

Diversity, ecology and vector potential of mosquito populations (Diptera: Culicidae) in two differentially structured zoological gardens in Germany

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1. Introduction

Mosquitoes are among the most researched arthropods worldwide, not least because of their role as pest species and vectors of disease agents (LANE & CROSSKEY 1993). Though much about the ecology of different mosquito species and transmission cycles of associated pathogens is still unknown. In Germany, mosquito research was neglected for many years (KAMPEN & WERNER 2015). One reason was the elimination of malaria in the 20th century, after which mosquitoes were not considered important vectors anymore. Recently, interest in mosquito research has increased again. One of the triggers was the outbreak of bluetongue in 2006 in Central Europe (MEHLHORN et al. 2008), although not a mosquito-borne but a biting midge-borne disease. Unexpectedly, native biting midges (Ceratopogonidae) of the genus *Culicoides* turned out to be the vectors of the bluetongue virus, which had previously only been known to circulate in Africa and, occasionally, southern Europe (WILSON & MELLOR 2009). This 2006 outbreak impressively demonstrated that native hematophagous insect species had the potential of transmitting disease agents, even if they had never before met with the specific pathogen. Similarly, the outbreak of West Nile fever (WNF) in the USA in 1999 has shown that pathogens can be transported over long distances (LANCIOTTI et al. 1999) and, once introduced, be transmitted by native potential vectors (HAYES et al. 2005). Although in Germany, no large outbreak of a mosquito-associated disease comparable to the WNF epidemic in the USA has occurred so far, mosquito-borne pathogens have repeatedly been detected in recent years in mosquitoes collected throughout the country. These included Batai-, Ťahyňa-, Sindbis- and Usutu viruses as well as filarial nematodes such as *Dirofilaria immitis*, *D. repens* and *Setaria tundra* (KAMPEN & WERNER 2015). Among these, Usutu virus (USUV) or *D. immitis* and *D. repens* are not endemic, but originate from Africa or southern Europe and were accidentally introduced (WILLIAMS et al. 1964; GENCHI et al. 2011a).

In addition to native mosquitoes, which appear to be able to transmit disease agents formerly not known in the country, invasive mosquito species are gaining importance in Germany. Some invasive species have already succeeded in establishing populations and spreading (KAMPEN et al. 2017). The invasive Asian tiger mosquito *Aedes albopictus* and the Asian bush or rock pool mosquito *Ae. japonicus*, for example, are known vectors of mosquito-borne pathogens such as chikungunya virus (CHIKV) or dengue virus (DENV) (GRATZ 2006). Autochthonous cases and outbreaks of chikungunya and dengue fever have been observed in several European countries following the establishment of invasive *Aedes* species (Italy, France, Croatia, Madeira) (TOMASELLO & SCHLAGENHAUF 2013). Should competent vectors

be present and the climatic conditions favourable, transmission of these or other mosquito-borne pathogens would also be conceivable in Germany.

Changes in temporal and spatial distribution of vectors and pathogens are attributed to several factors, including international trade and travel, anthropogenic activities and climate change in general (RANDOLPH & ROGERS 2010). To make accurate risk analyses for Germany, a basic understanding of the ecology of mosquito species is necessary. It is important to know, how diverse mosquito communities in Germany are, how populations change over the season, and if there are vertebrate species preferred by foraging mosquito species as blood hosts. To describe these complex processes and relationships, the heterogeneous environment of a zoological garden is an excellent location for research. Zoological gardens provide ideal living conditions for mosquitoes during their whole life cycle, including numerous breeding and resting sites and a variety of potential blood hosts within a confined area (TUTEN 2011b). The daily interaction of wild animals and zoo animals, humans and mosquitoes is unique in zoos, while findings are transferable to the environment outside the zoo area, since zoos are open systems which can be entered and left arbitrarily at least by humans, mosquitoes and wild native animals.

2. Literature review

2.1 Mosquitoes in zoos

Zoological gardens are defined as park-like grounds for the keeping and exhibition of animal species. Most modern zoos are concerned to create animal enclosures as close to natural habitats as possible. If possible, they present the animals to visitors without fences and bars and without a strict separation of different animal species (SUCHANEK 2012). As a result, zoos have become islands of high biodiversity, not only providing habitats for the kept animals, but also being attractive for wild native animals such as birds and arthropods like mosquitoes. This is of major importance, since 600 million people worldwide visit zoos and aquariums annually (GUSSET & DICK 2011).

Zoos provide numerous breeding sites for mosquitoes, mainly artificial ones, such as plastic containers or rain barrels, but also natural ones such as ponds, tree holes or bromeliads (TUTEN 2011a). Through the close contact between animals and humans in zoos, the accidental entry of a mosquito-borne pathogen, for example by migrating birds, could quickly lead to a disease outbreak (TUTEN 2011b). In addition to WNF and Usutu fever, avian malaria, eastern equine encephalomyelitis and dirofilariasis are well-known diseases occurring in zoos worldwide (ADLER et al. 2011). Affected species are mainly owls and penguins, which are susceptible to West Nile virus (WNV), USUV and avian malaria parasites (LUDWIG et al. 2002; HUIJEN et al. 2007; STEINMETZ et al. 2011). In 2006, however, WNV was also detected in a diseased polar bear, and in 2012 in two orcas in a zoo setting (DUTTON et al. 2009; JETT & VENTRE 2012), demonstrating that unexpected animal species can be infected by mosquito-borne pathogens, too. The isolation of USUV from two captive owls (*Strix nebulosa*) from the Zoological Garden Berlin in 2015 (ZIEGLER et al. 2016) underline the importance of zoos in the research and monitoring of mosquito-borne diseases in Germany.

Ecological studies in zoos regarding the general phenology of Culicidae as well as analysis of blood meal patterns can be decisive for a basic understanding of mosquito biology, transmission sources and pathways of associated pathogens. According to a study on the blood feeding ecology of mosquitoes collected in two zoos in South Carolina, USA, mosquitoes accept both captive and wild animals as well as humans as blood hosts (TUTEN et al. 2012). This emphasizes the risk of pathogen transmission by bridge vectors that have a low host preference and facultatively feed on different taxonomic groups of vertebrates such as birds and humans. The *Culex pipiens* complex which occurs widespread in Germany, contains such indiscriminate blood suckers. Essentially, unattended and often unknown artificial

breeding habitats are the origins of larger mosquito populations in zoos, while water sources designed for zoo animals are usually well monitored and less commonly serve as mosquito larval habitats (TUTEN 2011a).

Zoological gardens also gain importance as epidemiological sentinel stations, not least because diseased animals are discovered much faster in zoos than in nature. Many zoos have established collections of serum and tissue samples and have detailed medical records available that could be beneficial not only for animal health but also for public health (MCNAMARA 2007). Compared to mosquito monitoring outside zoos, monitoring inside zoos by pooling resources is more cost-effective, irrespective of the public and can be done routinely (H. Tuten, University of Illinois, Prairie Research Institute, personal communication). While several zoos in Europe have been studied in more detail, such as the Zuerich Zoo in Switzerland (SCHOENENBERGER et al. 2015) and the London Zoo in England (QUINTAVALLE PASTORINO et al. 2015), pertinent research in Germany is missing.

2.2 Mosquito biology and development

Although water is obligatory for immature mosquito development, life cycle strategies vary between species. Mosquito species can be classified according to the different types of breeding sites they use. One such breeding site classification was presented by LAIRD (1988) who differentiates between above-ground water and subterranean water breeding sites on the one hand and natural and artificial breeding sites on the other hand. This classification provides a good overview about the large range of potential habitats mosquitoes can use for larval development. Among above-ground waters, nine categories are defined:

1. Flowing streams
2. Ponded streams
3. Lake edges
4. Swamps and marshes
5. Shallow permanent ponds
6. Shallow temporary ponds
7. Intermittent ephemeral puddles
8. Natural containers
9. Artificial containers

Subterranean waters are divided into natural and artificial habitats. In a study by YEE et al. (2012) mosquito composition is different between tree-holes (natural container) and car tyres (artificial container), despite both being small and temporary habitats. Typical tree-hole

mosquitoes like *Aedes geniculatus* lay their eggs on the side of tree cavities above the water level, and larval development begins when the water level rises (MARSHALL 1938). Other container breeders are more indiscriminate in their acceptance of larval habitats, using not only natural tree-holes for larval development, but also a variety of small artificial containers such as flower pots, rain water barrels or discarded tyres. General container breeders often occur in the vicinity or right in the middle of human settlements, where they find a variety of breeding opportunities.

Another biological adaptation describes mosquitoes developing in shallow temporary ponds. These mosquitoes lay their eggs on the surface of humid substrates with immature development beginning when the pond fills with water, thus stimulating larval hatching. Some species are adapted to ponds which build after the snow-melt in forests, for example spring species such as *Aedes cataphylla* and *Ae. communis*. Other species like *Aedes vexans* and *Ae. sticticus* prefer floodplains and hatch at higher temperatures during the summer months (BECKER 1989). As a result, different landscape structures can produce different mosquito communities.

Based on the diversity of existing larval habitats, zoological gardens represent a distinct ecosystem for mosquitoes (TUTEN 2011a). In addition to a variety of artificial containers such as used tyres or buckets and bowls, zoos provide ponds, waterfalls and tropical rainforest plants right next to an arctic penguin enclosure. This results in a high diversity of possible mosquito breeding sites within a small space. Regardless of which breeding site is used for immature development, most non-predatory mosquito larvae feed on microorganisms and detritus at the water surface (mainly Anophelinae) or in the whole water column (Culicinae) (MERRITT et al. 1992). An exception are species of the genera *Coquillettidia* and *Mansonia*, which feed in plant root zones.

In laboratory studies, survival rates of mosquito larvae vary considerably. Of *Culex quinquefasciatus* and *Aedes aegypti*, between 3% and 92% survive, depending on species and temperature of the larval habitat (RUEDA et al. 1990).

Adult female and male mosquitoes obtain their energy from floral nectars (FOSTER & HANCOCK 1994), but sometimes also honeydew, plant phloem or damaged and rotting fruit are used as carbohydrate sources (YUVAL 1992). Since female mosquitoes are mainly anautogenous, they have to feed on blood for reproduction. Mating of mosquitoes typically takes place in swarms, but some species also mate on or near vertebrate hosts (TAKKEN et al. 2006). After blood feeding, mosquito females rest at shady sites for egg development. For blood digestion, some mosquito species like *Ae. vexans* withdraw into the vegetation, others

like *Cx. territans* can be found both in the vegetation and in artificial shelter-type habitats (BURKETT-CADENA et al. 2008). Again, zoological gardens are outstanding ecosystems for mosquitoes, since they provide to them a high diversity and density of possible natural and artificial resting sites (TUTEN 2011b).

Mosquitoes can be univoltine, having only one generation per year, or multivoltine with several generations per year. Favourable conditions for mosquito development include high temperatures and high humidity. A mosquito's life span can reach from a few days up to two months, depending on climatic conditions and species (BECKER et al. 2010; CHAVES 2016).

2.3 The German mosquito fauna

Worldwide, 3,555 mosquito species are described, belonging to two subfamilies and 122 genera (HARBACH 2019). Mosquito taxonomy is still under discussion, especially within the genus *Aedes*. The mosquito taxonomy used in this thesis follows WILKERSON et al. (2015), according to the recommendation of REISEN (2016).

In Germany, eight species are described in the subfamily Anophelinae, all belonging to the genus *Anopheles*, and 44 species in the subfamily Culicinae, distributed over five genera (ROBERT et al. 2019): *Aedes* (27 species), *Coquillettidia* (1), *Culex* (6), *Culiseta* (8) and *Uranotaenia* (1).

In addition to the native species, invasive mosquitoes have recently been collected in Germany. These include *Aedes albopictus*, *Ae. japonicus*, *Ae. aegypti*, *Ae. koreicus*, *Ae. berlandi*, *Ae. pulcritarsis*, *Anopheles petragrani* and *Culiseta longiareolata* (KAMPEN et al. 2017). *Aedes japonicus*, *Ae. albopictus* and *An. petragrani* are considered firmly established in Germany, as hibernation has been documented repeatedly (KAMPEN et al. 2017). Overwintering has also been shown for *Ae. koreicus* (PFITZNER et al. 2018; STEINBRINK et al. 2019). For *Cs. longiareolata* it is assumed that the species overwinters in Germany, but ultimate proof is still missing as annual collections do not come from the very same sites (KAMPEN et al. 2017).

2.4 Blood feeding patterns of mosquitoes

In contrast to permanent ectoparasites, mosquitoes do not live on their hosts but have to locate a potential blood source from the distance (SUTCLIFFE 1987). Three different phases of host location were observed by SUTCLIFFE (1987):

1. Intuitive search without targeted orientation to a specific host.
2. Active host location through host-associated stimuli (e.g. carbon dioxide, host evaporations containing ammonia, lactic and fatty acids, visual markers).
3. Attraction following accidental approach into the immediate vicinity of the potential blood source.

The distance from which hosts can be located by female mosquitoes differs, depending on both mosquito and host species, but seems to range from 7 to 30 m (LANE & CROSSKEY 1993). Larger host species or a higher density of potential blood hosts can enhance the distance from which attraction is possible (LANE & CROSSKEY 1993).

The time of the day of host seeking activity varies between species. Some species search for blood hosts primarily during dusk or dawn (crepuscular), others bite mainly during night time (nocturnal) or are active at day time (diurnal). Host preferences of mosquitoes can reach from strictly specialized to one single specific host species to generalists, feeding on any vertebrate host available. In general, four different feeding patterns are distinguished, although meant in a relative and not in an absolute sense (LANE & CROSSKEY 1993):

1. Anthropophagy (preference for human hosts)
2. Zoophagy (preference for non-human hosts)
3. Ornithophagy (preference for avian hosts)
4. Indiscriminative biters (no host preference)

The 'zoophagic' group does not only include mosquitoes that accept non-human mammals as hosts, but also those that feed exclusively on reptiles or amphibians. In fact, there are also mosquito species in mangrove forests using fish as blood source or others using insects like caterpillars as protein sources for egg development (LANE & CROSSKEY 1993), which is why a clear classification of mosquito blood hosts is difficult.

There are external host factors such as body heat, body mass, defensive behaviour or odors in general which could affect host preference (TAKKEN & VERHULST 2013). Furthermore, mosquito feeding patterns can change with the season. In North America, *Culex pipiens* prefers blood meals from avian hosts in spring, but shifts to human hosts in late summer, when birds migrate (KILPATRICK et al. 2006). Therefore, blood meal patterns may be influenced by host availability and not only by active mosquito choices (CHAVES et al. 2010;

SIMPSON et al. 2011). Another explanation for this shift from avian to human hosts in late summer could be caused by interbreeding of *Cx. pipiens* populations or biotypes. While anautogenous populations or biotypes, which occur in spring, feed either on birds or mammals, hybrids use both mammal and bird blood hosts (SPIELMAN 2001; REUSKEN et al. 2010; RIZZOLI et al. 2015). This indiscriminant feeding behaviour of *Cx. pipiens* hybrids can lead to an increased probability of transmission of zoonotic pathogens like WNV to humans (FONSECA et al. 2004), underlining that the analysis of blood feeding patterns is one of the major factors in describing transmission cycles of mosquito-borne pathogens.

Some mosquito species target certain vertebrate species irrespective of relative host abundance (UNNASCH et al. 2006). Second to host species, mosquitoes preferred to feed on nestlings, as their defensive behaviour against biting attacks is less pronounced (UNNASCH et al. 2006). A more broad classification of host selection was provided by BÖRSTLER et al. (2016), who describe two different feeding patterns. The first group of mosquitoes prefers non-human mammals and humans and the second the same two groups of hosts plus birds.

In contrast to the conditions in nature, where potential blood hosts are not permanently available, human and animal populations in zoological gardens are a stable blood source for foraging mosquitoes (ONYIDO et al. 2008). A constant interaction between blood hosts and mosquitoes could elevates the potential of transmission (ONYIDO et al. 2008), since the selection of a blood host is essential for a mosquito-borne pathogen to complete its life cycle. Furthermore, host choice can influence the vertebrate species exposed to mosquito-borne disease agents.

2.5 Public health importance of mosquitoes

2.5.1 Mosquito nuisance

Mosquitoes can become severe nuisances. After a mosquito bite, skin reactions like pain, itching, redness and wheals are common and may be accompanied by allergic reactions (SERVICE 1971). Questionnaire surveys have shown that people are more concerned about mosquitoes as pests than as vectors of pathogens (DICKINSON & PASKEWITZ 2012; FOUMANE et al. 2015). Nuisance species usually develop to high population densities, which are persistent for a longer period of time (RUSSELL 1999). Potential pest species are found in various landscape structures like rural or urban areas and on the coasts (MEDLOCK et al. 2005, 2012). Some species like *Cs. annulata* can become a pest throughout the year (MEDLOCK et al. 2012), other species only during spring or summer months under certain conditions. Spring species associated with snow-melt pools in forests can cause nuisance in wooded habitats

through their diurnal biting behaviour (MEDLOCK & VAUX 2011). Floodwater species such as *Ae. vexans* are serious nuisance pests in summer months in flood plains, with diurnal biting activity at shady places and aggressive biting at dusk (O'MALLEY 1990). Additionally, species formerly not known as nuisance species can become pests when occupying artificial breeding habitats that allow mass development. The tree-hole species *An. plumbeus* formerly not known to cause nuisance, tends to mass occurrence when using abandoned manure pits for immature development (DEKONINCK et al. 2011; HEYM et al. 2017). Since the species aggressively attacks humans, it can become a serious burden for people living near such breeding sources.

In some cases, nuisance can be stopped by sanitation, i.e. removal of the breeding site, as, for example, in the case of *An. plumbeus* mass occurrence (HEYM et al. 2017). Other species such as *Ae. vexans* which do not have a permanent breeding site need to be controlled on a regular basis (BECKER 1997).

Mosquito pests are also common in zoo settings because of the availability of shelter, food and water. Pests in a zoological garden cannot only be a burden for the kept animals, but could also lead to economic losses of the park, as the attractiveness for visitors significantly decreases (NATIONAL RESEARCH COUNCIL (US) COMMITTEE 2004).

2.5.2 Mosquitoes as vectors of diseases

Due to their hematophagous behaviour, mosquitoes are potential vectors of disease agents. In most cases, mosquitoes are biological vectors, being an obligatory host in the pathogen's life cycle (LANE & CROSSKEY 1993). Mosquitoes can be biological vectors for three different groups of pathogens:

1. Viruses (virus)
2. Protozoan parasites (protozoa)
3. Filarial nematodes (metazoa)

Vertebrate-pathogenic bacteria are not known to be biologically transmitted by mosquitoes, but mechanical transmission is possible (MULLEN & DURDEN 2009). The best-known example of mechanical transmission by mosquitoes is the tularemia-causing bacterium *Francisella tularensis* (HOPLA 1974). Contrasting this, *F. tularensis* has been found in unfed adult mosquitoes reared from field-collected larvae in Sweden, suggesting at least transstadial transmission (LUNDSTRÖM et al. 2011).

Developmental cycles of mosquito-borne pathogens include vertebrate and invertebrate hosts, which is why a close adaption between pathogen and vector is necessary for successful

transmission. Infection dynamics are complex and implicate various aspects such as the ability of the pathogen to develop in various host species or environmental factors like rainfall or temperature (UNNASCH et al. 2006).

2.5.2.1 Mosquito-borne viruses

Mosquito-borne viruses replicate in a variety of tissues, including ovaries, nerves and the salivary glands of the vector (MULLEN & DURDEN 2009). Transmission usually takes place via the saliva to the vertebrate host during blood feeding, but transovarial transmission to the female's progeny or venereal transmission from male to female may also occur (MULLEN & DURDEN 2009).

If the vertebrate host is susceptible to the virus, the pathogen will replicate in the tissues of the host (initially causing viremia) and can be retransmitted to the vector during the next blood meal. In some cases, when the concentration of the virus in the blood is not high enough to infect the blood-feeding mosquito, humans are dead-end hosts (DOBLER & ASPÖCK 2010).

In Europe, ten mosquito-borne viruses belonging to three families (Bunyaviridae, Flaviviridae, Togaviridae) are known to circulate. Of these, five have been demonstrated for Germany (BATV, TAHV, USUV, WNV, SINV; Table 1). European Bunyaviridae are generally of low virulence, causing mild to influenza-like symptoms in humans. While Inkoo virus and Ťahyňa virus are the most common California group viruses in Eurasia according to antibody prevalences found in humans (GRATZ 2006), TAHV has rarely been documented from German mosquitoes. After it had been demonstrated in the 1960s from *Ae. vexans* (SPIECKERMANN & ACKERMANN 1972), latest evidence came during the 1980s (PILASKI 1987). In recent studies, the virus was no longer found. By contrast, BATV turned out to be the most common virus in mosquitoes collected in Germany from 2011 to 2016 (SCHEUCH et al. 2018).

Of the Flaviviridae occurring in Europe, dengue virus (DENV) poses a major risk to human health, as the virus is transmitted almost exclusively by anthropophilic mosquito species (HUBÁLEK 2008). With the spread of the potential vector species *Ae. albopictus* in Europe, the threat of endemic dengue cases has risen, as travellers infected with DENV regularly enter from dengue-endemic countries (GRATZ 2006). If the infection is not detected in time and the patient is bitten by *Ae. albopictus* during the viremic phase, viral transmission is possible.

USUV seems to be a primarily avian pathogen. In Africa, where it was first isolated from a bird-biting mosquito species (WILLIAMS et al. 1964), it circulates between birds, and mammals are known as accidental hosts (GRATZ 2006). In 2013, the first human neuroinvasive infections with USUV were documented in Croatia, emphasizing the potential

public health significance of the virus (SANTINI et al. 2015). First evidence in Germany of USUV from a mosquito was obtained in 2010, when it was isolated from *Culex pipiens* biotype *pipiens* (JÖST et al. 2011).

Similar to USUV, reservoir hosts for WNV are bird species. Nevertheless, WNV was isolated from various mammals (Table 1), despite all of them being dead-end hosts and no competent reservoirs (GRATZ 2006). WNV infections were reported from Germany in 2018 for the first time (ZIEGLER et al. 2018), although WNV-antibodies were found in migratory birds in Germany before (LINKE et al. 2007; SEIDOWSKI et al. 2010). While dengue fever was once endemic in southern Europe and disappeared in the mid-20th century together with the main viral vector *Ae. aegypti* (SCHAFFNER & MATHIS 2014), USUV and WNV were apparently introduced to Europe only recently (USUV: 1996, Italy (WEISSENBÖCK et al. 2013); WNV: 1958, Albania (BARDOS et al. 1959)).

Another pathogen formerly not known to Europe is chikungunya virus (CHIKV), which belongs to the Togaviridae. It was described in Tanzania in 1953 (ROSS 1956) and has since been detected in several African countries, the Indian subcontinent and southeastern Asia (PIALOUX et al. 2007). In 2007, an outbreak of chikungunya fever occurred in Italy with more than 200 diseased patients (REZZA et al. 2007). The virus was also found in local *Ae. albopictus* mosquitoes (REZZA et al. 2007), which underlines that tropical mosquito-borne viruses have the potential to be transmitted in temperate regions if a competent vector is present.

Symptoms of SINV-infected patients are mild (TESH 1982). Humans are dead-end hosts of the virus, and the main reservoirs are birds, mainly Passeriformes (HUBÁLEK 2008). SINV is the most widely distributed virus causing arthritis in humans, occurring from Africa to Australia, the Philippines, Asia and India to Europe (International Catalogue of Arboviruses. US Dept.Health, Education, and Welfare, Public Health Service, (CDC) <https://wwwn.cdc.gov/Arbocat>).

Table 1: Mosquito-borne viruses in Germany according to GRATZ (2006), HUBÁLEK (2008) and KAMPEN & WALTHER (2018).

| family | pathogen | main vertebrate host | documented vectors |
|--------------|-----------------------|--|--|
| Bunyaviridae | Batai virus (BATV) | <i>mammals:</i> boar, deer, hare, human, sheep, human <i>birds:</i> house sparrow | <i>Aedes communis</i> , <i>Ae. punctor</i> , <i>Ae. vexans</i> , <i>Anopheles claviger</i> , <i>An. maculipennis</i> s.l., <i>An. messeae</i> , <i>Coquillettidia richiardii</i> , <i>Culex pipiens</i> , <i>Culiseta annulata</i> |
| | Ťahyňa virus (TAHV) | <i>mammals:</i> bear, boar, hare, cattle, goat, human, sheep <i>birds:</i> cormorant, duck, sparrow | <i>Aedes cantans</i> , <i>Ae. caspius</i> , <i>Ae. cinereus</i> , <i>Ae. communis</i> , <i>Ae. detritus</i> , <i>Ae. diantaeus</i> , <i>Ae. dorsalis</i> , <i>Ae. excrucians</i> , <i>Ae. flavescens</i> , <i>Ae. hexodontus</i> , <i>Ae. punctor</i> , <i>Ae. sticticus</i> , <i>Ae. vexans</i> , <i>Anopheles claviger</i> , <i>An. hyrcanus</i> , <i>An. maculipennis</i> s.l., <i>Coquillettidia richiardii</i> , <i>Culex modestus</i> , <i>Cx. pipiens</i> , <i>Culiseta annulata</i> |
| Flaviviridae | Usutu virus (USUV) | <i>birds:</i> blackbird, nuthatch, owl, sparrow, swallow, thrush | <i>Aedes albopictus</i> , <i>Ae. cantans</i> , <i>Ae. detritus</i> , <i>Ae. rossicus</i> , <i>Ae. vexans</i> , <i>Anopheles claviger</i> , <i>An. maculipennis</i> s.l., <i>Culex hortensis</i> , <i>Cx. modestus</i> , <i>Cx. pipiens</i> s.l., <i>Cx. pipiens</i> , <i>Cx. territans</i> , <i>Culiseta annulata</i> |
| | West Nile virus (WNV) | <i>mammals:</i> boar, cat, cattle, camel, dog, hare, human, horse, pig, sheep <i>birds:</i> coot, crow, garganey, goshawk, heron, ibis, lapwing, pigeon, seagull, starling <i>amphibians:</i> marsh frog | <i>Aedes albopictus</i> , <i>Ae. annulipes</i> , <i>Ae. cantans</i> , <i>Ae. caspius</i> , <i>Ae. cinereus</i> , <i>Ae. dorsalis</i> , <i>Ae. excrucians</i> , <i>Ae. flavescens</i> , <i>Ae. geniculatus</i> , <i>Ae. japonicus</i> , <i>Ae. punctor</i> , <i>Ae. rossicus</i> , <i>Ae. sticticus</i> , <i>Ae. vexans</i> , <i>Anopheles hyrcanus</i> , <i>An. maculipennis</i> s.l., <i>An. maculipennis</i> , <i>An. messeae</i> , <i>An. plumbeus</i> , <i>Coquillettidia richiardii</i> , <i>Culex modestus</i> , <i>Cx. pipiens</i> , <i>Cx. pipiens</i> biotype <i>molestus</i> , <i>Cx. territans</i> , <i>Cx. theileri</i> , <i>Cx. torrentium</i> , <i>Culiseta morsitans</i> , <i>Uranotaenia unguiculata</i> |
| Togaviridae | Sindbis virus (SINV) | <i>mammals:</i> human, rodents <i>birds:</i> Passeriformes, Anseriformes, chicken <i>amphibians:</i> marsh frog | <i>Aedes albopictus</i> , <i>Ae. caspius</i> , <i>Ae. cinereus</i> , <i>Ae. communis</i> , <i>Ae. diantaeus</i> , <i>Ae. excrucians</i> , <i>Ae. punctor</i> , <i>Ae. vexans</i> , <i>Anopheles maculipennis</i> s.l., <i>Coquillettidia richiardii</i> , <i>Culex modestus</i> , <i>Cx. pipiens</i> s.l., <i>Cx. theileri</i> , <i>Cx. torrentium</i> , <i>Culiseta morsitans</i> |

2.5.2.2 Filarial nematodes

In Europe, the most important filarial infections are caused by *Dirofilaria immitis* and *D. repens* (Table 2). *Dirofilaria immitis* infects pulmonary arteries and right heart chambers causing heartworm disease in dogs and cats, while *D. repens* has an affinity to subcutaneous tissues (GENCHI et al. 2009). Dirofilariae are transmitted to vertebrate hosts in their third larval stage during mosquito blood feeding (SIMÓN et al. 2012). As a result of reproduction in their vertebrate host, they release microfilariae into the blood, which are again infectious to a competent mosquito vector (GENCHI et al. 2009). In humans, who are not the definitive hosts of dirofilariae, the developmental cycle can normally not be completed (KAMPEN et al. 2012). Typical human infections are accompanied by an immune reaction, leading to the encapsulation of the worm in subcutaneous, pulmonary and intraorbital nodules (MCCALL et al. 2008). Most of the human dirofilarial infections worldwide are caused by *D. repens* (GRATZ 2004). However, the regionally high prevalence of canine infections with *D. immitis* suggests a huge dark number of human infections which are most often asymptomatic (GRATZ 2004).

