassitologia 36: 7-15.

De Zulueta, J., Ramsdale, C.D., Coluzzi, M. (1975) Receptivity to malaria in Europe. Bulletin of the World Health Organization 52: 109-111.

Di Luca, M., Boccolini, D., Marinucci, M., Romi, R. (2004) Intrapopulation polymorphism in *Anopheles messeae* (*An. maculipennis* complex) inferred by molecular analysis. Journal of Medical Entomology 41: 582-586.

Dobson, M.J. (1994) Malaria in England: A geographical and historical perspective. Parassitologia 36: 35-60.

Dorak, T. (2006) Real-time PCR. USA, New York: Taylor & Francis Group: 333 pp.

Doudier, B., Bogreau, H., DeVries, A., Ponçon, N., Stauffer, W.M., Fontenille, D., Rogier, C., Parola, P. (2007) Possible autochthonous malaria from Marseille to Minneapolis. Emerging Infectious Diseases 13: 1236-1238.

Duscher, G., Feiler, A., Wille-Piazzai, W., Bakonyi, T., Leschnik, M., Miterpáková, M., Kolodziejek, J., Nowotny, N., Joachim, A. (2009) Nachweis von Dirofilarien in österreichischen Hunden. Berliner und Münchener Tierärztliche Wochenschrift 122: 199-203.

ECDC (2014) Health Topics A-Z. Sindbis. (www.ecdc.europa.eu/en/healthtopics/Pages/health_topics_A_Z.aspx, accessed 19 February 2014).

Eichenlaub, D. (1979) Malaria in Deutschland. Bundesgesundheitsblatt 22: 8-13.

Eiden, M., Ziegler, U., Keller, M., Müller, K., Granzow, H., Jöst, H., Schmidt-Chanasit, J., Groschup, M.H. (2014) Vector-Borne and Zoonotic Diseases 14: 220-222.

EFSA, ECDC (2014) The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012. EFSA Journal 12: 3547.

Eling, W., van Gemert, G-J., Akinpelu, O., Curtis, J., Curtis, C.F. (2003) Production of *Plasmodium falciparum* sporozoites by *Anopheles plumbeus*. European Mosquito Bulletin 15: 12-13.

Engler, O., Savini, G., Papa, A., Figuerola, J., Groschup, M.H., Kampen, H., Medlock, J., Vaux, A., Wilson, A.J., Werner, D., Jöst, H., Goffredo, M., Capelli, G., Federici, V., Tonolla, M., Patocchi, N., Flacio, E., Portmann, J., Rossi-Pedruzzi, A., Mourelatos, S., Ruiz, S., Vázquez, A., Calzolari, M., Bonilauri, P., Dottori, M., Schaffner, M., Mathis, A., Johnson, N. (2013) European surveillance for West Nile virus in mosquito populations. International Journal of Environmental Research and Public Health 10: 4869-4895.

Eritja, R., Escosa, R., Kucientes, J., Marquès, E., Molina, R., Roiz, D., Ruiz, S. (2005) Worldwide invasion of vector mosquitoes: present European distribution and challenges for Spain. Biological Invasions 7: 87-97.

Fantini, B. (1994) Anophelism without malaria: an ecological and epidemiological puzzle. Parassitologia 36: 83-106.

Filipe, A.R. (1972) Isolation in Portugal of West Nile virus from *Anopheles maculipennis* mosquitoes. Acta Virologica 16: 361.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294-299.

Fontenille, D., Diallo, M., Mondo, M., Ndiaye, M., Thonnon, J. (1997) First evidence of natural vertical transmission of yellow fever virus in *Aedes aegypti*, its epidemic vector. Transactions of the Royal Society of Tropical Medicine and Hygiene 91: 533-535.

Frank, C., Faber, M., Hellenbrand, W., Wilking, H., Stark, K. (2014) Wichtige, durch Vektoren übertragene Infektionskrankheiten beim Menschen in Deutschland. Bundesgesundheitsblatt 57: 557-567.

Gaibani, P., Cavrini, F., Gould, E.A., Rossini, G., Pierro, A., Landini, M.P., Sambri, V. (2013) Comparative genomic and phylogenetic analysis of the first Usutu virus isolate from a human patient presenting with neurological symptoms. PLoS ONE 8: e64761.

Gärtner, H.-G. (1973) Neutralisierende Serumantikörper gegen Calovo-Virus bei der Bevölkerung Süddeutschlands. Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene, I. Abteilung, Originale A 225: 135-138.

Genchi, C., Bowman, D., Drake, J. (2014) Canine heartworm disease (*Dirofilaria immitis*) in Western Europe: survey of veterinary awareness and perceptions. Parasites & Vectors 7: 206.

Genchi, C., Kramer, L.H., Rivasi, F. (2011a) Dirofilarial infections in Europe. Vector-Borne and Zoonotic Diseases 11: 1307-1317.

Genchi, C., Mortarino, M., Rinaldi, L., Cringoli, G., Traldi, G., Genchi, M. (2011b) Changing climate and changing vector-borne disease distribution: the example of *Dirofilaria* in Europe. Veterinary Parasitology 176: 295-299.

Gjenero-Margan, I., Aleraj, B., Krajcar, D., Lesnikar, V., Klobučar, A., Pem-Novosel, I., Kurečić-Filipović, S., Komparak, S., Martić, R., Đuričić, S., Betica-Radić, L., Okmadžić, J., Vilibić-Čavlek, T., Babić-Erceg, A., Turković, B., Avšić-Županc, T., Radić, I., Ljubić, M., Šarac, K., Benić, N., Mlinarić-Galinović, G. (2011) Autochthonous dengue fever in Croatia, August-September 2010. Eurosurveillance 16: pii=19805.

Goddard, L.B., Roth, A.E., Reisen, W.K., Scott, T.W. (2003) Vertical transmission of West Nile virus by three California *Culex* (Diptera: Culicidae) species. Journal of Medical Entomology 40: 743-746.

Gould, E.A., Gallian, P., De Lamballerie, X., Charrel, R.N. (2010) First cases of autochthonous dengue fever and chikungunya fever in France: from bad dream to reality? Clinical Microbiology and Infection 12: 1702-1704.

Gordeev, M., Zsantsov, A., Goriacheva, I., Shaikevich, E., Bezzhonova, O. (2008) Mosquitoes of the genus *Anopheles* in countries of the WHO European Region having faced a recent resurgence of malaria. Copenhagen, Denmark: WHO Regional Office for Europe: 26 pp.

Gordeev, M.I., Zvantsov, A.B., Goriacheva, I.I., Shaĭkevich, E.V., Ezhov, M.N. (2005) Description of the new species *Anopheles artemievi* sp.n. (Diptera, Culicidae). Meditsinskaia Parazitologiia i Parazitarnye Bolezni 2: 4-5.

Grandadam, M., Caro, V., Plumet, S., Thiberge, J.M., Souarès, Y., Failloux, A.B., Tolou, H.J., Budelot, M., Cosserat, D., Leparc-Goffart, I., Desprès, P. (2011) Chikungunya virus, southeastern France. Emerging Infectious Diseases 17: 910-913.

Gratz, N.G. (1999) Emerging and resurging vector-borne diseases. Annual Review of Entomology 44: 51-75.

