

**Distribution and ecology of selected mosquito species
(Diptera: Culicidae) in Germany**

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Lisa Tippelt

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Dekan: Prof. Dr. Gerald Kerth

1. Gutachter: PD Dr. Helge Kampen

2. Gutachter: Prof. Dr. Jens Amendt

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1 Introduction

1.1 Mosquito systematics and taxonomy

The two-winged flies (Diptera) can be subclassified in the Brachycera, known as “higher Diptera” and the Nematocera, also denominated as “lower Diptera”, which includes the Culicidae (Schneeberg & Beutel, 2013). The Culicidae (mosquitoes) branch into the Anophelinae and Culicinae. The Anophelinae contain three genera, of which only the genus *Anopheles* occurs in Germany. The Culicinae are more diverse and include a total of 113 genera, five of which (*Aedes*, *Coquillettidia*, *Culex*, *Culiseta* and *Uranotaenia*) can be found in Germany (Harbach, 2007). Worldwide, more than 3,500 culicid species are known (Harbach, 2020), of which about 50 occur in Germany (see chapter 1.5).

The Culicidae are considered monophyletic, but the relationships between their subtaxa remain controversial. This is in particular the case for the Aedini (Becker et al., 2010). The extensive revisions in the last decade, suggesting the rise of 74 subgenera to generic rank (Reinert et al., 2004, 2006, 2008, 2009), have caused a lot of confusion among taxonomists and practitioners. Eventually, Wilkerson et al. (2015) reanalysed the data and decided to only preserve the generic rank of *Aedes* because of inconsistent and partly weakly supported phylogenies and missing phenotypic diagnoses. However, as many relationships within the Aedini remain unresolved, future nomenclatural revisions can be expected. In this thesis, the nomenclature of Wilkerson et al. (2015) will be applied.

1.2 Morphology and identification

Primarily, the Culicidae can be recognised in the adult stage by the presence of a long proboscis and their more or less slender body with long legs. They can vary in their body size from 2 to 19 mm (Service, 2012). For distinguishing the Anophelinae from the Culicinae, the length of the palpi is important. Anopheline females have palpi, which are as long as the proboscis, whereas culicid females have much shorter palpi (Fig. 1). For the differentiation of species, patterns of scales and setae on the dorsal and lateral part of the thorax, the tergum, the legs and the wings are diagnostic, but also the general colouration and size play decisive roles.

Introduction

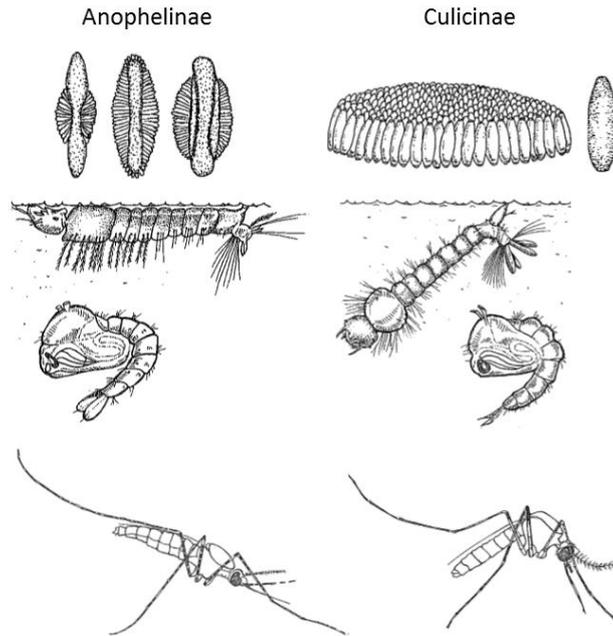


Figure 1: Differences between Anophelinae and Culicinae in all life stages (top: eggs, middle: larvae and pupae, bottom: female adults in resting position; note the difference between the length of the palpus in comparison to the antenna), from: Lehane, 2005 (modified).

Within the Culicidae, several species exist that cannot be told apart by morphological means in most developmental stages and form species complexes (sibling species). Usually, these are differentiated by genetic techniques specifically adapted to the respective species complex. In Germany, three different species complexes occur: the *Anopheles maculipennis* species complex, encompassing *Anopheles atroparvus* van Thiel, 1927, *Anopheles maculipennis* s.s. Meigen, 1818, *Anopheles messeae* Falleroni, 1926, and *Anopheles daciae* Linton, Nicolescu & Harbach, 2004; the *Culex pipiens* complex, which includes *Cx. pipiens* with its two biotypes *pipiens* Linnaeus, 1758 and *molestus* Forskål, 1775 as well as *Culex torrentium* Martini, 1925; and the *Anopheles claviger* complex, consisting of *Anopheles claviger* s.s. (Meigen, 1804) and *Anopheles petragrani* Del Vecchio 1939 (Becker et al., 2010).

1.3 Biology and life cycle

As culicids are holometabolic insects, they pass through a complete metamorphosis with only the imago being independent from water. Female mosquitoes lay their eggs singly (*Anopheles* and *Aedes*) or as egg batches (*Coquillettidia*, *Culex*, *Culiseta* and *Uranotaenia*) on the water surface or in moist substrates close to the water's surface. In the latter case, eggs do not

automatically hatch after completion of the larval development but enter dormancy, which can either be reversible by flooding at any time (quiescence) or is irreversible and has to be terminated before reacting to hatching stimuli is possible (diapause, see chapter 1.8).

After hatching, larvae pass through four instars and one pupal stage until metamorphosis to the adult mosquito (Becker et al., 2010). Eggs and larvae of anopheline mosquitoes can be easily distinguished from the culicine ones. The eggs of Anophelinae have air-filled chambers which allow them to float on the water surface (Fig. 1), often forming star-like structures (Lucius et al., 2018). The larvae lie parallel beneath the water surface because of a missing respiratory siphon at the rear end of their body which is present in Culicinae (Fig. 1).

Nearly in all species the culicid females need a blood meal as they use the proteins from the blood for the maturation of their eggs. However, there are also mosquito species completing the first gonotrophic cycle without a blood meal (autogeny). Olfactory stimuli, primarily carbon dioxide, but also thermal and visual stimuli, are relevant for finding a suitable host (Becker et al., 2010).

1.4 Medical importance

Hematophagous arthropods, such as mosquitoes, may act as biological vectors of parasites and viruses, thereby transmitting disease agents, leading to morbidity and mortality among humans and animals (Mehlhorn, 2017). The most prominent infectious disease mediated by mosquitoes is malaria, which is caused by protozoan parasites. Human malaria is restricted to the genus *Plasmodium*. Five different species are known: *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium falciparum* and *Plasmodium knowlesi* (Mehlhorn, 2017). The latter is the only zoonotic parasite of this group as it mainly infects macaques but can also be transmitted to humans (Cox-Singh et al., 2008). Human malaria parasites are exclusively vectored by mosquitoes of the genus *Anopheles*. About 70 species of *Anopheles* of the over 400 known ones are able to transmit *Plasmodium* parasites, and 40 of them are regarded as relevant vectors. In Europe, 18 different *Anopheles* species are considered competent vectors, predominantly representatives of the *An. maculipennis* complex. Other *Anopheles* species like *Anopheles algeriensis* Theobald, 1903 can also act as vectors but play a subordinate role (Piperaki & Daikos, 2016). Malaria is the most common and important infectious disease in humans and is endemic in over 100 countries, predominantly in tropical regions of Africa, Asia and Latin America (Reisen, 2009). In 2018, over 200 million cases, over 400,000 deaths and

circa 3.3 billion people living at risk were reported. The vast majority of the cases and deaths are attributed to infections with *P. falciparum* in African countries (WHO, 2018).

Historically, malaria did not only occur in tropical and subtropical but also in temperate regions of the world. Until the beginning of the 19th century, endemic malaria occurred in large parts of Europe, including Great Britain, Sweden and Finland (Bruce-Chwatt, 1988). Three species of malaria parasites circulated in Europe: *P. falciparum*, *P. malariae* and *P. vivax*. High incidences of malaria usually occurred in coastal and riverine areas. In Germany, the Upper Rhine Valley and the Danube Valley but also areas in Bavaria and Baden-Württemberg and the coastal regions along the North and Baltic Seas were affected (Wernsdorfer, 2002). Until the middle of the last century, malaria incidences receded due to vector control programmes, the increased availability of medication against malaria and the separation of human housing and livestock shelters which led to the deviation of preferentially zoophilic vectors from humans to animals (Bruce-Chwatt, 1988; Maier, 2004). With the exception of Turkey, Europe was declared malaria-free by the World Health Organisation in 1975 (WHO, 2016). However, cases of occasional autochthonous malaria have since been reported from several European countries, such as Spain, Germany and the United Kingdom (Krüger et al., 2001; Peralta et al., 2010; ECDC, 2017).