In Europe, highest *Dirofilaria* infection prevalences occur in the Mediterranean, although a northward expansion of the worms into formerly non-endemic areas can be observed (GENCHI et al. 2011a; MORCHÓN et al. 2012; SASSNAU et al. 2014). The main reason for that seem to be increasing summer temperatures linked to global warming, allowing the development of dirofilarial larvae and rendering natural transmission cycles more intense (GENCHI et al. 2011a; SASSNAU et al. 2014). In Germany, the first autochthonous *Dirofilaria* infection was observed in 2004, when *D. repens* was isolated from a dog which allegedly had never left the country (HERMOSILLA et al. 2006). Later, *D. repens* and *D. immitis* could also be demonstrated in mosquitoes collected in Germany (CZAJKA et al. 2014; KRONEFELD et al. 2014).

In contrast to *Dirofilaria*, the nematode *Setaria tundra* infects mainly cervids (Table 2). *Setaria tundra* has a significant veterinary importance, since it can cause serious disease, as it was the case in a Finnish reindeer population from 2003 to 2005 (LAAKSONEN et al. 2009). In Germany, *S. tundra* was isolated from mosquitoes several times, although no disease outbreak has become known so far (CZAJKA et al. 2012; KRONEFELD et al. 2014).

Table 2: Most important mosquito-borne filarial nematodes and the respective vectors in Germany according to GRATZ (2004), GENCHI et al. (2009, 2011b), LAAKSONEN et al. (2009), CZAJKA et al. (2012), MORCHÓN et al. (2012) and ANGELONE-ALASAAD et al. (2016).

| family | pathogen | main vertebrate hosts | documented vectors |
|---------------|-------------------|--|---|
| Onchocercidae | <i>D. immitis</i> | carnivores (domestic and wild, including dogs, cats and foxes) | <i>Aedes albopictus</i> <i>Ae. caspius</i> <i>Ae. geniculatus</i> <i>Ae. vexans</i> <i>Anopheles maculipennis</i> <i>Coquillettidia richiardii</i> <i>Culex pipiens</i> s.l. <i>Cx. pipiens</i> <i>Cx. theileri</i> |
| | <i>D. repens</i> | | <i>Aedes albopictus</i> <i>Ae. vexans</i> <i>Anopheles spp.</i> <i>An. daciae</i> <i>An. maculipennis</i> s.l. <i>Culex pipiens</i> <i>Culiseta annulata</i> |
| | <i>S. tundra</i> | cervids (reindeer, roe deer) | <i>Aedes cantans</i> <i>Ae. sticticus</i> <i>Ae. vexans</i> <i>Anopheles claviger</i> |

2.5.2.3 Protozoan parasites

Until the 20th century, human malaria was widespread in Europe. Agents of endemic human malaria were primarily the protozoan species *Plasmodium vivax* and *P. malariae* (BRUCE-CHWATT & DE ZULUETA 1980). In Germany, malaria disappeared at the end of the 19th century, although some endemic infection herds remained until the middle of the 1950s (TRAUTMANN 1913; EICHENLAUB 1979). The draining of marshes, improved hygiene, urbanization and the development of effective drugs are reasons for the decline of malaria in Germany (EICHENLAUB 1979). Until today, isolated autochthonous cases of human malaria occur in Europe, as demonstrated, for example, by two cases of *P. falciparum* malaria in Duisburg, Germany, in 1997 (KRÜGER et al. 2001). However, a re-emergence of malaria seems unlikely due to an effective health-care system, land use or construction codes (ROGERS & RANDOLPH 2000; SEMENZA & MENNE 2009).

In addition to plasmodia infecting humans, there are Haemosporidia species causing malaria-like diseases in other animal groups. One of them is avian malaria, which is caused by species

of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*. Avian malaria parasites are common throughout the world and can be detected approximately in 68 percent of the world's bird species (ATKINSON & VAN RIPER 1991). *Haemoproteus* sp. is mainly transmitted by biting midges (Ceratopogonidae) and *Leucocytozoon* sp. by black flies (Simuliidae) (ATKINSON & VAN RIPER 1991). The genus *Plasmodium*, which is transmitted by mosquitoes, also contains species adapted to birds (Table 3).

The infection cycle of avian malaria parasites is similar to the mosquito-human transmission cycle: A mosquito feeds on an infected bird and takes up the parasite with the host blood. If the mosquito is a competent vector, the parasite will be able to undergo its extrinsic developmental cycle and develop sporozoites infectious to a susceptible host during the next blood meal of the mosquito.

Natural infections in birds from wild native populations usually appear relatively harmless, whereas infections of exotic birds are often fatal (HUIJBEN et al. 2007). Of the 38 morphologically distinguishable avian *Plasmodium* species, *P. relictum* and *P. elongatum* are probably investigated best (HUIJBEN et al. 2007). Both species were involved in avian malaria cases in zoos worldwide (GRACZYK et al. 1994; BUENO et al. 2010; SIJBRANDA et al. 2017). Mosquito vectors of avian malaria parasites are often *Culex* species, as opposed to human malaria parasites which are only transmitted by species of the genus *Anopheles* (HUIJBEN et al. 2007). However, the vectors of many avian plasmodia are not known (see Table 4), since parasites are often only known from their vertebrate hosts.

Little is known about the prevalence of avian malaria in Germany. A study by KRONE et al. (2001) has found a rate of 11% in 1,149 analysed owls and raptors, including the genera *Haemoproteus*, *Leucocytozoon* and *Plasmodium*. The analysis of 369 passerine blood samples from Lower Saxony revealed 25% positive results, which could be attributed mainly to *Plasmodium* (15.4%) and *Haemoproteus* species (5.1%) (WIERSCH et al. 2007).

Table 3: Avian malaria *Plasmodium* species in Europe and their potential vectors according to HUFF (1965), BENNETT et al. (1993), GLAIZOT et al. (2012) and ZÉLÉ et al. (2014).

| <i>Plasmodium</i> species | potential vector |
|-------------------------------|--|
| <i>Plasmodium cathemerium</i> | <i>Culex territans</i> * |
| <i>P. circumflexum</i> | <i>Cx. pipiens</i> <i>Culiseta annulata</i> * |
| <i>P. elongatum</i> | <i>Cx. pipiens</i> * |
| | <i>Cx.. territans</i> * |
| <i>P. fallax</i> | <i>Aedes albopictus</i> * |
| <i>P. garnhami</i> | no data available |
| <i>P. giovannolai</i> | no data available |
| <i>P. hegneri</i> | no data available |
| <i>P. juxtannucleare</i> | no data available |
| <i>P. iophuræ</i> | <i>Ae. albopictus</i> * |
| | <i>Cx. pipiens</i> * |
| <i>P. matutinum</i> | <i>Cx. pipiens</i> * |
| <i>P. nucleophilum</i> | no data available |
| <i>P. polare</i> | <i>Cx. pipiens</i> |
| <i>P. relictum</i> | <i>Ae.s dorsalis</i> * |
| | <i>Ae. vexans</i> * |
| | <i>Cx. modestus</i> |
| | <i>Cx. pipiens</i> |
| | <i>Cx. territans</i> * |
| | <i>Cx. theileri</i> |
| <i>P. rouxi</i> | <i>Cx. pipiens</i> * |
| | <i>Cx. territans</i> * |
| <i>P. subpraecox</i> | no data available |
| <i>P. vauhani</i> | <i>Cx. pipiens</i> |

* reports on vector status contradictory

3. Project aims

According to the WHO (1975), effective mosquito control measurements start with the analysis of mosquito biology and their habitats in a defined area. In order to better understand mosquito biology, it is important to study them throughout their life cycle. Mosquito larvae may use different breeding habitats, while adult mosquito females may prefer different blood hosts and digest a blood meal in different kinds of shelters. Mosquito communities might even differ within small geographical scales, which could have an effect on potential host species. This is because the distinct blood feeding behaviour of different mosquito species can lead to different exposure to disease agents, as different vertebrate species can be reservoirs for different kinds of pathogens. Furthermore, different mosquito species could be vectors of different pathogens, which also needs to be considered.

From these considerations three questions arise that are important in the study of mosquito biology in a particular area:

1. Which mosquito species occur when and in which abundance?
2. Which hosts are used by the mosquito species for blood feeding?
3. Which mosquito-borne pathogens circulate in the area?

To analyse these questions zoological gardens are ideal locations. They provide mosquitoes a variety of breeding and resting sites as well as with a high diversity of potential blood hosts, including humans, mammals, birds, reptiles and amphibians. All these conditions are available in a zoo in a confined space and make the zoo a perfect place for ecological studies on mosquitoes. Furthermore, the study of mosquitoes in zoos is of public relevance. Zoos play a role in the conservation of endangered species and attract millions of visitors each year. Both animals and humans are affected by mosquitoes, be it as a nuisance or as potential pathogen vectors.

The present study addresses mosquito populations in two zoological gardens in Germany to analyse the ecology of mosquito communities and their vector potential. On the one hand, the results are meant to contribute to a better understanding of mosquito ecology in Germany in general, on the other hand may lead to a greater awareness of German zoological gardens for mosquitoes and the disease agents they possibly carry.

In the framework of the present dissertation, the following aspects were studied:

1. Mosquito communities in two zoological gardens in Germany – species composition and occurrence of potential vectors
2. Blood meal patterns of mosquitoes in two zoological gardens in Germany
3. Vector-borne pathogens of mosquitoes from two zoological gardens in Germany: screening for viruses, filarial nematodes and avian malaria parasites

Pictures of mosquito breeding- and resting sites as well as EVS-trap locations of both studied zoos are shown in the Supplementary Appendix.

4. Publications

4.1 Mosquito species composition and phenology (Diptera, Culicidae) in two German zoological gardens imply different risks of mosquito-borne pathogen transmission

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Mosquito species composition and phenology (Diptera, Culicidae) in two German zoological gardens imply different risks of mosquito-borne pathogen transmission

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ABSTRACT: Due to their large diversity of potential blood hosts, breeding habitats, and resting sites, zoological gardens represent highly interesting places to study mosquito ecology. In order to better assess the risk of mosquito-borne disease-agent transmission in zoos, potential vector species must be known, as well as the communities in which they occur. For this reason, species composition and dynamics were examined in 2016 in two zoological gardens in Germany. Using different methods for mosquito sampling, a total of 2,257 specimens belonging to 20 taxa were collected. Species spectra depended on the collection method but generally differed between the two zoos, while species compositions and relative abundances varied seasonally in both of them. As both sampled zoos were located in the same climatic region and potential breeding sites within the zoos were similar, the differences in mosquito compositions are attributed to immigration of specimens from surrounding landscapes, although the different sizes of the zoos and the different blood host populations available probably also have an impact. Based on the differences in species composition and the various biological characteristics of the species, the risk of certain pathogens to be transmitted must also be expected to differ between the zoos. *Journal of Vector Ecology* 43 (1): 80-88. 2018.

Keyword Index: Culicidae, biodiversity, urban, mosquito seasonality, vector, sampling methods.

INTRODUCTION

Zoological gardens represent unique ecosystems where exotic and native animal species coexist. These heterogeneous human-made environments are mostly located in urban areas and provide ideal living conditions for mosquito larvae and adults, with numerous opportunities for taking blood meals, mating, resting, and oviposition (Tuten 2011). Zoos also offer attractive habitats for wild native animals, such as squirrels, raccoons, and birds, both through the availability of food sources and the lack of natural predators (Nelder et al. 2009). If a potential mosquito vector population existed within the zoo area, the accidental entry of mosquito-associated pathogens could quickly lead to a disease outbreak among the zoo animals but might possibly also affect zoo visitors and workers.

The probability of disease-agent transmission, however, depends on many factors, such as the availability of vector-competent mosquito species, their population density, the presence of reservoir hosts, the proportion of infectious mosquitoes, and the rate at which people/animals are bitten, all of them contributing to the vector capacity (Smith et al. 2004). In addition to abiotic factors, like temperature and precipitation, the probability of disease transmission is strongly affected by biodiversity in the ecosystem. A study from the U.S.A. has shown that mosquitoes in zoos use captive animals as well as humans and free-roaming native animals as blood hosts, making a transmission of pathogens between the various groups possible (Tuten et al. 2012). A high diversity of the blood host population, which is typical for zoos, is assumed to reduce the risk of vector-borne disease, also known as the dilution effect (Ezenwa et al. 2006). The dilution effect, however, only takes effect if the mosquito is not host-specific and if the various possible blood host species are poor reservoir hosts.

If the particular host species of the mosquito population contains competent reservoirs, high species diversity could lead to an increase in disease prevalence (Ostfeld and Keesing 2000, Ezenwa et al. 2006). By contrast, a high biodiversity of the mosquito vector population may reduce the risk of pathogen transmission, as mosquito species-rich communities usually have a lower density as compared to species-poor communities (Chaves et al. 2011).

Mosquito abundance can vary considerably within small geographic areas, for example between two neighborhoods of a city (LaDeau et al. 2013). This can be attributed to dispersal barriers, such as streets or the availability of larval habitats, although mosquito ecology in urban environments is still poorly understood (LaDeau et al. 2015). In Wellington, New Zealand, it was found that the density of adult mosquitoes was substantially higher in the urban zoo area than in a natural forest only a few kilometers away (Derraik et al. 2003).

Due to the vulnerability of zoo animals in a non-natural and non-native environment where pathogens that they are not adapted to may circulate, infections often have life-threatening consequences. Cases of mosquito-borne diseases in zoological gardens have repeatedly been reported. During the 1999 West Nile outbreak in New York, numerous specimens of the Bronx Zoo/Wildlife Conservation Park bird collection died after infection with the virus (Ludwig et al. 2002). Cases of avian malaria, eastern equine encephalomyelitis, and dirofilariosis are also documented for zoos (Adler et al. 2011).

Species affected by mosquito-borne diseases in zoological gardens are mainly penguins and owls, which are susceptible to West Nile virus (WNV), Usutu virus (USUV), and avian malaria parasites (Ludwig et al. 2002, Huijben et al. 2007, Steinmetz et al. 2011). The detection of St. Louis encephalitis virus and WNV in orcas particularly emphasizes the vulnerability of captive animals

to mosquito-borne pathogens as compared to wild specimens of the same species which do not come into contact with mosquitoes in their natural habitat (Jett and Ventre 2012). In Germany, USUV was isolated from two perished captive great grey owls in the Zoological Garden Berlin in 2015 (Ziegler et al. 2016). Since such specific infections are often discovered in zoos sooner than in their natural surroundings, zoological gardens gain importance as epidemiological sentinel stations (McNamara 2007).

In order to better understand transmission cycles of mosquito-associated pathogens, to identify infection sources and to be able to successfully control the vectors, accurate investigations of vector communities and seasonal population dynamics are crucial. Detailed knowledge about mosquito species composition, however, is scarce in Germany, with hardly any data about mosquito communities in zoological gardens.

In order to assess differences between the mosquito faunas occurring in different kinds of zoological gardens, this study included a large zoo in a densely populated urban area and a smaller one that is surrounded by forest. Species composition and seasonal variation of mosquito populations in the two zoos were examined for a better understanding of mosquito community patterns and to identify potential vectors of mosquito-borne disease agents. To test if the collection method has an impact on the observed diversity, different methods for sampling were also compared.

MATERIALS AND METHODS

Study locations

Mosquitoes were collected in the Tierpark Berlin (Berlin, Germany, N52°49.8406', E13°53.0210') and the Zoological Garden Eberswalde (Brandenburg, Germany, N52°82.2664', E13°78.3025'). The distance between the two zoos is approximately 40 km (Figure 1).

The Tierpark Berlin is one of two zoological gardens in the city of Berlin. It covers 160 ha and is the largest so-called 'landscape' zoological garden in Europe. The zoo area is characterized by deciduous and coniferous trees, with artificial ponds and ditches traversing the park. The surroundings of the Tierpark are shaped by urban settlements, with the Spree River and the Treptower Park located at a linear distance of 4.2 km. The animal collection consists of a total of about 7,500 animals belonging to approximately 900 native and exotic species.

The Zoological Garden Eberswalde covers an area of 15 ha and is located in the southern outskirts of Eberswalde, federal state of Brandenburg. Small artificial ponds and ditches are present in the zoo area, but in contrast to the Tierpark Berlin, the Zoological Garden Eberswalde is adjacent to a nature reserve in the southeast, which is characterized by an alluvial forest, and to a forest in the northwest, which is dominated by beech and pine trees. The zoo is home to some 1,500 animals of approximately 150 native and exotic species.

Mosquito sampling

Mosquito sampling took place from May to September, 2016, in a four-week rhythm in each zoological garden. At both locations, eight EVS-traps (BioQuip Products, CA, U.S.A.; Rohe and Fall 1979) were evenly distributed at comparable locations

and heights between 160 cm and 200 cm, with a minimum of 50 m distance to ensure independence. As access to animal enclosures was restricted, traps could be located at freely accessible sites only. They were equipped with dry ice producing CO₂ as an attractant and were operated for 24 h. At the Zoological Garden Eberswalde, EVS-trapping could not be done in September.

In addition to trapping, mosquitoes were collected one day per month from selected potential resting sites using a battery-powered Improved Prokopack Aspirator (model 1419; John W. Hock, FL, U.S.A.). In each zoo, 15 natural and artificial sites were sampled. Aspirator collections were performed for five min per resting site. Natural resting sites were represented mainly by understory vegetation, artificial resting sites by eaves, and wooden or stone constructions with shaded hiding places. Mosquitoes approaching and trying to bite the collector during the fieldwork were also captured and later referred to as 'hand catches.' All collected adult mosquitoes were preserved on dry ice and stored frozen (-80° C) until further processing.

Larval stages were collected with a dipper in breeding sites like puddles, ponds, and ditches. Smaller artificial breeding sites, such as plastic containers, tree-holes or tires, were sampled with a tea strainer. Each potential breeding site was dipped ten times, or until the water of the breeding site was exhausted before ten samples could be taken. Since the number of possible breeding sites varied over the season, the number of sampled sites was not standardized between the two zoos. In the Tierpark Berlin, a maximum of 15 breeding sites, and in the Zoological Garden Eberswalde, a maximum of 19 sites was sampled per visit.

Mosquito identification

Immature mosquito stages collected were raised to adults in the laboratory for easier morphological identification. Adults were morphologically determined to species using the identification keys by Schaffner et al. (2001) and Becker et al. (2010). Identification of

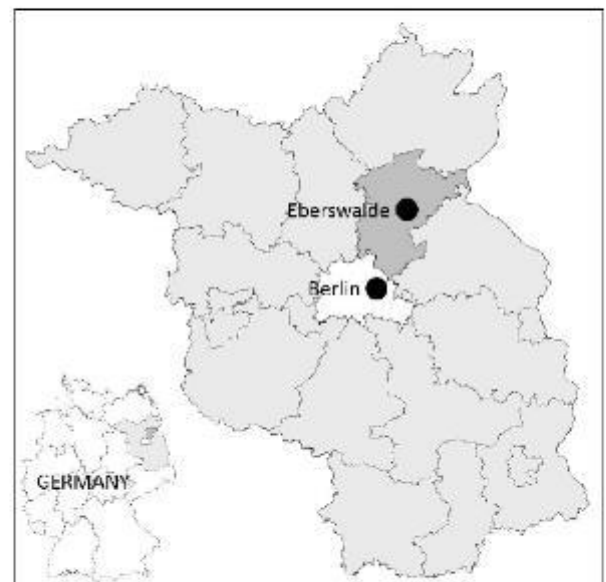


Figure 1. Location of the study areas in Germany.

Anopheles maculipennis complex specimens was done genetically by species-specific ITS2-PCR (Proft et al. 1999, Kronefeld et al. 2014), whereas identification of *Culex pipiens* complex specimens was conducted by a multiplex real-time PCR assay (Rudolf et al. 2013). Mosquitoes not belonging to these complexes but for some reason not morphologically identifiable (e.g., because of damage) were subjected to CO1 (cytochrome oxidase gene subunit 1) barcoding (Folmer et al. 1994, Hébert et al. 2003).

Females belonging to the *Aedes cinereus* and *Ae. annulipes* groups could neither be determined to species level morphologically nor by CO1 barcoding and were evaluated as groups. Also, *Culiseta morsitans* and *Cs. fumipennis* females were evaluated jointly, as no reliable morphological or molecular differentiation was possible.

Data analysis

Statistical analyses were conducted in R Version 3.2.0. Non-identified isomorphic specimens belonging to the *Cx. pipiens* or *An. maculipennis* complexes were not included in the statistical analysis. For comparing the biodiversity of the studied zoos, the Shannon-Wiener index ($H' = -\sum p_i \ln p_i$, where p_i is the proportion of the i -th species in the studied zoo) and the Shannon evenness ($E' = H'/H'_{\max}$, where H' is the Shannon-Wiener index and H'_{\max} is the maximum possible value of H') were calculated. Only adult mosquito samples were analyzed, as larval collections may be influenced by aggregate distributions of individuals, which could occur when large numbers of larvae of a single mosquito species are found in one breeding site (Medeiros-Sousa et al. 2015).

Because the observed number of species is often lower than the number of species actually present, sample adequacy was evaluated by a species accumulation curve, and total richness was estimated by the first-order jackknife method for each zoological garden (Burnham and Overton 1979, Medeiros-Sousa et al. 2015). For the analysis, 1,000 randomizations were implemented without replacement, and the 95% confidence intervals were estimated by the vegan package in R (Oksanen et al. 2016). This analysis was performed for both immature and adult forms according to Medeiros-Sousa et al. (2015), as it was assumed that species presence in the larval collections basically corresponded to their presence in the adult form.

To illustrate seasonal changes in the species composition, the five most common species of each zoo, as based on adult mosquito catches, were plotted in a radar chart according to month. Direct visual comparison was facilitated by \log_{10} transformation of the data.

Finally, non-metric multidimensional scaling (NMDS) was performed with the vegan package in R (Oksanen et al. 2016) to compare the species communities between the two zoological gardens with regard to the used adult collection methods, EVS-trapping and aspirating. Because in Eberswalde EVS-collections had not been conducted in September, trap data for both locations were analyzed only from May to August. Species which had only been captured once during the study period were not considered for NMDS. A stress test was performed to check whether the data were suitable for the analysis. The distance measure used was the Bray-Curtis index; the maximum number of random starts in search of a stable solution was 100.

RESULTS

Mosquito species diversity

In total, 2,257 specimens belonging to 20 mosquito species were collected, represented by 16 taxa in each zoo (Table 1). *Aedes punctor*, *Ae. rusticus*, *An. daciae*, and *Cs. morsitans/fumipennis* were only collected in Eberswalde, while *Ae. cataphylla*, *Ae. geniculatus*, *Cx. modestus*, and *Cx. pipiens* biotype *molestus* were only sampled in Berlin. Ten mosquito species were collected as immature forms. *Aedes geniculatus* was only detected in the larval form, but all other species collected as larvae were also found as adults. The majority of the 'hand-catches' consisted of *Ae. annulipes* group species in the Zoological Garden Eberswalde and of *Ae. vexans* in the Tierpark Berlin (Table 1).

Regarding adult mosquitoes, species diversity obtained by EVS-traps was higher than by aspirator collections. As the Shannon evenness values were 0.63 ($H' = 1.82$) for the Zoological Garden Eberswalde and 0.65 ($H' = 1.76$) for the Tierpark Berlin, respectively, the abundance of different species was more even in Berlin than in Eberswalde. This can be explained by the much higher number of *Ae. annulipes* group mosquitoes ($n = 425$) collected with EVS-traps in the Zoological Garden Eberswalde as compared to all other mosquitoes (second most frequently collected species: 129 *Cs. annulata*; Table 1).

The species accumulation curve of the Zoological Garden Eberswalde reaches an asymptote after 40 collections, demonstrating a good sample adequacy (Figure 2a). First-order jackknife total richness estimation indicates approximately 18 species (standard error (SE) = 0.99) for the Zoological Garden Eberswalde, which is close to the observed 16 species. For the Tierpark Berlin, the curve (Figure 2b) is still not stable at the end of the collection period, suggesting that more samples are needed to cover the whole biodiversity of this location. It also indicates that in the Tierpark Berlin more common and less rare mosquito species occur. The jackknife total richness estimator gives a higher probability for 21 species to occur than for the observed 16 taxa, although a high standard error (SE = 1.98) was measured.

Seasonal variation of mosquito species

Figure 3 visualizes the seasonal variation of mosquito taxa at the two locations. In Eberswalde, high numbers of *Ae. annulipes* group mosquitoes were observed in May. Altogether, specimens of this group accounted for 45.2% of all captured adult mosquitoes at this location. The second most frequently collected species in Eberswalde was *Cs. annulata* (17.2% of all adult collections), dominating from June until August. Starting in June, high numbers of *Cq. richiardii* (6.0% of all adult collections) were recorded, but this species was not present in the September collection. In September, *Cx. p.* biotype *pipiens* (4.2% of all adult collections in Eberswalde) predominated over *Cs. annulata*. High numbers of specimens were constantly collected for *An. messeae* (4.8% of all adults in Eberswalde).

The species pattern was different in Berlin, as the numbers of specimens of the dominant species remained relatively constant, confirming the results of the species accumulation curves and the higher evenness value (Figure 3). Here, *Cs. annulata*, *An. maculipennis* s.s., and *Cx. p.* biotype *pipiens* were collected with relatively constant high numbers over the whole season. *Culiseta*

Table 1. Species composition in the sampled zoos according to collection method.

| Species | Tierpark Berlin | | | | Zoological Garden Eberswalde | | | |
|--|-----------------|------------|--------------|-------------------|------------------------------|------------|--------------|-------------------|
| | Aspirator | EVS-trap | Hand catches | Larval collection | Aspirator | EVS-trap | Hand catches | Larval collection |
| <i>Ae. annulipes</i> group ¹ | - | 3 | 3 | - | 11 | 425 | 19 | 2 |
| <i>Ae. cataphylla</i> | - | 1 | - | - | - | - | - | - |
| <i>Ae. cinereus</i> group ¹ | - | 2 | - | - | - | 16 | 1 | - |
| <i>Ae. geniculatus</i> | - | - | - | 2 | - | - | - | - |
| <i>Ae. punctor</i> | - | - | - | - | - | 6 | 1 | - |
| <i>Ae. rusticus</i> | - | - | - | - | - | 1 | - | - |
| <i>Ae. vexans</i> | 2 | 194 | 16 | - | 1 | 5 | - | - |
| <i>An. claviger</i> | - | 2 | - | - | 28 | 19 | 1 | 1 |
| <i>An. maculipennis</i> complex ² | 173 | 2 | - | 15 | 9 | 3 | 1 | 13 |
| <i>An. daciae</i> | - | - | - | - | 8 | 11 | - | - |
| <i>An. maculipennis</i> s.s. | 96 | 8 | - | 20 | 15 | 2 | - | 10 |
| <i>An. messeae</i> | 4 | 2 | - | - | 21 | 27 | - | 2 |
| <i>An. plumbeus</i> | 33 | 1 | - | - | 4 | 2 | - | 1 |
| <i>Cq. richiardii</i> | 1 | - | - | - | 1 | 58 | 1 | - |
| <i>Cs. annulata</i> | 124 | 16 | - | 13 | 48 | 129 | - | - |
| <i>Cs. morsitans/fumipennis</i> ¹ | - | - | - | - | 14 | - | - | - |
| <i>Cx. pipiens</i> complex ² | 44 | 30 | - | 73 | 30 | 6 | - | 119 |
| <i>Cx. p. biotype molestus</i> | - | 1 | - | - | - | - | - | - |
| <i>Cx. p. biotype pipiens</i> | 28 | 79 | - | 13 | 40 | 3 | 6 | 4 |
| <i>Cx. torrentium</i> | 5 | 18 | - | 6 | 10 | 7 | - | 31 |
| <i>Cx. modestus</i> | - | - | 1 | - | - | - | - | - |
| <i>Cx. territans</i> | 3 | 1 | - | 12 | 16 | - | - | 20 |
| Total number of species¹ | 9 | 13 | 3 | 6 | 13 | 14 | 6 | 8 |
| Total collected | 513 | 361 | 20 | 154 | 256 | 720 | 30 | 203 |

¹ reliable morphological or genetic differentiation not possible.² not identified to species.³ *An. maculipennis* and *Cx. pipiens* complex specimens only considered if species differentiation was done.

annulata accounted for 15.7%, *Cx. p. biotype pipiens* for 12.0%, and *An. maculipennis* s.s. for 11.3% of all adult mosquitoes. In contrast to Eberswalde, high numbers of *Ae. vexans* were recorded in Berlin in July. Altogether, *Ae. vexans* specimens made up for 23.7% of adult mosquitoes collected in the Tierpark Berlin.