Gratz, N.G. (2004) The mosquito-borne infections of Europe. European Mosquito Bulletin 17: 1-7.

Gresiková, M., Sekeyova, M., Tempera, G., Guglielmino, S., Castro, A. (1978) Identification of a Sindbis virus strain isolated from *Hyalomma marginatum* ticks in Sicily. Acta Virologica 22: 231-232.

Grober, J.A. (1903) Die deutsche Malaria. Naturwissenschaftliche Wochenschrift 18: 601-603.

Hackett, L.W. (1934) The present status of our knowledge of the subspecies of *Anopheles maculipennis*. Transactions of the Royal Society of Tropical Medicine and Hygiene 28: 109-128.

Hackett, L.W., Martini, E., Missiroli, A. (1932) The races of *Anopheles maculipennis*. American Journal of Hygiene 16: 137-162.

Hackett, L.W., Missiroli, A. (1935) The varieties of *Anopheles maculipennis* and their relation to the distribution of malaria in Europe. Rivista di Malariologia 14: 45-109.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95-98.

Halouzka, J., Pejčoch, M., Hubálek, Z., Knoz, J. (1991) Isolation of Tahyna virus from biting midges (Diptera: Ceratopogonidae) in Czechoslovakia. Acta Virologica 35: 247-251.

Harbach, R.E. (2004) The classification of genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. Bulletin of Entomological Research 94: 537-553.

Harbach, R.E. (2014) Mosquito Taxonomic Inventory. (http://mosquito-taxonomic-inventory.info, accessed 06 February 2014).

Harizanov, R.N., Jordanova, D.P., Bikov, I.S (2014) Some aspects of the epidemiology, clinical manifestations, and diagnosis of human dirofilariasis caused by *Dirofilaria repens*. Parasitology Research 113: 1571-1579.

Hebert, P.D., Ratnasingham, S., de Waard, J.R. (2003) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings. Biological Sciences 270(Suppl 1): 96-99.

Hermosilla, C., Pantchev, N., Dyachenko, V., Gutmann, M., Bauer, C. (2006) First autochthonous case of canine ocular *Dirofilaria repens* infection in Germany. Veterinary Record 158: 134-135.

Hoffmann, B., Depner, K., Schirrmeier, H., Beer, M. (2006) A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. Journal of Virological Methods 136: 200-209.

Hubálek, Z. (2008) Mosquito-borne viruses in Europe. Parasitology Research 130 (Suppl 1): 29-43.

Huber, K., Pluskota, B., Jöst, A., Hoffmann, K., Becker, N. (2012) Status of the invasive species *Aedes japonicus japonicus* (Diptera: Culicidae) in southwest Germany in 2011. Journal of Vector Ecology 37: 462-465.

Iglódyová, A., Miterpáková, M., Hurníková, Z., Antolová, D., Dubinský, P., Letková, V. (2012) Canine dirofilariosis under specific environmental conditions of the eastern Slovak Lowland. Annals of Agricultural and Environmental Medicine 19: 57-60.

Jacsó, O., Mándoki, M., Majoros, G., Pétsch, M., Mortarino, M., Genchi, C., Fok, É. (2009) First autochthonous *Dirofilaria immitis* (Leidy, 1856) infection in a dog in Hungary. Helminthologia 46: 159-161.

Jetten, T.H., Takken, W. (eds.) (1994) Anophelism without malaria in Europe: a review of the ecology and distribution of the genus *Anopheles* in Europe. Wageningen, The Netherlands: Agricultural University Papers 94-5: 69 pp.

Jöst, H, Bialonski, A., Maus, D., Sambri, V., Eiden, M., Groschup, M.H., Günther, S., Becker, N., Schmidt-Chanasit, J. (2011b) Isolation of Usutu virus in Germany. The American Journal of Tropical Medicine and Hygiene 85: 551-553.

Jöst, H., Bialonski, A., Schmetz, C., Günther, S., Becker, N., Schmidt-Chanasit, J. (2011a) Short report: Isolation and phylogenetic analysis of Batai virus, Germany. The American Journal of Tropical Medicine and Hygiene 84: 241-243.

Jöst, H., Bialonski, A., Storch, V., Günther, S., Becker, N., Schmidt-Chanasit, J. (2010) Isolation and phylogenetic analysis of Sindbis viruses from mosquitoes in Germany. Journal of Clinical Microbiology 48: 1900-1903.

Kampen, H. (2005) Integration of *Anopheles beklemishevi* (Diptera: Culicidae) in a PCR assay diagnostic for palaearctic *Anopheles maculipennis* sibling species. Parasitology Research 97: 113-117.

Kampen, H., Kronefeld, M., Werner, D. (2012a) Culicid mosquitoes as vectors of disease agents in Europe. In: Mehlhorn, H. (ed.), Arthropods as Vectors of Emerging Diseases. Parasitology Research Monographs 3. Berlin Heidelberg, Germany: Springer-Verlag: 1-30.

Kampen, H., Kronefeld, M., Zielke, D., Werner, D. (2013a) Three rarely encountered and one new *Culiseta* species (Diptera: Culicidae) in Germany. Journal of the European Mosquito Control Association 31: 36-39.

Kampen, H., Kronefeld, M., Zielke, D., Werner, D. (2013b) Further specimens of the Asian tiger mosquito *Aedes albopictus* (Diptera, Culicidae) trapped in southwest Germany. Parasitology Research 112: 905-907.

Kampen, H., Maltezos, E., Pagonaki, M., Hunfeld, K.P., Maier, W.A., Seitz, H.M. (2002) Individual cases of autochthonous malaria in Evros Province, northern Greece: serological aspects. Parasitology Research 88: 261-266.

Kampen, H., Proft, J., Etti, S., Maltezos, E., Pagonaki, M., Maier, W.A., Seitz, H.M. (2003) Individual cases of autochthonous malaria in Evros Province, northern Greece: entomological aspects. Parasitology Research 89: 252-258.

Kampen, H., Werner, D. (2014) Out of the bush: the Asian bush mosquito *Aedes japonicus japonicus* (Theobald, 1901) (Diptera, Culicidae) becomes invasive. Parasites & Vectors 7: 59.

Kampen, H., Zielke, D., Werner, D. (2012b) A new focus of *Aedes japonicus japonicus* (Theobald, 1901) (Diptera, Culicidae) distribution in western Germany: rapid spread or a further introduction event? Parasites & Vectors 5: 284.

Kilpatrick, A.M., Kramer, L.D., Jones, M.J., Marra, P.P., Daszak, P., Fonseca, D.M. (2007) Genetic Influences on mosquito feeding behavior and the emergence of zoonotic pathogens. The American Journal of Tropical Medicine and Hygiene 77: 667-671.

Kitzmiller, J.B., Frizzi, G., Baker, R.H. (1967) Evolution and speciation within the Maculipennis complex of the genus *Anopheles*. In: Pal, R., Wright, J.W. (eds) Genetics of Insect Vectors of Disease. Amsterdam, London, New York: Elsevier: pp 151-210.