Mosquitoes are also known to transmit over 200 different arboviruses. These are viruses needing an arthropod vector for their replication and transmission to a vertebrate host. The majority of the known arboviruses belong to three virus families: Togaviridae, Flaviviridae and Bunyaviridae (Hubálek, 2008). With a few exceptions, mosquito-borne viruses were formerly restricted to the tropics and did not occur in Europe. However, with increasing globalisation, both in terms of travel and trade, and the ameliorating climatic suitability of European regions to competent vectors due to climate change, these viruses have found their way to Europe, with the infections they cause gaining attention as so-called “emerging diseases” (Gould & Higgs, 2009; Randolph & Rogers, 2010). These are diseases which are novel in a certain population or experience a sudden increase in incidence and/or a geographical shift or expansion (Morse, 1995).

The composition of local mosquito populations has tremendously changed during the last decades as non-indigenous mosquitoes like *Aedes koreicus* (Edwards 1917), *Aedes japonicus* (Theobald 1901), and *Aedes albopictus* (Skuse 1895) but also other *Aedes* species were introduced and even have established populations in Europe (see chapter 1.6). Many invasive species are competent vectors of a variety of vector-borne disease agents and thus pose a serious

threat to public and animal health. Outbreaks of emerging diseases like chikungunya, dengue, Zika or West Nile fever have demonstrated that the theoretical risk has become a realistic scenario (Rezza et al., 2007; Gjenero-Margan et al., 2011; Merdić et al., 2013).

An example for an arbovirus showing a recent northward spread is the West Nile virus (WNV) causing West Nile fever (WNF). WNV is considered a re-emerging zoonotic arbovirus which has birds as its amplifying hosts. Predominant vectors are various *Culex* species, among them the widespread members of the *Cx. pipiens* complex, but several *Aedes* species can also transmit the virus (Higgs et al., 2004; Medlock et al., 2005; Service, 2012). Several additional species like *Uranotaenia unguiculata* Edwards, 1913 were found infected in the field, but their vector status is unknown (Zeller & Schuffenecker, 2004; Engler et al., 2013; Camp et al., 2018). In Europe, WNV has been circulating since the 1960s (Zeller & Schuffenecker, 2004). In summer 2018, many countries in Europe, especially in the southeast, reported a huge increase in cases of WNF in both animals and humans, and the first cases in Germany were reported in wild and captive birds as well as in horses (ECDC, 2018; Ziegler et al., 2019). Demonstrations of infections in several bird species and horses in northern and eastern Germany in 2019 strongly suggest local overwintering of the virus. Furthermore, the number of cases was much higher in 2019 than in 2018, leading to the first epidemic of WNF in Germany, accompanied by the first human cases (Wilking et al., 2019; Ziegler et al., 2020). The positive findings in mosquitoes collected in Berlin also account for a much higher circulation of the West Nile virus in Germany than in 2018 (Kampen et al., 2020). Recently, several cases of WNF were reported in horses in Germany and also 13 human cases occurred (ECDC, 2020).

Before the arrival of WNV, four different arboviruses had been documented to circulate in Germany: Ťahyňa, Sindbis, Batai and Usutu virus. All of them are able to infect humans and were detected in mosquitoes in Germany (Spieckermann & Ackermann, 1972; Jöst et al., 2010, 2011a, b; Scheuch et al., 2018). Ťahyňa, Sindbis and Batai viruses usually cause mild flu-like symptoms in humans (Hubálek, 2008), whereas Usutu virus rarely infects humans. Symptoms of an infection with the Usutu virus were described as rash or fever but also severe neurological infections are possible as reported for two patients representing the first human cases in Europe (Pecorari et al., 2009; Ashraf et al., 2015). Both patients were immunocomprised but there are also reports about infections in patients without pre-existing conditions (Pecorari et al., 2009; Simonin et al., 2018). Apart from the few reported cases in humans the Usutu virus plays a predominant role as an epizootic virus in wild bird populations (Becker et al., 2012; Ashraf et al., 2015; Cadar et al., 2017; Lühken et al., 2017).

Mosquitoes cannot only transmit protozoa and arboviruses but also filarioid worms. In Europe, two filarioid species pose a risk to humans and domestic animals: *Dirofilaria repens* Railliet and Henry, 1911 (dog skinworm) and *Dirofilaria immitis* Leidy, 1856 (dog heartworm). Both *Dirofilaria* species can infect canines, rarely felines and humans, and the associated diseases are considered emerging diseases because of their recent considerable spread towards northern European regions, like Germany, due to climate change and the trade with infected dogs (Genchi et al., 2009).

1.5 Importance of mosquito monitoring

After the eradication of malaria in Germany by the mid-20th century, research on mosquitoes had been neglected for many decades. Comprehensive works dealing with the diversity and ecology of the indigenous culicid fauna date back to the late 1960s or early 1970s (Mohrig, 1969; Peus, 1972). However, the ongoing process of global warming and the increase in globalisation, which have led to changes in distribution areas and the introduction of non-native species into Germany (see chapter 1.6), urgently required a revision of knowledge about the occurrence and phenology of species. The importance of up-to-date data could be demonstrated during the bluetongue outbreak in 2006 for the Ceratopogonidae, commonly known as biting midges. These rather small nematocerans are competent vectors of many viruses, amongst them the viruses causative for bluetongue disease and Schmallenberg disease (Mehlhorn, 2012). Both viral diseases affect ruminants, and especially the bluetongue virus has caused considerable economic loss due to morbidity, mortality, massive cullings and trade restrictions (Hateley, 2009). In the course of the epidemics, it was found out that not invasive but native biting midge species acted as vectors of the viruses (Mehlhorn et al., 2007; Rasmussen et al., 2012). However, as research on this dipteran group had also been neglected for a long time, the identification and management of the vectors of these disease agents needed time and cost further losses in livestock.

For more than two decades the compilation prepared by Dahl et al. (1999) have been used when referring to the species inventory of Germany. Recently, an updated inventory of culicid species could confirm the existence of 44 of the 46 species listed by Dahl et al. (1999). However, the detection of *Cs. subochrea* stays ambiguous but an existence still in present can be assumed (Werner et al., 2020). Five further species have been introduced to and established in Germany: *An. petragrani*, *Culiseta longiareolata* (Macquart, 1838), *Ae. japonicus*, *Ae. albopictus* and,

quite recently, *Ae. koreicus* (Werner et al., 2020). Furthermore, the member of the *An. maculipennis* complex identified last, *An. daciae*, was also be found in Germany (Kronefeld et al., 2012). Mosquitoes were collected passively by the citizen science project “Mückenatlas” (Kampen et al., 2015) and actively for instance through dipping, netting but also trapping in the course of two monitoring projects (Werner et al., 2020).

The first monitoring programme covering the whole of Germany was conducted by the Friedrich-Loeffler-Institut (FLI) and the Leibniz-Centre for Agricultural Landscape Research (ZALF) in 2011-2014. Within its framework, mosquitoes were monitored by trapping to establish the status quo of the German mosquito fauna. During the monitoring activities, the majority of the culicid species documented for Germany were detected, among them also rare species like *Culiseta alaskaensis* (Ludlow, 1906) and *Culiseta glaphyroptera* (Schiner, 1864). Furthermore, the invasive species *Cs. longiareolata*, which could be recently detected in Germany for the first time, could also be found during this monitoring programme (Kampen et al., 2013).

In 2015, the successor of the first programme started within a cooperation network of different institutions distributed over the whole of Germany. The FLI was responsible for the northeastern part of Germany. During that monitoring project, it was possible to detect nearly all species sampled in the previous project in the same area. Furthermore, mosquito species that had not been detected in Germany for decades were also encountered (see chapters 3.1 and 3.2), and the existence of rare species only recently re-discovered, like *Aedes refiki* (Medschid, 1928) and *Culiseta ochroptera* (Peus, 1935) was confirmed (own data) (Kuhlish et al., 2017; Kuhlish et al., 2019). Both monitoring projects have led to a considerable growth in knowledge regarding the occurrence and distribution of mosquito species in Germany.

1.6 Invasive mosquitoes

One of the many definitions describes an invasive species as a non-indigenous species spreading after its introduction and having an impact on the environment in terms of interactions with other species and ecosystems but also on humans, agriculture, conservation and public health (Juliano & Lounibos, 2005). In the last decades, especially species of the genus *Aedes* have spread in Europe, among them *Aedes aegypti* (Linnaeus 1762), *Aedes atropalpus* (Coquillett 1902), *Ae. koreicus*, *Ae. japonicus* and *Ae. albopictus*. Whereas the first two species

mentioned do not play a role in Germany, *Ae. japonicus*, *Ae. albopictus* and, most recently, *Ae. koreicus* have not only been introduced but also established in Germany.