Differences in species composition between the zoos

Different community patterns at both locations and differences in species composition depending on aspirator or EVS-collections are visualized in the NMDS plot in Figure 4. Mosquito collections in the Tierpark Berlin were more similar to each other, while collections made in the Zoological Garden Eberswalde were more variable (Figure 4). Aspirator and EVS-collections in Eberswalde from May are characterized by a completely different species composition, attributed both to a high proportion of *Ae. annulipes* group mosquitoes in EVS-collections and high numbers of aspirator-collected *Cs. morsitans/fumipennis*. Monthly aspirator

and EVS-collections from June to August from Eberswalde are more similar to each other than the May collections. From June to August, *An. messeae* and *Cq. richiardii* had a great influence on the observed species composition. Eberswalde aspirator collections from September are more similar to Tierpark Berlin EVS-collections from June. At both locations, *Cx. torrentium* was caught in high numbers. Monthly aspirator collections from Berlin were quite similar among each other, with a predominance of *An. maculipennis* s.s., *Cx. p. biotype pipiens*, *Cs. annulata*, and *An. plumbeus*. Berlin EVS-collections from June and July were dominated by *Ae. vexans* and from August by *Cx. territans*. Tierpark Berlin EVS-collections from May were dissimilar to the other collections, as *Ae. annulipes* group and *An. claviger* mosquitoes significantly influenced the mosquito composition but were not found in later samples.

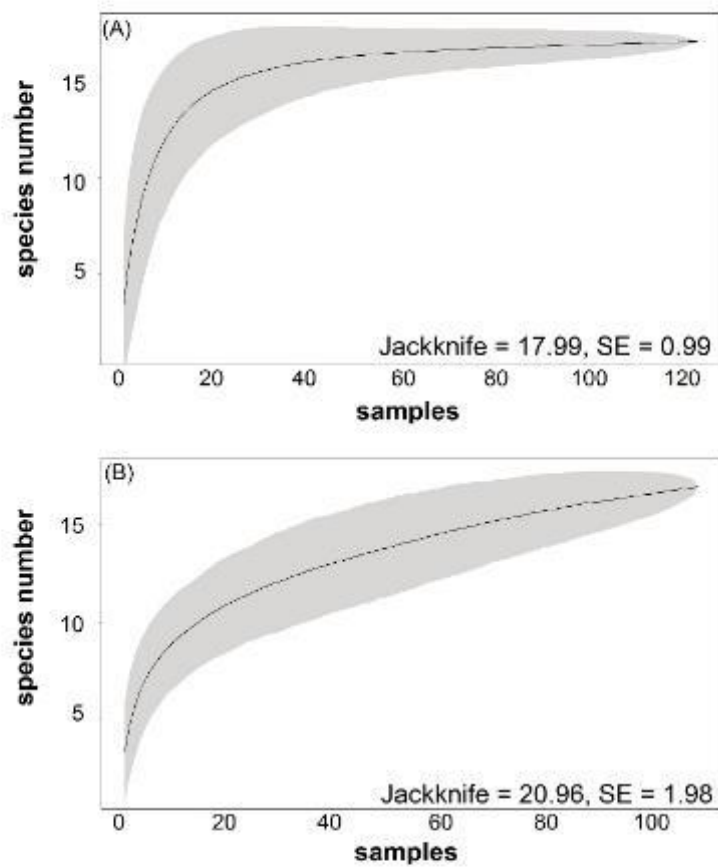


Figure 2. Species accumulation curves (SAC) for the Zoological Garden Eberswalde (A) and the Tierpark Berlin (B).

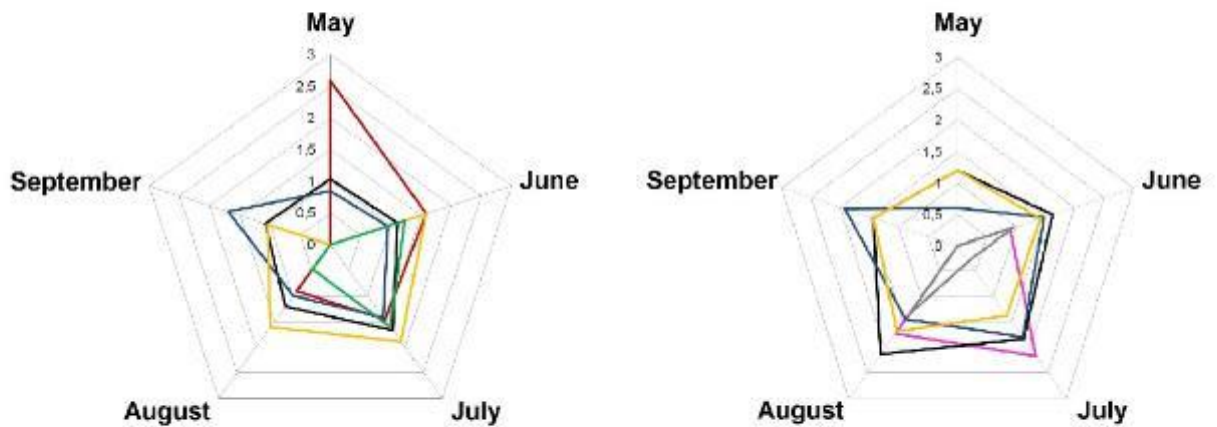


Figure 3. Seasonal variation of the five most frequently collected species of the Zoological Garden Eberswalde (left) and the Tierpark Berlin (right). Red: *Ae. annulipes* group, black: *An. messeae*, blue: *Cx. pipiens* biotype *pipiens*, yellow: *Cs. annulata*, green: *Cq. richiardii*, pink: *Ae. vexans*, grey: *An. maculipennis* s.s., brown: *An. plumbeus*.

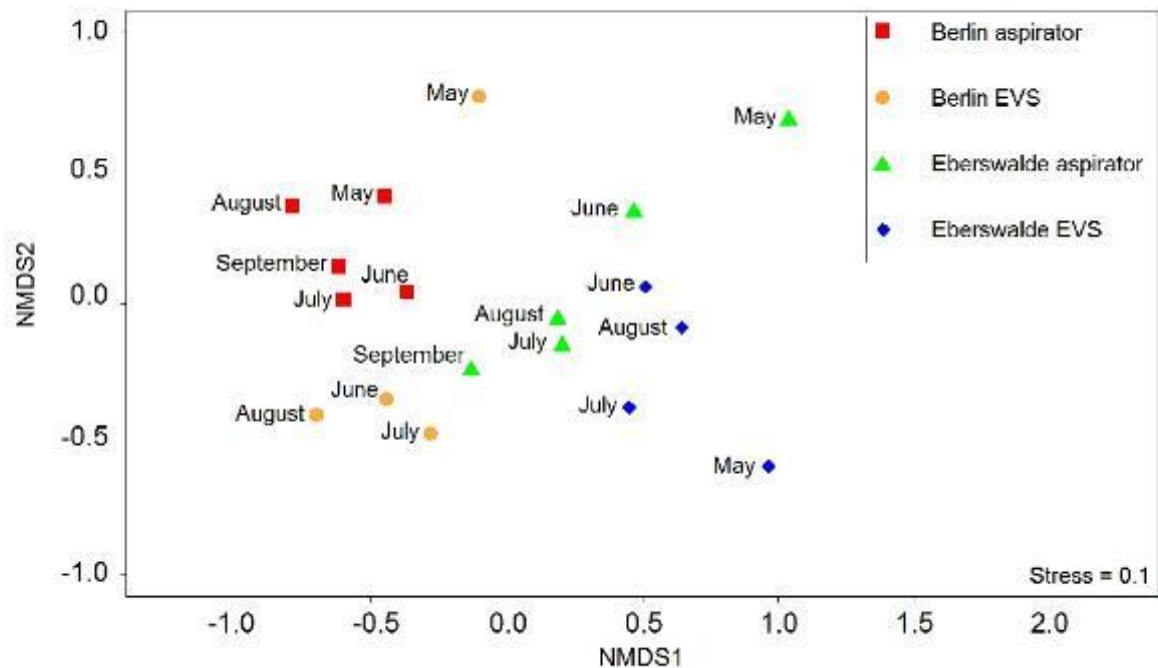


Figure 4. Non-metric multidimensional scaling (NMDS) plot showing mosquito species composition similarity between collections by different methods and at different locations. Each of the 18 dots corresponds to all samples collected at the same time with the same method.

DISCUSSION

Although the Tierpark Berlin and the Zoological Garden Eberswalde are located in the same climatic region, mosquito species compositions were different. These results agree with a study from the U.S.A. from 2013, where the composition of species varied substantially between two sites located in the same ecological region with the same regional pool of species (LaDeau et al. 2013). In the present study, the surveyed zoos differed in size and in surrounding landscapes but also in the spectrum of available blood hosts. Heterogeneous environments across space and time affect the demography and composition of mosquito populations and thus may influence the risk of mosquito-borne diseases (Smith et al. 2004). Consequently, disease risks in zoos embedded in different landscape structures have to be estimated according to the specific situation, as different species may have different significance as potential vectors of different pathogens. Factors that could make a mosquito species a relevant vector in zoos, in addition to its innate vector competence, include a wide spatial distribution at high population densities, host preferences for captive animals and humans, and an extended seasonal occurrence (Smith et al. 2004; Meyer Steiger et al. 2016). Of the 50 mosquito species native to Germany, 27 have been found associated with pathogens, although not necessarily with vector competence (Kampen and Werner 2015). Five of them were detected in the present study in one or both zoos with at least hundred captured specimens: *Ae. annulipes* group, *Ae. vexans*, *Cs. annulata*, *An. maculipennis* s.s., and *Cx. p. biotype pipiens*. Of note,

the proportion and influence of isomorphic *An. maculipennis* and *Cx. pipiens* complex species could be underestimated, since not all individuals were identified to species level.

The *Ae. annulipes* group was not only the most frequently captured taxon in the Zoological Garden Eberswalde but is also widespread in Germany in general (Mohrig 1969). One member of the group, *Ae. cantans*, is suspected to be involved in the transmission of WNV, Tahyna virus (TAHV), Usutu virus (USUV), and of the filarial nematodes *Dirofilaria repens* and *Setaria tundra* (Czjaka et al. 2012; Kampen and Walther 2018). Preferred hosts of *Ae. cantans* are mammals and birds (Medlock et al. 2007), but blood-feeding on exotic animals has not been documented. In the present study, most of the hand-caught mosquitoes belonged to the *Ae. annulipes* group, confirming an association with human hosts. Controlling *Ae. annulipes* group mosquitoes in the zoo area could be difficult, as the preferred breeding sites, shaded ponds, and swampy waters which may develop after the snow melt or heavy rainfalls (Becker et al. 2010), do not only exist on the premises of the zoological garden but also in the surrounding forest.

As opposed to the Zoological Garden Eberswalde, spring species were less abundant in the Tierpark Berlin. Instead, high numbers of the floodwater species *Ae. vexans* were recorded in July. These had mainly been captured by EVS-traps, not excluding the possibility of attraction from outside the study area. A study by Burkett-Cadena et al. (2008) has shown that *Ae. vexans* prefers understory vegetation for rest, where mosquitoes have a higher probability of escaping from being caught than at shelter-type resting sites. The species is also known from urban environments

(Lebl et al. 2014), and is one of few mosquito species actively dispersing over longer distances (Verdonschot and Besse-Lototskaya 2014).

As a typical floodwater mosquito species, *Ae. vexans* could play an important role in the epidemiology of viral mosquito-borne diseases, as immature stages may develop in tremendous numbers in floodplains, the resting sites of resident and migratory birds which can be reservoirs of the causative viruses (Kampen et al. 2012). The species has been shown to be vector-competent for Rift Valley fever virus, various equine encephalitis viruses, TAHV, WNV, and Zika virus (ZIKV) and has been incremented as a potential vector of Batai virus (BATV), CHIKV, USUV, and filarial worms (Kampen and Walther 2017). Avian malaria parasites like *P. circumflexum* and *P. elongatum*, which frequently infect anseriform and galliform birds but also bird species in zoological gardens (Bennett et al. 1993), were also isolated from *Ae. vexans* (Huff 1965). *Aedes vexans* is a primarily ornithophilic mosquito species, but blood meals are occasionally also taken from mammals and exotic zoo animals (Becker et al. 2010; Schoenenberger et al. 2015). Additionally, this species seems to accept human hosts, as demonstrated by the attacks and biting attempts on the mosquito collector during the fieldwork of this study. *Aedes vexans* is not active throughout the complete vegetation period but mainly during the summer months when it was observed in the Tierpark Berlin. Since humans prefer to visit the zoos in summer and most zoo animals are only outdoors during the warmer periods of the year, both are more exposed to mosquitoes in summer. *Aedes vexans* therefore would have a higher probability to transmit pathogens than typical spring species. In addition, the extrinsic incubation period is shorter at summer temperatures.

Although *Cs. annulata* was caught regularly at both survey sites as adult and immature forms, its abundance was higher in the Zoological Garden Eberswalde. The species prefers the proximity to human settlements and hibernates as adult females in houses or cellars (Peus 1929). Preferred blood hosts are birds and mammals, including exotic zoo animals (Medlock et al. 2005; Schoenenberger et al. 2015). Field-collected *Cs. annulata* specimens were found infected with BATV, USUV, and *D. repens*, and transmission was shown for TAHV (Becker et al. 2014; Kampen and Walther 2017). Additionally, avian malaria parasites have been isolated from this species (Huff 1965). All of these findings give rise to the assumption that *Cs. annulata* might be a vector in both zoos.

Studies on the vector competence of isomorphic species belonging to species complexes are rare. Therefore, species of the *An. maculipennis* and the *Cx. pipiens* complexes are discussed on a complex level. Both species complexes were regularly found at the two study sites as adult and immature forms. Species of the *An. maculipennis* complex were one of the most frequently caught taxa in the Tierpark Berlin. Vector competence has been demonstrated for malaria parasites, BATV, Sindbis virus (SINV), and WNV, and *An. maculipennis* complex species are probably also involved in the transmission of dirofilariæ (Kampen and Walther 2017). *Dirofilaria repens* was isolated from an *An. daciae* specimen captured in Germany in 2012 (Kronefeld et al. 2014), a mosquito species included in the collections from the Zoological Garden Eberswalde. *Anopheles maculipennis* complex species are described to be opportunistic feeders and have been shown to take blood meals on both mammals and birds (Danabalan et al.

2014). Also, feeding on exotic species in zoos has been described (Schoenenberger et al. 2015).

Various viruses have been detected in members of the *Cx. pipiens* complex, for example BATV, SINV, WNV, TAHV, and USUV (Kampen and Walther 2017). Avian malaria parasites, mainly *P. relictum*, were isolated from *Cx. pipiens* complex mosquitoes (Huff 1965). Also, *D. immitis* was demonstrated in species of this complex in Germany in 2012 (Kronefeld et al. 2014). Despite a preference for birds, *Cx. p. biotype pipiens* mosquitoes may take advantage of human hosts and thus increase transmission risk to humans (Kilpatrick et al. 2006). Furthermore, *Cx. pipiens* complex species accept exotic zoo vertebrates as hosts (Schoenenberger et al. 2015).

According to Smith (2004), mosquito biting rates are highest near breeding sites where adult emergence takes place. By contrast, vector infection rates are highest far from the breeding sites, where older mosquitoes can be found. Thus, the distance of adults to the breeding place reflects the age structure of mosquito populations. According to this hypothesis, species whose larvae develop within the zoo area would be more likely to become a mere nuisance, while species developing outside the zoo area have a higher probability to be infectious when collected within the zoo. For this reason, breeding site management in the zoo could reduce the nuisance situation but not necessarily the transmission risk of mosquito-associated pathogens. These aspects require further research, as the infection risk is also influenced by mosquito densities and biting rates, landscape, and, above all, blood and reservoir host densities (Tsuda et al. 2016), which are probably higher in the zoo than outside.

The results also demonstrate that the obtained mosquito spectra depend on the collection method applied. Therefore, the sampling methodology may have an impact on which species is perceived as a relevant vector as, for example, attractant EVS-traps collect higher numbers of mosquitoes and species compared to aspirator collections. On the other hand, species like *Cx. territans*, which is not or poorly attracted to commonly used lure traps, are more likely to be collected by aspiration or larval sampling (Küpper et al. 2006; Burkett-Cadena et al. 2008). Furthermore, different mosquito species prefer different types of resting sites (Burkett-Cadena et al. 2008), causing a bias in the species collected by the selection of sampled sites. Finally, the sampling season is crucial as the biodiversity is subject to change with progressive seasons.

According to the vector potential of the most commonly collected species in the Zoological Garden Eberswalde, TAHV, USUV, WNV, filariae, and avian malaria parasites seem to have the highest risk of being transmitted to zoo animals or humans. Given the vector potential of *Ae. vexans*, a theoretical risk for the transmission of a much broader spectrum of pathogens can be deduced for the Tierpark Berlin. Regarding transmission to zoo animals, Disease agents like BATV, SINV, TAHV, USUV, and filarial worms are of major importance, as these have recently been shown to circulate in Germany. Additionally, avian malaria parasites present a considerable risk to susceptible exotic bird species.

Although potential mosquito vectors occur in both zoos surveyed, in neither of them are mosquitoes actively controlled, although prophylactic treatment for the prevention of avian malaria is administered. Should control be implemented, it is

important to consider mosquito breeding sites both within and outside the zoo areas.

As the vector potential of native mosquito populations is hardly known, detailed risk analyses are difficult, the more so as the transmission of mosquito-borne pathogens depends on many additional abiotic and biotic factors. The surveillance of mosquito species is only a first step for further research. The study demonstrates that in addition to the special structure of the zoo, the environment in which it is embedded needs to be considered when it comes to risk analysis, mosquito control, and mosquito-borne disease management. While the present study focused on the mosquito fauna within zoos, the special situation in the area surrounding the zoo should be included in future analyses.

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REFERENCES CITED

- Adler, P.H., H.C. Tuten, and M.P. Nelder. 2011. Arthropods of medicoveterinary importance in zoos. *Annu. Rev. Entomol.* 56: 123-142.
- Becker, N., D. Petric, M. Zgomba, C. Boase, C. Dahl, M. Madon, and A. Kaiser. 2010. *Mosquitoes and Their Control*. Springer, Heidelberg, 577 pp.
- Becker, N., A. Krüger, C. Kuhn, A. Plenge-Bönig, S.M. Thomas, J. Schmidt-Chanasit, and E. Tannich. 2014. [Mosquitoes as vectors for exotic pathogens in Germany]. *Bundesgesundheitsbl. Gesundheitsforsch. Gesundheitssch.* 57: 531-540. [In German]
- Bennett, G.F., M.A. Peirce, and R.W. Ashford. 1993. Avian haematozoa: mortality and pathogenicity. *J. Nat. Hist.* 27: 993-1001.
- Burnham, K.P. and W.S. Overton. 1979. Robust estimation of population size when capture probabilities vary among animals. *Ecology* 60: 927-936.
- Burkett-Cadena, N.D., M.D. Eubanks, and T.R. Urmasch. 2008. Preference of female mosquitoes for natural and artificial resting sites. *J. Am. Mosq. Contr. Assoc.* 24: 228-235.
- Chaves, L.F., G.L. Hamer, E.D. Walker, W.M. Brown, M.O. Ruiz, and U.D. Kitron. 2011. Climatic variability and landscape heterogeneity impact urban mosquito diversity and vector abundance and infection. *Ecosphere* 2: 1-21.
- Czajka, C., N. Becker, S. Poppert, H. Jöst, J. Schmidt-Chanasit, and A. Krüger. 2012. Molecular detection of *Setaria tundra* (Nematoda: Filarioidea) and an unidentified filarial species in mosquitoes in Germany. *Parasit. Vectors* 5: e14.
- Danabalan, R., M.T. Monaghan, D.J. Ponsonby, and Y.M. Linton. 2014. Occurrence and host preferences of *Anopheles maculipennis* group mosquitoes in England and Wales. *Med. Vet. Entomol.* 28: 169-178.
- Derai, J.G.B., D. Slaney, P. Weinstein, P. Lester, and G. Purdie. 2003. Presence of adult *Ochlerotatus (Pinella) notoscriptus* (Skuse) and *Culex (Culex) pervigilans* Bergroth (Diptera: Culicidae) in tree canopy in Wellington, New Zealand. *N. Z. Entomol.* 26: 105-107.
- ECDC (European Centre for Disease Control and Prevention). 2014. *Guidelines for the Surveillance of Native Mosquitoes in Europe*. Technical Report. Stockholm, Sweden: 111 pp.
- Ezenwa, V.O., M.S. Godsey, R.J. King, and S.C. Guptill. 2006. Avian diversity and West Nile virus: testing associations between biodiversity and infectious disease risk. *Proc. R. Soc. Lond. B* 273: 109-117.
- Folmer O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* 3: 294-299.
- Hébert, P.D.N., A. Cywinska, S.L. Ball, and J.R. deWaard. 2003. Biological identifications through DNA barcodes. *Proc. R. Soc. Lond. B* 270: 313-321.
- Huff, C.G. 1965. Susceptibility of mosquitoes to avian malaria. *Exp. Parasitol.* 16: 107-132.
- Huijben, S., W. Schaftenaar, A. Wijsman, K.P. Paaijman, and W. Takken. 2007. Avian malaria in Europe: an emerging infectious disease? In: W. Takken and B.G. Knols (eds.) *Emerging Pests and Vector-borne Diseases in Europe*. Wageningen Academic Publishers, Netherlands, pp. 59-74.
- Jett, J. and J. Ventre. 2012. Orca (*Orcinus orca*) captivity and vulnerability to mosquito-transmitted viruses. *J. Mar. Anim. Ecol.* 5: 9-16.
- Kampen, H., M. Kronefeld, and D. Werner. 2012. Culicid mosquitoes as vectors of disease agents in Europe. In: H. Mehlhorn (ed.) *Arthropods as Vectors of Emerging Diseases*. Parasitol. Res. Monogr. 3: 1-30.
- Kampen, H. and D. Werner. 2015. [The recurring necessity of mosquito surveillance and research]. *Bundesgesundheitsbl. Gesundheitsforsch. Gesundheitssch.* 58: 1101-1109. [In German]
- Kampen H. and D. Walther. 2018. Vector potential of mosquito species (Diptera: Culicidae) occurring in Central Europe. In: G. Benelli and H. Mehlhorn (eds.) *Mosquito-borne Diseases: Implications for Public Health*. Parasitol. Res. Monogr. 10: (in press).
- Kilpatrick, A.M., L.D. Kramer, M.J. Jones, P.P. Marra, and P. Daszak. 2006. West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior. *PLoS Biol.* 4: e82.
- Kronefeld, M., D. Werner, and H. Kampen. 2014. PCR identification and distribution of *Anopheles daciae* (Diptera, Culicidae) in Germany. *Parasitol. Res.* 113: 2079-2086.
- Küpper, S., S. Schulze, W.A. Maier, and H. Kampen. 2006. Beitrag zum Vorkommen und zur Verbreitung von Stechmücken (Diptera: Culicidae) in Nordrhein-Westfalen mit besonderer Berücksichtigung des Großraums Bonn. *Mitt. Dtsch. Ges. Allg. Angew. Entomol.* 15: 337-344.
- LaDeau, S., P. Leisnham, D. Biehler, and D. Bodner. 2013. Higher mosquito production in low-income neighborhoods of Baltimore and Washington, DC: understanding ecological

- drivers and mosquito-borne disease risk in temperate cities. *Int. J. Environ. Res. Publ. Hlth.* 10: 1505.
- LaDeau, S.L., B.F. Allan, P.T. Leisnham, and M.A. Levy. 2015. The ecological foundations of transmission potential and vector-borne disease in urban landscapes. *Funct. Ecol.* 29: 889-901.
- Lebl, K., C. Zittira, K. Silbermayr, A. Obwaller, D. Berer, K. Brugger, M. Walter, B. Pinior, H.-P. Fuehrer, and F. Rubel. 2014. Mosquitoes (Diptera: Culicidae) and their relevance as disease vectors in the city of Vienna, Austria. *Parasitol. Res.* 114: 707-713.
- Ludwig, G.V., P.P. Calle, J.A. Mangialico, B.L. Raphael, D.K. Danner, J.A. Hile, T.L. Clippinger, J.F. Smith, R.A. Cook, and T. McNamara. 2002. An outbreak of West Nile virus in a New York city captive wildlife population. *Am. J. Trop. Med. Hyg.* 67: 67-75.
- McNamara, T. 2007. The role of zoos in biosurveillance. *Int. Zoo Yearbook* 41: 12-15.
- Medeiros-Sousa, A.R., W. Ceretti-Júnior, G.C. de Carvalho, M.S. Nardi, A.B. Araujo, D.P. Vendrami, and M.T. Marrelli. 2015. Diversity and abundance of mosquitoes (Diptera: Culicidae) in an urban park: larval habitats and temporal variation. *Acta Trop.* 150: 200-209.
- Medlock, J., K. Snow, and S. Leach. 2005. Potential transmission of West Nile virus in the British isles: an ecological review of candidate mosquito bridge vectors. *Med. Vet. Entomol.* 19: 2-21.
- Medlock, J.M., K.R. Snow, and S. Leach. 2007. Possible ecology and epidemiology of medically important mosquito-borne arboviruses in Great Britain. *Epidemiol. Infect.* 135: 466-482.
- Meyer Steiger, D.B., S.A. Ritchie, and S.G.W. Laurance. 2016. Mosquito communities and disease risk influenced by land use change and seasonality in the Australian tropics. *Parasit. Vectors* 9: e387.
- Mohrig, W. 1969. *Die Culiciden Deutschlands*. Gustav Fischer Verlag, Jena. *Parasitol. Schriftenr.* 18: 1-260.
- Nelder, M.P., W.K. Reeves, P.H. Adler, A. Wozniak, and W. Wills. 2009. Ectoparasites and associated pathogens of free-roaming and captive animals in zoos of South Carolina. *Vector-borne Zoonotic Dis.* 9: 469-477.
- Oksanen, J., F.G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGinn, P.R. Minchin, R.B. O'Hara, G.L. Simpson, P. Solymos, M. Henry, H. Stevens, and E. Szoecs. 2016. *Vegan: Community ecology*. <http://CRAN.R-project.org/package=vegan>.
- Ostfeld, R.S. and F. Keesing. 2000. Biodiversity series: the function of biodiversity in the ecology of vector-borne zoonotic diseases. *Can. J. Zool.* 78: 2061-2078.
- Peus, F. 1929. Die Stechmückenfauna des südwestlichen Gebietes von Groß-Berlin. *Z. Desinfekt.* 21: 67-72.
- Profl, J., W.A. Maier, and H. Kampen. 1999. Identification of six sibling species of the *Anopheles maculipennis* complex (Diptera: Culicidae) by a polymerase chain reaction assay. *Parasitol. Res.* 85: 837-843.
- Rohe, D.L. and R.P. Fall. 1979. A miniature battery powered CO₂ baited light trap for mosquito borne encephalitis surveillance. *J. Vector Ecol.* 4: 24-27.
- Rudolf, M., C. Czajka, J. Böstler, C. Melaun, H. Jöst, H. von Thien, M. Badusche, N. Becker, J. Schmidt-Chanasit, A. Krüger, E. Tannich, and S. Becker. 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.
- Schaffner F, G. Angel, B. Geoffroy, J. P. Hervy, A. Rhaïem, and J. Brunhes. 2001. The mosquitoes of Europe. An identification and training programme. IRD Editions (CD-Rom). Montpellier, France.
- Schoenenberger, A.C., S. Wagner, H.C. Tuten, F. Schaffner, P. Torgerson, S. Furrer, A. Mathis, and C. Silaghi. 2015. Host preferences in host-seeking and blood-fed mosquitoes in Switzerland. *Med. Vet. Entomol.* 30: 39-52.
- Silver, J.B. 2008. *Mosquito Ecology: Field sampling methods*. Springer, New York: 1494 pp.
- Smith, D.L., J. Dushoff, and E.E. McKenzie. 2004. The risk of a mosquito-borne infection in a heterogeneous environment. *PLoS Biol.* 2: e368.
- Steinmetz, H.W., T. Bakonyi, H. Weissenböck, J.-M. Hatt, U. Eulenberger, N. Robert, R. Hoop, and N. Nowotny. 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. *Vet. Microbiol.* 148: 207-212.
- Tsuda, Y., Y. Maekawa, K. Ogawa, K. Itokawa, O. Komagata, T. Sasaki, H. Isawa, T. Tomita, and K. Sawabe. 2016. Biting density and distribution of *Aedes albopictus* during the September 2014 outbreak of dengue fever in Yoyogi Park and the vicinity of Tokyo metropolis, Japan. *Jpn. J. Infect. Dis.* 69: 1-5.
- Tuten, H.C. 2011. Habitat characteristics of larval mosquitoes in zoos of South Carolina, USA. *J. Am. Mosq. Contr. Assoc.* 27: 111-119.
- Tuten, H.C., W.C. Bridges, K.S. Paul, and P.H. Adler. 2012. Blood-feeding ecology of mosquitoes in zoos. *Med. Vet. Entomol.* 26: 407-416.
- Verdonschot, P.F.M. and A.A. Besse-Lototskaya. 2014. Flight distance of mosquitoes (Culicidae): a metadata analysis to support the management of barrier zones around rewetted and newly constructed wetlands. *Limnologia* 45: 69-79.
- Ziegler, U., C. Fast, M. Eiden, S. Bock, C. Schulze, D. Hoepfer, A. Ochs, P. Schlieben, M. Keller, D.E. Zielke, R. Luehken, D. Cadar, D. Walther, J. Schmidt-Chanasit, and M.H. Groschup. 2016. Evidence for an independent third Usutu virus introduction into Germany. *Vet. Microbiol.* 192: 60-66.