Knuth, T., Liebermann, H., Urbaneck, D. (1990) Für die DDR neuartige Virusinfektionen der Haustiere, 4. Mitteilung: Die Tahyna-Virusinfektion – ein Überblick. Archiv für Experimentelle Veterinärmedizin 44: 265-277.

Kronefeld, M., Dittmann, M., Zielke, D., Werner, D., Kampen, H. (2012) Molecular confirmation of the occurrence in Germany of *Anopheles daciae* (Diptera, Culicidae). Parasites & Vectors 5: 250.

Kronefeld, M., Kampen, H., Sassnau, R., Werner, D. (2014) Molecular evidence for the occurrence of *Dirofilaria immitis*, *Dirofilaria repens* and *Setaria tundra* in mosquitoes from Germany. Parasites & Vectors 7: 30.

Krüger, A., Rech, A., Su, X-Z., Tannich, E. (2001) Two cases of autochthonous *Plasmodium falciparum* malaria in Germany with evidence for local transmission by indigenous *Anopheles plumbeus*. Tropical Medicine and International Health 6: 983-985.

Kurkela, S., Rätti, O., Huhtamo, E., Uzcátegui, N.Y., Nuorti, J.P., Laakkonen, J., Manni, T., Helle, P., Vaheri, A., Vapalahti, O. (2008) Sindbis virus infection in resident birds, migratory birds, and humans, Finland. Emerging Infectious Diseases 14: 41-47.

Kutzer, E., Hinaidy, H.K. (1969) Die Parasiten der wildlebenden Wiederkäuer Österreichs. Zeitschrift für Parasitenkunde 32: 354-368.

Laaksonen, S. (2010) *Setaria tundra*, an emerging parasite of reindeer, and an outbreak it caused in Finland in 2003-2006. Finland: Ph.D. thesis, University of Helsinki.

Laaksonen, S., Kuusela, J., Nikander, S., Nylund, M., Oksanen, A. (2007) Outbreak of parasitic peritonitis in reindeer in Finland. The Veterinary Record 160: 835-841.

Laaksonen, S., Oksanen, A. (2009) Status and review of the vector-borne nematode *Setaria tundra* in Finnish cervids. Alces 45: 81-84.

Laaksonen, S., Solismaa, M., Kortet, R., Kuusela, J., Oksanen, A. (2009) Vectors and transmission dynamics for *Setaria tundra* (Filarioidea; Onchocercidae), a parasite of reindeer in Finland. Parasites & Vectors 2: 3.

La Ruche, G., Souarès, Y., Armengaud, A., Peloux-Petiot, F., Delaunay, P., Desprès, P., Lenglet, A., Jourdain, F., Leparc-Goffart, I., Charlet, F., Ollier, L., Mantey, K., Mollet, T., Fournier, J.P., Torrents, R., Leitmeyer, K., Hilairet, P., Zeller, H., Van Bortel, W., Dejour-Salamanca, D., Grandadam, M., Gastellu-Etchegorry, M. (2010) First two autochthonous dengue virus infections in metropolitan France, September 2010. Eurosurveillance 15: pii=19676.

Latrofa, M.S., Montarsi, F., Ciocchetta, S., Annoscia, G., Dantas-Torres, F., Ravagnan, S., Capelli, G., Otrando, D. (2012) Molecular xenomonitoring of *Dirofilaria immitis* and *Dirofilaria repens* in mosquitoes from north-eastern Italy by real-time PCR coupled with melting analysis. Parasites & Vectors 5: 76-82.

Lecová, L., Letková, V. (2009) The prevalence of *Dirofilaria* spp. in domesticated and wild carnivores in Slovakia. Folia Veterinaria 53: 160-161.

LeDuc, J.W. (1979) The ecology of California group viruses. Journal of Medical Entomology 16: 1-17.

Linke, S., Niedrig, M., Kaiser, A., Ellerbrok, H., Müller, K., Müller, T., Conraths, F.J., Mühle, R.U., Schmidt, D., Köppen, U., Bairlein, F., Berthold, P., Pauli, G. (2007) Serologic evidence of West Nile virus infections in wild birds captured in Germany. The American Journal of Tropical Medicine and Hygiene 77: 358-364.

Linton, Y.M., Lee, A.S., Curtis, C. (2005) Discovery of a third member of the Maculipennis group in SW England. European Mosquito Bulletin 19: 5-9.

Linton, Y.M., Smith, L., Koliopoulos, G., Samanidou-Voyadjoglou, A., Zounos, A.K., Harbach, R.E (2003) Morphological and molecular characterisation of *Anopheles (Anopheles) maculipennis* Meigen, type species of the genus and nominotypical member of the Maculipennis complex. Systematic Entomology 28: 39-55.

Lord, C.C., Alto, B.W., Anderson, S.L., Connelly, C.R., Day, J.F., Richards, S.L., Smartt, C.T., Tabachnick, W.J. (2014) Can Horton hear the whos? The importance of scale in mosquito-borne disease. Journal of Medical Entomology 51: 297-313.

Lundström, J.O. (1999) Mosquito-borne viruses in western Europe: A review. Journal of Vector Ecology 24: 1-39.

Lundström, J.O., Pfeffer, M. (2010) Phylogeographic structure and evolutionary history of Sindbis virus. Vector-Borne and Zoonotic Diseases 10: 889-907.

Macini, P., Squintani, G., Finarelli, A.C., Angelini, P., Martini, E., Tamba, M., Dottori, M., Bellini, R., Santi, A., Loli Piccolomini, L., Po, C. (2008) Detection of West Nile virus infection in horses, Italy, September 2008. Eurosurveillance 13: pii=18990.

Madani, T.A., Al-Mazrou, Y.Y., Al-Jeffri, M.H., Mishkhas, A.A., Al-Rabeah, A.M., Turkistani, A.M., Al-Sayed, M.O., Abodahish, A.A., Khan, A.S., Ksiazek, T.G., Shobokshi, O. (2003) Rift Valley fever epidemic in Saudi Arabia: epidemiological, clinical, and laboratory characteristics. Clinical Infectious Diseases 37: 1084-1092.

Magi, M., Calderini, P., Gabrielli, S., Dell'Omodarme, M., Macchioni, F., Prati, M.C., Cancrini, G. (2008) *Vulpes vulpes*: a possible wild reservoir for zoonotic filariae. Vector-Borne and Zoonotic Diseases 8: 249-252.

Maier, W.A. (2004) Das Verschwinden des Sumpffiebers in Europa: Zufall oder Notwendigkeit? Denisia 13: 515-527.

Majori, G., Sabatinelli, G., Kondrachine, A.V. (1999) Re-emerging malaria in the WHO European region: control priorities and constraints. Parassitologia 41: 327-328.

Maltezos, E., Christakidis, D., Nikolaidis, P., Gotsis, N. (1995) Autochthonous malaria in Evros, Greece, during the last two years. Medical Education 3: 41-45.

Manarolla, G., Bakonyi, T., Gallazzi, D., Crosta, L., Weissenböck, H., Dorrestein, G.M., Nowotny, N. (2010) Usutu virus in wild birds in northern Italy. Veterinary Microbiology 141: 159-163.

Manson, P. (1878) On the development of *Filaria sanguinis hominis*, and on the mosquito considered as a nurse. Zoological Journal of the Linnean Society of London 14: 304-311.