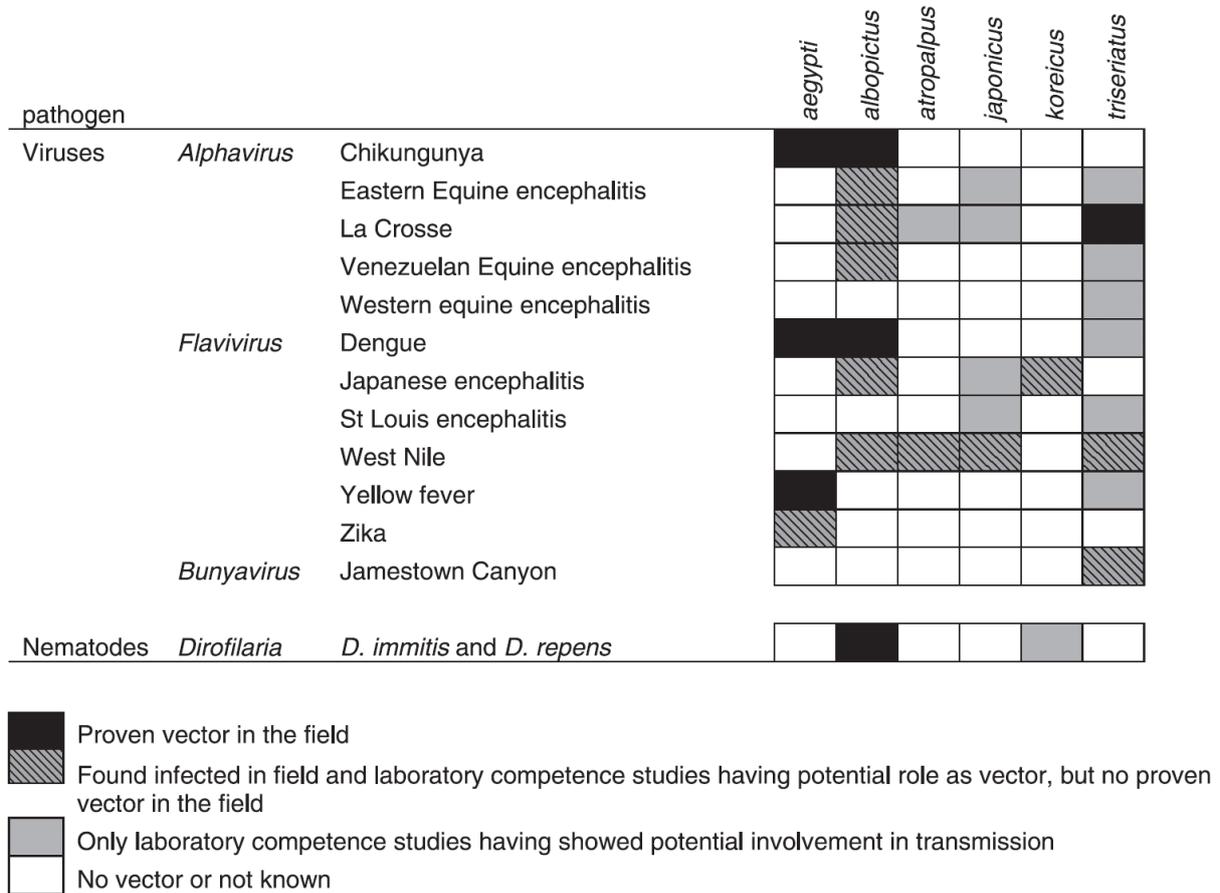


Figure 2: Overview about the vector status of invasive mosquito species of the genus *Aedes* (from: Medlock et al., 2012).

Aedes koreicus was first found in Germany in 2015 (Werner et al., 2016). Thus, after several detections in the years 2016-2018, it is assumed to be established in Germany (Pfitzner et al., 2018; Steinbrink et al., 2019). This invasive species is a competent vector of arboviruses, like chikungunya virus, and of dirofilariiae (Fig. 2) (Ciocchetta et al., 2018; Montarsi et al., 2015). *Aedes koreicus* has a high potential of further spreading as demonstrated by the recent dispersal in other European countries (Kampen et al., 2017).

The Asian bush mosquito, *Ae. japonicus*, is closely related to *Ae. koreicus* but is much more widely distributed in Germany. *Aedes japonicus* was first found in 2008 (Schaffner et al., 2009). Further findings in southwestern, western and northern Germany documented further introductions and spread (Schneider, 2011; Huber et al., 2012; Kampen et al., 2012; Werner et al., 2012; Werner & Kampen, 2013; Zielke et al., 2016). At present, there are two populations

in Germany (Koban et al., 2019). The first belongs to the largest European population spanning over southwestern Germany, Liechtenstein, parts of France, Switzerland and Austria and is the result of the fusion of expanding separate populations. The second occurs in northern Germany and has not shown a tendency of expansion since its initial detection (Kampen et al., 2016). In the future, it is expected that the two German populations will merge (Koban et al., 2019). *Aedes japonicus* is a vector of numerous arboviruses, among them the emerging chikungunya, dengue and West Nile viruses (Fig. 2) (Sardelis & Turell, 2001; Schaffner et al., 2011). In contrast to *Ae. aegypti* and *Ae. albopictus*, *Ae. japonicus* is no thermophilic species but tolerant towards low temperatures which would allow a distribution northwards. Models referring to climatic conditions agreed with such a further northward spread (Früh et al., 2018).

Of all invasive mosquito species mentioned, the Asian tiger mosquito *Ae. albopictus* shows the widest expansion throughout Europe. This species originates from temperate and tropical regions in southeastern Asia and the Pacific (Nawrocki & Hawley, 1987) and has spread to North and South America, Africa, Australia and Europe as well as numerous islands in the Pacific and Indian Ocean within a few decades (Paupy et al., 2009). The displacement of eggs and larvae via used tyres and ornamental plants of the genus *Dracaena* have played a major role in the possible introduction route of this species (Madon et al., 2002; Hofhuis et al., 2009). In Europe, *Ae. albopictus* was initially introduced into Albania in 1979, but did not spread further (Adhami & Murati, 1987). Instead, another introduction into the north of Italy in 1990 paved the way for the further spread in Europe (Sabatini et al., 1990; Scholte & Schaffner, 2007). Presently, *Ae. albopictus* is distributed in 28 European countries with established populations in 20 of them including Germany (Robert et al., 2019). Although it is a thermophilic mosquito, its presence and spread in temperate areas has demonstrated that it is capable of reproducing and overwintering in these regions (see chapter 1.8). After its initial finding in Germany in 2008, *Ae. albopictus* has managed to spread to several regions, especially in the southern and western parts of Germany (Walther et al., 2017). The northernmost population occurs in Jena in Central Germany, where the first larvae were found in 2015, probably introduced via exotic plants into a garden centre. The special geographic location in a valley is supposed to provide climatically suitable conditions for *Ae. albopictus* (Kuhlish et al., 2018a). Nowadays, *Ae. albopictus* is considered established in Germany (Becker et al., 2017; Walther et al., 2017), and a much wider spread into southern and western areas is projected (Kraemer et al., 2015; Thomas et al., 2018). This mosquito species is a competent vector of plenty of arboviruses and filarial worms (Fig. 2) and was responsible for repeated outbreaks of emerging

diseases like chikungunya or dengue in several South European countries (Rezza et al., 2007; Gjenero-Margan et al., 2011; Delisle et al., 2015; Succo et al., 2016; Calba et al., 2017; Venturi et al., 2017). Furthermore, laboratory infections (Di Luca et al., 2016; Heitmann et al., 2017) and field infections with the Zika virus demonstrated the comprehensive vector competence of this species (Grard et al., 2014). With regard to the first autochthonous case of Zika in France, another formerly tropical disease has recently managed the leap to Europe and it is most likely that a local *Ae. albopictus* population played a major role in the transmission of the virus (Giron et al., 2019).

Aedes albopictus and other invasive species are container breeders. They are able to use minute collections of water for oviposition and preimaginal development and a huge variety of natural and artificial containers (Paupy et al., 2009; Medlock et al., 2015). There are also clues suggesting a displacement of native species when occurring concurrently or emerging earlier than other species (Alto, 2011; Hardstone & Andreadis, 2012; Damiens et al., 2014; Kampen & Werner, 2014; Kaufman & Fonseca, 2014).

1.7 Thermophilic mosquitoes

Like all insects, Culicidae are poikilothermic, i.e. highly dependent on the ambient temperature. This is not only the case for the adults, in terms of seasonal activity, biting frequency, fecundity and survival, but also for larval hatching from eggs and the development of preimaginal stages. This development is prolonged with decreasing water temperature in breeding habitats and cannot be accomplished when reaching critical thermal limits. These critical temperature limits and developmental time frames are species-specific (Becker et al., 2010). Therefore, temperature plays a crucial role when modelling the potential geographic or spatial occurrence of a species based on the suitability of certain habitats.

Roughly, three types of temperature tolerance can be differentiated: species with a low, a median and a high tolerance against high temperatures. The latter group is called thermophilic mosquitoes and can be found in subtropical and tropical regions. However, there are different graduations in temperature tolerance. For instance, *Ae. aegypti* has a higher tolerance against high temperatures than *Ae. albopictus* (Kamimura et al., 2002).