4.2 Mosquito bloodmeal preferences in two zoological gardens in Germany

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Mosquito bloodmeal preferences in two zoological gardens in Germany

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Abstract. Because they provide a high density and diversity of vertebrate species, small water pools and shaded environments, zoological gardens offer ideal living conditions for numerous mosquito species. Depending on their host preferences and vector competencies, these species may be able to transmit pathogens between native and non-adapted exotic blood host species, thereby causing morbidity and mortality among valuable zoo animals. To determine the extent to which native mosquito species feed on captive and wild animals, as well as on humans, in two German zoological gardens, mosquitoes were collected over two seasons by trapping and aspirating. A total of 405 blood-fed specimens belonging to 16 mosquito taxa were collected. Genetic bloodmeal analysis revealed 56 host species, mainly representing mammals of the zoo animal population, including exotic species previously not known as blood hosts of the mosquito species collected. These results indicate opportunistic feeding patterns with low host-specificity in the analysed mosquitoes, although these could be grouped, according to their bloodmeals, into 'amphibian-', 'non-human mammal-' and 'non-human mammal and human-' feeding species. As the blood-feeding preferences of vector-competent mosquito species are major determinants of vector capacity, information on the blood-feeding behaviour of mosquitoes in zoos is crucial to the success of targeted vector management.

Key words. Culicidae, bloodmeal, host preferences, host species, zoo.

Introduction

The haematophagous behaviour of a vector-competent mosquito species is essential to the lifecycle of a mosquito-borne pathogen (Takken & Verhulst, 2013). The choice of blood host determines the pathogens to which the mosquito is exposed during feeding and vice versa. Studying mosquito bloodmeal patterns is therefore crucial to understanding the transmission cycles of mosquito-borne pathogens.

Although mosquitoes vary according to their specialization in host species, their mouthparts are quite similar across species and theoretically enable them to feed on a wide range of vertebrates (Hocking, 1971). The development of a generalist feeding behaviour is supported in environments with low densities of vertebrates and low levels of biodiversity because waiting for an

optimal host involves the risk of dying before a bloodmeal can be taken (Lyimo & Ferguson, 2009). Accordingly, specialization in blood feeding might develop in an environment with a high density and diversity of potential hosts. Vertebrate biodiversity is therefore likely to influence the host choice of mosquitoes. The extent to which blood-feeding patterns are affected by the availability of diverse vertebrate species, however, remains unclear (Chaves *et al.*, 2010).

In addition to the availability of acceptable hosts, blood-feeding success may also depend on the defensive behaviour of potential hosts and the density of the mosquito population (Thiemann *et al.*, 2011; Takken & Verhulst, 2013). A seasonal shift from avian to mammalian hosts is also possible, as observed in the *Culex pipiens* complex (Diptera: Culicidae) (Kilpatrick *et al.*, 2006).

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The unique environment of the zoological garden offers ideal conditions in which to study this complexity of environmental, host and mosquito factors and their interactions within a limited area (Tuten *et al.*, 2012). Zoos are characterized by a high biodiversity of exotic animals. In addition to animal species living in captivity, zoos are attractive habitats for free-roaming animals, such as squirrels, raccoons and birds, providing food sources and relative security from natural predators (Nelder *et al.*, 2009). At the same time, the mosquito fauna in zoos can be highly diverse as a result of the various types of breeding sites within the zoo area and also because mosquitoes are attracted from surrounding areas (Heym *et al.*, 2018).

A study conducted in two zoos in South Carolina, U.S.A., found that the bloodmeal preferences of mosquitoes reflected those observed in nature, although some differences occurred (Tuten *et al.*, 2012). For example, *Culex erraticus* (Diptera: Culicidae) showed a preference for avian hosts in a zoo setting, although it is described as an indiscriminate feeder (Tuten *et al.*, 2012). Similarly, a large portion of mammalophilic *Aedes japonicus* (Diptera: Culicidae) fed on birds in a zoo in Switzerland (Schönenberger *et al.*, 2015). In both studies, native mosquito species included exotic animals into their host spectra.

With respect to the circulation of agents of mosquito-borne disease, infections in zoo animals are likely to be detected at an early stage as a result of thorough health monitoring in zoos (McNamara, 2007). This is of major public health importance because humans, in addition to captive and wild animals, have been shown to be common mosquito blood hosts in zoos (Greenberg *et al.*, 2012; Tuten *et al.*, 2012; Schönenberger *et al.*, 2015). The identification of vectors in cases of emerging mosquito-borne diseases is only possible if the foraging behaviours and the blood-host preferences of the occurring mosquito species are known (Chaves *et al.*, 2010).

In Germany, knowledge of the bloodmeal patterns of native mosquito species is scant. A recent study has shown that the host spectra of mosquitoes in Germany are broad and do not necessarily reflect the restricted preferences described in the literature (Börstler *et al.*, 2016).

To better understand mosquito host preferences, blood-fed mosquitoes, collected over 2 years in two zoological gardens in Germany, were examined to determine the origins of their bloodmeals. The aim was to evaluate the extent to which native mosquito species accept captive zoo animals as hosts, in addition to free-roaming wild animals and humans. Moreover, the study investigated whether the species collected were generalists, feeding on any available host, or specialists, selecting specific vertebrate groups or species for blood feeding, regardless of the broad range of potential hosts available.

Materials and methods

Study locations

Mosquitoes were collected in the Tierpark Berlin (Berlin, Germany; 52°49.8406' N, 13°53.0210' E) and the Zoological Garden Eberswalde (Brandenburg, Germany; 52°42.2664' N, 13°78.3025' E). The 160-ha Tierpark Berlin is surrounded by urban settlements, with the River Spree and the Treptower Park at a linear distance of 4.2 km. A total of 7500 animals

belong to the Tierpark animal collection, representing approximately 900 native and exotic species. Most of the animals are birds (30.2%), invertebrates (28.4%) and mammals (18.8%). With 15 ha, the Zoological Garden Eberswalde is much smaller and is located in a forested area. The zoo is home to approximately 1500 animals, representing some 150 native and exotic species. Birds again make up the majority of the kept animals (32.0%), followed by mammals (27.3%) and reptiles (8.8%).

Mosquito sampling

Mosquito sampling took place from May to September 2016 and from April to September 2017. Collections were performed for 24 h in a 4-week cycle in each zoological garden. Eight EVS traps (BioQuip Products, Inc., Rancho Dominguez, CA, U.S.A.) (Rohe & Fall, 1979) were placed at comparable sites in both zoos at heights of 160–200 cm, with a minimum distance of 50 m between traps to ensure independence. As access to animal enclosures was limited, traps could be placed only at freely accessible sites. The traps were operated with dry ice evaporating carbon dioxide as an attractant. At the Zoological Garden Eberswalde, the use of EVS traps was not possible in September 2016.

Additionally, adult mosquitoes were actively collected from selected potential resting sites using a battery-powered Improved Prokopack Aspirator (model 1419; John W. Hock Co., Gainesville, FL, U.S.A.). In total, 15 natural and artificial resting sites were sampled once per month in both zoos for 5 min per session. Natural resting sites were represented mainly by understory vegetation, artificial resting sites by caves and wooden or stone constructions with shaded hiding places. The heights of the sampled sites varied from 1 m to 3 m.

Any mosquitoes that tried to attack the collector during fieldwork were also captured and later referred to as 'hand catches'. Collected mosquitoes were conserved on dry ice and stored frozen (−80 °C) until further processing.

Mosquito identification

Morphological species determination was performed using the identification keys of Schaffner *et al.* (2001) and Becker *et al.* (2010). Complex species were identified genetically. *Anopheles maculipennis* complex (Diptera: Culicidae) specimens (including *Anopheles atroparvus*, *Anopheles daciae*, *Anopheles maculipennis* and *Anopheles messeae*) were analysed by species-specific internal transcribed spacer 2 (ITS2) polymerase chain reaction (PCR) (Proft *et al.*, 1999; Kronefeld *et al.*, 2014), whereas the identification of *Cx. pipiens* complex specimens (including *Cx. pipiens* biotype *pipiens*, *Cx. pipiens* biotype *molestus* and *Culex torrentium*) was carried out using a multiplex real-time PCR assay (Rudolf *et al.*, 2013). Because of damage to or the absence of characteristic identification cues, some mosquitoes not belonging to a species complex were not identifiable morphologically. In these cases, specimens were subjected to cytochrome oxidase gene subunit I (COI) barcoding (Folmer *et al.*, 1994; Hebert *et al.*, 2003). Females belonging to the *Aedes cinereus* and *Aedes annulipes* (Diptera: Culicidae) groups were evaluated at group level as species determination was possible

neither morphologically nor genetically. For the same reason, *Caliseta morsitans* (Diptera: Culicidae) and *Caliseta fumipennis* females (Diptera: Culicidae) were jointly analysed.

Bloodmeal identification

Bloodmeals were identified based on PCR amplification and sequencing of a polymorphic region of the vertebrate mitochondrial 16S ribosomal DNA (rDNA). DNA was prepared from individual blood-fed mosquitoes using the NucleoMag[®] VET Kit (Macherey-Nagel GmbH & Co. AG, Dürren, Germany), following the manufacturer's protocol. The universal PCR primers and protocol were those described by Kitano *et al.* (2007). When the 16S rDNA PCR assay failed, another PCR, targeting a specific part of the tRNA^{Glu}/cytochrome b gene of vertebrate DNA, was conducted using primers and protocols according to Wolf *et al.* (1999).

Each PCR assay included a 'no-template control' (NTC) and a positive control (rat DNA) run in parallel with the test samples (e.g. Burkert-Cadena *et al.*, 2008a; Barrera *et al.*, 2012; Stevens *et al.*, 2012). Previous studies have shown that the PCR protocol consistently amplifies vertebrate DNA of any origin, which is why only one DNA sample (rat) was routinely used as a positive control.

In order to avoid contamination, the various experimental steps were carried out in separate rooms or laboratory hoods. The PCR products were checked by electrophoresis on 1.5% agarose gels, excised and recovered using the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). Products were subsequently analysed by Sanger DNA sequencing using the BigDye Terminator Version 1.1 Cycle Sequencing Kit (Applied Biosystems Deutschland GmbH, Darmstadt, Germany). Products were purified using NucleoSEQ Columns (Macherey-Nagel GmbH & Co. AG) before they were loaded onto a 3130 Genetic Analyzer (Applied Biosystems Deutschland GmbH). The data obtained were analysed with the bioedit programme Chromas (<http://www.technelysium.com.au/chromas2.html>), and high-quality sequences (i.e. sequences without artefacts and signs of contamination) were aligned against sequences available in GenBank using the BLAST algorithm in the National Center for Biotechnology Information (NCBI) database (Altschul *et al.*, 1990).

The best fitting vertebrate species that was reasonable was chosen as the blood host species. Sequence similarities lower than 95% indicated questionable identities and were not considered. Sometimes, more than one species fitted by more than 95% and were geographically plausible. In such cases, however, the possible host species belonged to the same family, such as in the cervids *Dama dama* (Artiodactyla: Cervidae) and *Cervus elaphus* (Artiodactyla: Cervidae), and hence these hosts were evaluated only at the family level.

Data analysis

As not every specimen belonging to the *An. maculipennis* and *Cx. pipiens* complexes could be identified to species level, statistical analysis included both only as complexes.

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Post-bloodmeal flight distances were calculated only for mosquito species feeding on exotic host species found at the Tierpark Berlin or the Zoological Garden Eberswalde, but not normally present in Germany. The distance from the centre of the exotic animal's enclosure to the mosquito sampling site was determined to the nearest metre using GPS, as described by Greenberg *et al.* (2012). A one-way analysis of variance (ANOVA) was conducted using R (R Core Team, 2017) to test if flight distances varied significantly among mosquito species. Before analysis, measured distances were Box–Cox transformed for normal distribution with a λ -value of 0.5. The level of significance was set at $P < 0.05$.

A co-occurrence analysis according to Chaves *et al.* (2010) was conducted to test if mosquito feeding patterns are random or structured. Analysis was performed in R, using the package EcoSimR (Gotelli *et al.*, 2015). For both zoos, the null hypothesis that mosquito species were equally likely to feed on any host species was tested. To do so, the checkerboard score (c-score) (Stone & Roberts, 1990) was applied to determine the randomness of the distribution of two or more species feeding on different host species.

In order to use the c-score for the analysis of host preferences, its formula was interpreted as stated by Chaves *et al.* (2010). The data consisted of a binary presence–absence matrix in which each row is a mosquito species and each column is one of the observed host species. The entries represent 'feeding' (1) or 'not feeding' (0) on the host. As some of the bloodmeal hosts could be identified only to family level and exemplary analysis of data available at species level produced no added value, all hosts were analysed at the family level. The applied null models were fixed-equiprobable. For the test, 1000 randomizations of the null models were calculated. In the interpretation of the results, the standardized effect size (SES), which converts the P -value into a standardized deviate, was applied. Non-significant tail probabilities fall between -2.0 and $+2.0$, which would mean that the simulated distribution of mosquito species and hosts does not differ significantly from their observed distribution pattern. This would indicate a random feeding pattern and no pronounced host specificity.

To illustrate whether mosquito species can be subsumed under larger groups according to taxonomically related blood hosts, a two-way cluster analysis was performed with PAST Version 3.x (<https://folk.uio.no/ohammer/past/>), using the unweighted pair group with arithmetic mean (UPGMA) method with Euclidean distance. For this analysis, blood hosts were grouped into 'non-human mammal', 'human', 'avian' and 'amphibian', and data were \log_{10} -transformed for normal distribution. Mosquito species for which only one analysed bloodmeal was available were not included in the cluster analysis.

Results

Mosquito collection

A total of 4605 mosquito specimens belonging to 21 taxa were collected, of which only five taxa were not represented by blood-fed specimens (*Aedes caspius*, *Aedes punctor*, *Aedes*

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Table 1. Blood-fed mosquito species in which the host source was successfully identified in the Tierpark Berlin and the Zoological Garden Eberswalde and average flight distances from the enclosures of identified hosts (2016, 2017).

| Blood-fed species | Tierpark Berlin | | | | Zoological Garden Eberswalde | | | |
|---|-------------------------------|-----------------------------------|--------------------------|---|-------------------------------|-----------------------------------|--------------------------|---|
| | Specimens collected, <i>n</i> | Blood-fed specimens, <i>n</i> (%) | Amplification success, % | Flight distance (m) mean \pm SE (distances measured, <i>n</i>) | Specimens collected, <i>n</i> | Blood-fed specimens, <i>n</i> (%) | Amplification success, % | Flight distance (m) mean \pm SE (distances measured, <i>n</i>) |
| <i>Aedes annulipes</i> group | 8 | 0 | — | — | 718 | 88 (12.3) | 96.6 | 88.98 \pm 97.3 (53) |
| <i>Aedes cataphylla</i> | 1 | 0 | — | — | 8 | 2 (25.0) | 50.0 | 0.0 (1) |
| <i>Aedes cinereus</i> group | 7 | 1 (14.3) | 100 | 200.0 (1) | 51 | 5 (9.8) | 80.0 | 0.0 \pm 0.0 (2) |
| <i>Aedes sticticus</i> | 10 | 0 | — | — | 10 | 3 (30.0) | 100 | 0.0 \pm 0.0 (3) |
| <i>Aedes vexans</i> | 352 | 6 (1.7) | 100 | 129.3 \pm 75.0 (3) | 29 | 8 (27.6) | 87.5 | 29.7 \pm 48.2 (6) |
| <i>Anopheles claviger</i> | 2 | 0 | — | — | 127 | 32 (25.2) | 87.5 | 60.6 \pm 44.4 (25) |
| <i>Anopheles maculipennis</i> complex | 175 | 24 (13.7) | 87.5 | 149.6 \pm 58.7 (14) | 13 | 3 (23.1) | 66.7 | 110.0 \pm 110.0 (2) |
| <i>Anopheles daciae</i> | — | — | — | — | 47 | 15 (31.9) | 86.7 | 72.5 \pm 72.5 (2) |
| <i>Anopheles maculipennis</i> s.s. | 250 | 47 (18.8) | 95.7 | 181.9 \pm 143.1 (45) | 89 | 27 (30.3) | 81.5 | 72.4 \pm 53.3 (21) |
| <i>Anopheles messeae</i> | 8 | 2 (25.0) | 100 | 101.0 \pm 13.0 (2) | 98 | 15 (15.3) | 86.7 | 17.9 \pm 42.2 (12) |
| <i>Anopheles plumbeus</i> | 66 | 4 (6.1) | 100 | 134.0 \pm 35.6 (4) | 8 | 1 (12.5) | 100 | — |
| <i>Coquilletidia richiardii</i> | 1 | 1 (100) | 100 | — | 98 | 0 | — | — |
| <i>Culiseta annulata</i> | 226 | 21 (9.3) | 100 | 235.9 \pm 169.9 (20) | 310 | 37 (11.9) | 91.9 | 65.0 \pm 66.1 (31) |
| <i>Culiseta morsitans/pipiens</i> | — | — | — | — | 42 | 2 (4.8) | 50.0 | — |
| <i>Culex pipiens</i> complex | 75 | 8 (10.6) | 75.0 | 272.5 \pm 227.5 (2) | 526 | 7 (1.3) | 100 | — |
| <i>Culex pipiens</i> biotype <i>pipiens</i> | 305 | 21 (6.9) | 71.0 | 362.3 \pm 156.2 (3) | 205 | 16 (7.8) | 43.8 | 132.2 \pm 123.9 (6) |
| <i>Culex torrentium</i> | 34 | 2 (5.9) | 100 | — | 55 | 2 (3.6) | 100 | 83 (1) |
| <i>Culex territans</i> | 16 | 3 (18.8) | 100 | — | 53 | 2 (3.8) | 100 | — |
| Total | 1536 | 140 (9.1) | 88.6 | 193.2 \pm 142.7 (131) | 2487 | 265 (10.7) | 90.9 | 71.2 \pm 77.6 (181) |

SE, standard error.

nesticus, *Culex modestus*, *Cx. pipiens* biotype *molestus*). Collections in the Tierpark Berlin amounted to a total of 140 blood-fed specimens, representing 12 taxa, and those in the Zoological Garden Eberswalde totalled 265 blood-fed specimens, representing 16 taxa (Table 1).

All mosquito species with blood-fed specimens were collected by aspirating, but EVS traps were also able to catch some blood-fed specimens (Fig. 1). In the case of *Ae. annulipes* group mosquitoes, EVS traps collected more blood-fed specimens ($n = 53$) than the aspirator ($n = 32$).

Mosquito blood hosts

The amplification success of mosquito bloodmeal DNA was generally high (90.1%), although a considerable percentage of PCRs of blood host DNA from *Cx. pipiens* biotype *pipiens* collected in the Zoological Garden Eberswalde failed (Table 1). The NTC reactions included always correctly yielded negative results and the positive control reactions revealed the vertebrate species tested (rat).

Fifty-six different host species belonging to 23 vertebrate families were identified, including exotic species not native to

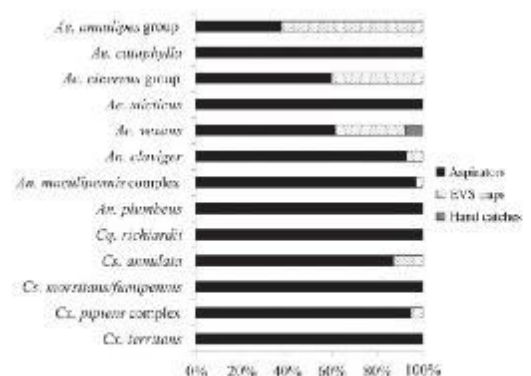


Fig. 1. Methods of collecting blood-fed mosquito taxa. *Ae.*, *Aedes*; *An.*, *Anopheles*; *Cq.*, *Coquilletidia*; *Cs.*, *Culiseta*; *Cx.*, *Culex*.

Germany (Table 2). In both sampled zoos, the host species were mainly mammals (Tierpark Berlin: 96.8%, $n = 120$; Zoological Garden Eberswalde: 97.5%, $n = 235$). Avian (Tierpark Berlin:

1.6%, $n=2$; Zoological Garden Eberswalde: 1.7%, $n=4$) and amphibian (Tierpark Berlin: 1.6%, $n=2$; Zoological Garden Eberswalde: 0.8%, $n=2$) blood hosts were rarely identified.

Identified blood host species were mainly represented by the captive zoo animal population at both locations (Table 2 and Fig. 2). This contrasts with the findings of an earlier study conducted in the U.S.A., in which mosquitoes containing bloodmeals from captive and wild animals were collected in almost equal proportions (Fig. 2). In the Tierpark Berlin, major hosts belonged to the families Bovidae (38.9%, $n=37$), Camelidae (35.8%, $n=34$) and Cervidae (7.4%, $n=7$). Similarly, in Eberswalde, most bloodmeals that could be assigned to zoo animals were from Bovidae (33.0%, $n=60$), Equidae (25.3%, $n=46$) and Cervidae (16%, $n=40$). All avian bloodmeals originating from the zoo animal population were from ostriches [*Struthio camelus* (Struthioniformes: Struthionidae)], which was also the most common avian host species (57.1%, $n=4$). Humans were the second most frequent blood hosts at both locations, whereas bloodmeals from free-roaming wild animals were rarely detected (Fig. 2). Identified blood hosts belonging to the wild animal population at the Tierpark Berlin were foxes [*Vulpes vulpes* (Carnivora: Canidae), $n=2$], common toad [*Bufo bufo* (Anura: Bufonidae), $n=1$], wild boar [*Sus scrofa* (Artiodactyla: Suidae), $n=1$], great tit [*Parus major* (Passeriformes: Paridae), $n=1$] and house sparrow [*Passer domesticus* (Passeriformes: Passeridae), $n=1$], in addition to a frog [*Rana* sp. (Anura: Ranidae)] which could not be identified to species. In Eberswalde, two bloodmeals from wild boar (*S. scrofa*), one from a fox (*V. vulpes*) and two from the genus *Rana* were identified.

Blood-feeding preferences

To analyse if mosquito taxa had preferences for certain host families, a co-occurrence analysis was conducted (Table 3). According to the SES value, the results indicate no aggregated patterns of mosquito species with regard to host family in either zoo as the observed pattern showed no statistical difference from what would be expected for a random feeding pattern.

Nevertheless, the mosquitoes sampled could be categorized into three groups based on their blood-feeding behaviours according to cluster analysis (Fig. 3). *Culex territans* is the only species that fed on amphibians, which is why this species has its own branch. The other mosquito species are divided into two groups: the first includes specimens that had mainly fed on non-human mammals and the second includes large portions of specimens that had ingested either human or non-human mammal blood (Fig. 3).

Post-bloodmeal flight distances

Calculated flight distances varied between 0 m and 770 m, with generally larger distances observed in the Tierpark Berlin than in the Zoological Garden Eberswalde (Table 1). The longest distance measured applied to *An. maculipennis* s.s. (770 m), but ANOVA showed no significant differences between species ($F_{(5,119)} = 2.2$, $P = 0.06$).

Discussion

The mosquito feeding patterns found mainly confirm the use of host vertebrates reported as favoured in the literature, indicating that blood-feeding behaviour in zoos does not differ from that observed in nature (Table 2). Deviations were observed for the mammalophilic *Anopheles claviger* (Diptera: Culicidae), which used an avian host (*S. camelus*), and amphibiphilic *Culex territans* (Diptera: Culicidae), which fed on a human host, in addition to their documented preferred hosts (Table 2).

Irrespective of mosquito species, most mosquitoes mainly used humans and non-human mammals of the zoo animal populations as hosts. The study protocol was designed to ensure human-origin results did not derive from experimental contamination by field collectors. Moreover, only samples showing clear sequence electropherograms without evidence of signals from two or more DNA fragments were analysed. Unfortunately, however, this approach excluded mosquitoes that had fed on more than one host.

Among non-human mammals, large herbivorous mammals, such as those of the Camelidae, Bovidae and Equidae, were primarily identified. This is in agreement with earlier studies on mosquito bloodmeal patterns in zoos, in which New World camelids [*Lama glama*, *Lama guanicoe*, *Vicugna pacos* (all: Artiodactyla: Camelidae)] (Schöneberger *et al.*, 2015) or equids [*Equus caballus* (Perissodactyla: Equidae)] (Tuten *et al.*, 2012) were the most common hosts, in addition to humans. One reason for this could be that large host species attract mosquitoes from a wider distance than do smaller vertebrates as they emit more odorants (Lane & Crosskey, 1993). In addition, larger vertebrate species may have more difficulty in repelling insects by defence behaviour (Edman & Scott, 1987). Both body size and the difficulty of preventing insect bites may explain why ostriches were the most common avian blood hosts and were also selected by mammalophilic *An. claviger*.

Regardless of that, avian blood was rarely identified in the collected mosquitoes, although both zoological gardens keep more avian than mammal specimens. This is in contrast with findings in previous zoo studies, in which birds were frequently identified as hosts. For example, Tuten *et al.* (2012) collected a high ratio of wild animal bloodmeals compared with the numbers found in Berlin and Eberswalde, most of which were of avian origin. Hence, if only the abundance of potential host species was the decisive factor, more avian bloodmeals should have been expected. One cause of the present contrary finding may refer to the small proportion of nestlings during the study period. Nestlings are particularly prone to mosquito bites as they display much less defence behaviour than older birds, and their protective plumage is not pronounced (Kale *et al.*, 1972; Unnasch *et al.*, 2006). In addition, the mosquito species sampled were considered predominantly to be mammalophilic or at least indiscriminate feeders, rather than ornithophilic feeders (Table 2).

Further, the aspirator collected mosquito species selectively and blood-fed specimens most effectively. Thus, some mosquito species were collected more often in a blood-fed status than others. For example, *Aedes vexans* (Diptera: Culicidae) prefers to rest in understory vegetation (Burkett-Cadena *et al.*, 2008b) from which escape is easier than from shelter-type resting sites.