Marchand, E., Prat, C., Jeannin, C., Lafont, E., Bergmann, T., Flusin, O., Rizzi, J., Roux, N., Busso, V., Deniau, J., Noel, H., Vaillant, V., Leparc-Goffart, I., Six, C., Paty, M.C. (2013) Autochthonous case of dengue in France, October 2013. Eurosurveillance 18: pii=20661.

Marchant, P, Rling, W., Van Gemert, G.J., Leake, C.J., Curtis, C.F. (1998) Could british mosquitoes transmit falciparum malaria? Parasitology Today 14: 344-345.

Masny, A., Golab, E., Cielecka, D., Sałamatin, R.V. (2013) Vector-borne helminths of dogs and humans - focus on central and eastern parts of Europe. Parasites & Vectors 6: 38.

McCall, J.W., Genchi, C., Kramer, L.H., Guerrero, J., Venco, L. (2008) Heartworm disease in animals and humans. Advances in Parasitology 66: 193-285.

Medlock, J.M., Hansford, K.M., Schaffner, F., Versteirt, V., Hendrickx, G., Zeller, H., Van Bortel, W. (2012) A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options. Vector-Borne and Zoonotic Diseases 12: 435-447.

Meister, T., Lussy, H., Bakonyi, T., Sikutová, S., Rudolf, I., Vogl, W., Winkler, H., Frey, H., Hubálek, Z., Nowotny, N., Weissenböck, H. (2008) Serological evidence of continuing high Usutu virus (Flaviviridae) activity and establishment of herd immunity in wild birds in Austria. Veterinary Microbiology 127: 237-248.

Mircean, V., Dumitrache, M.O., Györke, A., Pantchev, N., Jodies, R., Mihalca, A.D., Cozma, V. (2012) Seroprevalence and geographic distribution of *Dirofilaria immitis* and tick-borne infections (*Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato, and *Ehrlichia canis*) in dogs from Romania. Vector-Borne and Zoonotic Diseases 12: 595-604.

Miterpáková, M., Antolová, D., Hurníková, Z., Dubinský, P., Pavlacka, A., Németh, J. (2010) *Dirofilaria* infections in working dogs in Slovakia. Journal of Helminthology 84: 173-176.

Miterpáková, M., Hurníková, Z., Zaleśny, G., Chovancová, B. (2013) Molecular evidence for the presence of *Dirofilaria repens* in beech marten (*Martes foina*) from Slovakia. Veterinary Parasitology 196: 544-546.

Mohrig W (1969) Die Culiciden Deutschlands - Untersuchungen zur Taxonomie, Biologie und Ökologie der einheimischen Stechmücken. Parasitologische Schriftenreihe 18. Jena, Germany: VEB Gustav Fischer Verlag: 260 pp.

Morchón, R., Bargues, M.D., Latorre, J.M., Melero-Alcíbar, R., Pou-Barreto, C., Mas-Coma, S., Simón, F. (2007) Haplotype H1 of *Culex pipiens* implicated as a natural vector of *Dirofilaria immitis* in an endemic area of Western Spain. Vector-Borne and Zoonotic Diseases 7: 653-658.

Morchón, R., Carretón, E., González-Miguel, J., Mellado-Hernández, I. (2012) Heartworm disease (*Dirofilaria immitis*) and their vectors in Europe - new distribution trends. Frontiers in Physiology 3: 196.

Morens, D.M., Folkers, G.K., Fauci, A.S. (2004) The challenge of emerging and re-emerging infectious diseases. Nature 430: 242-249.

Murgue, B., Murri, S., Zientara, S., Durand, B., Durand, J.P., Zeller, H. (2001) West Nile outbreak in southern France, 2000: the return after 35 years. Emerging Infectious Diseases 7: 692-696.

Nasci, R.S., Savage, H.M., White, D.J., Miller, J.R., Cropp, B.C., Godsey, M.S., Kerst, A.J., Bennett, P., Gottfried, K., Lanciotti, R.S. (2001) West Nile virus in overwintering *Culex* mosquitoes, New York City, 2000. Emerging Infectious Diseases 7: 742-744.

Nicolescu, G., Linton, Y.M., Vladimirescu, A., Howard, T.M., Harbach, R.E. (2004) Mosquitoes of the *Anopheles maculipennis* group (Diptera: Culicidae) in Romania, with the discovery and formal recognition of a new species based on molecular and morphological evidence. Bulletin of Entomological Research 94: 525-535.

Nikolaeva, N. (1996) Resurgence of malaria in the former Soviet Union (FSU). Society For Vector Ecology 27: 10-11.

Nikolay, B., Diallo, M., Boye, C.S., Sall, A.A. (2011) Usutu virus in Africa. Vector-Borne and Zoonotic Diseases 11: 1417-1423.

Orihel, T.C., Eberhard, M.L. (1998) Zoonotic filariasis. Clinical Microbiology Reviews 11: 366-381.

Otranto, D., Capelli, G., Genchi, C. (2009) Changing distribution patterns of canine vector borne diseases in Italy: leishmaniosis vs. dirofilariosis. Parasites & Vectors 2 (Suppl 1): 2.

Otranto, D., Dantas-Torres, F., Brianti, E., Traversa, D., Petric, D., Genchi, C., Capelli, G. (2013a) Vector-borne helminths of dogs and humans in Europe. Parasites & Vectors 6: 16.

Otranto, D., Dantas-Torres, F., Giannelli, A., Latrofa, M.S., Papadopoulos, E., Cardoso, L., Cortes, H. (2013b) Zoonotic *Onchocerca lupi* infection in dogs, Greece and Portugal, 2011-2012. Emerging Infectious Diseases 19: 2000-2003.

Overgaauw, P., van Dijk, E. (2009) Autochthonous case of *Dirofilaria repens* in a dog in the Netherlands. The Veterinary Record 164: 158.

Pampiglione, S., Rivasi, F., Gustinelli, A. (2009) Dirofilarial human cases in the Old World, attributed to *Dirofilaria immitis*: a critical analysis. Histopathology 54: 192-204.

Panaitescu, D., Freda, A., Bain, O., Vasile-Bugarin, A.C. (1999) Four cases of human filariosis due to *Setaria labiatopapillosa* found in Bucharest, Romania. Roumanian Archives of Microbiology and Immunology 58: 203-207.

Panning, M., Grywna, K., van Esbroeck, M., Emmerich, P., Drosten, C. (2006) Chikungunya fever in travelers returning to Europe from the Indian Ocean region. Emerging Infectious Diseases 14: 416-422.

Pantchev, N., Norden, N., Lorentzen, L., Rossi, M., Rossi, U., Brand, B., Dyachenko, V. (2009) Current surveys on the prevalence and distribution of *Dirofilaria* spp. in dogs in Germany. Parasitology Research 105: 63-74.

Papa, A., Xanthopoulou, K., Gewehr, S., Mourelatos, S. (2011) Detection of West Nile virus lineage 2 in mosquitoes during a human outbreak in Greece. Clinical Microbiology and Infection 17: 1176-1180.