In Germany, seven mosquito species, all of which are relatively rare, are considered thermophilic: *An. algeriensis*, *Culex hortensis* Ficalbi, 1889, *Culex martinii* Medschid, 1930,

Ur. unguiculata, *Cs. longiareolata*, *An. petragrani* and *Ae. albopictus*. The three species mentioned first have been known to occur in Germany for a long time (Mohrig, 1969) and were confirmed to be still present during the monitoring activities in 2015-2017 (own data, see chapters 3.1 and 3.2) (Kuhlisch et al., 2018b; Werner et al., 2020). By contrast, the other three species have become members of the German mosquito fauna only in the last and present decade after introduction and establishment (Pluskota et al., 2008; Becker & Hoffmann, 2011; Becker et al., 2016; Kampen et al., 2017). Furthermore, *Aedes berlandi* (Seguy 1921) and *Aedes pulcritarsis* (Rondani 1872), which are not indigenous in Germany, were also detected in Germany, although these findings were restricted to a single individual each (Kampen et al., 2017).

1.8 Hibernation

Just as in the case of high temperatures, mosquitoes have to be able to withstand low temperatures to overwinter. Hibernation or overwintering is defined as the endurance of unfavoured low temperature conditions (usually in a certain dormant state) which allows the survival and breeding after conditions have improved. Often, hibernation is associated with a retreat to hibernacula, sheltered and usually frost-free overwintering sites, or the elevation of cold-hardiness (Lee, 2009).

One possibility of increasing cold-hardiness is diapausing. Together with quiescence, this is a form of dormancy in which the metabolic activity is decreased for surviving either hot conditions, e.g. certain seasons in tropical regions (aestivation), or cold conditions (hibernation). Both processes are initiated by environmental factors and aim to increase survival. However, unlike quiescence, which can be initiated at any time and stage, diapause is a dormant stage-specific state. It is initiated a long time before unfavourable conditions affect the population and is triggered by the exposure of sensitive stages to certain environmental cues like photoperiod and temperature (Tauber et al., 1986; Denlinger, 2009). In mosquitoes, hibernation through diapausing occurs at different species-specific stages, but also depends on latitude. In temperate areas, species like *Cx. pipiens* or *Cs. alaskaensis* hibernate as adults, whereas others, like *Anopheles plumbeus* Stephens, 1828 or *Coquillettidia richiardii* (Ficalbi, 1889), overwinter in the larval stage. Furthermore, there are species, like *Culiseta morsitans* (Theobald, 1901), which are able to overwinter in different stages (Becker et al., 2010).

Introduction

Although pupal stages are quite common overwintering stages in other Diptera, they do not contribute to hibernation in the Culicidae (Denlinger & Armbruster, 2014).

Within the genus *Aedes*, the hibernation via egg stage predominates (Becker et al., 2010). One well-known representative of species overwintering in the egg stage is *Ae. albopictus*, which earns much attention because of its vector status and invasion biology (see chapters 1.4 and 1.6). This mosquito species is able to produce drought-resistant diapausing eggs which can tolerate much lower temperatures than larvae or adults (Hawley et al., 1989; Sota & Mogi, 1992). The ability to produce diapausing eggs enables the spread and establishment in a variety of suitable habitats in subtropical and temperate areas. By contrast, *Ae. aegypti*, cannot produce diapausing eggs and therefore is restricted to subtropical and tropical regions. It is assumed that the ability to diapause was decisive for *Ae. albopictus* in spreading to temperate areas of North America and Europe (Medlock et al., 2015).

A short photoperiod and temperatures around 21 °C trigger diapause in *Ae. albopictus* (Pumpuni et al., 1992). Diapause is maternally induced, and the sensitive stages are the 4th larval instar and the pupa of the previous generation. Once diapause is initiated, it can only be terminated after a certain time period, linked with an extension of photoperiod and a rise of temperature (Vinogradova, 2007). After termination, eggs enter quiescence and then are sensitive to hatching stimuli (Lacour et al., 2015).

There are different functions of mosquito diapause in nature. First, diapause decreases larval mortality by preventing accidental hatching after rainfall in late autumn and subsequent dying in winter (Mori et al., 1981). Second, when favourable conditions are met at the beginning of spring, there is a certain stock of eggs ready to hatch that allows a nearly contemporaneous hatching and the fast build-up of a population. This can affect not only population dynamics itself, but also invasion biology and the circulation of pathogens (Nasci et al., 2001; Reisen, 2013; Denlinger & Armbruster, 2014).

2 Objectives

The first part of the thesis is about the detections of *Ur. unguiculata* and *An. algeriensis* in the course of a monitoring project conducted in northeastern Germany. Both species were known to occur in Germany but were only rarely documented. Therefore, each of these species was considered in this thesis (see chapters 3.1 and 3.2).

The second part of the thesis deals with one mosquito species only: *Ae. albopictus*. Field as well as laboratory studies in Asia, North America and Europe have demonstrated that this species exhibits a remarkable ecological plasticity as tolerance against low temperatures is highly adaptive throughout strains from different geographic regions (Hanson & Craig, 1994, 1995a; Mogi, 2011; Thomas et al., 2012). Furthermore, successful overwintering could be shown for a natural population in southern Germany (Pluskota et al., 2016; Walther et al., 2017). Hence, the ability of different strains of *Ae. albopictus* to withstand wintry temperatures in northern Germany should be examined in the field (see chapter 3.3). Laboratory experiments analysing temperature thresholds for hatching in different strains exposed to low temperatures for a maximum of 24 hours (Thomas et al., 2012) were used to supplement the data. The laboratory study contributing to this thesis elucidates survival capabilities for exposure times much longer than a day and also includes experiments with fluctuating temperatures (see chapter 3.4). Consequently, both the field and laboratory studies contribute to a better understanding of low temperature tolerance of *Ae. albopictus*, in general. The findings are also especially relevant for Germany as this country faced an imminent establishment of *Ae. albopictus* at the beginning of the experiments.

The objective of this thesis was to gain further knowledge about the distribution, phenology and ecology of mosquitoes with emphasis on thermophilic species. Therefore, the following studies were conducted:

- (I) Monitoring survey of the occurrence of *Ur. unguiculata* in northeastern Germany (chapter 3.1)
- (II) Monitoring survey of the occurrence and phenology of *An. algeriensis* in northeastern Germany (chapter 3.2)
- (III) Examination of low temperature tolerance of *Ae. albopictus* in field experiments (chapter 3.3)
- (IV) Examination of low temperature tolerance of *Ae. albopictus* in laboratory experiments (chapter 3.4)

3 Publications

3.1 The thermophilic mosquito species *Uranotaenia unguiculata* Edwards, 1913 (Diptera: Culicidae) moves north in Germany

Lisa Tippelt, Doreen Walther, Helge Kampen

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SHORT COMMUNICATION

The thermophilic mosquito species *Uranotaenia unguiculata* Edwards, 1913 (Diptera: Culicidae) moves north in Germany

Lisa Tippelt¹ · Doreen Walther² · Helge Kampen¹Received: 8 September 2017 / Accepted: 11 October 2017 / Published online: 4 November 2017
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Abstract *Uranotaenia unguiculata* is a thermophilic mosquito species frequently occurring in the Mediterranean. Its first detection in the southern German Upper Rhine Valley in 1994 represented its northernmost distribution limit for a long time. During recent mosquito monitoring activities, two specimens of the species were trapped at different localities, about 70 km apart, in northeastern Germany, some 300-km latitude north of previous collection sites. It is not known whether *Ur. unguiculata* is vector-competent for disease agents although specimens collected in the field were found infected with West Nile virus and *Dirofilaria repens*. The finding of the species in northern Germany is probably a further example of mosquito species spreading northwards as a consequence of climate warming.

Keywords *Uranotaenia unguiculata* · Culicidae · Germany · Mosquito monitoring · Climate change · Vector

During the production process of the article, larval collections carried out at the more northern of the two trapping sites in mid-October 2017, i.e. one year after the described trapping of the two *U. unguiculata* adults, also contained *U. unguiculata* specimens. This finding suggests that even if the trapping of the two adults were the result of accidental displacement of single individuals, and establishment, including overwintering, has probably been accomplished.

✉ Lisa Tippelt
lisa.tippelt@fli.de

¹ Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Insel Riems, Greifswald, Germany

² Leibniz Centre for Agricultural Landscape Research, Muencheberg, Germany

Introduction

Climatic changes cause plant and animal species to perpetually adjust their geographical distribution. As a northward shift of climates is supposed to be ongoing in the northern hemisphere in the context of global warming (Rubel and Kottek 2010), thermophilic species are expected to spread to more northern regions previously not suitable for colonisation. A recent northward spread has been observed in Europe for various mosquito species, including *Aedes albopictus* (Skuse, 1894) (Walther et al. 2017), *Anopheles hyrcanus* (Pallas, 1771) (Votýpka et al. 2008; Lebl et al. 2013), *Anopheles petragrani* Del Vecchio, 1939 and *Culiseta longiareolata* (Macquart, 1838) (Kampen et al. 2017), with all of them now considered established north of the Alps and the first two being documented vectors of disease agents.