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Table 2. Blood hosts of mosquito taxa sampled in the two German zoos over 2 years (2016, 2017) and host preferences according to the literature. Animal species considered native and free-roaming are underlined.

| Mosquito taxon | Blood host species identified | Hosts according to literature | References (e.g.) |
|--|---|-------------------------------|--|
| <i>Aedes annulipes</i> group | Mammal: <i>Axis axis</i> , <i>Bison bonasus</i> , <i>Bos mutus</i> , <i>Bovidae</i> , <i>Camelus bactrianus</i> , <i>Capra hircus</i> , <i>Dama dama</i> , <i>Equus asinus</i> , <i>Homo sapiens</i> , <i>Hydrochaeris hydrochaeris</i> , <i>Leptailurus serval</i> , <i>Procyon lotor</i> , <i>Tupirus terrestris</i> , <i>Vulpes vulpes</i> | Mammals and birds | Medlock <i>et al.</i> , 2007 |
| <i>Aedes cataphylla</i> | Mammal: <i>Dama dama</i> | Mammals | Murdock <i>et al.</i> , 2010 |
| <i>Aedes cinereus</i> group | Mammal: <i>Equus asinus</i> , <i>Homo sapiens</i> , <i>Rangifer tarandus</i> | Predominantly mammals | Schönenberger <i>et al.</i> , 2015 |
| <i>Aedes sticticus</i> | Mammal: <i>Capra hircus</i> , <i>Equus asinus</i> | Predominantly mammals | Schönenberger <i>et al.</i> , 2015 |
| <i>Aedes vexans</i> | Mammal: <i>Bovidae</i> , <i>Camelidae</i> , <i>Camelus bactrianus</i> , <i>Capra hircus</i> , <i>Cervus elaphus</i> , <i>Equus asinus</i> , <i>Homo sapiens</i> , <i>Vulpes vulpes</i> | Predominantly mammals | Schönenberger <i>et al.</i> , 2015 |
| <i>Anopheles claviger</i> | Mammal: <i>Axis axis</i> , <i>Bison bonasus</i> , <i>Bos grunniens</i> , <i>Camelus bactrianus</i> , <i>Capra hircus</i> , <i>Cervidae</i> , <i>Dama dama</i> , <i>Equidae</i> , <i>Homo sapiens</i> , <i>Hydrochaeris hydrochaeris</i> , <i>Panthera pardus</i> , <i>Rangifer tarandus</i> , <i>Sus scrofa</i> , <i>Tragelaphus speki</i> Avian: <i>Struthio camelus</i> | Mammals | Schönenberger <i>et al.</i> , 2015 Börsler <i>et al.</i> , 2016 |
| <i>Anopheles maculipennis</i> complex | Mammal: <i>Bos taurus</i> , <i>Bovidae</i> , <i>Budorcas taxicolor</i> , <i>Camelidae</i> , <i>Camelus bactrianus</i> , <i>Camelus dromedarius</i> , <i>Canis lupus</i> , <i>Capra hircus</i> , <i>Equidae</i> , <i>Equus asinus</i> , <i>Equus caballus</i> , <i>Macropodidae</i> , <i>Sus scrofa</i> , <i>Tayassu pecari</i> | Mammals and birds | Schönenberger <i>et al.</i> , 2015 |
| <i>Anopheles daciae</i> | Mammal: <i>Bison bonasus</i> , <i>Camelus bactrianus</i> , <i>Capra hircus</i> , <i>Felidae</i> , <i>Panthera leo</i> , <i>Panthera tigris</i> , <i>Rangifer tarandus</i> , <i>Sus scrofa</i> | Mammals and birds | Danabalan <i>et al.</i> , 2014 |
| <i>Anopheles maculipennis</i> s.s. | Mammal: <i>Bison bonasus</i> , <i>Bos mutus</i> , <i>Bos taurus</i> , <i>Bovidae</i> , <i>Budorcas taxicolor</i> , <i>Camelidae</i> , <i>Camelus bactrianus</i> , <i>Camelus dromedarius</i> , <i>Capra hircus</i> , <i>Capromys wagneri</i> , <i>Caviidae</i> , <i>Cervidae</i> , <i>Cervus nippon</i> , <i>Cuon alpinus</i> , <i>Equus asinus</i> , <i>Equus zebra</i> , <i>Homo sapiens</i> , <i>Hystrix indica</i> , <i>Lama guanicoe</i> , <i>Lama pacos</i> , <i>Martes flavigula</i> , <i>Mephitis mephitis</i> , <i>Oreamnos americanus</i> , <i>Ovis aries</i> , <i>Rangifer tarandus</i> , <i>Syncerus caffer</i> Avian: <i>Struthio camelus</i> | See complex | See complex |
| <i>Anopheles messeae</i> | Mammal: <i>Axis axis</i> , <i>Bos grunniens</i> , <i>Camelidae</i> , <i>Camelus bactrianus</i> , <i>Capra hircus</i> , <i>Caviidae</i> , <i>Dama dama</i> , <i>Equus asinus</i> , <i>Homo sapiens</i> , <i>Lama guanicoe</i> , <i>Macropus rufogriseus</i> , <i>Rangifer tarandus</i> Avian: <i>Struthio camelus</i> | Mammals and birds | Danabalan <i>et al.</i> , 2014 |
| <i>Anopheles plumbeus</i> | Mammal: <i>Camelidae</i> , <i>Camelus dromedarius</i> , <i>Homo sapiens</i> , <i>Ovis aries</i> | Mammals and birds | Medlock <i>et al.</i> , 2005 Börsler <i>et al.</i> , 2016 |
| <i>Coquillettidia richiardii</i> | Mammal: <i>Homo sapiens</i> | Mammals and birds | Schönenberger <i>et al.</i> , 2015 Börsler <i>et al.</i> , 2016 |
| <i>Culiseta annulata</i> | Mammal: <i>Axis axis</i> , <i>Bison bonasus</i> , <i>Bos mutus</i> , <i>Bos taurus</i> , <i>Bovidae</i> , <i>Bubalus bubalis</i> , <i>Camelidae</i> , <i>Camelus bactrianus</i> , <i>Camelus dromedarius</i> , <i>Capra hircus</i> , <i>Cervinae</i> , <i>Cervus canadensis</i> , <i>Dama dama</i> , <i>Equidae</i> , <i>Equus asinus</i> , <i>Equus burchelli</i> , <i>Giraffa camelopardalis</i> , <i>Homo sapiens</i> , <i>Macropodidae</i> , <i>Macropus rufogriseus</i> , <i>Ovis ammon</i> , <i>Ovis aries</i> , <i>Panthera leo</i> , <i>Rangifer tarandus</i> , <i>Syncerus caffer</i> | Predominantly mammals | Schönenberger <i>et al.</i> , 2015 |
| <i>Culiseta morsitans</i> / <i>flumpeana</i> | Mammal: <i>Homo sapiens</i> | Mammals and birds* | Medlock <i>et al.</i> , 2005 |
| <i>Culex pipiens</i> complex | Mammal: <i>Bovidae</i> , <i>Camelus dromedarius</i> , <i>Homo sapiens</i> | Mammals and birds | Apperson <i>et al.</i> , 2004 |
| <i>Culex pipiens</i> biotype <i>pipiens</i> | Mammal: <i>Capra hircus</i> , <i>Homo sapiens</i> , <i>Mephitis mephitis</i> , <i>Ovis aries</i> , <i>Rangifer tarandus</i> , <i>Sus scrofa</i> Avian: <i>Parus major</i> , <i>Passer domesticus</i> | Mammals and birds | Börsler <i>et al.</i> , 2016 |
| <i>Culex torrentium</i> | Mammal: <i>Camelidae</i> , <i>Homo sapiens</i> | Mammals and birds | Börsler <i>et al.</i> , 2016 |
| <i>Culex territans</i> | Mammal: <i>Homo sapiens</i> Amphibian: <i>Bufo bufo</i> , <i>Rana</i> sp. | Predominantly amphibians | Savage <i>et al.</i> , 2007 |

*Data available for *Culiseta morsitans* only.

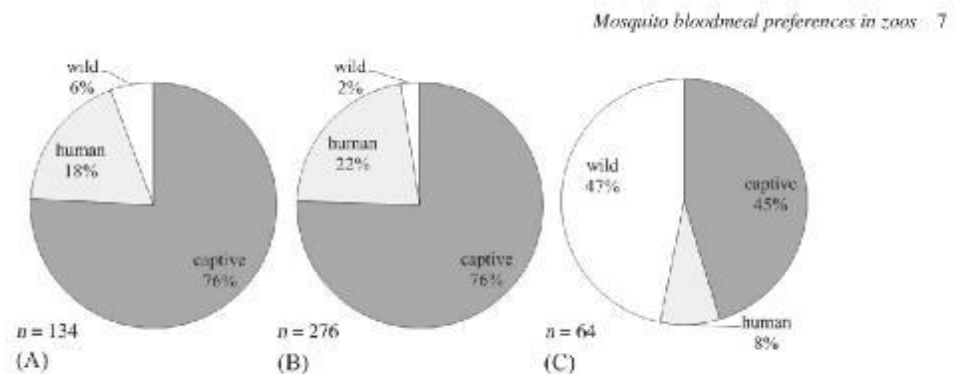


Fig. 2. Origins of bloodmeals according to captive zoo animal population, wild animal population and humans in (A) the Tierpark Berlin, (B) the Zoological Garden Eberswalde and (C) the zoos studied by Tuten *et al.* (2012): Greenville Zoo and Riverbanks Zoo (South Carolina, U.S.A.).

Table 3. Co-occurrence analysis of mosquito blood-feeding patterns in the two sampled zoos, 2016 and 2017.

| Location | c-score | SES | Pattern |
|------------------------------|----------|-------|---------|
| | observed | | |
| Tierpark Berlin | 4.2 | -1.66 | Random |
| Zoological Garden Eberswalde | 1.39 | 0.78 | Random |

c-score, checkerboard score; SD, standard deviation; SES, standardized effect size.

Therefore, it must be noted that the outcome of the blood host analysis is influenced not only by the relative abundances of the vertebrates available, but also by the relative abundances of the mosquito species in the collections, together with the relative levels of defence behaviour in the vertebrate species available at the time the study was conducted, and hence may not reflect the use of the most preferred host species (Chaves *et al.*, 2010; Simpson *et al.*, 2012; Takken & Verhulst, 2013).

On a statistical basis, a random feeding pattern was identified by co-occurrence analysis, which indicates that the mosquito species collected had no pronounced preferences for certain host species. The present data do not allow for clarification of the extent to which animal species not found as host species had been purposely avoided by the mosquitoes or were not present in the sample by chance, particularly as the analysis could include only animal families from which at least one bloodmeal was available. The generalist feeding behaviour found possibly reduces the risk for the transmission of mosquito-borne disease agents, as the dilution effect might prevent the spread of a pathogen (Ostfeld & Keesing, 2000). However, the risk for transmission may be enhanced if the preferred host species are equally competent pathogen reservoirs and a 'diversity amplification' occurs (Miller & Huppert, 2013). Such reinforcement may be particularly prevalent in zoos, in which stressed captive animals may be equally at risk of infection with locally occurring pathogens. Nonetheless, cluster analysis identified three groups of mosquitoes according to blood-feeding behaviour. One group contains one mosquito species feeding on amphibians (*Cx. territans*), the second group consists of mosquito taxa that fed on high proportions on non-human mammals (*Ae. cinereus* group, *Aedes sticticus*, *Ae. vexans*, *An. claviger*, *Anopheles plumbeus*), and the third cluster includes mosquito

taxa of which the majority used both humans and non-human mammals as blood hosts and a smaller proportion used birds as blood hosts (*Ae. annulipes* group, *An. maculipennis* complex, *Culiseta annulata*, *Cx. pipiens* complex). These groups correspond to host-feeding groups of German mosquito species identified by Börstler *et al.* (2016). Thus, the overlap of host species as recognized by Börstler *et al.* (2016) for different mosquito species collected all over Germany applies even within the smaller scale of the zoological garden, despite the use of non-native vertebrate species as main hosts in the latter context.

Culex territans is known to prefer amphibians as blood hosts, but has been found to feed on reptiles, birds and mammals as well (Savage *et al.*, 2007); this was confirmed by the finding of one human bloodmeal in this study. The mosquito group feeding mainly on human and non-human mammal blood also included the species most frequently collected in a blood-fed status. All of these are considered potential vectors of mosquito-borne disease agents and may be able to transmit zoonotic agents. Species of the group that fed mainly on non-human mammals were less abundant in the samples, which suggests they may show a broader host range with a larger sample size and explains why the data pertaining to this group should not be overestimated. Notwithstanding, these species may play a role in the transmission of pathogens within the zoo population, as well as between captive and wild animals.

Bloodmeals originating from free-roaming wild animals were rarely detected. Among these, a house sparrow and a great tit, which belong to the avian order Passeriformes, were identified in the Tierpark Berlin. Passeriform birds are known to be hosts of Batai, Sindbis, Tuffyña and Usutu viruses (Gratz, 2006; Hubálek, 2008), all of which have been demonstrated to circulate in Germany (Pilaski, 1987; Jöst *et al.*, 2010, 2011a,

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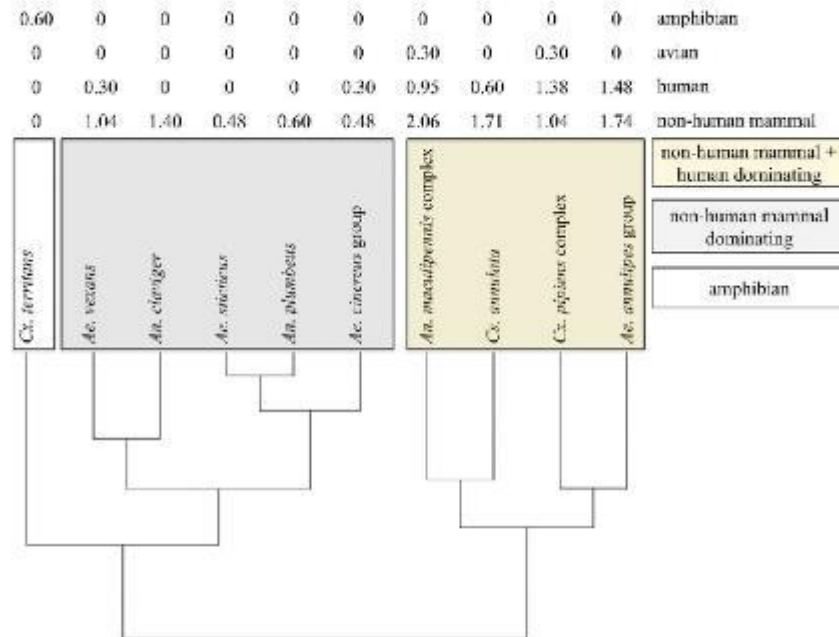


Fig. 3. Two-way cluster analysis grouping mosquito species according to blood source (amphibian, avian, human, non-human mammal). *Ae.*, *Aedes*; *An.*, *Anopheles*; *Cs.*, *Caliseta*; *Cx.*, *Culex*. [Colour figure can be viewed at wileyonlinelibrary.com].

2011b). Thus, the potential for introducing these viruses into the zoo population exists.

A previous study on the mosquito fauna of the Tierpark Berlin and the Zoological Garden Eberswalde found that the locations differ in their species composition, with partially different mosquito species acting as possible vectors (Heym *et al.*, 2018). Accordingly, the analysis of blood-fed mosquitoes also showed differences between the zoos in terms of the number of blood-fed specimens collected. The number of engorged mosquitoes caught in the smaller Zoological Garden Eberswalde was higher than that in the larger Tierpark Berlin, which may be attributable to a higher host density in Eberswalde. This may have led to a higher mosquito density in the smaller zoo, which would increase the level of nuisance caused by mosquitoes. However, as almost equal ratios of humans, and captive and wild animals served as blood hosts at both locations and feeding patterns were random for both zoos, mosquito foraging behaviour appears to be similar, irrespective of mosquito abundances.

Flight distances varied between the zoos, with longer distances measured in the Tierpark Berlin (up to 770 m) in comparison with those in other studies conducted in zoological gardens. Ejiri *et al.* (2011) measured a maximum distance of 350 m, Greenberg *et al.* (2012) of 170 m and Tuten *et al.* (2012) of 327 m. Indeed, the Tierpark Berlin is much larger than the Zoological Garden Eberswalde and the other zoos cited and therefore longer distances are more likely in Berlin. According to the literature, mosquito flight capacity is strongly related to the ecological preferences of a species and to its physiology, but

also to abiotic factors such as landscape structure and climatic conditions (Verdonschot & Besse-Lototskaya, 2014). In this study, no significant differences among species were observed.

Conclusions

The mosquito species collected in the present study displayed a generalist feeding behaviour, with no statistically significant preference for certain taxonomic families, although mainly mammal hosts were identified. As free-roaming wild animals were also detected as blood hosts, the introduction of a mosquito-borne pathogen into the zoo animal population is possible, and chronically stressed animals living in captivity are probably particularly vulnerable. Because humans have closer contact with wild animals in zoos than outside zoos and mosquitoes are particularly likely to bite visitors in the zoo areas, a risk for pathogen transmission to humans should be considered. Not to be underestimated is the mere nuisance to humans and animals caused by biting mosquitoes. The results of this work demonstrate that mosquito blood feeding can be influenced by the body size and defensive behaviour of potential hosts, and that mosquitoes do not discriminate between native and exotic vertebrate species when choosing a bloodmeal host. There are still many factors influencing mosquito blood feeding that require further investigation. For example, it is still not clear if a change in or absence of defensive behaviour against mosquitoes in captive animals can affect mosquito bloodmeal

preferences. Additionally, the spatial contexts in which zoo animals are kept may influence mosquito host choice. Future studies might also focus on determining the optimum method of collecting blood-fed mosquitoes in order to provide a more balanced spectrum of mosquito species and to obtain a differentiated picture of the local annoyance caused by mosquitoes. More detailed knowledge about the factors determining mosquito blood-feeding behaviour can lead to a better assessment of the risks for the transmission of mosquito-borne disease in general and in zoos in particular.

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References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403–410.
- Apperson, C.S., Hassan, H.K., Harrison, B.A. *et al.* (2004) Host feeding patterns of established and potential mosquito vectors of West Nile virus in the eastern United States. *Vector-Borne and Zoonotic Diseases*, **4**, 71–82.
- Barrera, R., Bingham, A.M., Hassan, H.K. *et al.* (2012) Vertebrate hosts of *Aedes aegypti* and *Aedes mediopinnatus* (Diptera: Culicidae) in rural Puerto Rico. *Journal of Medical Entomology*, **49**, 917–921.
- Becker, N., Petric, D., Zgomba, M. *et al.* (2010). *Mosquitoes and their Control*. Springer, Heidelberg.
- Börstler, J., Jöst, H., Garms, R. *et al.* (2016) Host-feeding patterns of mosquito species in Germany. *Parasites & Vectors*, **9**, 1–14.
- Burkett-Cadena, N.D., Graham, S.P., Hassan, H.K. *et al.* (2008a) Blood feeding patterns of potential arbovirus vectors of the genus *Culex* targeting ectothermic hosts. *American Journal of Tropical Medicine and Hygiene*, **79**, 809–815.
- Burkett-Cadena, N.D., Eubanks, M.D. & Unnasch, T.R. (2008b) Preference of female mosquitoes for natural and artificial resting sites. *Journal of the American Mosquito Control Association*, **24**, 228–235.
- Chaves, L.F., Harrington, L.C., Koogh, C.L., Nguyen, A.M. & Kitron, U.D. (2010) Blood feeding patterns of mosquitoes: random or structured? *Frontiers in Zoology*, **7**, 3.

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- Danabalan, R., Monaghan, M.T., Ponsonby, D.J. & Linton, Y.M. (2014) Occurrence and host preferences of *Anopheles maculipennis* group mosquitoes in England and Wales. *Medical and Veterinary Entomology*, **28**, 169–178.
- Edman, J.D. & Scott, T.W. (1987) Host defensive behaviour and the feeding success of mosquitoes. *Insect Science and its Application*, **8**, 617–622.
- Ejiri, H., Sato, Y., Kim, K.-S. *et al.* (2011) Entomological study on transmission of avian malaria parasites in a zoological garden in Japan: bloodmeal identification and detection of avian malaria parasite DNA from blood-fed mosquitoes. *Journal of Medical Entomology*, **48**, 600–607.
- Folmer, O., Black, M., Hoch, 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.
- Gotelli, N.J., Hart, E.M. & Ellison, A.M. (2015) *EcoSimR: Null Model Analysis for Ecological Data*. R Package, Version 0.1.0.5.0. URL: <https://cran.r-project.org/web/packages/EcoSimR/index.html> [accessed on 17 November 2018].
- Gratz, N. (2006) *Vector- and Rodent-Borne Diseases in Europe and North America*. Cambridge University Press, New York, NY.
- Greenberg, J.A., Dimenna, M.A., Hanelt, B. & Hofkin, B.V. (2012) Analysis of post-blood meal flight distances in mosquitoes utilizing zoo animal blood meals. *Journal of Vector Ecology*, **37**, 83–89.
- Hebert, P.D.N., Ratnasingham, S. & De Waard, J.R. (2003) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London B: Biological Sciences*, **270**, S96–S99.
- Heym, E.C., Kampen, H. & Walther, D. (2018) Mosquito species composition and phenology (Diptera, Culicidae) in two German zoological gardens imply different risks of mosquito-borne pathogen transmission. *Journal of Vector Ecology*, **43**, 80–88.
- Hocking, B. (1971) Blood-sucking behavior of terrestrial arthropods. *Annual Review of Entomology*, **16**, 1–26.
- Huhälek, Z. (2008) Mosquito-borne viruses in Europe. *Parasitology Research*, **103**, 29–43.
- Jöst, H., Bialonski, A., Storch, V., Gunther, 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.
- Jöst, H., Bialonski, A., Schmetz, C., Gunther, S., Becker, N. & Schmidt-Chanasit, J. (2011a) Isolation and phylogenetic analysis of Batai virus, Germany. *American Journal of Tropical Medicine and Hygiene*, **84**, 241–243.
- Jöst, H., Bialonski, A., Maus, D. *et al.* (2011b) Isolation of Usutu virus in Germany. *American Journal of Tropical Medicine and Hygiene*, **85**, 551–553.
- Kale, H.W., Edman, J.D. & Webber, L.A. (1972) Effect of behavior and age of individual ciconiiform birds on mosquito feeding success. *Mosquito News*, **32**, 343–350.
- Kilpatrick, A.M., Kramer, L.D., Jones, M.J., Marra, P.P. & Daszak, P. (2006) West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior. *PLoS Biology*, **4**, e82.
- Kitano, T., Umetsu, K., Tian, W. & Osawa, M. (2007) Two universal primer sets for species identification among vertebrates. *International Journal of Legal Medicine*, **121**, 423–427.
- Kronefeld, M., Werner, D. & Kampen, H. (2014) PCR identification and distribution of *Anopheles daciae* (Diptera, Culicidae) in Germany. *Parasitology Research*, **113**, 2079–2086.

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- Lane, R.P. & Crosskey, R.W. (1993) *Medical Insects and Arachnids*. Chapman & Hall, London.
- Lyimo, I.N. & Ferguson, H.M. (2009) Ecological and evolutionary determinants of host species choice in mosquito vectors. *Trends in Parasitology*, **25**, 189–196.
- McNamara, T. (2007) The role of zoos in biosurveillance. *International Zoo Yearbook*, **41**, 12–15.
- Medlock, J.M., Snow, K.R. & Leach, S. (2005) Potential transmission of West Nile virus in the British Isles: an ecological review of candidate mosquito bridge vectors. *Medical and Veterinary Entomology*, **19**, 2–21.
- Medlock, J.M., Snow, K.R. & Leach, S. (2007) Possible ecology and epidemiology of medically important mosquito-borne arboviruses in Great Britain. *Epidemiology and Infection*, **135**, 466–482.
- Miller, E. & Huppert, A. (2013) The effects of host diversity on vector-borne disease: the conditions under which diversity will amplify or dilute the disease risk. *PLoS One*, **8**, e80279.
- Murdock, C.C., Olival, K.J. & Perkins, S.L. (2010) Molecular identification of host feeding patterns of snow-melt mosquitoes (Diptera: Culicidae): potential implications for the transmission ecology of Jamestown canyon virus. *Journal of Medical Entomology*, **47**, 226–229.
- Nelder, M.P., Reeves, W.K., Adler, P.H., Wozniak, A. & Wills, W. (2009) Ectoparasites and associated pathogens of free-roaming and captive animals in zoos of South Carolina. *Vector-Borne and Zoonotic Diseases*, **9**, 469–477.
- Ostfeld, R.S. & Keesing, F. (2000) Biodiversity series: the function of biodiversity in the ecology of vector-borne zoonotic diseases. *Canadian Journal of Zoology*, **78**, 2061–2078.
- Pilaski, J. (1987) Contribution to the ecology of Tahyna virus in Central Europe. *Bulletin of the Society for Vector Ecology*, **12**, 544–553.
- 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.
- R Core Team (2017) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna.
- Rohe, D.L. & Fall, R.P. (1979) A miniature battery powered CO₂ baited light trap for mosquito borne encephalitis surveillance. *Bulletin of the Society of Vector Ecology*, **4**, 24–27.
- Rudolf, M., Czajka, C., Böseler, J. et al. (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.
- Savage, H.M., Aggarwal, D., Apperson, C.S. et al. (2007) Host choice and West Nile virus infection rates in blood-fed mosquitoes, including members of the *Culex pipiens* complex, from Memphis and Shelby County, Tennessee, 2002–2003. *Vector-Borne and Zoonotic Diseases*, **7**, 365–386.
- Schaffner, F., Angel, G., Geoffroy, B., Hervy, J.P., Rhaïem, A. & Brunhes, J. (2001) *The Mosquitoes of Europe. An Identification and Training Programme* [CD-Rom]. IRD Editions, Montpellier.
- Schönenberger, A.C., Wagner, S., Tuten, H.C. et al. (2015) Host preferences in host-seeking and blood-fed mosquitoes in Switzerland. *Medical and Veterinary Entomology*, **30**, 39–52.
- Simpson, J.E., Hurtado, P.J., Medlock, J. et al. (2012) Vector host-feeding preferences drive transmission of multi-host pathogens: West Nile virus as a model system. *Proceedings of the Royal Society B: Biological Sciences*, **279**, 925–933.
- Stevens, L., Dom, P.L., Hobson, J. et al. (2012) Vector blood meals and Chagas disease transmission potential, United States. *Emerging Infectious Diseases*, **18**, 646–649.
- Stone, L. & Roberts, A. (1990) The checkerboard score and species distributions. *Oecologia*, **85**, 74–79.
- Takken, W. & Verhulst, N.O. (2013) Host preferences of blood-feeding mosquitoes. *Annual Review of Entomology*, **58**, 433–453.
- Thiemann, T.C., Wheeler, S.S., Barker, C.M. & Reisen, W.K. (2011) Mosquito host selection varies seasonally with host availability and mosquito density. *PLoS Neglected Tropical Diseases*, **5**, e1452.
- Tuten, H.C., Bridges, W.C., Paul, K.S. & Adler, P.H. (2012) Blood-feeding ecology of mosquitoes in zoos. *Medical and Veterinary Entomology*, **26**, 407–416.
- Unnasch, R.S., Cupp, E.W. & Unnasch, T.R. (2006) Host selection and its role in transmission of arboviral encephalitides. *Disease Ecology: Community Structure and Pathogen Dynamics* (ed. by S.K. Collinge & C. Ray), pp. 73–89. Oxford University Press, New York, NY.
- Verdonschot, P.F.M. & Besse-Lototskaya, A.A. (2014) Flight distance of mosquitoes (Culicidae): a metadata analysis to support the management of barrier zones around rewetted and newly constructed wetlands. *Limnologia – Ecology and Management of Inland Waters*, **45**, 69–79.
- Wolf, C., Rentsch, J. & Hübner, P. (1999) PCR-RFLP analysis of mitochondrial DNA: a reliable method for species identification. *Journal of Agricultural and Food Chemistry*, **47**, 1350–1355.

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**4.3 Molecular detection of vector-borne pathogens from mosquitoes
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ARTHROPODS AND MEDICAL ENTOMOLOGY - ORIGINAL PAPER



Molecular detection of vector-borne pathogens from mosquitoes collected in two zoological gardens in Germany

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Abstract

In Germany, knowledge of disease agents transmitted by arthropods in zoological gardens is scarce. In the framework of ecological studies, mosquitoes were therefore collected in German zoological gardens and examined for mosquito-borne pathogen DNA and RNA. In total, 3840 mosquitoes were screened for filarial nematodes and three groups of viruses (orthobunyaviruses, flaviviruses, alphaviruses) while 405 mosquitoes were tested for avian malaria parasites. In addition to the filarial nematode species *Dirofilaria repens* ($n = 1$) and *Setaria tundra* ($n = 8$), Sindbis virus ($n = 1$) and the haemosporidian genera *Haemoproteus* ($n = 8$), *Leucocytozoon* ($n = 10$) and *Plasmodium* ($n = 1$) were demonstrated. Identified pathogens have the potential to cause disease in zoo and wild animals, but some of them also in humans. Positive mosquitoes were collected most often in July, indicating the highest infection risk during this month. Most of the pathogens were found in mosquito specimens of the *Culex pipiens* complex, suggesting that its members possibly act as the most important vectors in the surveyed zoos, although the mere demonstration of pathogen DNA/RNA in a homogenised complete mosquito is not finally indicative for a vector role. Outcomes of the study are not only significant for arthropod management in zoological gardens, but also for the general understanding of the occurrence and spread of mosquito-borne disease agents.

Keywords Avian malaria · *Dirofilaria* · *Haemoproteus* · *Leucocytozoon* · *Plasmodium* · Sindbis virus

Introduction

Through globalisation, both invasive mosquito species and mosquito-borne pathogens can be introduced into temperate climate zones such as central Europe. Climate change might facilitate survival of the mosquitoes in the newly invaded regions but also lead to conditions allowing pathogen development or increasing the efficiency of pathogen replication in vector-competent mosquito species (Weissenböck et al. 2010). As both globalisation and climate change significantly increase the risk of mosquito-borne disease outbreaks (Suk

2017), surveillance of mosquitoes and mosquito-borne pathogens become more and more important.

Monitoring mosquito-borne pathogens faces difficulties since not all potential vectors are known, and most infections, particularly of animals, remain undetected. This is different in zoological gardens, where mosquito-borne diseases are likely to be detected in an early stage as the health status of zoo animals is regularly and thoroughly monitored. In addition, stressed captive vertebrate species can be vulnerable for disease agents, rendering the zoo animals into sentinels for mosquito-borne diseases in urban settings (McNamara 2007). In parallel, zoological gardens offer ideal living conditions for mosquitoes through numerous breeding and resting sites and provision of a high diversity of vertebrate species for blood feeding (Tuten 2011).