Pecorari, M., Longo, G., Gennari, W., Grottola, A., Sabbatini, A.M., Tagliazucchi, S., Savini, G., Monaco, F., Simone, M.L., Lelli, R., Rumpianesi, F. (2009) First human case of Usutu virus neuroinvasive infection, Italy, August-September 2009. Eurosurveillance 14: pii=19446.

Pilaski, J., Mackenstein, H. (1985) Nachweis des Tahyna-Virus in zwei verschiedenen europäischen Naturherden. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, I. Abteilung, Originale B 180: 394-420.

Pluskota, B., Storch, V., Braunbec, T., Beck M., Becker, N. (2008) First record of *Stegomyia albopicta* (Skuse) (Diptera: Culicidae) in Germany. European Mosquito Bulletin 26: 1-5.

Poppert, S., Hodapp, M., Krüger, A., Hegasy, G., Niesen, W.D., Kern, W.V., Tannich, E. (2009) *Dirofilaria repens* infection and concomitant meningoencephalitis. Emerging Infectious Diseases 15: 1844-1846.

Praetorius, F., Altrock, G., Blees, N., Schuh, N., Faulde, M. (1999) Importierte *Anopheles*: im Gepäck oder aus dem Flugzeug? Ein Fall von schwerer autochthoner Malaria tropica in Flughafennähe. Deutsche Medizinische Wochenschrift 124: 998-1002.

Proft, J., Maier, W.A., Kampen, H. (1999) Identification of six sibling species of the *Anopheles maculipennis* complex (Diptera: Culicidae) by a polymerase chain reaction assay. Parasitology Research 85: 837-843.

ProMED-mail (2012) Usutu virus - Germany (03) 1st case. Archive Number: 20120821.1255556. (www.promedmail.org, accessed 28 February 2014).

Putkuri, N., Kurkela, S., Levanov, L., Huhtamo, E., Vaheri, A., Sironen, T., Vapalahti, O. (2014) Isolation and characterization of a California encephalitis serogroup orthobunyavirus from Finnish mosquitoes. Infection, Genetics and Evolution 22: 164-173.

Ramsdale, C.D., Coluzzi, M. (1975) Studies on the infectivity of tropical African strains of *Plasmodium falciparum* to some southern European vectors of malaria. Parassitologia 17: 39-48.

Ramsdale, C.D., Snow, K.R. (2000) Distribution of the genus *Anopheles* in Europe. European Mosquito Bulletin 7: 1-26.

Randolph, S.E., Rogers, D.J (2010) The arrival, establishment and spread of exotic diseases: patterns and predictions. Nature reviews. Microbiology 8: 361-371.

Rappole, J.H., Derrickson, S.R., Hubálek, Z. (2000) Migratory birds and spread of West Nile virus in the western hemisphere. Emerging Infectious Diseases 6: 319-328.

Reed, K.D., Meece, J.K., Henkel, J.S., Shukla, S.K. (2003) Birds, migration and emerging zoonoses: West Nile virus, Lyme disease, influenza A and enteropathogens. Clinical Medicine and Research 1: 5-12.

Rehbein, S., Lutz, W., Visser, M., Winter, R. (2000) Beiträge zur Kenntnis der Parasitenfauna des Wildes in Nordrhein-Westfalen. 1. Der Endoparasitenbefall des Rehwildes. Zeitschrift für Jagdwissenschaft 46: 248-269.

Rehbein, S., Visser, M., Jekel, I., Silaghi, C. (2014) Endoparasites of the fallow deer (*Dama dama*) of the Antheringer Au in Salzburg, Austria. Wiener Klinische Wochenschrift 10: 1-5.

Reinert, J.F. (2000) New classification for the composite genus *Aedes* (Diptera : Culicidae: Aedini), elevation of subgenus *Ochlerotatus* to generic rank, reclassification of the other subgenera, and notes on certain subgenera and species. Journal of the American Mosquito Control Association 16: 175-188.

Reinert, J.F., Harbach, R.E., Kitching, I.J. (2004) Phylogeny and classification of Aedini (Diptera: Culicidae) based on morphological characters of all life stages. Zoological Journal of the Linnean Society 142: 289-368.

Reinert, J.F., Harbach, R.E., Kitching, I.J. (2006) Phylogeny and classification of *Finlaya* and allied taxa (Diptera: Culicidae: Aedini) based on morphological data from all life stages. Zoological Journal of the Linnean Society 142: 1-101.

Reinert, J.F., Harbach, R.E., Kitching, I.J. (2008) Phylogeny and classification of *Ochlerotatus* and allied taxa (Diptera: Culicidae: Aedini) based on morphological data from all life stages. Zoological Journal of the Linnean Society 153: 29-114.

Reinert, J.F., Harbach, R.E., Kitching, I.J. (2009) Phylogeny and classification of tribe Aedini (Diptera: Culicidae). Zoological Journal of the Linnean Society 157: 700-794.

Reiter, P. (2010) Yellow fever and dengue: a threat to Europe?. Eurosurveillance 15: pii=19509.

Rizzo, C., Vescio, F., Declich, S., Finarelli, A.C., Macini, P., Mattivi, A., Rossini, G., Piovesan, C., Barzon, L., Palù, G., Gobbi, F., Macchi, L., Pavan, A., Magurano, F., Ciufolini, M.G., Nicoletti, L., Salmaso, S., Rezza, G. (2009) West Nile virus transmission with human cases in Italy, August-September 2009. Eurosurveillance 14: pii=19353.

RKI (2014) SurvStat. (http://www3.rki.de/SurvStat, accessed: 11 April 2014).

Rocconi, F., Di Tommaso, M., Traversa, D., Palmieri, C., Pampurini, F., Boari, A. (2012) Allergic dermatitis by *Dirofilaria repens* in a dog: clinical picture and treatment. Parasitology Research 111: 493-496.

Rödl, P., Bárdoš, V., Ryba, J. (1979) Experimental transmission of Ťahyňa virus (California group) to wild rabbits (*Oryctolagus cuniculus*) by mosquitoes. Folia Parasitologica 26: 61-64.

Roiz, D., Vazquez, A., Rosso, F., Arnoldi, D., Girardi, M., Cuevas, L., Perez-Pastrana, E., Sanchez-Seco, M.P., Tenorio, A., Rizzoli, A. (2012) Detection of a new insect flavivirus and isolation of *Aedes* flavivirus in Northern Italy. Parasites & Vectors 5: 223.

Rosen, L. (1986) Dengue in Greece in 1972 and 1982 and the pathogenesis of DHF: New data and a different conclusion. The American Journal of Tropical Medicine and Hygiene 35: 642-653.

Rudolf, M., Czajka, C., Börstler, J., Melaun, C., Jöst, H., von Thien, H., Badusche, M., Becker, N., Schmidt-Chanasit, J., Krüger, A., Tannich, E., Becker, S. (2013) First nationwide surveillance of *Culex pipiens* complex and *Culex torrentium* mosquitoes demonstrated the presence of *Culex pipiens* biotype *pipiens/molestus* hybrids in Germany. PLoS ONE 8: e71832.

Sałamatin, R.V., Pavlikovska, T.M., Sagach, O.S., Nikolayenko, S.M., Kornyushin, V.V., Kharchenko, V.O., Masny, A., Cielecka, D., Konieczna-Sałamatin, J., Conn, D.B., Golab, E. (2013) Human dirofilariasis due to *Dirofilaria repens* in Ukraine, an emergent zoonosis: epidemiological report of 1465 cases. Acta Parasitologica 135: 592-598.