Uranotaenia unguiculata Edwards, 1913 is a thermophilic mosquito species commonly found in the Mediterranean, i.e. North Africa, the Middle East and southern Europe, with records also from central and southern Asia (Becker et al. 2010; Kurucz et al. 2017). In Europe, it has been found from the Iberian Peninsula in the west to southern Russia (North Caucasus) in the east (Ramsdale and Snow 2001). In 1973, Ryba et al. (1974) described the species from southern Moravia (Czech Republic) and, thus, marked a new northern distribution limit. Two decades later, in 1994, a larva was collected even slightly further north in southwestern Germany by Becker and Kaiser (1995). The same authors reported that they have since come across *Ur. unguiculata* specimens in the German Upper Rhine Valley repeatedly, although the species seems to be very infrequent (Becker and co-workers, pers. communication).

Ecological data on *Ur. unguiculata* are scarce. The species is active throughout the warm season (May to October) and has its activity peak in late summer (Becker et al. 2010; Rudolf et al.

2015). Breeding sites are often found in floodplain forests and include various kinds of pools and ponds, marshes, swamps, shallow shores of lakes, rice fields, hoof prints, artificial containers and even ditches and canals with slowly flowing water (Becker et al. 2010; Kurucz et al. 2017). Contrary to the description by Gutsevich et al. (1971) that developmental life stages do not occur in waters with a salinity higher than 0.1–0.2‰, larvae were found in Africa in water bodies with salinities of up to 10‰ (Senevet and Andarelli 1959). Dense aquatic vegetation, e.g. reed (*Phragmites* sp.), seems to be important (Becker et al. 2010; Šebesta et al. 2013). There is evidence that hibernation takes place in the adult (female) stage (Mouchet and Rageau 1965; Rudolf et al. 2015).

Uranotaenia species appear to prefer amphibians and reptiles as blood-hosts, but *Ur. unguiculata* has been shown to at least occasionally feed on birds and mammals including horses and humans (Beier et al. 1987; Braverman et al. 1991; Baghirov et al. 1997; Toma et al. 2014). Autogeny has also been described (Babaiants and Karapet'ian 1970; Brunhes et al. 2000). The species is mainly crepuscular (Baghirov et al. 1997).

Specimens of *Ur. unguiculata* collected in the field have been found infected with West Nile virus in southern Russia, Hungary, Austria and Romania (Shopenskaya et al. 2008; Kemenesi et al. 2014; Pachler et al. 2014; Dinu et al. 2015) and with *Dirofilaria repens* Railliet & Henry (1911) in Moldova (Suleşco et al. 2016). Vector competency for these pathogens, however, has not been demonstrated yet.

We here report the trapping of *Ur. unguiculata* specimens much farther north in Germany than previously described.

Materials and methods

Mosquito collection

As part of a nationwide monitoring project, mosquitoes were trapped with BG sentinel traps (Biogents, Regensburg, Germany), equipped with carbon dioxide and BG Lure (Biogents) as attractants, at 21 sites in northeastern Germany in 2016. Traps were run for 24 h twice per month during the mosquito season (April and October). After each operation unit, captured specimens were collected and stored at –20 °C until further processing.

Mosquito identification

Mosquitoes were morphologically identified using the determination keys by Schaffner et al. (2001), Becker et al. (2010) and Gunay et al. (2017). Genetic characterisation was done by COI (cytochrome c oxidase subunit I) barcoding (Folmer et al. 1994; Hébert et al. 2003). Barcode sequences were subjected to a GenBank search using the Blast algorithm (Altschul et al. 1990).

Results and discussion

One female each of *Ur. unguiculata* was trapped at two sites in northeastern Germany, west of Berlin. The collection sites were about 70 km distant from each other, and both of them were very rural (Fig. 1). Both specimens were captured in October 2016, one in early, one in mid-October. Further mosquitoes included in the trapping samples belonged to the *Culex pipiens* complex.

The females in question were morphologically determined as belonging to the genus *Uranotaenia*. This genus is easily identifiable by the characteristic formation of their anal wing vein which is sharply bent and ends anteriorly to or at the base of the mediocubital crossvein (Becker et al. 2010). Being the only species of the genus *Uranotaenia* described for Europe, the trapped specimens were supposed to be *Ur. unguiculata*. A morphological comparison of the specimens with *Uranotaenia* species of other faunal regions for excluding non-European *Uranotaenia* species (e.g. Gunay et al. 2017) supported the preliminary assumption.

Genetic species identification by DNA barcoding confirmed the morphological identification. A GenBank search showed 100% correspondence of the COI-DNA sequences generated for the two specimens with *Ur. unguiculata* sequences. The DNA sequences obtained in this study have been deposited in GenBank (accession nos. MF432126 and MF432127).

The region in which the two specimens were found is characterised by relatively high annual average temperatures compared to the rest of Germany and ranks second in the warmth categories of the country (Fig. 1). The Upper Rhine Valley where *Ur. unguiculata* had previously been observed is one of the warmest regions in the country.

In terms of latitude, the species has now emerged more than 300 km farther to the north. However, despite its documented thermophily, the preferred temperature range of *Ur. unguiculata* is not known.

It cannot be excluded that *Ur. unguiculata* had been present that far north already before 2016 but remained unnoticed due to its being rare and inconspicuous in terms of only exceptionally biting humans. As mosquito collection activities have been busily conducted in northeastern Germany since 2011 without ever detecting this species, however, its emergence that far north is more likely a recent event than previously missing the species.

Admittedly, the finding of two adults is not sufficient to document a spread linked to climate change. The emergence of this species in northern Germany may well be attributed to passive displacement, although it appears more than accidental that the two specimens were found at different places but in the same region, and far away from major traffic routes. Given active spread, the species must be expected to occur in other temperature-favoured German regions as well,

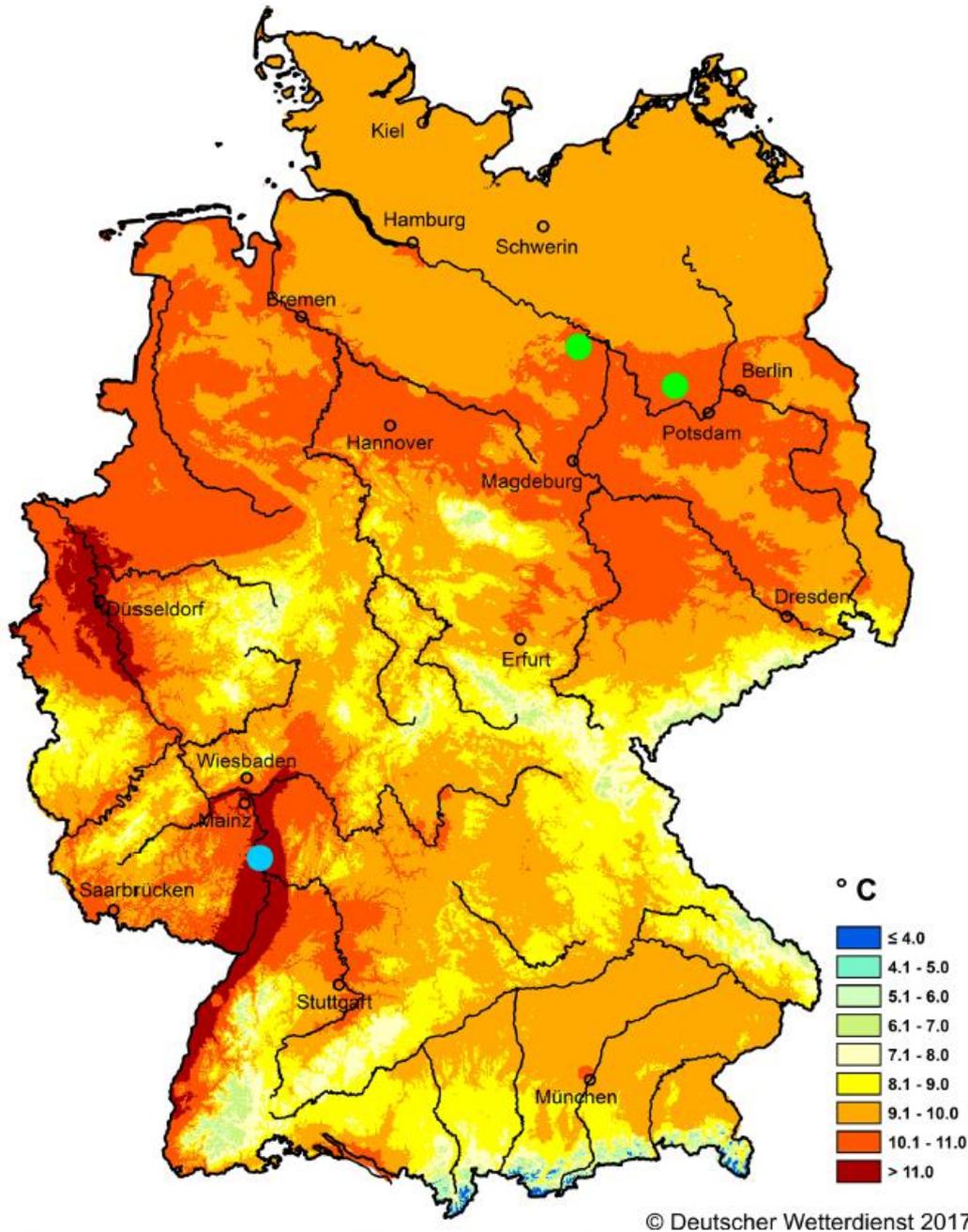


Fig. 1 Map of Germany showing previously documented collection site of *Ur. unguiculata* (blue dot) and new collection sites (green dots), before the background of average air temperatures for Germany, January to