Mosquito-borne diseases have been repeatedly documented from zoo animals worldwide (Adler et al. 2011). The agents of these include viruses, filarial nematodes and avian malaria parasites infecting non-adapted animals. For example, the first avian infection by *Dirofilaria immitis* Leidy, 1856 was from a Humboldt penguin living in a zoo in Japan (Sano et al. 2005). Likewise, the avian malaria parasites *Plasmodium relictum*

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Grassi & Feletti, 1891 and *Plasmodium elongatum* Huff, 1930 were involved in various fatal cases in zoos (e.g. Graczyk et al. 1994; Bueno et al. 2010; Sijbranda et al. 2017).

In addition to known pathogens infecting vulnerable zoo animals, pathogens previously not known to occur in the studied area have been documented from diseased animals in zoos. In 1999, for example, the Bronx Zoo/Wildlife Conservation Park, New York, was one of the first institutions recognising unusually high bird mortality that was later attributed to West Nile virus (WNV), which was not known from the USA at that time (Ludwig et al. 2002). Also in Germany, the first documentation ever of WNV was from zoological gardens, where six fatal cases of infection occurred in wild and captive birds (Ziegler et al. 2018). In the Zoological Garden Berlin, Germany, a Usutu virus strain was detected in two deceased juvenile great grey owls, which could be distinguished from strains previously known to circulate in Germany, demonstrating the mobility of mosquito-borne viruses (Ziegler et al. 2016).

Other mosquito-borne viruses documented for Germany are Batai virus (BATV), Sindbis virus (SINV) and Tahyna virus (TAHV) (Pilaski 1987; Jöst et al. 2010, 2011). BATV occurs widespread in Europe and was the most often detected virus in mosquitoes collected in Germany from 2011 to 2016 (Scheuch et al. 2018). Human infections by BATV seem to be rare and associated with mild symptoms, but little is known on the pathogenicity to animals (Hubálek 2008). In contrast, SINV can cause acute disease in humans and birds (Hubálek 2008; Adouchief et al. 2016). Passeriform birds are the main vertebrate hosts, but the virus has occasionally been isolated also from rodents and amphibians (Hubálek 2008). According to antibody prevalence studies in humans, TAHV is among the most common California group viruses in Eurasia (Gratz 2006), although infection rates in here, which are the principal vertebrate hosts next to hedgehogs and rodents, are low in Germany (Dobler et al. 2006). Whether infections produce disease in animals is unknown but humans may develop influenza-like symptoms (Hubálek 2008).

The first evidence of an autochthonous *Dirofilaria* infection from Germany is from 2004, when *Dirofilaria repens* Railliet & Henry, 1911 was isolated from a diseased dog which had never left Germany (Hermosilla et al. 2006). *Dirofilaria repens* was later also demonstrated in mosquitoes from Germany (Czajka et al. 2014; Kronefeld et al. 2014a), just as *Dirofilaria immitis*, the latter being the first evidence of this nematode in mosquitoes collected outside the Mediterranean (Kronefeld et al. 2014a). *Dirofilaria repens* and *D. immitis* are endemic in southern Europe, where they mainly infect canines, felines and other carnivores, but human infections have increasingly been reported (Genchi et al. 2011). Due to improving climatic conditions, but also through the introduction of dogs and cats from endemic countries, both nematode species have successfully established in northern and eastern European countries (Genchi et al. 2011).

The nematode *Setaria tundra* Rajewsky, 1929 is generally believed to be asymptomatic in its natural hosts, which are mainly cervids, although severe disease outbreaks have been reported in reindeer populations in Finland (Laaksonen et al. 2007). There are no documented human infections with *S. tundra*.

Avian malaria is a parasitic disease caused by haemosporidian protozoans. Haemosporidians infecting birds include the genera *Plasmodium* Marchiafava & Celli, 1885, *Haemoproteus* Kruse, 1890 and *Leucocytozoon* Ziemann, 1898. There is little information about haemosporidian prevalence in Germany, although all three genera were previously documented (Krone et al. 2001, 2008; Wiersch et al. 2007). While infections of native birds with indigenous haemosporidian strains are usually harmless or asymptomatic, mortality rates are high in infected captive non-native birds which are immunologically naive (Huijben et al. 2007).

Although mosquito-associated pathogens have been detected repeatedly in Germany, it remains difficult to assess transmission risks at a local level. A recent study about the mosquito fauna of two zoological gardens in Germany showed that differences in mosquito species composition can occur even within small geographic scales (Heym et al.). Due to varying biological characteristics of the most frequent mosquito species, different pathogens could therefore become locally relevant. Additionally, blood meal analyses in the same zoological gardens demonstrated that humans and captive zoo animals were the most frequent blood-hosts of the collected mosquito species (Heym et al. 2019), suggesting that transmission of circulating mosquito-borne microorganisms between zoo animals and humans cannot be excluded, although most of them might be non-pathogenic to humans.

To better estimate the transmission risk of mosquito-borne pathogens in a zoo setting, the aim of this study was to analyse pathogen species/group, time and locality of circulation, as well as mosquito species carrying them.

Material and methods

Study locations and mosquito collection

Mosquitoes were collected in a 4-week rhythm from May to September 2016 and from April to September 2017 in the Tierpark Berlin (Berlin, Germany, N 52° 49.8406', E 13° 53.0210') and the Zoological Garden Eberswalde (Brandenburg, Germany, N 52° 82.2664', E 13° 78.3025'). The Tierpark Berlin covers an area of ca. 160 ha, is surrounded by urban area and harbours some 7500 animals. The Zoological Garden Eberswalde comprises only 15 ha, is located in a forested area and is home to ca. 1500 animals.

Mosquito collections were conducted with eight EVS-traps (BioQuip Products, CA, USA; Rohe and Fall 1979), placed at

comparable locations in both zoos at 1.6–2 m height, with a minimum of 50 m distance to ensure independence. EVS-traps were baited with dry ice producing CO₂ as an attractant and were operated 24 h per field visit. In the Zoological Garden Eberswalde, EVS-trapping was not possible in September 2016.

In addition, adult mosquitoes were collected from their resting sites using a battery-powered Improved Prokopack Aspirator (model 1419; John W. Hock, FL, USA). Aspiration took place at a total of 15 resting sites per zoo, which were sampled once during every zoo visit for 5 min each. Resting sites consisted of shaded hiding places, which included understorey vegetation as well as eaves and wooden or stone constructions. Sampled sites were at a height of 1–3 m. Mosquitoes trying to bite the collector during fieldwork were also captured, later defined as ‘hand catches’. All collected mosquito specimens were conserved on dry ice and stored frozen at –80 °C.

Mosquito identification

Mosquito species determination was conducted morphologically using the identification keys by Schaffner et al. (2001) and Becker et al. (2010).

Specimens belonging to the *Anopheles maculipennis* and *Culex pipiens* species complexes (*An. maculipennis* s.l., *Cx. pipiens* s.l.) were identified genetically. Species-specific ITS2-PCR was conducted to analyse *An. maculipennis* complex specimens (Proft et al. 1999; Kronefeld et al. 2014b). *Culex pipiens* complex specimens were determined by a multiplex real-time PCR assay (Rudolf et al. 2013).

Mosquitoes not belonging to a species complex, but not identifiable morphologically due to missing identification cues, were subjected to COI (cytochrome oxidase gene subunit 1) barcoding (Folmer et al. 1994; Hébert et al. 2003).

Females belonging to the *Aedes cinereus* Meigen, 1818 and *Aedes annulipes* Meigen, 1830 groups, where species identification is possible neither morphologically nor genetically, were evaluated at the group level. Also, *Culiseta morsitans* Theobald, 1901 and *Culiseta funipennis* Stephens, 1825 females were not separated in the evaluation, due to not being reliably distinguishable morphologically or genetically.

Pathogen screening

Virus diagnostics

Mosquito pools were screened using quantitative real-time PCRs following the protocols of Lambert and Lanciotti (2009) for orthobunyaviruses, Chao et al. (2007) for flaviviruses and Eshoo et al. (2007) for alphaviruses.

Filarial diagnostics

Mosquito screening for filarial nematodes was performed with a filarioid-specific real-time PCR assay according to Kronefeld et al. (2014a), targeting a 90 bp fragment of the mitochondrial 16S rRNA gene. Real-time PCR dissociation curves were analysed with the BioRad CFX-Manager software (www.bio-rad.com). If a signal was detected, a second conventional PCR was conducted on the sample, targeting 650 bp of the filarioid COI gene (Casiraghi et al. 2001). The resulting PCR products were processed and sequenced as described by Kronefeld et al. (2014a). Species identification of obtained sequences was conducted by standard BLAST programme search against the GenBank nucleic acid sequence database (Altschul et al. 1990).

Haemosporidian diagnostics

Screening for haemosporidian parasites of the genera *Haemoproteus*, *Leucocytozoon* and *Plasmodium* was performed with a real-time PCR assay targeting a 182 bp fragment of mitochondrial rDNA followed by high-resolution melting-analysis (Bell et al. 2015). Briefly, DNA was prepared from individual engorged mosquitoes using the QIAamp DNA Blood Mini Kit (Qiagen). Five microlitre of DNA was then used as a template in a 25 µl amplification reaction using the 2× QuantiTect SYBR Green PCR Master Mix (Applied Biosystems, Germany), as well as 0.4 µM forward (R330F) and reverse primer (R480RL), 8 µl sterile RNase-free water and 5 µl control or test DNA. Each real-time PCR assay systematically included a no template control (NTC) reaction and two positive control reactions (DNA from *Plasmodium ovale* Stephens, 1922 and *Haemoproteus* sp.) run in parallel with the test samples. Reactions started with an incubation step at 95 °C for 15 min, followed by 39 cycles at 94 °C for 15 s, 51 °C for 30 s and 72 °C for 30 s, and were completed by a dissociation curve ranging from 60 to 95 °C in steps of 5 °C. All data were analysed using the BioRad CFX-Manager software.

To confirm positive PCR results, two nested PCRs targeting the cytochrome b gene were conducted, which amplify a 477 bp fragment in the case of *Haemoproteus/Plasmodium* and a 526 bp fragment in the case of *Leucocytozoon* (Bell et al. 2015). PCR products were visualised by electrophoresis on a 1.5% agarose gel, excised and purified by means of the QIAquick PCR Purification Kit (Qiagen, Germany). This was followed by one-directional sequencing with the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) using the primer FIFI for sequencing *Haemoproteus* and *Plasmodium* DNA and the primer L545F for *Leucocytozoon* DNA (Bell et al. 2015). Products were then purified by SigmaSpin Sequencing Reaction Clean-Up Columns (Sigma Aldrich, Germany) before loading onto a 3130 Genetic Analyser (Applied Biosystems). The genus

assignment of the unknown samples was determined by comparing with sequences at GenBank.

Results

From the Zoological Garden Eberswalde, 2407 mosquito females belonging to 20 taxa were screened for filarial nematodes and viruses. Of these, 265 blood-fed specimens, including 17 taxa, were additionally screened for avian malaria parasites.

From the Tierpark Berlin, a total of 1402 mosquito females belonging to 16 taxa were analysed for filarial nematodes and viruses. The haemosporidian screening was conducted on 140 blood-fed specimens belonging to 12 taxa. Table 1 gives an overview of the collected mosquito species and analysed specimens.

Filarial nematodes

In the Zoological Garden Eberswalde, filarial nematode DNA was detected in four different mosquito species (Table 2). In addition to one nematode which could not be determined to species level, *D. repens* and *S. tundra* were identified. *Dirofilaria repens* was found in *Anopheles messeae* Falleroni, 1926 in July 2016, while evidence for *S. tundra* came from *Ae. annulipes* group mosquitoes, which had been caught in June and August 2016.

Setaria tundra was also detected in five *Aedes vexans* Meigen, 1830 pools collected in the Tierpark Berlin in July 2016. In addition, a *Cx. pipiens* biotype *pipiens* Linnaeus, 1758 specimen was found positive for a filarial nematode that could not be identified to species level (Table 2).

No filarial nematode DNA could be detected in mosquitoes collected in 2017.

Mosquito-borne viruses

SINV-RNA was demonstrated in July 2016 in the Tierpark Berlin in a mosquito pool (12 individuals) belonging to the *Cx. pipiens* complex (Table 2). Mosquito-borne viruses were neither detected in mosquitoes collected in 2016 in the Zoological Garden Eberswalde nor in 2017 in any of the zoos.

Haemosporidian protozoans

DNA of haemosporidian parasites of the genera *Haemoproteus*, *Leucocytozoon* and *Plasmodium* was demonstrated in mosquitoes from both zoos (Table 2).

Haemoproteus sp. DNA was detected in the Zoological Garden Eberswalde in one *Cx. pipiens* biotype *pipiens* mosquito collected in July 2016 and two *Cx. pipiens* biotype *pipiens* mosquitoes collected in August 2017 (Table 2). *Haemoproteus* sp. was also identified in one *Cx. pipiens*

Table 1 Analysed number of specimens per mosquito species collected in the two sampled German zoos

| Species | Tierpark Berlin Total no. of specimens (no. of blood-fed specimens) analysed | Zoological Garden Eberswalde Total no. of specimens (no. of blood-fed specimens) analysed |
|---|---|---|
| <i>Ae. annulipes</i> group ¹ | 8 (0) | 699 (88) |
| <i>Ae. caspius</i> | 0 (0) | 1 (0) |
| <i>Ae. cataphylla</i> | 1 (0) | 6 (2) |
| <i>Ae. cinereus</i> group ¹ | 7 (1) | 44 (5) |
| <i>Ae. punctator</i> | 0 (0) | 15 (0) |
| <i>Ae. rusticus</i> | 0 (0) | 1 (0) |
| <i>Ae. sticticus</i> | 10 (0) | 10 (3) |
| <i>Ae. vexans</i> | 352 (6) | 29 (8) |
| <i>An. claviger</i> | 2 (0) | 117 (32) |
| <i>An. maculipennis</i> complex ² | 175 (24) | 4 (3) |
| <i>An. daciae</i> | 2 (0) | 46 (15) |
| <i>An. maculipennis</i> s.s. | 245 (47) | 89 (27) |
| <i>An. messeae</i> | 7 (2) | 98 (15) |
| <i>An. plumbeus</i> | 15 (4) | 4 (1) |
| <i>Cq. richiardii</i> | 1 (1) | 98 (0) |
| <i>Cx. annulata</i> | 142 (21) | 290 (37) |
| <i>Cx.</i> <i>morsitans</i> s. <i>fumipennis</i> ¹ | 0 (0) | 42 (2) |
| <i>Cx. pipiens</i> complex ² | 75 (8) | 504 (7) |
| <i>Cx. p.</i> biotype <i>molestus</i> | 2 (0) | 0 (0) |
| <i>Cx. p.</i> biotype <i>pipiens</i> | 305 (21) | 202 (16) |
| <i>Cx. torrentium</i> | 34 (2) | 53 (2) |
| <i>Cx. modestus</i> | 3 (0) | 2 (0) |
| <i>Cx. territans</i> | 16 (3) | 53 (2) |
| Total no. of species | 17 | 20 |
| Total no. of specimens analysed | 1402 (140) | 2407 (265) |

¹ Reliable morphological or genetic differentiation not possible

² Not identified to species

biotype *pipiens* and one *Cx. torrentium* Martini, 1925 specimen, respectively, collected in the Tierpark Berlin in 2017.

Leucocytozoon sp. was demonstrated in the Zoological Garden Eberswalde in a mosquito belonging to the *An. maculipennis* complex (Table 2). The positive sample originated from June 2016. In the Tierpark Berlin, *Leucocytozoon* sp. was detected in one *Cx. pipiens* biotype *pipiens* mosquito collected in 2016 and in three *Cx. pipiens* biotype *pipiens* mosquitoes and one *Culex torrentium* collected in 2017. The positive *Cx. pipiens* biotype *pipiens* mosquito collected in June 2016 in the Tierpark Berlin was co-infected with *Haemoproteus* sp. and *Plasmodium* sp. (Table 2), while three *Cx. pipiens* biotype *pipiens* mosquitoes collected in May and July 2017 were found

Table 2 Mosquito-borne pathogens found in mosquitoes in the two sampled German zoos (mosquito collections May–September 2016, April–September 2017)

| Pathogen | Mosquito species | Number of specimens/pools tested positive | Collection month, year | Location ^{1,2} |
|--|---|---|------------------------|-------------------------|
| Filarial nematodes | | | | |
| <i>Dirofilaria repens</i> | <i>An. messeae</i> | 1 | July 2016 | ZE |
| Filarioidea sp. | <i>Cx. torrentium</i> | 1 | June 2016 | ZE |
| Filarioidea sp. | <i>Cx. pipiens</i> biotype <i>pipiens</i> | 1 | June 2016 | TB |
| <i>Setaria tundra</i> | <i>Ae. annulipes</i> group | 2 pools (<i>n</i> = 5, 7) | June 2016 | ZE |
| | | 1 | August 2016 | ZE |
| <i>Setaria tundra</i> | <i>Ae. vexans</i> | 5 pools (<i>n</i> = 3, 10, 10, 14, 7) | July 2016 | TB |
| Viruses | | | | |
| Sindbis virus | <i>Cx. pipiens</i> complex | 1 pool (<i>n</i> = 12) | July 2016 | TB |
| Haemosporidia | | | | |
| <i>Haemoproteus</i> sp. | <i>Cx. pipiens</i> biotype <i>pipiens</i> | 1 | July 2017 | TB |
| <i>Haemoproteus</i> sp. | <i>Cx. torrentium</i> | 1 | July 2017 | TP |
| <i>Haemoproteus</i> sp. | <i>Cx. pipiens</i> biotype <i>pipiens</i> | 1 | July 2016 | ZE |
| <i>Haemoproteus</i> sp. | <i>Cx. pipiens</i> biotype <i>pipiens</i> | 2 | August 2017 | ZE |
| <i>Leucocytozoon</i> sp. | <i>Cx. pipiens</i> biotype <i>pipiens</i> | 1 | June 2016 | TB |
| <i>Leucocytozoon</i> sp. | <i>Cx. pipiens</i> biotype <i>pipiens</i> | 2 | June 2017 | TB |
| <i>Leucocytozoon</i> sp. | <i>Cx. pipiens</i> biotype <i>pipiens</i> | 1 | September 2017 | TB |
| <i>Leucocytozoon</i> sp. | <i>Cx. torrentium</i> | 1 | June 2017 | TB |
| <i>Leucocytozoon</i> sp. | <i>An. maculipennis</i> complex | 1 | July 2016 | ZE |
| <i>Haemoproteus</i> sp./ <i>Leucocytozoon</i> sp. co-infection | <i>Cx. pipiens</i> biotype <i>pipiens</i> | 1 | May 2017 | TB |
| <i>Haemoproteus</i> sp./ <i>Leucocytozoon</i> sp. co-infection | <i>Cx. pipiens</i> biotype <i>pipiens</i> | 1 | July 2017 | TB |
| <i>Haemoproteus</i> sp./ <i>Leucocytozoon</i> sp. co-infection | <i>Cx. pipiens</i> biotype <i>pipiens</i> | 1 | August 2017 | ZE |
| <i>Plasmodium</i> sp./ <i>Leucocytozoon</i> sp. co-infection | <i>Cx. pipiens</i> biotype <i>pipiens</i> | 1 | June 2016 | TB |

¹ ZE, Zoological Garden Eberswalde² TB, Tierpark Berlin

co-infected with *Haemoproteus* sp. and *Leucocytozoon* sp. One *Cx. pipiens* biotype *pipiens* co-infected with *Haemoproteus* sp. and *Leucocytozoon* sp. from the Zoological Garden Eberswalde was collected in August 2017.

Discussion

In the past, infections in zoo animals were mostly imported together with their hosts, which had been captured in the field

(Canavan 1929). Nowadays, zoos are increasingly able to maintain their animal collections by own breeding or exchange with other wildlife parks, indicating linkage of potential infections to transmission within the zoo area. By analysing haematophagous arthropods in the zoo area, it can be examined to what extent vector-borne pathogens circulate, even if there are no acute disease cases. Knowing circulating disease agents helps better assess risks that humans and animals are exposed to in zoological gardens.

While *D. repens* has not been documented from a zoo animal yet, the parasite had already been detected in Germany in 2011 and 2012 in mosquitoes caught only 30 km away from the Zoological Garden Eberswalde (Czajka et al. 2014), and, apparently, autochthonous infections had been diagnosed in dogs in the same federal state of Brandenburg (Sassnau et al. 2009). An established transmission cycle of this filarial species in that area can therefore be assumed. It is conceivable, that the nematode found in this study came from a fox, which is a possible reservoir host (Magi et al. 2008). At this point, it is unclear if zoo animals in the Zoological Garden Eberswalde are asymptotically infected with *D. repens*, as happened to a snow leopard in Japan infected by *D. immitis* (Murata et al. 2003). Awareness for *D. repens* is needed not only regarding the zoo animals but also regarding zoo visitors since this filarial species is the main agent of human dirofilariasis in Europe (Gratz 2004). Principal vectors of *D. repens* are supposed to be *Aedes albopictus* Skuse, 1895, *Cx. pipiens* s.l. and *An. maculipennis* s.l. (Cancrini and Gabrielli 2007), the latter including *An. messeae*, a species found infected in this study and shown to feed on humans in a previous study in the Zoological Garden Eberswalde (Heym et al. 2019).

The main vectors of *S. tundra* are *Aedes* species (Laaksonen et al. 2009), corresponding with the finding of the worm in the *Ae. annulipes* group in this study. However, *S. tundra* has been shown to be not very vector-specific, which could increase the probability of the nematode to expand its geographical range (Laaksonen et al. 2009). In Germany, the worm was detected in mosquitoes collected in the federal states of Baden-Wuerttemberg, Bavaria, Rhineland-Palatinate and Saxony (Czajka et al. 2012; Kronefeld et al. 2014a). Despite no known *S. tundra* infection of a zoo animal, there is a certain disease risk for cervid zoo animals if infected with the nematode at higher doses, which had led to an outbreak of peritonitis in reindeer in Finland (Laaksonen et al. 2007).

All filarial species that could not be identified in the present study were demonstrated in *Culex* species collected in June 2016. This is in agreement with the study of Czajka et al. (2012), who had also found indeterminable filarial species exclusively in *Culex* mosquitoes. Due to the omithophilic blood feeding behaviour of most *Culex* species, Czajka et al. (2012) assume that these species most likely use birds as vertebrate hosts. This might also apply to the findings of this

study, indicating that there are probably numerous unrecognised filarial species in the field, including some with disease potential for exotic birds held captive in zoos.

SINV had previously been isolated in Germany from *Cx. pipiens* s.l. and *An. maculipennis* s.l. mosquitoes collected in 2009, 2013 and 2015 (Jöst et al. 2010; Scheuch et al. 2018). The detection of SINV in a mosquito pool in the Tierpark Berlin was from a July sample, which is consistent with Jöst et al. (2010), who measured the highest mosquito infection rate with SINV in Germany in the same month in 2009. Main hosts of SINV are migratory birds, which could spread the virus over long distances (Jöst et al. 2010). For this reason, it is likely that the mosquitoes tested positive in this study had obtained the virus from a wild bird and not from the zoo animal population. Although there are no SINV cases of captive animals documented until now, the potential of the virus to infect humans makes this pathogen an important arbovirus in Germany.

Since most studies on avian malaria parasites focus on vertebrate hosts, evidence of these blood parasites from invertebrate species is of major importance to better understand disease epidemiology. Avian malaria parasites of the genus *Haemoproteus* are the most common and least pathogenic haemosporidians infecting wild birds (Atkinson and van Riper 1991). Transmission of *Haemoproteus* species has been shown to be continuous throughout the mosquito season, although main vectors are supposed to be biting midges (Atkinson and van Riper 1991). The detection of *Haemoproteus* sp. in this study is the first documentation of this protozoan genus from German mosquitoes. A study by Valkiunas et al. (2013), analysing *Aedes cantans* Meigen, 1818, has shown that *Haemoproteus* species indeed undergo sexual processes in mosquitoes, and DNA can be detected in the head, thorax and abdomen of an infected mosquito, but sporogonic development is terminated in the oocyst stage without the formation of sporozoites. Gutiérrez-López et al. (2016) demonstrated *Cx. pipiens* to be a competent vector of avian plasmodia, but not of *Haemoproteus* sp.

The blood parasite *Leucocytozoon* is known to infect mainly domestic poultry and waterfowl (Atkinson and van Riper 1991), but was recently also detected at a very high prevalence rate (85.3%) in crows from southern Germany (Schmid et al. 2017). Infections occur mainly in spring and fall (Atkinson and van Riper 1991), corresponding to the demonstration of *Leucocytozoon* sp. in this study in June and September. Similar to *Haemoproteus* sp., mosquitoes are no proven vectors of *Leucocytozoon* sp.; instead, black flies are considered the main vectors (Atkinson and van Riper 1991).

Haemoproteus sp. and *Leucocytozoon* sp. were the only pathogens in this study documented in both project years, indicating a high prevalence of the parasites at both locations. Both groups of parasites had been detected in birds from zoological gardens before, and a study conducted in the

Oklahoma City zoo revealed that 14% of wild and captive bird species harboured them (Halpern and Bennett 1983; Chagas et al. 2016). Also in the Zoological Garden Eberswalde, massive infections of snow owls with species of the *Haemoproteus* subgenus *Parahaemoproteus* had previously occurred, subsequently resulting in regular malaria prophylaxis in owls and penguins (Valentin et al. 1994).

Avian malaria parasites of the genus *Plasmodium* were only detected in the Tierpark Berlin in 2016. Main vectors of avian *Plasmodium* species are *Culex* mosquitoes (Huijben et al. 2007), in which the parasite was also detected in this study. *Plasmodium* infections in evolutionarily adapted wild birds appear to be relatively harmless, but infections of captive non-adapted birds are often fatal (Huijben et al. 2007). Thus, avian malaria is one of the major causes of captive penguin mortality (Grilo et al. 2016). Infections of penguins are a worldwide problem in zoos, with recent reports from Japan (Ejiri et al. 2009), Brazil (Bueno et al. 2010) and Israel (Lublin et al. 2018). Also in the Tierpark Berlin, avian malaria is well known, and penguins are subjected to routine malaria prophylaxis.

Despite a study on blood meal patterns of mosquitoes collected in the Tierpark Berlin and the Zoological Garden Eberswalde showing low numbers of avian blood meals (Heym et al. 2019), the detection of avian pathogens in this study suggests *Culex*/avian interactions in both zoos. This is of major importance, since birds are especially important as reservoirs and amplifiers of zoonotic pathogens transmitted by mosquitoes. Hamer et al. (2012) showed that birds trapped in urban sites were more often seropositive for WNV than birds from less urban locations. This could have also been the case in the present study, as the only mosquito-borne virus was detected in the urban Tierpark Berlin. Additionally, with six mosquito pools and 11 single mosquito specimens positive for vector-borne pathogens, disease agents were more prevalent in Berlin than in Eberswalde, where two mosquito pools and eight single specimens tested positive for one or more pathogens. Among the pathogens, *D. repens* was only detected in the Zoological Garden Eberswalde, while SINV was only detected in the Tierpark Berlin, indicating that different pathogens can be important at different locations, depending on various ecosystemic factors, as already discussed by Heym et al.

Most of the pathogen-positive mosquitoes were collected in July. This is in agreement with earlier studies that analysed mosquito-borne viruses and filarial nematodes in Germany and obtained most pathogen-positive mosquitoes from July to September (Kronefeld et al. 2014b; Scheuch et al. 2018).

Since some of the detected disease agents were only demonstrated in one of the project years, it cannot be clarified whether the pathogens are firmly established at the study sites.

As no infection of a zoo animal with one of the pathogens detected in the mosquitoes was registered over the course of the project, the prevalence of circulating pathogens still seems to be rather low. As for viruses and filarial worms, this assumption is supported by other studies (Kronefeld et al. 2014a; Scheuch et al. 2018). More prevalent are certainly avian malaria parasites of the genera *Haemoproteus* sp. and *Leucocytozoon* sp. which were not only detected in this study in both zoos in both project years but have been repeatedly demonstrated in Germany previously from infected birds (Krone et al. 2001, 2008; Wiersch et al. 2007).