Sambri, V., Capobianchi, M., Charrel, R., Fyodorova, M., Gaibani, P., Gould, E., Niedrig, M., Papa, A., Pierro, A., Rossini, G., Varani, S., Vocale, C., Landini, M.P. (2013) West Nile virus in Europe: emergence, epidemiology, diagnosis, treatment, and prevention. Clinical Microbiology and Infection 19: 699-704.

Sane, J., Guedes, S., Ollgren, J., Kurkela, S., Klemets, P., Vapalahti, O., Kela, E., Lyytikainen, O., Nuorti, J.P. (2011) Epidemic Sindbis virus infection in Finland: a population-based case-control study of risk factors. Journal of Infectious Diseases 204: 459-466.

Santa-Olalla Peralta, P., Vazquez-Torres, M.C., Latorre-Fandós, E., Mairal-Claver, P., Cortina-Solano, P., Puy-Azón, A., Adiego Sancho, B., Leitmeyer, K., Lucientes-Curdi, J., Sierra-Moros, M.J. (2010) First autochthonous malaria case due to *Plasmodium vivax* since eradication, Spain, October 2010. Eurosurveillance 15: pii=19684.

Sartori, M., La Terra, G., Pozzoli, G., Franzini, C. (1989) A case of autochthonous malaria in Italy. Scandinavian Journal of Infectious Diseases 21: 357-358.

Sassnau, R., Dyachenko, V., Pantchev, N., Stöckel, F., Dittmar, K., Daugschies, A. (2009) *Dirofilaria-repens*-Befall in einem Schlittenhunde-Rudel im Land Brandenburg. Diagnose und Therapie der kaninen kutanen Dirofilariose. Tierärztliche Praxis 37: 95-101.

Sassnau, R., Kohn, M., Demeler, J., Kohn, B., Müller, E., Krücken, J., von Samson-Himmelstjerna, G. (2013) Is *Dirofilaria repens* endemic in the Havelland district in Brandenburg, Germany? Vector-Borne and Zoonotic Diseases 13: 888-891.

Sassnau, R., Kronefeld, M., Werner, D., Genchi, C., Tannich, E., Czajka, C., Kampen, H. (2014) *Dirofilaria repens* and *Dirofilaria immitis* DNA findings in mosquitoes in Germany: temperature data allow autochthonous extrinsic development. Parasitology Research, doi: 10.1007/s00436-014-3970-1.

Schaffner, F., Angel, G., Geoffroy, B., Hervy, J.P., Rhaiem, A., Brunhes, J. (2001) The Mosquitoes of Europe (CD-Rom). Montpellier: IRD Éditions & EID Méditerrannée.

Schaffner, F., Thiéry, I., Kaufmann, C., Zettor, A., Lengeler, C., Mathis, A., Bourgouin, C. (2012) *Anopheles plumbeus* (Diptera: Culicidae) in Europe: a mere nuisance mosquito or potential malaria vector? Malaria Journal 26: 393.

Schneider, K. (2011) Breeding of *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) 80 km north of its known range in southern Germany. European Mosquito Bulletin 29: 129-132.

Scholte, E.J., Den Hartog, W., Dik, M., Schoelitsz, B., Brooks, M., Schaffner, F., Foussadier, R., Braks, M., Beeuwkes, J. (2010) Introduction and control of three invasive mosquito species in the Netherlands, July-October 2010. Eurosurveillance 15: pii=19710.

Schuffenecker, I., Iteman, I., Michault, A., Murri, S., Frangeul, L., Vaney, M.C., Lavenir, R., Pardigon, N., Reynes, J.M., Pettinelli, F., Biscornet, L., Diancourt, L., Michel, S., Duquerroy, S., Guigon, G., Frenkiel, M., Bréhin, A., Cubito, N., Desprès, P., Kunst, F., Rey, F.A., Zeller, H., Brisse, S. (2006) Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. PLoS Medicine 3: e263.

Sedaghat, M.M., Howard, T., Harbach, R.E. (2009) Morphological study and description of *Anopheles (Anopheles) persiensis*, a member of the Maculipennis Group (Diptera: Culicidae: Anophelinae) in Iran. Journal of Entomological Society of Iran 28: 25-35.

Sérandour, J., Rey, D., Raveton, M. (2006) Behavioural adaptation of *Coquillettidia* (*Coquillettidia*) *richiardii* larvae to underwater life: environmental cues governing plant–insect interaction. Entomologia Experimentalis et Applicata 120: 195-200.

Service, M.W. (2001) The Encyclopedia of Arthropod-transmited Infections of Man and Domesticated Animals, 1th Ed. London, United Kingdom: CABI: 608 pp.

Service, M.W. (2008) Medical Entomology for Students, 4th Ed. Cambridge, United Kingdom: University Press: 306 pp.

Shah, K.V., Johnson, H.N., Rao, T.R. (1960) Isolation of five strains of Sindbis virus in India. The Indian Journal of Medical Research 48: 300-308.

Shute, P.G. (1940) Failure to infect english specimens of *Anopheles maculipennis*, var. *atroparvus*, with certain strains of *Plasmodium falciparum*, of tropical origin. Journal of Tropical Medicine and Hygiene 43: 175-178.

Simón, F., López-Belmonte, J., Marcos-Atxutegi, C., Morchón, R., Martín-Pacho, J.R. (2005) What is happening outside North America regarding human dirofilariasis? Veterinary Parasitology 133: 181-189.

Simón, F., Siles-Lucas, M., Morchón, R., González-Miguel, J., Mellado, I., Carretón, E., Montoya-Alonso, J.A. (2012) Human and animal dirofilariasis: the emergence of a zoonotic mosaic. Clinical Microbiology Reviews 25: 507-544.

Smith, J.L., Fonseca, D.M. (2004) Rapid assay for identification of members of the *Culex* (*Culex*) *pipiens* complex, their hybrids, and other sibling species (Diptera: Culicidae). The American Journal of Tropical Medicine and Hygiene 70: 339-345.

Snow, K.R., Ramsdale, C.D. (2013) Fauna Europaea: Aedini. In: Beuk, P. & Pape, T. (2013) Fauna Europaea: Culicidae. Fauna Europaea version 2.6, http://www.faunaeur.org.

Sousa, C.A., Clairouin, M., Seixas, G., Viveiros, B., Novo, M.T., Silva, A.C., Escoval, M.T., Economopoulou, A. (2012) Ongoing outbreak of dengue type 1 in the Autonomous Region of Madeira, Portugal: preliminary report. Eurosurveillance 17: pii=20333.

Spickler, A.R., Roth, J.A., Galyon, J., Lofstedt, J. (eds.) (2010) Emerging and Exotic Diseases of Animals, 4th Ed. Iowa State University, College of Veterinary Medicine, Ames, USA: Center for Food Security and Public Health and the Institute for International Cooperation in Animal Biologics: 383 pp.

Spieckermann, D., Ackennann, R. (1972) Isolierung von Viren der California-Enzephalitis-Gruppe aus Stechmücken in Nord-Bayern. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, I. Abteilung, Originale A 221: 283-295.