December 2016 (map: German Weather Service, 2017; <http://www.dwd.de/DE/leistungen/klimakartendeutschland/klimakartendeutschland.html>)

particularly between the Upper Rhine Valley and the collection sites in northern Germany, which is not the case according to the monitoring data of the recent years. On the other hand, climate change as a crucial factor supporting the establishment of *Ur. unguiculata* in more northern regions is not at all far-

etched considering the recent emergence, establishment and dispersal of other non-indigenous thermophilic mosquito species in Germany such as *A. albopictus*, *C. longiareolata* and *Anopheles petragrani* (Kampen et al. 2017).

The spread of mosquitoes is particularly alarming when potential vectors of disease agents are affected. To recognise this and to be able to implement adequate measures to prevent the establishment of vector species in due time, surveillance activities should be continued.

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3.2 Further reports of *Anopheles algeriensis* Theobald, 1903 (Diptera: Culicidae) in Germany, with evidence of local mass development

Lisa Tippelt, Doreen Walther, Dorothee E. Scheuch, Mandy Schäfer, Helge Kampen

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Further reports of *Anopheles algeriensis* Theobald, 1903 (Diptera: Culicidae) in Germany, with evidence of local mass development

Lisa Tippelt¹ · Doreen Walther² · Dorothee E. Scheuch¹ · Mandy Schäfer¹ · Helge Kampen¹Received: 9 April 2018 / Accepted: 15 May 2018 / Published online: 26 May 2018
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Abstract

Anopheles algeriensis, a thermophilic mosquito species widely distributed in the Mediterranean, is supposed to be extremely rare and to occur in very low abundances in central and northern Europe. Being one of seven native *Anopheles* species, it has been reported from Germany a few times only, with all but one report several decades ago. Only in 2013, the endemic persistence of the species was confirmed when two larval specimens were found north of Hamburg. We here report the trapping of *An. algeriensis* adults at three additional sites in northeastern Germany, with one of them representing two thirds of all mosquitoes collected over two monitored seasons, 2015 and 2017, and a second one with still 12.3% of all specimens caught during the mosquito season 2016. At a third site, one single female was trapped in 2015. Despite considerable efforts, breeding sites could not be identified at the two locations characterised by the high abundances. *Anopheles algeriensis* has been shown to be vector-competent for *Plasmodium* parasites and might locally play a role in malaria epidemiology when abundance is high.

Keywords *Anopheles algeriensis* · Germany · Mass development · Mosquito monitoring

Introduction

According to Ramsdale & Snow (2000), 18 *Anopheles* species occur in Europe. A nineteenth, *Anopheles daciae* Linton, Nicolescu & Harbach, 2004 was added in 2004 after genetic separation from a close relative (Nicolescu et al. 2004). Eight of them have been documented for Germany, including *An. petragani* Del Vecchio, 1939 which was reported as a newly emerging species only in 2016 (Kampen 2014; Becker et al. 2016). Together with this invader, *Anopheles algeriensis*, Theobald, 1903 which was first found in 1931 (Martini 1931), is by far the rarest *Anopheles* species in Germany and has been demonstrated a few times only since its detection (Table 1 and Fig. 1). Except for the last finding (Krüger & Tannich 2014), all published reports date back at least 60 years.

Anopheles algeriensis morphologically resembles *Anopheles claviger* (Meigen, 1804) except that scales and setae on its scutum, anterior acrostichal area and vertex are brownish as opposed to *An. claviger* where these body areas are grey or cream-coloured. In addition, the anterior acrostichal area of *An. claviger* has a conspicuous tuft of setae reaching over the anterior part of the vertex which is not existent in *An. algeriensis*. The male genitalia of the two species are unambiguously distinct (Krüger & Tannich 2014).

Anopheles algeriensis is a thermophilic mosquito species frequently occurring in the Mediterranean, including northern Africa (Senevet & Andarelli 1956; Becker et al. 2010). In southern Europe, it has been described to occur from Portugal in the west to Caucasia in the east (Ramsdale & Snow 2000). The species becomes increasingly rare in more northern parts of Europe but has been found up to the British Isles in the west (Edwards 1932; Hart 1954; Morgan 1987; Rees & Rees 1989; Ashe et al. 1991) and Estonia in the east (Remm 1957), with the latter location being the northernmost distribution area known. More recently, specimens were encountered in the Netherlands (Scholte et al. 2011).

Many ecological data on *An. algeriensis* are contradictory. Breeding sites have been described to be swamps and marshes, ponds, silting zones of lakes and even sluggish streams and rivulets, usually shaded by vegetation, e.g.

✉ Lisa Tippelt
lisa.tippelt@fli.de

¹ Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Suedufer 10, 17493 Greifswald, Insel Riems, Germany

² Leibniz Centre for Agricultural Landscape Research, Muencheberg, Germany

Table 1 Present and previous demonstrations of *An. algeriensis* in Germany

Year of collection	Site in Fig. 1	Collection site	Developmental stage and site specifics	Reference
1931	1	'Haus Bey'/Hinsbeck (close to Krefeld, NW)	Adults, stable; eggs, close to stable	Martini (1931)
1933	2	Nature reserve 'Schildow' (north of Berlin, BB)	Adults	Peus collection (not published)
1935	3	Kleiner-Klobich Lake, Münchehofe (BB)	Adults	Peus collection (not published)
Not provided	4	Müritz (MP) (= Waren, according to Rioux et al. 1966)	Not provided	Weyer (1939)
	5	Spreewald (BB)		
Not provided	2?	Berlin	Not provided	Weyer (1951)
1955	6	Mecklenburg (MP), most probably identical to 'Serrahn' (close to Carpin, MP)	Numerous adults, forester's house	Peus, cited in Baer (1960)
1956, 1957, 1958	7	Neu-Isenburg (close to Frankfurt/M., HE)	48 adults in a stable	Peus collection (not published)
1957			Larvae, garden pond	Scherpner (1960)
1967	8	Eschenloher Moos, Eschenlohe, Upper Bavaria (BV)	Overwintering female, greenhouse	
1967, 1968, 1970	9	Mumauer Moos, Mumau, Upper Bavaria (BV)	Adults	Peus collection (not published)
2013	10	Brenner Moor, Bad Oldesloe (SH)	Adults	Peus collection (not published)
2015	11	Güstrow (MP)	Larvae, inland salt meadow	Krüger & Tannich (2014)
2015, 2017	12	Wustrow (MP)	Adults, city outskirts, pond and meadows nearby	This study
2016	13	Klein Behnitz (BB)	Adults, inhabited clearing in forested area, lake present	This study
			Adults, rural settlement, adjacent to meadows, forest and bushy landscape, large lake nearby	This study

BB Brandenburg, BV Bavaria, HE Hesse, MP Mecklenburg-Western Pomerania, NW North Rhine Westphalia, SH Schleswig-Holstein)

reed (*Phragmites* sp.), but larvae have also been found in wells and cisterns (Russel et al., 1943; Berberian 1946; Hedeem 1957; Rioux et al. 1966; Gutsevich et al. 1971; Postiglione et al. 1973; Becker et al. 2010). Owing to the vegetation, the water is relatively cool (Peus 1942). A preference for cool water is confirmed by findings from Palestine, where breeding is more intense in winter and spring than in summer, resulting in a distinct cold season activity peak (Kligler 1930). Also in Turkey, cool waters are preferred (Postiglione et al. 1973), and in Caucasia, the species starts to appear in spring waters at temperatures around 5 °C, with larval development taking at least 22–23 days (Enikolopov 1944). Contrasting this, *An. algeriensis* larvae have been reported from sunlit ditches and margins of streams in northern Africa, with shaded habitats only in mountainous regions (Senevet, cited in Edwards 1932; Tari et al. 2004).