Although a relatively low number of mosquitoes were tested compared with other studies analysing mosquito-borne pathogens, it can be stated that several pathogen-positive mosquito specimens collected in the zoos were demonstrated in this study. This is of major importance, since operators of zoological gardens are pretty much aware of the risk of arthropod-borne diseases in zoo animals, such as avian malaria, but there is still little attention given to the vectors which transmit the disease-causing pathogens. In a closer collaboration between entomologists and zoological gardens, pathogen circulation could be detected in an early stage and vector control measures be implemented if considered necessary. This could be particularly relevant in Germany where mosquito-borne pathogens and their vectors have been neglected for decades. Only recently, activities to screen potential vertebrate hosts and mosquitoes for disease agents have been resumed (e.g. Jöst et al. 2010, 2011a, 2011b; Michel et al. 2018; Scheuch et al. 2018). In 2018, WNV emerged in Germany for the first time, with the first evidence coming from zoos and wildlife parks (Ziegler et al. 2018). Thus, zoological gardens can be excellent locations to detect and analyse such incidents, since here potential vertebrate hosts and various mosquito species live close together within a defined space, and previous disease cases should be known. Mosquito and mosquito-borne disease agent surveillance in zoological gardens would not only help zoos to protect their animals, but could, as a by-product, also contribute to public health surveillance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

- Adler PH, Tuten HC, Nelder MP (2011) Arthropods of medicoveterinary importance in zoos. *Annu Rev Entomol* 56:123–142
- Adouchief S, Smura T, Sane J, Vapalahti O, Kurkela S (2016) Sindbis virus as a human pathogen – epidemiology, clinical picture and pathogenesis. *Rev Med Virol* 26:221–241
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410
- Atkinson CT, Van Riper C (1991) Pathogenicity and epizootiology of avian haematophagous: *Plasmodium*, *Leucocytozoon* and *Haemoproteus*. In: Loye JE, Zuk M (eds) Bird-parasite interactions: ecology, evolution and behaviour. Oxford Ornithology Series, pp 19–48
- Becker N, Petric D, Zgomba M, Boase C, Madon M, Dahl C, Kaiser A (2010) Mosquitoes and their control. Springer, Heidelberg
- Bell JA, Wackstein JD, Fecchio A, Tkach VV (2015) A new real-time PCR protocol for detection of avian haemosporidians. *Parasit Vectors* 8:e383
- Bueno MG, Lopez RPG, Menezes RMTD, Costa-Nascimento MDJ, Lima GFMD, Araújo RDS, Guida FJV, Kirchgatter K (2010) Identification of *Plasmodium relictum* causing mortality in penguins (*Spheniscus magellanicus*) from São Paulo Zoo, Brazil. *Vet Parasitol* 173:123–127
- Canavan WPN (1929) Nematode parasites of vertebrates in the Philadelphia Zoological Garden and vicinity. *Parasitology* 21:63–102
- Cancrini G, Gabriellini S (2007) Vectors of *Dirofilaria* nematodes: biology, behaviour and host/parasite relationships. In: Cringoli G (ed.) *Dirofilaria immitis* and *D. repens* in dog and cat, and human infections. *Mappe Parasitologiche*, Naples, pp 48–59
- Casaghi M, Anderson TJC, Bandi C, Bazzocchi C, Genchi C (2001) A phylogenetic analysis of filarial nematodes: comparison with the phylogeny of *Wolbachia* endosymbionts. *Parasitology* 122:93–103
- Chagas CRF, Guimarães LDO, Monteiro EF, Valkiūnas G, Katayama MV, Santos SV, Guida FJV, Simões RF, Kirchgatter K (2016) Hemosporidian parasites of free-living birds in the São Paulo Zoo, Brazil. *Parasitol Res* 115:1443–1452
- Chao D-Y, Davis BS, Chang GJJ (2007) Development of multiplex real-time reverse transcriptase PCR assays for detecting eight medically important flaviviruses in mosquitoes. *J Clin Microbiol* 45:584–589
- Czajka C, Becker N, Poppert S, Jöst H, Schmidt-Chanasit J, Krüger A (2012) Molecular detection of *Setaria tundra* (Nematoda: Filarioidea) and an unidentified filarial species in mosquitoes in Germany. *Parasit Vectors* 5:e14
- Czajka C, Becker N, Jöst H, Poppert S, Schmidt-Chanasit J, Krüger A, Tanich E (2014) Stable transmission of *Dirofilaria repens* nematodes, northern Germany. *Emerg Infect Dis* 20:328–331
- Dobler G, Wölfel R, Schmüser H, Essbauer S, Pfeffer M (2006) Seroprevalence of tick-borne and mosquito-borne arboviruses in European brown hares in northern and western Germany. *Int J Med Microbiol* 296:80–83
- Ejiri H, Sato Y, Sawai R, Sasaki E, Matsumoto R, Ueda M, Higa Y, Tsuda Y, Omori S, Murata K, Yukawa M (2009) Prevalence of avian malaria parasite in mosquitoes collected at a zoological garden in Japan. *Parasitol Res* 105:629–633
- Eshoo MW, Whitehouse CA, Zoll ST, Massire C, Pennella T-TD, Blyn LB, Sampath R, Hall TA, Ecker JA, Desai A, Wasieleski LP, Li F, Turell MJ, Schink A, Rudnick K, Otero G, Weaver SC, Ludwig GV, Hofstadler SA, Ecker DJ (2007) Direct broad-range detection of alphaviruses in mosquito extracts. *Virol J* 368:286–295
- 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. *Mol Mar Biol Biotechnol* 3:294–299
- Genchi C, Kramer LH, Rivasi F (2011) Dirofilaria infections in Europe. *Vector-borne Zoonot Dis* 11:1307–1317
- Graczyk TK, Cranfield MR, Meechan TF, Bicknese EJ (1994) Characteristics of naturally acquired avian malaria infections in naive juvenile African black-footed penguins (*Spheniscus demersus*). *Parasitol Res* 80:634–637
- Gratz N (2004) The vector-borne human infections of Europe – their distribution and burden on public health. World Health Organization, Regional Office for Europe, Copenhagen, Denmark
- Gratz N (2006) Vector- and rodent-borne diseases in Europe and North America. Cambridge University Press, New York, USA
- Grilo ML, Vanstreels RE, Wallace R, Garcia-Parraga D, Braga EM, Chitty J, Cato-Dias JL, Madeira De Carvalho LM (2016) Malaria in penguins – current perceptions. *Avian Pathol* 45:393–407
- Guillemez-López R, Martínez-De La Puente J, Gangoso L, Yan J, Sorriquer RC, Figuerola J (2016) Do mosquitoes transmit the avian malaria-like parasite *Haemoproteus*? An experimental test of vector competence using mosquito saliva. *Parasit Vectors* 9:e609
- Halpern N, Bennett GF (1983) *Haemoproteus* and *Leucocytozoon* infections in birds of the Oklahoma city zoo. *J Wildl Dis* 19:330–332
- Hamer SA, Lehrer E, Magle SB (2012) Wild birds as sentinels for multiple zoonotic pathogens along an urban to rural gradient in greater Chicago, Illinois. *Zoonoses Public Health* 59:355–364
- Hébert PDN, Ratnasingham S, De Waard JR (2003) Barcoding animal life: cytochrome c oxidase subunit I divergences among closely related species. *Proc R Soc Lond B Biol Sci* 270:S96–S99
- Hermosilla C, Pantchev N, Dyachenko V, Gutmann M, Bauer C (2006) First autochthonous case of canine ocular *Dirofilaria repens* infection in Germany. *Vet Rec* 158:134–135
- Heyn EC, Kampen H, Walther D (2018) Mosquito species composition and phenology (Diptera, Culicidae) in two German zoological gardens imply different risks of mosquito-borne pathogen transmission. *J Vector Ecol* 43:80–88
- Heyn EC, Kampen H, Schäfer M, Walther D (2019) Mosquito bloodmeal preferences in two zoological gardens in Germany. *Med Vet Entomol* 33, 203–212
- Hübäck Z (2008) Mosquito-borne viruses in Europe. *Parasitol Res* 103:29–43
- Huijben S, Schaftenaar W, Wijsman A, Paaijmans KP, Takken W (2007) Avian malaria in Europe: an emerging infectious disease? In: Takken W, Knols BG (eds) *Emerging pests and vector-borne diseases in Europe*. Wageningen Academic Publishers, Wageningen, Netherlands, pp 59–74
- Jöst H, Bialonski A, Storch V, Gunther S, Becker N, Schmidt-Chanasit J (2010) Isolation and phylogenetic analysis of Sindbis viruses from mosquitoes in Germany. *J Clin Microbiol* 48:1900–1903
- Jöst H, Bialonski A, Schmetz C, Gunther S, Becker N, Schmidt-Chanasit J (2011b) Isolation and phylogenetic analysis of Batai virus, Germany. *Am J Trop Med Hyg* 84:241–243
- Krone O, Priemer J, Streich J, Sommer P, Langgemach T, Lessow O (2001) Haemosporidia of birds of prey and owls from Germany. *Acta Protozool* 40:281–289
- Krone O, Waldenström J, Valkiūnas G, Lessow O, Müller K, Iezhova TA, Fickel J, Bensch S (2008) Haemosporidian blood parasites

- (Haemosporida, Haemoproteidae) in European birds of prey and owls. *J Parasitol* 94:709–715
- Kronfeld M, Kampen H, Sassnau R, Werner D (2014a) Molecular detection of *Dirofilaria immitis*, *Dirofilaria repens* and *Setaria tundra* in mosquitoes from Germany. *Parasit Vectors* 7:e30
- Kronfeld M, Werner D, Kampen H (2014b) PCR identification and distribution of *Anopheles daciae* (Diptera, Culicidae) in Germany. *Parasitol Res* 113:2079–2086
- Laaksonen S, Kuusela J, Nikander S, Nylund M, Oksanen A (2007) Outbreak of parasitic peritonitis in reindeer in Finland. *Vet Rec* 160:835–841
- Laaksonen S, Solismaa M, Kortte R, Kuusela J, Oksanen A (2009) Vectors and transmission dynamics for *Setaria tundra* (Filarioidea; Onchocercidae), a parasite of reindeer in Finland. *Parasit Vectors* 2:e3
- Lambert AJ, Lanciotti RS (2009) Consensus amplification and novel multiplex sequencing method for S segment species identification of 47 viruses of the *Orthobunyavirus*, *Phlebovirus* and *Nairovirus* genera of the family *Bunyaviridae*. *J Clin Microbiol* 47:2398–2404
- Lublin A, Lapid R, Mechani S, Famoushi Y, Goldbach C, Edery N (2018) Unusual prevalence of avian malaria (*Plasmodium* sp.) in the adult population of Humboldt penguins (*Spheniscus humboldti*) at an Israeli zoo between years 2012 and 2015. *Isr J Vet Med* 73:18–23
- Ludwig GV, Calle PP, Mangiafico JA, Raphael BL, Daner DK, Hile JA, Clippinger TL, Smith JF, Cook RA, McNamara T (2002) An outbreak of West Nile virus in a New York city captive wildlife population. *Am J Trop Med Hyg* 67:67–75
- Magi M, Calderini P, Gabrielli S, Dell’Omodarme M, Macchioni F, Prati MC, Cancrini G (2008) *Vulpes vulpes*: a possible wild reservoir for zoonotic filariae. *Vector-borne Zoonot Dis* 8:249–252
- McNamara T (2007) The role of zoos in biosurveillance. *Int Zoo Yearb* 41:12–15
- Michel F, Fischer D, Eiden M, Fast C, Reuschel M, Müller K, Rinder M, Urbanik S, Brandes F, Schween R, Lühken R, Groschup MH, Ziegler U (2018) West Nile virus and Usutu virus monitoring of wild birds in Germany. *Int J Environ Res Public Health* 15:e171
- Murata K, Yanai T, Agatsuma T, Uni S (2003) *Dirofilaria immitis* infection of a snow leopard (*Uncia uncia*) in a Japanese zoo with mitochondrial DNA analysis. *J Vet Med Sci* 65:945–947
- Pilaski J (1987) Contribution to the ecology of Tahyna virus in Central Europe. *J Vector Ecol* 12:544–553
- Proft J, Maier WA, Kampen H (1999) Identification of six sibling species of the *Anopheles maculipennis* complex (Diptera: Culicidae) by a polymerase chain reaction assay. *Parasitol Res* 85:837–843
- Robe DL, Fall RP (1979) A miniature battery powered CO₂ baited trap for mosquito borne encephalitis surveillance. *Bull Soc Vector Ecol* 4:24–27
- Rudolf M, Czajka C, Böstler J, Melaun C, Jöst H, von Thien H, Badusche M, Becker N, Schmidt-Chanasit J, Krüger A, Tannich F, 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
- Sano Y, Aoki M, Takahashi H, Miura M, Komatsu M, Abe Y, Kakino J, Itagaki T (2005) The first record of *Dirofilaria immitis* infection in a Humboldt penguin, *Spheniscus humboldti*. *J Parasitol* 91:1235–1237
- Sassnau R, Dyachenko V, Panichev N, Stöckel F, Dittmar K, Dausgries A (2009) *Dirofilaria repens* infestation in a sled dog kennel in the federal state of Brandenburg (Germany). Diagnosis and therapy of canine cutaneous dirofilariosis. *Tierärztl Prax* 37:95–101 [In German]
- Schaffner F, Angel G, Geoffroy B, Hervy JP, Rhaïem A, Brunhes J (2001) The mosquitoes of Europe (CD-ROM). In: EID Méditerranée. Montpellier, France
- Scheuch DE, Schäfer M, Eiden M, Heym EC, Ziegler U, Walther D, Schmidt-Chanasit J, Keller M, Groschup MH, Kampen H (2018) Detection of Usutu, Sindbis and Batai viruses in mosquitoes (Diptera: Culicidae) collected in Germany, 2011–2016. *Viruses* 10:e389
- Schmid S, Facht K, Dinkel A, Mackenstedt U, Woog F (2017) Carrion crows (*Corvus corone*) of southwest Germany: important hosts for haemosporidian parasites. *Malar J* 16:369
- Sijbrandt DC, Hunter S, Howe L, Lenting B, Argilla L, Gattrell BD (2017) Cases of mortality in little penguins (*Eudyptula minor*) in New Zealand associated with avian malaria. *N Z Vet J* 65:332–337
- Suk J (2017) Preparedness for mosquito-borne diseases in Europe: the ECDC perspective. *Eur J Pub Health* 27(Suppl 3):230–231
- Tuten HC (2011) Zoos as experiment environments: biology of larval and adult mosquitoes (Diptera: Culicidae). Ph.D. thesis, Clemson University, USA
- Valentin A, Haberkorn A, Hensch B, Jakob W (1994) Massive Malaria-Infektionen mit *Parahaemoproteus* spec. in Schnee-Eulen (*Nyctea scandiaca*) und deren Behandlung mit Primaquin. *Verhandlungsber Erkrank Zootiere* 36:401–404
- Valkunas G, Kazlauskienė R, Bernotienė R, Palinauskas V, Iezhova TA (2013) Abortive long-lasting sporogony of two *Haemoproteus* species (Haemosporida, Haemoproteidae) in the mosquito *Ochlerotatus cantans*, with perspectives on haemosporidian vector research. *Parasitol Res* 112:2159–2169
- 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. *Vet Microbiol* 140:271–280
- Wiersch SC, Lubjuhn T, Maier WA, Kampen H (2007) Haemosporidian infection in passerine birds from Lower Saxony. *J Ornithol* 148:17–24
- Ziegler U, Fast C, Eiden M, Bock S, Schulze C, Hoepfer D, Ochs A, Schlieben P, Keller M, Zielke DE, Lühken R, Cadar D, Walther D, Schmidt-Chanasit J, Groschup MH (2016) Evidence for an independent third Usutu virus introduction into Germany. *Vet Microbiol* 192:60–66
- Ziegler U, Lühken R, Keller M, Cadar D, van der Grinten E, Michel F, Albrecht K, Eiden M, Rinder M, Lachmann L, Höper D, Vina-Rodriguez A, Gaede W, Pohl A, Schmidt-Chanasit J, Groschup MH (2018) West Nile virus epizootic in Germany. *Antivir Res* 162:39–43

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5. Eigenanteil

1.

HEYM EC, KAMPEN H and WALTHER D, 2018. Mosquito species composition and phenology (Diptera, Culicidae) in two German zoological gardens imply different risks of mosquito-borne pathogen transmission. *Journal of Vector Ecology*, 43:80-88. doi.org/10.1111/jvec.12286.

Eva C. Heym: Konzeption und Koordination der Studie, Probenahmen im Feld, morphologische und molekulare Bestimmung der Stechmücken, statistische Auswertung der Daten, Erstellung des Manuskripts inkl. Abbildungen

Helge Kampen: Konzeption der Studie und Interpretation aller Daten, Optimierung und Hilfe bei der Erstellung des Manuskripts

Doreen Walther: Konzeption der Studie und Interpretation aller Daten, Optimierung und Hilfe bei der Erstellung des Manuskripts

2.

HEYM EC, KAMPEN H, SCHÄFER M and WALTHER D, 2019. Mosquito bloodmeal preferences in two zoological gardens in Germany. *Medical and Veterinary Entomology*, 33: 203-2012. doi:10.1111/mve.12350.

Eva C. Heym: Konzeption und Koordination der Studie, Probenahmen im Feld, morphologische und molekulare Bestimmung der Stechmücken, molekulare Analyse der Blutmahlzeiten und Auswertung der Sequenzen, statistische Auswertung der Ergebnisse, Erstellung des Manuskripts inkl. Abbildungen

Helge Kampen: Konzeption der Studie und Interpretation aller Daten, Optimierung und Hilfe bei der Erstellung des Manuskripts

Mandy Schäfer: Molekulare Analyse der Blutmahlzeiten und Auswertung der Sequenzen, Hilfe bei der Erstellung des Manuskripts

Doreen Walther: Konzeption der Studie und Interpretation aller Daten, Optimierung und Hilfe bei der Erstellung des Manuskripts

3.

HEYM EC, KAMPEN H, KRONE O, SCHÄFER M and WERNER D, 2019. Molecular detection of vector-borne pathogens from mosquitoes collected in two zoological gardens in Germany. *Parasitology Research*, 118: 2097-2105. doi.org/10.1007/s00436-019-06327-5.

Eva C. Heym: Konzeption und Koordination der Studie, Probenahmen im Feld, morphologische und molekulare Bestimmung der Stechmücken, Pathogen-Screening (Filarien, Viren), Auswertung der Ergebnisse, Erstellung des Manuskripts

Helge Kampen: Konzeption der Studie und Interpretation aller Daten, Optimierung und Hilfe bei der Erstellung des Manuskripts

Oliver Krone: Hilfe bei der Etablierung einer qPCR für das Screening nach Vogelmalaria-Parasiten, Hilfe bei der Erstellung des Manuskripts

Mandy Schäfer: Pathogen-Screening (Filarien, Viren, Vogelmalaria-Parasiten) inklusive der Etablierung einer qPCR für das Screening nach Vogelmalaria-Parasiten, Hilfe bei der Erstellung des Manuskripts

Doreen Werner: Konzeption der Studie und Interpretation aller Daten, Optimierung und Hilfe bei der Erstellung des Manuskripts

Obige Angaben werden bestätigt:

.....
PD Dr. Helge Kampen

.....
Eva C. Heym

6. Summary

The present work focusses on the mosquito populations of two zoological gardens in Germany with the aim to better understand mosquito biology of native species and to contribute to a greater awareness of mosquito and mosquito-borne disease agent surveillance in zoos. For this purpose, data on species composition, blood meal patterns and mosquito-borne pathogens were analysed. The investigated zoological gardens differed not only in their sizes and animal stocks, but also in their surrounding environments. The 160 ha Tierpark Berlin is located in a densely populated urban area, while the 15 ha Zoological Garden Eberswalde is surrounded by forest.

To gain an overview about the mosquito fauna of both zoos, adult specimens were caught by aspirating and EVS-trapping during the 2016 season. In addition, larval stages were collected from their breeding sites located in the zoo areas. In total, 2,257 mosquitoes were sampled, belonging to 20 taxa. Seasonal differences between the zoos were documented, both in terms of species composition and the relative abundance of mosquito species collected. As the studied zoos were located in the same climatic region and both locations provided similar breeding sites, differences in species composition were attributed to the entry of mosquitoes from surrounding landscapes. Influencing factors could have been the different sizes of the zoos and variations in the potential host animal populations.

According to the vector potential of most frequently collected taxa in the Zoological Garden Eberswalde (Annulipes Group, *Culiseta annulata*), TAHV, USUV, WNV, filariae and avian malaria parasites appear to have the highest risk of being transmitted at this location. In the Tierpark Berlin, *Aedes vexans* was the most frequently collected mosquito species, suggesting a theoretical risk for the transmission of a broader spectrum of pathogens due to covered vector competences. Pathogens such as BATV, SINV, TAHV, USUV and filarial worms could be of major importance regarding transmission risk to zoo animals, as they had previously been found to circulate Germany. In addition, avian malaria parasites represent a considerable risk for susceptible exotic bird species in Berlin.

Since the blood-feeding behaviour of vector-competent mosquito species has a major influence on the transmission of a mosquito-associated pathogen, the analysis of blood meal patterns is crucial to better understand vector-pathogen cycles. Therefore, blood meals of blood-fed mosquitoes caught in 2016 and 2017 by aspirating and EVS-trapping in the Tierpark Berlin and the Zoological Garden Eberswalde were analysed. The aim was to

investigate to what extent native mosquito species accept exotic zoo animals, wild native animals and humans as blood hosts. In addition, it was examined whether the collected species are generalists or specialists when selecting vertebrates for blood feeding.

A total of 405 blood-fed mosquitoes from 16 taxa were collected. The genetic analysis of blood meals identified 56 host species, which – in addition to humans – mainly originated from mammals of the zoo animal populations. In agreement with the previous study on the mosquito fauna of the Tierpark Berlin and the Zoological Garden Eberswalde, the analysis of blood meals also showed differences between the two zoos. In the smaller Zoological Garden Eberswalde, a higher number of blood-fed mosquitoes was collected than in the Tierpark Berlin, probably caused by a higher host density in Eberswalde, which may have led to an overall higher mosquito density. However, no differences between both zoos were observed with respect to the blood feeding behaviour of the analysed mosquito species: Mosquitoes of both locations were rather generalistic, although species could be grouped according their blood meals into 'amphibian', 'non-human mammal' and, 'non-human mammal and human' feeding species. The more random selection of hosts could indicate a low probability of effective pathogen transmission by applying the 'dilution effect'. Notwithstanding, since wild animals have also been accepted as hosts, pathogen transmission by bridge vectors from one vertebrate group to another could be relevant in the sampled zoos.

Adult mosquito specimens collected in 2016 and 2017 were screened for filarial nematodes, avian Haemosporidia and mosquito-borne viruses. *Dirofilaria repens* was detected in a mosquito from the Zoological Garden Eberswalde. Mosquitoes from Berlin and Eberswalde were tested positive for the nematode species *S. tundra*. Sindbis virus was found in a mosquito pool collected in the Tierpark Berlin, while no mosquito-associated viruses were detected in specimens collected in the Zoological Garden Eberswalde. Mosquitoes from both zoos were positive for the haemosporidian parasites *Haemoproteus* sp. and *Leucocytozoon* sp., and one documentation was made for avian *Plasmodium* sp. in the Tierpark Berlin.

The identified pathogens have the potential to cause disease in captive and wild animals, and some of them also in humans. Most of the mosquitoes tested positive had been collected in July, suggesting a high infection risk during this month. Since most pathogen detections were made from species belonging to the *Cx. pipiens* complex, species of this complex seem to be most relevant in the studied zoos when it comes to mosquito-borne pathogen transmission. Although mosquitoes are no proven vectors of most of the avian malaria parasite genera found, evidence for *Haemoproteus* sp. and *Leucocytozoon* sp. demonstrated a high prevalence of avian malaria parasites in the zoos.

In summary, the results of the three studies indicate regional differences both in the mosquito species composition and in the occurrence of mosquito-borne pathogens. However, no differences were found between the mosquito communities of both zoos concerning their blood feeding behaviour, suggesting that the general behaviour of the insects is location-independent.

Several potential disease agents were found in the collected mosquitoes, although not at high abundances. Whether these pathogens were found by chance in the two zoos or whether the particular zoo environment is a hot spot of arthropod-borne pathogens cannot be determined with the studies conducted. Nonetheless, it seems clear that zoological gardens are attractive to mosquito females not only in their search for breeding sites, but also when looking for blood hosts and places for mating or resting. These advantageous conditions also attract mosquito species that have their larval habitats outside the zoological gardens, which is why elimination of breeding sites on the zoo premises alone will not necessarily keep away all mosquitoes.

A closer collaboration between zoological gardens and entomologists could be beneficial for both. Zoo officials could benefit from being able to identify potential arthropod vectors on the zoo grounds and receiving information on circulating arthropod-borne disease agents, as well as on the animal species susceptible to those. For entomologists, zoological gardens are ideal research locations, as they provide an environment with a high diversity of habitats and potential blood hosts for haematophagous arthropods in a confined space.

Studying mosquito biology will become even more significant in the future, since in a world that is getting smaller, both potential vectors and pathogens are regularly introduced into areas where they did not occur before. Therefore, it would be desirable if more studies targeting ecological as well as infectiological aspects of vector species in zoological gardens in Germany were carried out.

7. References

- ADLER PH, TUTEN HC and NELDER MP, 2011. Arthropods of medicoveterinary importance in zoos. *Annual Review of Entomology*, 56: 123-142.
- ANGELONE-ALASAAD S, JOWERS MJ, PANADERO R, PÉREZ-CREO A, PAJARES G, DÍEZ-BAÑOS P, SORIGUER RC and MORRONDO P, 2016. First report of *Setaria tundra* in roe deer (*Capreolus capreolus*) from the Iberian peninsula inferred from molecular data: epidemiological implications. *Parasites & Vectors*, 9: e521.
- ATKINSON CT and VAN RIPER C, 1991. 2. Pathogenicity and epizootiology of avian haematozoa: *Plasmodium*, *Leucocytozoon*, and *Haemoproteus*. In: LOYE JE and ZUK M (eds), *Bird-Parasite Interactions: Ecology, Evolution and Behaviour*. Oxford Ornithology Series: 19-48.
- BARDOS V, ADAMCOVÁ J, DEDEI S, GJINI N, ROSICKÝ B and SIMKOVÁ A, 1959. Neutralizing antibodies against some neurotropic viruses determined in human sera in Albania. *Journal of Hygiene, Epidemiology, Microbiology and Immunology*, 3: 277-282.
- BECKER N, 1989. Life strategies of mosquitoes as an adaptation to their habitats. *Bulletin of the Society for Vector Ecology*, 14: 6-25.
- 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, PETRIC D, ZGOMBA M, BOASE C, DAHL C, MADON M and KAISER A, 2010. *Mosquitoes and their Control*, 2nd Edition. Springer Verlag, Heidelberg, Germany: 577 pp.
- BENNETT GF, BISHOP MA and PEIRCE A, 1993. Checklist of the avian species of *Plasmodium* Marchiafava & Celli, 1885 (Apicomplexa) and their distribution by avian family and Wallacean life zones. *Systematic Parasitology*, 26: 171-179.
- BÖRSTLER J, JÖST H, GARMS R, KRÜGER A, TANNICH E, BECKER N, SCHMIDT-CHANASIT J and LÜHKEN R, 2016. Host-feeding patterns of mosquito species in Germany. *Parasites & Vectors*, 9: e318.
- BRUCE-CHWATT LJ and DE ZULUETA J, 1980. *The Rise and Fall of Malaria in Europe: A Historico-epidemiological Study*. Oxford, United Kingdom: Oxford University Press: 256 pp.
- BUENO MG, LOPEZ RPG, TORINO DE MENEZES RM, COSTA-NASCIMENTO MdJ, LIMA GFMdC, ARAÚJO RAdS, GUIDA FJV and KIRCHGATTER K, 2010. Identification of *Plasmodium relictum* causing mortality in penguins (*Spheniscus magellanicus*) from São Paulo Zoo, Brazil. *Veterinary Parasitology*, 173: 123-127.