Steinmetz, H.W., Bakonyi, T., Weissenböck, H., Hatt, J.M., Eulenberger, U., Robert, N., Hoop, R., Nowotny, N. (2011) Emergence and establishment of Usutu virus infection in wild and captive avian species in and around Zurich, Switzerland - Genomic and pathologic comparison to other central European outbreaks. Veterinary Microbiology 148: 207-212.

Stiasny, K., Aberle, S.W., Heinz, F.X. (2013) Retrospective identification of human cases of West Nile virus infection in Austria (2009 to 2010) by serological differentiation from Usutu and other flavivirus infections. Eurosurveillance 18: pii=20614.

Svobodová, Z., Svobodová, V., Genchi, C., Forejtek, P. (2006) The first report of autochthonous dirofilariosis in dogs in the Czech Republic. Helminthologia 43: 242-245.

Świątalska, A., Demiaszkiewicz, A.W. (2012) First autochthonous case of *Dirofilaria immitis* invasion in dog in Poland. Życie Weterynaryjne 87: 685-686.

Szénási, Z., Kovács, A.H., Pampiglione, S., Fioravanti, M.L., Kucsera, I., Tánczos, B., Tiszlavicz, L. (2008) Human dirofilariosis in Hungary: an emerging zoonosis in central Europe. Wiener Klinische Wochenschrift 120: 96-102.

Takken, W., Geene, R., Adam, W., Jetten, T.H., van der Velden, J.A. (2002) Distribution and dynamics of larval populations of *Anopheles messeae* and *A. atroparvus* in the delta of the rivers Rhine and Meuse, The Netherlands. AMBIO 31: 212-218.

Takken, W., Kager, P.A., Verhave, J.P. (2007) Will malaria return to North-West Europe? In: Takken, W., Knols, B.J.G. (eds.), Emerging Pests and Vector-Borne Diseases in Europe, Volume 1. Wageningen, The Netherlands: Wageningen Academic Publishers: 500 pp.

Takken, W., Knols, B.G.J. (2007) Epilogue: emerging pests and vector-borne disease are a reality. In: Takken, W., Knols, B.J.G. (eds.), Emerging Pests and Vector-Borne Diseases in Europe, Volume 1. Wageningen, The Netherlands: Wageningen Academic Publishers: 500 pp.

Ţălu, S.D., Ştefănuţ, A.C., Mihalca, A.D., Coroiu, Z. (2012) Subconjunctival infestation with *Setaria*. Helminthologia 49: 119-121.

Tappe, D., Plauth, M., Bauer, T., Muntau, B., Dießel, L., Tannich, E., Herrmann-Trost, P. (2014) A case of autochthonous human *Dirofilaria* infection, Germany, March 2014 . Eurosurveillance 19: pii=20790.

Tomasello, D., Schlagenhauf, P. (2013) Chikungunya and dengue autochthonous cases in Europe, 2007-2012. Travel Medicine and Infectious Disease 11: 274-284.

Tsai, T.F., Popovici, F., Cernescu, C., Campbell, G.L., Nedelcu, N.I. (1998) West Nile encephalitis epidemic in southeastern Romania. Lancet 352: 767-771.

Vázquez, A., Jiménez-Clavero, M.A., Franco, L., Donoso-Mantke, O., Sambri, V., Niedrig, M., Zeller, H., Tenorio, A. (2011b) Usutu virus – potential risk of human disease in Europe. Eurosurveillance 16: pii=19935.

Vázquez, A., Ruiz, S., Herrero, L., Moreno, J., Molero, F., Magallanes, A., Sánchez-Seco, M.P., Figuerola, J., Tenorio, A. (2011a) West Nile and Usutu viruses in mosquitoes in Spain, 2008-2009. The American Journal of Tropical Medicine and Hygiene 85: 178-181.

Vázquez, A., Sánchez-Seco, M.P., Palacios, G., Molero, F., Reyes, N., Ruiz, S., Aranda, C., Marqués, E., Escosa, R., Moreno, J., Figuerola, J., Tenorio, A. (2012). Novel flaviviruses detected in different species of mosquitoes in Spain. Vector-Borne and Zoonotic Diseases 12: 223-229.

Víchová, B., Miterpáková, M., Iglódyová, A. (2014) Molecular detection of co-infections with *Anaplasma phagocytophilum* and/or *Babesia canis canis* in *Dirofilaria*-positive dogs from Slovakia. Veterinary Parasitology 203: 167-172.

Weaver, S.C., Reisen W.K. (2010) Present and future arboviral threats. Antiviral Research 85: 328-345.

Weidmann, M., Rudaz, V., Nunes, M.R., Vasconcelos, P.F., Hufert, F.T. (2003) Rapid detection of human pathogenic orthobunyaviruses. Journal of Clinical Microbiology 41: 3299-3305.

Weissenböck, H., Bakonyi, T., Rossi, G., Mani, P., Nowotny, N. (2013) Usutu virus, Italy, 1996. Emerging Infectious Diseases 19: 274-277.

Weissenböck, H., Chvala-Mannsberger, S., Bakonyl, T., Nowotny, N. (2007) Emergence of Usutu virus in Central Europe: diagnosis, surveillance and epizootiology. In: Takken, W., Knols, B.J.G. (eds.), Emerging Pests and Vector-Borne Diseases in Europe, Volume 1. Wageningen, The Netherlands: Wageningen Academic Publishers: 500 pp.

Weissenböck, H., Hubálek, Z., Bakonyi, T., Nowotny, N. (2010) Zoonotic mosquito-borne flaviviruses: worldwide presence of agents with proven pathogenicity and potential candidates of future emerging diseases. Veterinary Microbiology 140: 271-280.

Weissenböck, H., Kolodziejek, J., Url, A., Lussy, H., Rebel-Bauder, B., Nowotny, N. (2002) Emergence of Usutu virus, an African mosquito-borne flavivirus of the Japanese encephalitis virus group, Central Europe. Emerging Infectious Diseases 8: 652-656.

Weitzel, T., Gauch, C., Becker, N. (2012) Identification of *Anopheles daciae* in Germany through ITS2 sequencing. Parasitology Research 111: 2431-2438.

Werner, D., Kampen, H. (2013) The further spread of *Aedes japonicus japonicus* (Diptera, Culicidae) towards northern Germany. Parasitology Research 112: 3665-3668.

Werner, D., Kronefeld, M., Schaffner, F., Kampen, H. (2012) Two invasive mosquito species, *Aedes albopictus* and *Aedes japonicus japonicus*, trapped in south-west Germany, July to August 2011. Eurosurveillance 17: pii=20067.

Weyer, F. (1934) Über die *Anopheles* Mecklenburgs, insbesondere die Verbreitung der Rassen von *Anopheles maculipennis*. Sitzungsberichte und Abhandlungen der Naturforschenden Gesellschaft zu Rostock 3: 59-75.

Weyer, F. (1938) Die geographische Verbreitung der Rassen von *Anopheles maculipennis* in Deutschland. Zeitschrift für Parasitenkunde 10: 437-463.

Weyer, F. (1948) Die "Rassen" von *Anopheles maculipennis* in Deutschland. Zeitschrift für Parasitenkunde 14: 38-49.