The species is generally found in freshwater, but is obviously tolerant to some degree of salinity as it has been described from brackish water in northern Africa, France and, more recently, Germany (Senevet & Andarelli 1956; Hedeem 1957; Guy 1959; Rioux et al. 1966; Krüger & Tannich 2014). Ashe et al. (1991) emphasised that all reports from the British Isles are from calcareous waters. According to Enikolopov (1944) and Bates et al. (1949), adults stay close to their

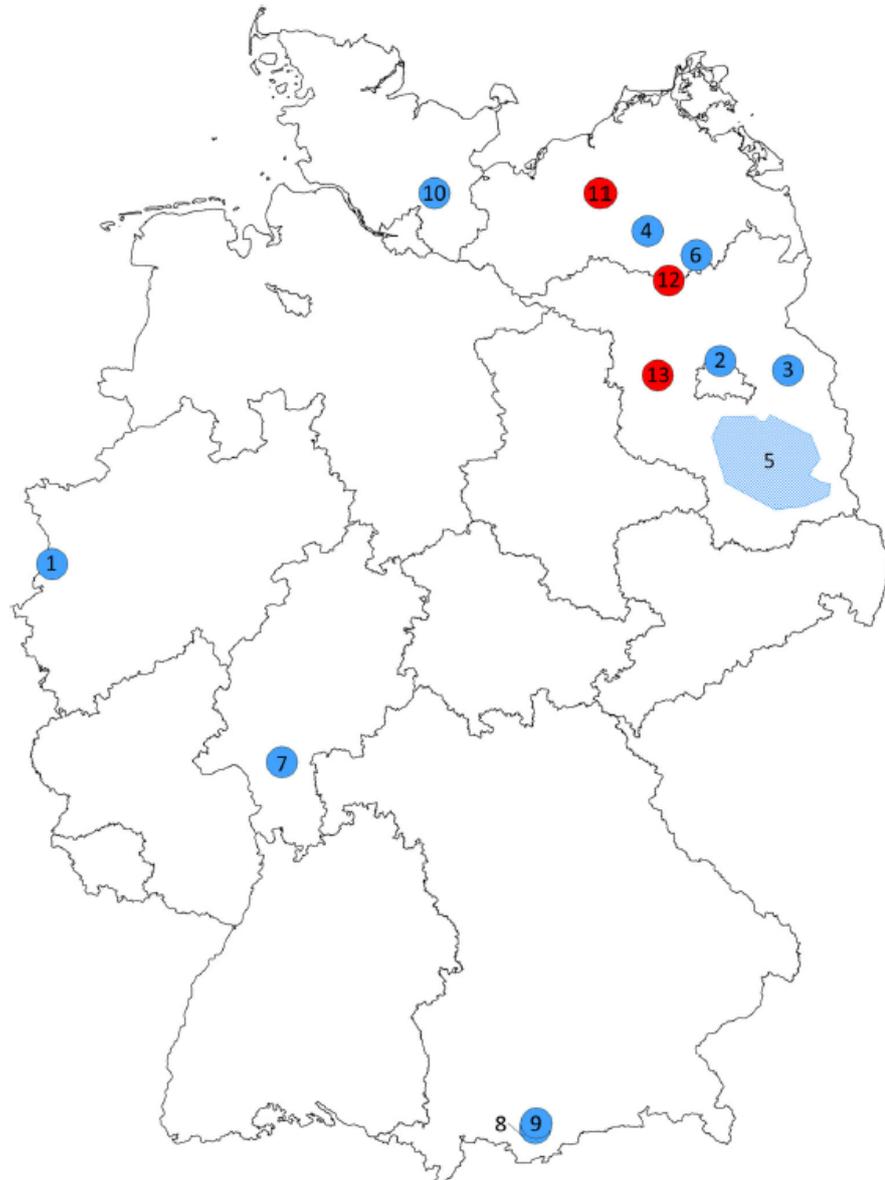
breeding sites, with a maximal dispersal range of 1.5 km. At least in its eastern distribution range, the species has never been found at altitudes higher than 900 m (Bates et al. 1949). Rees & Rees (1989) demonstrated an adult survival time in captivity of 63 days.

Anopheles algeriensis is most active at dusk and dawn (e.g. Edwards 1932; Hart 1954; Remm 1957) and is said to occur in Europe from early summer onwards (Becker et al. 2010). For northeastern Caucasia, which is characterised by a humid continental climate, the first occurrence of adults was observed already in late April (Enikolopov 1937). On Sardinia, the species reaches its seasonal larval peak in late summer and early autumn (Aitken 1953).

Overwintering seems to occur mainly in the larval stage (Livadas & Sphangos 1941; Bates et al. 1949; Rioux et al. 1966; Peus 1970; Rees & Rees 1989) but adult females have repeatedly been observed in wintertime both in northern Africa and Europe (Senevet & Andarelli 1956; Guy 1959; Scherpner 1960). Enikolopov (1937) described larvae to survive in completely frozen water. However, under laboratory conditions, these larvae died after thawing before pupation.

Anopheles algeriensis feeds on both animals and humans (Senevet & Andarelli 1956; Gutsevich et al. 1971), but not on reptiles and amphibians (Enikolopov 1944). Edwards (1932)

Fig. 1 Map of Germany showing previously documented (blue dots) and new (red dots) collection sites of *An. algeriensis* (for site numbers and details, see Table 1)



and Rioux et al. (1966) attest an aggressive behaviour. The species is generally supposed to be strongly exophilic (Becker et al. 2010), although Trari et al. (2004) call it, perhaps by mistake, endophilic. According to the majority of authors, it hardly ever enters buildings (e.g. Sergent & Sergent 1905; Klügler 1930; Gutsevich et al. 1971; Becker et al. 2010), whilst others claim that they have collected considerable numbers of specimens in houses, tents and stables (e.g. Edwards 1932; Enikolopov 1944; Logan et al. 1953; Senevet & Andarelli 1956; Peus, in Baer 1960). Enikolopov (1937) described that this species could not be found within a distance of 3 to 5 km

to any settlement. Temperatures of adult occurrence have been reported to be between 8 and 26 °C (Enikolopov 1944).

There is evidence that *An. algeriensis* has some competence in transmitting human malaria parasites. After a malaria outbreak in Algeria in 1904, *An. algeriensis* was the only mosquito species occurring at the affected locality whilst later salivary glands of two specimens collected in the field at two other sites in Algeria were found positive for plasmodial sporozoites (Sergent & Sergent 1905). Moreover, according to Barber & Rice (1935), 86% (six out of seven specimens) developed oocysts after feeding on a human *Plasmodium*

falci-parum (Welch, 1897) gametocyte carrier. Lissowa (1932) succeeded in infecting *An. algeriensis* with *Plasmodium vivax* (Grassi & Feletti, 1890). Recently, *An. algeriensis* collected in Austria were found infected with *Dirofilaria repens* Railliet & Henry, 1911 (Silbermayr et al. 2014), although a vector competence for this nematode could not be demonstrated so far.

We here report the finding of *An. algeriensis* at three sites in northeastern Germany, with relatively high abundances at two of them.

Materials and methods

Mosquito collection

In the framework of monitoring activities, mosquitoes were collected at 42 sites in northeastern Germany from 2015 to 2017 by BG sentinel traps (Biogents, Germany) equipped with BG Lure™ (Biogents) and a CO₂ source as odour attractants. Routinely, traps were activated for a period of 24 h per week for at least one vegetative season (April to October as the maximum annual collection period), with catches collected from the traps after every period of trap activity. At one additional location, a BG sentinel trap was operated continuously from May to October 2015 and 2016, each, with yields being recovered once a week. Collections were stored at –20 °C until further processing.

Mosquito identification

Mosquitoes were identified morphologically using the determination keys by Schaffner et al. (2001) and Becker et al. (2010). Cryptic species were, in part, subjected to species-specific PCR assays (Kampen et al. 2003; Rudolf et al. 2013; Kronefeld et al., 2014a, b). For confirmation of morphologically identified *An. algeriensis*, at least one specimen per collection site was subjected to bidirectional cytochrome oxidase subunit I (COI) gene sequencing, following the procedures described by Ibáñez-Justicia et al. (2014), except that DNA/RNA extraction was done using the NucleoMag VET Kit (Macherey & Nagel, Germany) according to the manufacturer's instructions.

Pathogen screening

DNA/RNA extracted from single mosquito specimens or pools with up to 30 specimens, covering the whole collection periods, was subjected to PCR assays targeting alphaviral, flaviviral and bunyaviral RNA, and filarial DNA, respectively, following published protocols (Chao et al. 2007; Eshoo et al. 2007; Lambert and Lanciotti, 2009; Becker et al. 2012; Kronefeld et al., 2014a, b).

Results

Amongst the mosquito collections carried out from 2015 to 2017, three trapping sites were positive for *An. algeriensis*, with only females collected.