- BURKETT-CADENA ND, EUBANKS MD and UNNASCH TR, 2008. Preference of female mosquitoes for natural and artificial resting sites. *Journal of the American Mosquito Control Association*, 24: 228-235.
- CHAVES LF, 2016. Mosquito species (Diptera: Culicidae) persistence and synchrony across an urban altitudinal gradient. *Journal of Medical Entomology*, 54: 329-339.
- CHAVES LF, HARRINGTON LC, KEOGH CL, NGUYEN AM and KITRON UD, 2010. Blood feeding patterns of mosquitoes: random or structured? *Frontiers in Zoology*, 7: e3.
- CZAJKA C, BECKER N, POPPERT S, JÖST H, SCHMIDT-CHANASIT J and KRÜGER A, 2012. Molecular detection of *Setaria tundra* (Nematoda: Filarioidea) and an unidentified filarial species in mosquitoes in Germany. *Parasites & Vectors*, 5: e14.
- CZAJKA C, BECKER N, JÖST H, POPPERT S, SCHMIDT-CHANASIT J, KRÜGER A and TANNICH E, 2014. Stable transmission of *Dirofilaria repens* nematodes, northern Germany. *Emerging Infectious Diseases* 20: 328-331.
- DEKONINCK W, HENDRICKX F, BORTEL WV, VERSTEIRT V, COOSEMANS M, DAMIENS D, HANCE T, DE CLERCQ EM, HENDRICKX G, SCHAFFNER F and 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.
- DICKINSON K and PASKEWITZ S, 2012. Willingness to pay for mosquito control: how important is West Nile virus risk compared to the nuisance of mosquitoes? *Vector-borne and Zoonotic Diseases*, 12: 886-892.
- DOBLER G and ASPÖCK H, 2010. Durch Stechmücken übertragene Arboviren als Erreger von Infektionen des Menschen. In: ASPÖCK H (ed.), *Krank durch Arthropoden*. Denisia 30, Linz, Österreich: 501-553.
- DUTTON CJ, QUINNELL M, LINDSAY R, DELAY J and BARKER IK, 2009. Paraparesis in a polar bear (*Ursus maritimus*) associated with West Nile virus infection. *Journal of Zoo and Wildlife Medicine*, 40: 568-571.
- EICHENLAUB D, 1979. Malaria in Deutschland. *Bundesgesundheitsblatt*, 22: 8-13.
- FONSECA DM, KEYGHOBADI N, MALCOLM CA, MEHMET C, SCHAFFNER F, MOGI M, FLEISCHER RC and WILKERSON RC, 2004. Emerging vectors in the *Culex pipiens* complex. *Science*, 303: 1535-1538.
- FOSTER WA and HANCOCK RG, 1994. Nectar-related olfactory and visual attractants for mosquitoes. *Journal of the American Mosquito Control Association*, 10: 288-296.

- FOUMANE V, BESNARD P, LE MIRE J, FORTES F, DOS SANTOS M, CARNEVALE P and MANGUIN S, 2015. First knowledge, attitude and practices (KAP) survey of mosquitoes and malaria vector control at household level in Lobito town (Angola). *Journal of Infectious Diseases and Therapy*, 3: 230.
- GENCHI C, RINALDI L, MORTARINO M, GENCHI M and CRINGOLI G, 2009. Climate and *Dirofilaria* infection in Europe. *Veterinary Parasitology*, 163: 286-292.
- GENCHI C, KRAMER LH and RIVASI F, 2011a. *Dirofilaria* infections in Europe. *Vector-borne and Zoonotic Diseases*, 11: 1307-1317.
- GENCHI C, MORTARINO M, RINALDI L, CRINGOLI G, TRALDI G and GENCHI M, 2011b. Changing climate and changing vector-borne disease distribution: the example of *Dirofilaria* in Europe. *Veterinary Parasitology*, 176: 295-299.
- GLAIZOT O, FUMAGALLI L, IRITANO K, LALUBIN F, ROOYEN J and CHRISTE P, 2012. High prevalence and lineage diversity of avian malaria in wild populations of great tits (*Parus major*) and mosquitoes (*Culex pipiens*). *PLoS One*, 7: e34964.
- GRACZYK TK, CRANFIELD MR, MCCUTCHAN TF and BICKNESE EJ, 1994. Characteristics of naturally acquired avian malaria infections in naive juvenile African black-footed penguins (*Spheniscus demersus*). *Parasitology Research*, 80: 634-637.
- GRATZ N, 2004. The Vector-borne Human Infections of Europe – their Distribution and Burden on Public Health. WHO Regional Office for Europe, Copenhagen, Denmark: 144 pp.
- GRATZ N, 2006. Vector- and Rodent-borne Diseases in Europe and North America. Cambridge University Press, Cambridge, United Kingdom: 393 pp.
- GUSSET M and DICK G, 2011. The global reach of zoos and aquariums in visitor numbers and conservation expenditures. *Zoo Biology*, 30: 566-569.
- HARBACH RE, 2019. Mosquito Taxonomic Inventory. (<http://mosquito-taxonomic-inventory.info>, accessed 21 January 2019).
- HAYES EB, KOMAR N, NASCI RS, MONTGOMERY SP, O'LEARY DR and CAMPBELL GL, 2005. Epidemiology and transmission dynamics of West Nile virus disease. *Emerging Infectious Diseases*, 11: 1167-1173.
- HERMOSILLA C, PANTCHEV N, DYACHENKO V, GUTMANN M and BAUER C, 2006. First autochthonous case of canine ocular *Dirofilaria repens* infection in Germany. *Veterinary Record* 158: 134-135.
- HEYM EC, KAMPEN H, FAHLE M, HOHENBRINK TL, SCHÄFER M, SCHEUCH DE and WALTHER D, 2017. *Anopheles plumbeus* (Diptera: Culicidae) in Germany: updated geographic

- distribution and public health impact of a nuisance and vector mosquito. *Tropical Medicine & International Health*, 22: 103-112.
- HOPLA CE, 1974. The ecology of tularemia. *Advances in Veterinary Science and Comparative Medicine*, 18: 25-53.
- HUBÁLEK Z, 2008. Mosquito-borne viruses in Europe. *Parasitology Research*, 103: 29-43.
- HUFF CG, 1965. Susceptibility of mosquitoes to avian malaria. *Experimental Parasitology*, 16: 107-132.
- HUIJBEN S, SCHAFTENAAR W, WIJSMAN A, PAAIJMANS KP and TAKKEN W, 2007. Avian malaria in Europe: an emerging infectious disease? In: TAKKEN W and KNOLS BG (eds), *Emerging Pests and Vector-borne Diseases in Europe*. Wageningen Academic Publishers, Wageningen, Netherlands: 59-74.
- JETT J and VENTRE J, 2012. Orca (*Orcinus orca*) captivity and vulnerability to mosquito-transmitted viruses. *Journal of Marine Animals and their Ecology*, 5: 9-16.
- JÖST H, BIALONSKI A, MAUS D, SAMBRI V, EIDEN M, GROSCHUP MH, GÜNTHER S, BECKER N and SCHMIDT-CHANASIT J, 2011. Isolation of Usutu virus in Germany. *American Journal of Tropical Medicine and Hygiene*, 85: 551-553.
- JÖST H, BIALONSKI A, SCHMETZ C, GUNTHER S, BECKER N and SCHMIDT-CHANASIT J, 2011. Isolation and phylogenetic analysis of Batai virus, Germany. *American Journal of Tropical Medicine and Hygiene*, 84: 241-243.
- KAMPEN H, KRONEFELD M and WERNER D, 2012. Culicid mosquitoes as vectors of disease agents in Europe. In: MEHLHORN H (ed.), *Arthropods as Vectors of Emerging Diseases*. Parasitology Research Monographs 3, Springer Verlag, Heidelberg, Germany: 1-30.
- KAMPEN H and SCHAFFNER F, 2008. Mosquitoes. In: Bonnefoy, X, Kampen H, Sweeney K (eds), *Public Health Significance of Urban Pests*. WHO Regional Office for Europe, Copenhagen, Denmark: 347-386.
- KAMPEN H, SCHUHBAUER A and WALTHER D, 2017. Emerging mosquito species in Germany – a synopsis after 6 years of mosquito monitoring (2011-2016). *Parasitology Research*, 116: 3253-3263.
- KAMPEN H and WALTHER D, 2018. Vector potential of mosquito species (Diptera: Culicidae) occurring in Central Europe. In: BENELLI G and MEHLHORN H (eds), *Mosquito-borne Diseases: Implications for Public Health*. Parasitology Research Monographs 10, Springer Verlag, Heidelberg, Germany: 41-68.

- KAMPEN H and WERNER D, 2015. Die wiederkehrende Notwendigkeit von Stechmücken-Surveillance und -Forschung. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz, 58: 1101-1109.
- KILPATRICK AM, KRAMER LD, JONES MJ, MARRA PP and DASZAK P, 2006. West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior. PLoS Biology, 4: e82.
- KRONE O, PRIEMER J, STREICH J, SOMMER P, LANGGEMACH T and LESSOW O, 2001. Haemosporidia of birds of prey and owls from Germany. Acta Protozoologica, 40: 281-289.
- KRONEFELD M, KAMPEN H, SASSNAU R and WERNER D, 2014. Molecular detection of *Dirofilaria immitis*, *Dirofilaria repens* and *Setaria tundra* in mosquitoes from Germany. Parasites & Vectors, 7: e30.
- KRÜGER A, RECH A, SU XZ and TANNICH E, 2001. Two cases of autochthonous *Plasmodium falciparum* malaria in Germany with evidence for local transmission by indigenous *Anopheles plumbeus*. Tropical Medicine & International Health, 6: 983-985.
- LAAKSONEN S, SOLISMAA M, KORTET R, KUUSELA J and OKSANEN A, 2009. Vectors and transmission dynamics for *Setaria tundra* (Filarioidea; Onchocercidae), a parasite of reindeer in Finland. Parasites & Vectors, 2: e3.
- LAIRD M, 1988. The Natural History of Larval Mosquito Habitats. London, United Kingdom: Academic Press: 555 pp.
- LANCIOTTI RS, ROEHRIG JT, DEUBEL V, SMITH J, PARKER M, STEELE K, CRISE B, VOLPE KE, CRABTREE MB, SCHERRET JH, HALL RA, MACKENZIE JS, CROPP CB, PANIGRAHY B, OSTLUND E, SCHMITT B, MALKINSON M, BANET C, WEISSMAN J, KOMAR N, SAVAGE HM, STONE W, MCNAMARA T and GUBLER DJ, 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science, 286: 2333-2337.
- LANE RP and CROSSKEY RW, 1993. Medical Insects and Arachnids. Chapman & Hall, London, United Kingdom: 723 pp.
- LINKE S, NIEDRIG M, KAISER A, ELLERBROK H, MÜLLER K, MÜLLER T, CONRATHS FJ, MÜHLE RU, SCHMIDT D, KÖPPEN U, BAIRLEIN F, BERTHOLD P and PAULI G, 2007. Serologic evidence of West Nile virus infections in wild birds captured in Germany. American Journal of Tropical Medicine and Hygiene, 77: 358-364.
- LUDWIG GV, CALLE PP, MANGIAFICO JA, RAPHAEL BL, DANNER DK, HILE JA, CLIPPINGER TL, SMITH JF, COOK RA and MCNAMARA T, 2002. An outbreak of West Nile virus in

- a New York city captive wildlife population. *American Journal of Tropical Medicine and Hygiene*, 67: 67-75.
- LUNDSTRÖM JO, ANDERSSON AC, BÄCKMAN S, SCHÄFER ML, FORSMAN M and THELAUS J, 2011. Transstadial transmission of *Francisella tularensis holarctica* in mosquitoes, Sweden. *Emerging Infectious Diseases*, 17: 794-799.
- MARSHALL JF, 1938. *The British Mosquitoes*. The British Museum, London, United Kingdom: 341 pp.
- MCCALL JW, GENCHI C, KRAMER LH, GUERRERO J and VENCO L, 2008. Heartworm disease in animals and humans. *Advances in Parasitology*, 66: 193-285.
- MCNAMARA T, 2007. The role of zoos in biosurveillance. *International Zoo Yearbook*, 41: 12-15.
- MEDLOCK J, HANSFORD K, ANDERSON M, MAYHO R and SNOW K, 2012. Mosquito nuisance and control in the UK – a questionnaire-based survey of local authorities. *European Mosquito Bulletin*, 30: 15-29.
- MEDLOCK J and VAUX A, 2011. Assessing the possible implications of wetland expansion and management on mosquitoes in Britain. *European Mosquito Bulletin*, 29: 38-65.
- MEDLOCK JM, SNOW KR and LEACH SA, 2005. Potential transmission of West Nile virus in the British isles: an ecological review of candidate mosquito bridge vectors. *Medical and Veterinary Entomology*, 19: 2-21.
- MEHLHORN H, WALLDORF V, KLIMPEL S, SCHAUB G, KIEL E, FOCKE R, LIEBISCH G, LIEBISCH A, WERNER D, BAUER C, CLAUSEN H, BAUER B, GEIER M, HÖRBRAND T, BÄTZA H-J, CONRATHS FJ, HOFFMANN B and BEER M, 2009. Bluetongue disease in Germany (2007–2008): monitoring of entomological aspects. *Parasitology Research*, 105: 313-319.
- MEHLHORN H, WALLDORF V, KLIMPEL S and SCHMAHL G, 2008. Outbreak of bluetongue disease (BTD) in Germany and the danger for Europe. *Parasitology Research*, 103: 79-86.
- MERRITT RW, DADD RH and WALKER ED, 1992. Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. *Annual Review of Entomology*, 37: 349-376.
- MORCHÓN R, CARRETÓN E, GONZÁLEZ-MIGUEL J and MELLADO-HERNÁNDEZ I, 2012. Heartworm disease (*Dirofilaria immitis*) and their vectors in Europe – new distribution trends. *Frontiers in Physiology*, 3: e196.

- MULLEN GR and DURDEN LA, 2009. Medical and Veterinary Entomology, 2nd Edition. Academic Press, San Diego, USA: 637 pp.
- NATIONAL RESEARCH COMMITTEE (US), 2004. Pest management. In: Animal Care and Management at the National Zoo: Interim Report. National Academies Press, Washington DC, USA: 55-58.
- O'MALLEY CM, 1990. *Aedes vexans* (Meigen): an old foe. Proceedings of the Annual Meeting of the New Jersey Mosquito Extermination Association, 14: 90-95.
- ONYIDO AE, OZUMBA NA, EZIKE VI, CHUKWUEKEZIE OC, NWOSU EO, NWAORGU OC and IKPEZE OO, 2008. Mosquito fauna of a tropical museum and zoological garden complex. Animal Research International, 5: 852-858.
- PFITZNER WP, LEHNER A, HOFFMANN D, CZAJKA C and BECKER N, 2018. First record and morphological characterization of an established population of *Aedes* (*Hulecoeteomyia*) *koreicus* (Diptera: Culicidae) in Germany. Parasites & Vectors, 11: e662.
- PIALOUX G, GAÜZÈRE B-A, JAURÉGUIBERRY S and STROBEL M, 2007. Chikungunya, an epidemic arbovirosis. Lancet Infectious Diseases, 7: 319-327.
- PILASKI J, 1987. Contributions to the ecology of Ťahyňa virus in Central Europe. Bulletin of the Society of Vector Ecology, 12: 544-553.
- QUINTAVALLE PASTORINO G, ALBERTINI M, CARLSEN F, CUNNINGHAM AA, DANIEL BA, FLACH E, HOFER H, JUNHOLD J, KOCK R, LEATHER S, MASTERS N, PREZIOSI R, ROUTH A, SAINSBURY AW, SATO Y, SPENCER W and PEARCE-KELLY P, 2015. Project MOSI: rationale and pilot-study results of an initiative to help protect zoo animals from mosquito-transmitted pathogens and contribute data on mosquito spatio-temporal distribution change. International Zoo Yearbook, 49: 172-188.
- RANDOLPH SE and ROGERS DJ, 2010. The arrival, establishment and spread of exotic diseases: patterns and predictions. Nature Reviews Microbiology, 8: 361-371.
- REISEN WK, 2016. Update on journal policy of aedine mosquito genera and subgenera. Journal of Medical Entomology, 53: 249-249.
- REUSKEN CBEM, DE VRIES A, BUIJS J, BRAKS MAH, DEN HARTOG W and SCHOLTE EJ, 2010. First evidence for presence of *Culex pipiens* biotype *molestus* in the Netherlands, and of hybrid biotype *pipiens* and *molestus* in northern Europe. Journal of Vector Ecology, 35: 210-212.
- REZZA G, NICOLETTI L, ANGELINI R, ROMI R, FINARELLI AC, PANNING M, CORDIOLI P, FORTUNA C, BOROS S, MAGURANO F, SILVI G, ANGELINI P, DOTTORI M, CIUFOLINI

- MG, MAJORI GC and CASSONE A, 2007. Infection with chikungunya virus in Italy: an outbreak in a temperate region. *Lancet*, 370: 1840-1846.
- RIZZOLI A, BOLZONI L, CHADWICK EA, CAPELLI G, MONTARSI F, GRISENTI M, MARTINEZ DE LA PUENTE J, MUÑOZ J, FIGUEROLA J, SORIGUER R, ANFORA G, DI LUCA M and ROSÀ R, 2015. Understanding West Nile virus ecology in Europe: *Culex pipiens* host feeding preference in a hotspot of virus emergence. *Parasites & Vectors*, 8: 213.
- ROBERT V, GÜNAY F, LE GOFF G, BOUSSÈS P, SULESCO T, KHALIN A, MEDLOCK JM, KAMPEN H, PETRIĆ D and SCHAFFNER F, 2019. Distribution chart for Euro-Mediterranean mosquitoes (western Palaearctic region). *Journal of the European Mosquito Control Association*, 37: 1-28.
- ROGERS DJ and RANDOLPH SE, 2000. The global spread of malaria in a future, warmer world. *Science*, 289: 1763-1766.
- ROSS RW, 1956. The Newala epidemic: III. The virus: isolation, pathogenic properties and relationship to the epidemic. *Journal of Hygiene*, 54: 177-191.
- RUEDA LM, PATEL KJ, AXTELL RC and STINNER RE, 1990. Temperature-dependent development and survival rates of *Culex quinquefasciatus* and *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology*, 27: 892-898.
- RUSSELL RC, 1999. Constructed wetlands and mosquitoes: health hazards and management options – an Australian perspective. *Ecological Engineering*, 12: 107-124.
- SANTINI M, VILIBIC-CAVLEK T, BARSIC B, BARBIC L, SAVIC V, STEVANOVIC V, LISTES E, DI GENNARO A and SAVINI G, 2015. First cases of human Usutu virus neuroinvasive infection in Croatia, August-September 2013: clinical and laboratory features. *Journal of Neurovirology*, 21: 92-97.
- SASSNAU R, CZAJKA C, KRONEFELD M, WERNER D, GENCHI C, TANNICH E and KAMPEN H, 2014. *Dirofilaria repens* and *Dirofilaria immitis* DNA findings in mosquitoes in Germany: temperature data allow autochthonous extrinsic development. *Parasitology Research*, 113: 3057-3061.
- SCHAFFNER F and MATHIS A, 2014. Dengue and dengue vectors in the WHO European region: past, present, and scenarios for the future. *The Lancet Infectious Diseases*, 14: 1271-1280.
- SCHOENENBERGER AC, WAGNER S, TUTEN HC, SCHAFFNER F, TORGERSON P, FURRER S, MATHIS A and SILAGHI C, 2015. Host preferences in host-seeking and blood-fed mosquitoes in Switzerland. *Medical and Veterinary Entomology*, 30: 39-52.

- SEIDOWSKI D, ZIEGLER U, RÖNN JA, MÜLLER K, HÜPPOP K, MÜLLER T, FREULING C, MÜHLE RU, NOWOTNY N, ULRICH RG, NIEDRIG M and GROSCHUP MH, 2010. West Nile virus monitoring of migratory and resident birds in Germany. *Vector-borne and Zoonotic Diseases*, 10: 639-647.
- SEMENZA JC and MENNE B, 2009. Climate change and infectious diseases in Europe. *Lancet Infectious Diseases*, 9: 365-375.
- SERVICE MW, 1971. Feeding behaviour and host preferences of British mosquitoes. *Bulletin of Entomological Research*, 60: 653-661.
- SIJBRANDA DC, HUNTER S, HOWE L, LENTING B, ARGILLA L and GARTRELL BD, 2017. Cases of mortality in little penguins (*Eudyptula minor*) in New Zealand associated with avian malaria. *New Zealand Veterinary Journal*, 65: 332-337.
- SIMÓN F, SILES-LUCAS M, MORCHÓN R, GONZÁLEZ-MIGUEL J, MELLADO I, CARRETÓN E and MONTOYA-ALONSO JA, 2012. Human and animal dirofilariasis: the emergence of a zoonotic mosaic. *Clinical Microbiology Reviews*, 25: 507-544.
- SIMPSON JE, HURTADO PJ, MEDLOCK J, MOLAEI G, ANDREADIS TG, GALVANI AP and DIUK-WASSER MA, 2011. Vector host-feeding preferences drive transmission of multi-host pathogens: West Nile virus as a model system. *Proceedings of the Royal Society B: Biological Sciences*, 279: 1471-2954.
- SPIECKERMANN D and ACKERMANN R, 1972. Isolierung von Viren der California-Enzephalitis-Gruppe aus Stechmücken in Nordbayern. *Zentralblatt für Bakteriologie, Parasitenkrankheiten, Infektionskrankheiten und Hygiene, Abteilung I, Originale A*, 221: 283-295.
- SPIELMAN A, 2001. Structure and seasonality of nearctic *Culex pipiens* populations. *Annals of the New York Academy of Sciences*, 951: 220-234.
- STEINBRINK A, ZOTZMANN S, CUNZE S and KLIMPEL S, 2019. *Aedes koreicus* – a new member of the genus *Aedes* establishing in Germany? *Parasitology Research*, 118: 1073-1076.
- STEINMETZ HW, BAKONYI T, WEISSENBOCK H, HATT J-M, EULENBERGER U, ROBERT N, HOOP R and 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.

- SUCHANEK D, 2012. Zoo und Natur: Interaktionsräume zwischen Mensch und Tier und damit verbundene Naturvorstellungen: Ergebnisse einer empirischen Erhebung im Tiergarten Schönbrunn. Ph.D. thesis, University of Vienna: 109 pp.
- SUTCLIFFE JF, 1987. Distance orientation of biting flies to their hosts. *International Journal of Tropical Insect Science*, 8: 611-616.
- TAKKEN W, CONSTANTINI C, DOLO G, HASSANALI A, SAGNON N and OSIR E, 2006. Mosquito mating behaviour. In: KNOLS BG and LOUIS C (eds), *Bridging Laboratory and Field Research for Genetic Control of Disease Vectors*, Springer, Wageningen, Netherlands: 183-188.
- TAKKEN W and VERHULST NO, 2013. Host preferences of blood-feeding mosquitoes. *Annual Review of Entomology*, 58: 433-453.
- TESH RB, 1982. Arthritides caused by mosquito-borne viruses. *Annual Review of Medicine*, 33: 31-40.
- TOMASELLO D and SCHLAGENHAUF P, 2013. Chikungunya and dengue autochthonous cases in Europe, 2007–2012. *Travel Medicine and Infectious Disease*, 11: 274-284.
- TRAUTMANN A, 1913. Die Verbreitung der einheimischen Malaria in Deutschland in Vergangenheit und Gegenwart. *Archiv für Hygiene und Bakteriologie*, 80: 84-108.
- TUTEN HC, 2011a. Habitat characteristics of larval mosquitoes in zoos of South Carolina, USA. *Journal of the American Mosquito Control Association*, 27: 111-119.
- TUTEN HC, 2011b. Zoos as experiment environments: biology of larval and adult mosquitoes (Diptera: Culicidae). Ph.D. thesis, Clemson University: 163 pp.
- TUTEN HC, BRIDGES WC, PAUL KS and ADLER PH, 2012. Blood-feeding ecology of mosquitoes in zoos. *Medical and Veterinary Entomology*, 26: 407-416.
- UNNASCH RS, CUPP EW and UNNASCH TR, 2006. Host selection and its role in transmission of arboviral encephalitides. In: COLLINGE SK and RAY C (eds), *Disease Ecology: Community Structure and Pathogen Dynamics*. Oxford University Press, New York, USA: 240 pp.
- WEISSENBOCK H, BAKONYI T, ROSSI G, MANI P and NOWOTNY N, 2013. Usutu virus, Italy, 1996. *Emerging Infectious Diseases*, 19: 274-277.
- WHO, 1975. *Manual on Practical Entomology in Malaria, Part II: Methods and Techniques*. World Health Organization, Geneva, Switzerland: 192 pp.
- WIERSCH SC, LUBJUH T, MAIER WA and KAMPEN H, 2007. Haemosporidian infection in passerine birds from lower Saxony. *Journal of Ornithology*, 148: 17-24.

- WILKERSON RC, LINTON Y-M, FONSECA DM, SCHULTZ TR, PRICE DC and STRICKMAN DA, 2015. Making mosquito taxonomy useful: a stable classification of tribe Aedini that balances utility with current knowledge of evolutionary relationships. PLoS One, 10: e0133602.
- WILLIAMS MC, SIMPSON DIH, HADDOW AJ and KNIGHT EM, 1964. The isolation of West Nile virus from man and of Usutu virus from the bird-biting mosquito *Mansonia aurites* (Theobald) in the Entebbe area of Uganda. Annals of Tropical Medicine and Parasitology, 58: 367-374.
- WILSON AJ and MELLOR PS, 2009. Bluetongue in Europe – past, present and future. Philosophical Transactions of the Royal Society B, 364: 2669-2681
- YEE DA, ALLGOOD D, KNEITEL JM and KUEHN KA, 2012. Constitutive differences between natural and artificial container mosquito habitats: vector communities, resources, microorganisms, and habitat parameters. Journal of Medical Entomology, 49: 482-491.
- YUVAL B, 1992. The other habit: sugar feeding by mosquitoes. Bulletin of the Society of Vector Ecology, 17: 150-156.
- ZÉLÉ F, VÉZILIER J, L'AMBERT G, NICOT A, GANDON S, RIVERO A and DURON O, 2014. Dynamics of prevalence and diversity of avian malaria infections in wild *Culex pipiens* mosquitoes: the effects of *Wolbachia*, filarial nematodes and insecticide resistance. Parasites & Vectors, 7: e437.
- ZIEGLER U, FAST C, EIDEN M, BOCK S, SCHULZE C, HOEPER D, OCHS A, SCHLIEBEN P, KELLER M, ZIELKE DE, LUEHKEN R, CADAR D, WALTHER D, SCHMIDT-CHANASIT J and GROSCHUP MH, 2016. Evidence for an independent third Usutu virus introduction into Germany. Veterinary Microbiology, 192: 60-66.
- ZIEGLER U, LÜHKEN R, KELLER M, CADAR D, VAN DER GRINTEN E, MICHEL F, ALBRECHT K, EIDEN M, RINDER M, LACHMANN L, HOEPER D, VINA-RODRIGUEZ A, GAEDE W, POHL A, SCHMIDT-CHANASIT J and GROSCHUP MH, 2018. West Nile virus epizootic in Germany, 2018. Antiviral Research, 162: 39-43.

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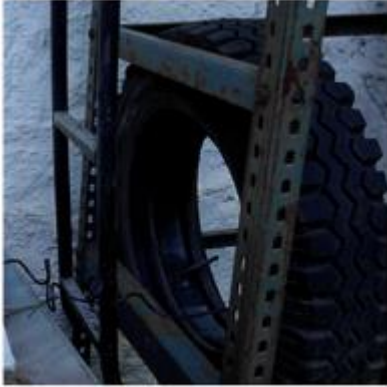
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9. Appendix

9.1 Pictures of mosquito breeding sites

9.1.1 Mosquito breeding sites of Zoological Garden Eberswalde

(1)



(2)



(3)



(4)



(5)



(6)



(7)



(8)



(9)



(10)



(11)



(12)



(13)



(14)



(15)



(16)



(17)



(18)



(19)



9.1.2 Mosquito breeding sites of Tierpark Berlin

(1)



(2)



(3)



(4)



(5)



(6)



(7)



(8)



(9)



(10)



(11)



(12)



(13)



(14)



(15)



9.2 Pictures of mosquito resting sites

9.2.1 Mosquito resting sites of Zoological Garden Eberswalde

(1)



(2)



(3)



(4)



(5)



(6)



(7)



(8)



(9)



(10)



(11)



(12)



(13)



(14)



(15)

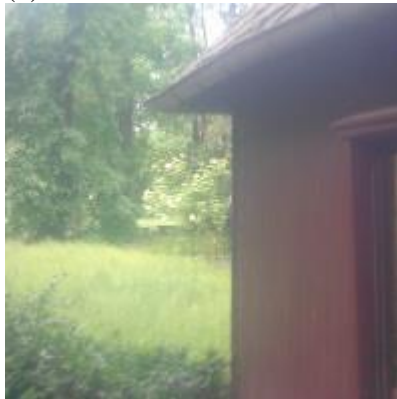


9.2.2 Mosquito resting sites of Tierpark Berlin

(1)



(2)



(3)



(4)



(5)



(6)



(7)



(8)



(9)



(10)



(11)



(12)



(13)



(14)



(15)



9.3 Pictures of EVS-trap locations

9.3.1 EVS-trap locations of Zoological Garden Eberswalde

(1)



(2)



(3)



(4)



(5)



(6)



(7)



(8)



9.3.2 EVS-trap locations of Tierpark Berlin

(1)



(2)



(3)



(4)



(5)



(6)



(7)



(8)