Weyer, F. (1951) Neuere Beobachtungen über *Anopheles* in Deutschland. Tropenmedizin und Parasitologie 2: 367-401.

White, G.B. (1978) Systematic reappraisal of the *Anopheles maculipennis* complex. Mosquito Systematics Journal 10: 13-44.

White G.B. (1982) Malaria vector ecology and genetics. British Medical Journal 38: 207-212.

WHO (2013) World malaria report 2013. Geneva, Switzerland: World Health Organization: 255 pp.

Yildirim, A., Inci, A., Duzlu, O., Biskin, Z., Ica, A., Sahin, I. (2011) *Aedes vexans* and *Culex pipiens* as the potential vectors of *Dirofilaria immitis* in Central Turkey. Veterinary Parasitology 178: 143-147.

Yunicheva, Y.U., Ryabova, T.E., Markovich, N.Y., Bezzhonova, O.V., Ganushkina, L.A., Semenov, V.B., Tarkhov, G.A., Vasilenko, L.E., Guzeeva, T.M., Shevereva, T.V., Sergiev, V.P. (2008) First data on the presence of breeding populations of the *Aedes aegypti* L. mosquito in Greater Sochi and various cities of Abkhazia. Medicinskaja Parazitilogija i Parazitarnye Bolezni 3: 40-43.

Zeller, H., Marrama, L., Sudre, B., Van Bortel, W., Warns-Petit, E. (2013) Mosquito-borne disease surveillance by the European Centre for Disease Prevention and Control. Clinical Microbiology and Infection 19: 693-698.

Zoller, T., Naucke, T.J., May, J., Hoffmeister, B., Flick, H., Williams, C.J., Frank, C., Bergmann, F., Suttorp, N., Mockenhaupt, F.P. (2009) Malaria transmission in non-endemic areas: case report, review of the literature and implications for public health management. Malaria Journal 8: 71.

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II. ABBREVIATIONS

°C	Degrees centigrade
µg	microgram
µl	microlitre
µM	micromolar
28S	rRNA species in the large eukaryotic ribosomal subunit
28S	universal primer in ITS2 PCR
5.8S	rRNA species in the large eukaryotic ribosomal subunit
5.8S-UN	universal primer in ITS2 PCR
A	Adenin
AAT	species-specific primer for *An. atroparvus* in ITS2 PCR
ADA	species-specific primer for *An. daciae* in ITS2 PCR
AE	elution buffer for genomic DNA preparations
Ae.	*Aedes*
ALA	species-specific primer for *An. labranchiae* in ITS2 PCR
AMA	species-specific primer for *An. maculipennis* in ITS2 PCR
AML	species-specific primer for *An. melanoon* in ITS2 PCR
AMS	species-specific primer for *An. messeae* in ITS2 PCR
An.	*Anopheles*
ASA	species-specific primer for *An. sacharovi* in ITS2 PCR
ATL	tissue lysis buffer for use in purification of nucleic acids
BATV	Batai virus
bp	basepair
C	Cytosin
CO_2	carbon dioxide
COI	cytochrome c oxidase subunit I
CPE	cytopathic effect
Cs.	*Culiseta*
Cx.	*Culex*
D.	*Dirofilaria*
DNA	deoxyribonucleic acid
dNTP	nucleoside triphosphate
E	eastern longitude
e.g.	exempli gratia, for example
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EGFP	enhanced Green Fluorescent Protein
et al.	et alii, and others
EU	European Union
FLI	Friedrich-Loeffler-Institut

g	standard gravity
G	Guanin
h	hour
Hz	Hertz
ITS2	internal transcribed spacer 2
L3	metacyclic, infective third larval stage of nematodes
MEM	minimum essential medium
mg	milligram
$MgCl_2$	magnesium chloride
min	minute/s
ml	millilitre
mm	millimetre
mtDNA	mitochondrial deoxyribonucleic acid
N	northern latitude
nm	nanometer
nsP1	nonstructural protein 1
O.	*Onchocerca*
P.	*Plasmodium*
PCR	polymerase chain reaction
qRT-PCR	quantitative reverse-transcriptase polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RVFV	Rift Valley fever virus
s	second/s
S.	*Setaria*
s.l.	sensu lato
SINV	Sindbis virus
spp.	species pluralis, serveral species
S-segment	small RNA segment of virus genome
T	Thymin
TAHV	Tahyna virus
U	Unit
USSR	Union of Soviet Socialist Republics
USUV	Usutu virus
V	Volt
W.	*Wuchereria*
WHO	World Health Organization
WNV	West Nile virus

III. LIST OF PUBLICATIONS

Kronefeld, M., Schaffner, F., Kampen, H., Werner, D. (2014) Gynandromorphism and intersexualism in Culicidae (Diptera: Culicomorpha: Culicidea): description of five individual cases and a literature review. Studia dipterologica 20: 239–253.

Kronefeld, M., Werner, D., Kampen, H. (2014) PCR identification and distribution of *Anopheles daciae* (Diptera, Culicidae) in Germany. Parasitology Research 113: 2079-2086.

Sassnau, R., **Kronefeld, M.**, Werner, D., Genchi, C., Tannich, E., Czajka, C., Kampen, H. (2014) *Dirofilaria repens* and *D. immitis* DNA findings in mosquitoes in Germany: temperature data allow autochthonous extrinsic development. Parasitology Research 113: 3057-3061.

Kampen, H., **Kronefeld, M.**, Zielke, D., Werner, D. (2014) Some new, less frequent and rare mosquito species (Diptera, Culicidae) recently collected in Germany. Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie 19: 123-130.

Kronefeld, M., Kampen, H., Sassnau, R.,Werner, D. (2013) Molecular detection of *Dirofilaria immitis*, *Dirofilaria repens* and *Setaria tundra* in mosquitoes from Germany. Parasites & Vectors 7: 30.

Kampen, H., **Kronefeld, M.**, Zielke, D., Werner, D. (2013) Three rarely encountered and one new *Culiseta* species (Diptera: Culicidae) in Germany. Journal of the European Mosquito Control Association 31: 36-39.

Kampen, H., **Kronefeld, M.**, Zielke, D., & Werner, D. (2013) Further specimens of the Asian tiger mosquito *Aedes albopictus* (Diptera, Culicidae) trapped in southwest Germany. Parasitology Research 112: 905-907.

Kronefeld, M., Dittmann, M., Zielke, D., Werner, D., Kampen, H. (2012) Molecular confirmation of the occurrence in Germany of *Anopheles daciae* (Diptera, Culicidae). Parasites & Vectors 5: 250.

Kampen, H., **Kronefeld, M.**, Werner, D. (2012) Culicid Mosquitoes as Vectors of Disease Agents in Europe. In: Mehhorn, H. (ed.), Arthropods as Vectors of Emerging Diseases. Springer Berlin Heidelberg. Parasitology Research Monographs 3: 1-30.

Werner, D., **Kronefeld, M.**, Schaffner, F., Kampen, H. (2012) Two invasive mosquito species, *Aedes albopictus* and *Aedes japonicus japonicus*, trapped in south-west Germany, July to August 2011. Eurosurveillance 17: pii=20067.

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