Collection site Güstrow (site 11 in Table 1 and Fig. 1)

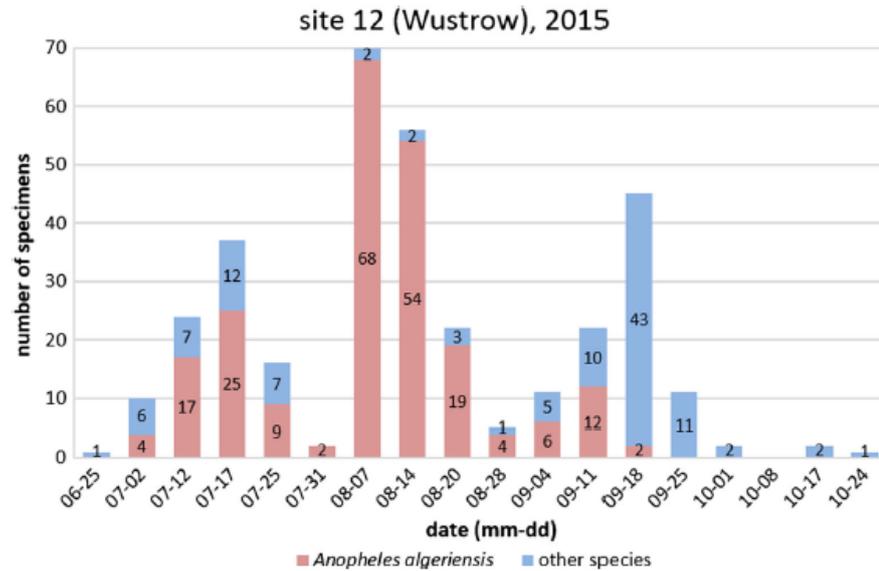
At this site, a BG sentinel trap was run permanently from late May to late September 2015 and from mid-May to late October 2016, with collections sampled once a week. Amongst some 2000 mosquitoes trapped in 2015, one single specimen in an early June sample was identified as *An. algeriensis*. No *An. algeriensis* was included in about 4000 mosquito specimens caught in 2016. Other mosquito taxa trapped at this site were *Aedes annulipes* group (not identified to species), *Aedes cataphylla* (Dyar, 1916), *Aedes cinereus* group (not identified to species), *Aedes leucomelas* (Meigen, 1804), *Aedes punctor* (Kirby, 1837), *Aedes rusticus* (Rossi, 1790), *An. claviger* s.s. (Meigen, 1804), *Anopheles maculipennis* s.l. (not identified to species), *Anopheles plumbeus* Stephens, 1828, *Culex pipiens* s.l. (not identified to species) and *Culiseta annulata* (Theobald, 1905).

The collection site is situated on the outskirts of the city of Güstrow (federal state of Mecklenburg-Western Pomerania), with a large pond and a meadow landscape nearby.

Collection site Wustrow (site 12 in Table 1 and Fig. 1)

At this site, an inhabited clearing in a forested area in the same federal state of Mecklenburg-Western Pomerania, traps were operated for 24 h once a week. Collections included *An. algeriensis* from early July until mid-September 2015, with a peak from early to mid-August (Fig. 2). Percentages of specimens of this species related to all specimens in the same 24 h-collection periods ranged from 4.4% (2 out of 45 specimens by mid-September) to 100% (2 out of 2 specimens by late July). In early and mid-August, 68 and 54 *An. algeriensis* specimens (97.1 and 96.4%), respectively, were trapped. In total, this species represented 65.9% of all mosquito specimens (222 out of 337) collected at this site and 69.4% of all mosquitoes collected whenever *An. algeriensis* was present in the catches (222 out of 320). Culicid taxa trapped together with *An. algeriensis* were *Ae. annulipes* group (not identified to species), *Aedes cinereus* group (not identified to species), *An. claviger* s.s. (Meigen, 1804), *Anopheles messeae* Falleroni, 1926, *An. plumbeus*, *Coquillettidia richiardii* (Ficalbi, 1889), *Culex modestus* Ficalbi, 1890, *Cx. pipiens* biotype *pipiens* Linnaeus, 1758, *Culex torrentium* Martini, 1925 and *Culiseta morsitans* (Theobald, 1901). As not all *Cx. pipiens* s.l. were identified to species and biotype, it remained unclear whether *Cx. pipiens* biotype *molestus* Forskål, 1775 occurred in Wustrow.

Fig. 2 Numbers of specimens of *An. algeriensis* and other species collected at site 12, 2015



As the collections were mainly processed during the winter 2015/2016, attempts to identify potential breeding sites had to be postponed to 2016. In that year, all artificial and natural water sources that could be identified within a diameter of ca. 1.5 km inside and outside of the forest, including rain water barrels, garden ponds, swampy areas, water pools, the reed belt of a lake and tree holes, were checked for *Anopheles* larvae, with findings of *An. maculipennis* s.l. and *An. claviger* s.l. only.

In 2017, the BG sentinel trap, which was run at the very same site again from early June to early September, collected *An. algeriensis* from early June to mid-August (Fig. 3). As in 2015, considerable portions of the catches (up to 92.3% in mid-July; 55.6% in total until mid-August) consisted of this species but efforts to identify the breeding sites were unsuccessful again.

Collection site Klein Behnitz (site 13 in Table 1 and Fig. 1)

Also at Klein Behnitz, federal state of Brandenburg, the BG sentinel was operated for 24 h per week, but only in 2016. Over the whole season, *An. algeriensis* was not as abundant as in Wustrow but still constituted 12.3% of the total number of specimens caught (15.4% of the catches with *An. algeriensis* presence), with a peak of 66.7% in late May (six out of nine individuals) (Fig. 4). Most *An. algeriensis* individuals per 24 h were captured in early September (19 out of 64 total). By-catches consisted of *Ae. annulipes* group (not identified to species), *Ae. cinereus* group (not identified to species), *Ae. leucomelas*, *Aedes vexans* (Meigen 1830), *An. claviger* s.s., *An. messeae*, *Cs. annulata*, *Cx. pipiens* biotype *molestus*, *Cx. pipiens* biotype *pipiens* and *Cx. torrentium*. Breeding sites

were only searched for in 2016, but as in Wustrow, all *Anopheles* larvae found belonged to the *An. maculipennis* and *An. claviger* complexes.

Molecular analyses

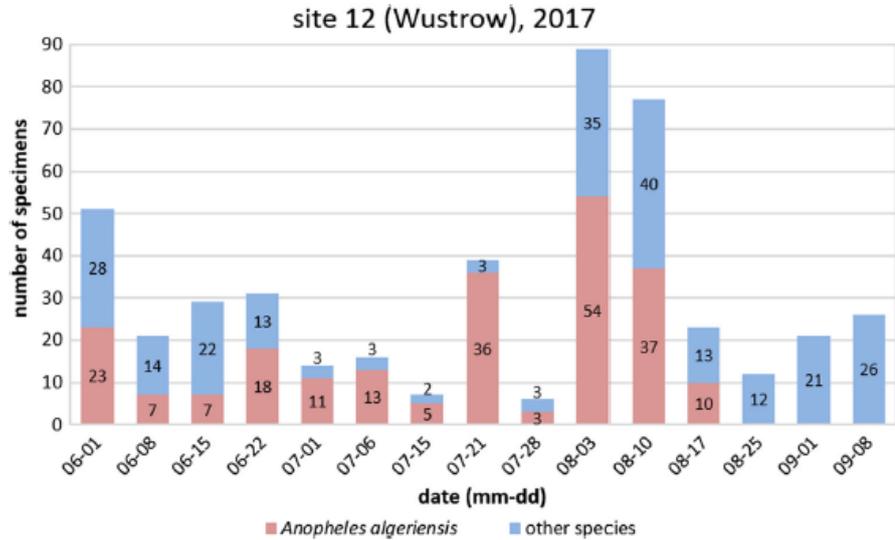
DNA barcode sequences of selected *An. algeriensis* specimens (one each of sites 11 and 13, six of site 12) have been deposited in GenBank under accession no. MG808414–21.

All 264 *An. algeriensis* specimens (38 pools) subjected to viral and filarial screening were tested negative.

Discussion

In the historic literature, only five articles refer to findings of *An. algeriensis* in Germany (Martini 1931; Weyer 1939, 1951; Scherpner 1960; Baer 1960). Unfortunately, some of these completely lack the year of collection whilst others only mention large areas, but no specific sites, as the origin (Table 1). We were lucky to have the dipteran collection (nowadays property of the Senckenberg Museum in Frankfurt/Main) by the renowned German entomologist Fritz Peus (1904–1978) at our disposal, which contained numerous *An. algeriensis* specimens properly labelled with collection date and site. After thorough consideration, we are quite certain that the unspecified collection site ‘Berlin’, referred to by Weyer (1951), but obviously not sampled by himself, corresponds to the collection site ‘Schildow’ (a village on the northern outskirts of Berlin) as found in the Peus collection. Moreover, Peus is cited in Baer (1960) to have found numerous *An. algeriensis* in a forester’s house in ‘Mecklenburg’, which is a huge North German region,

Fig. 3 Numbers of specimens of *An. algeriensis* and other species at site 12, 2017

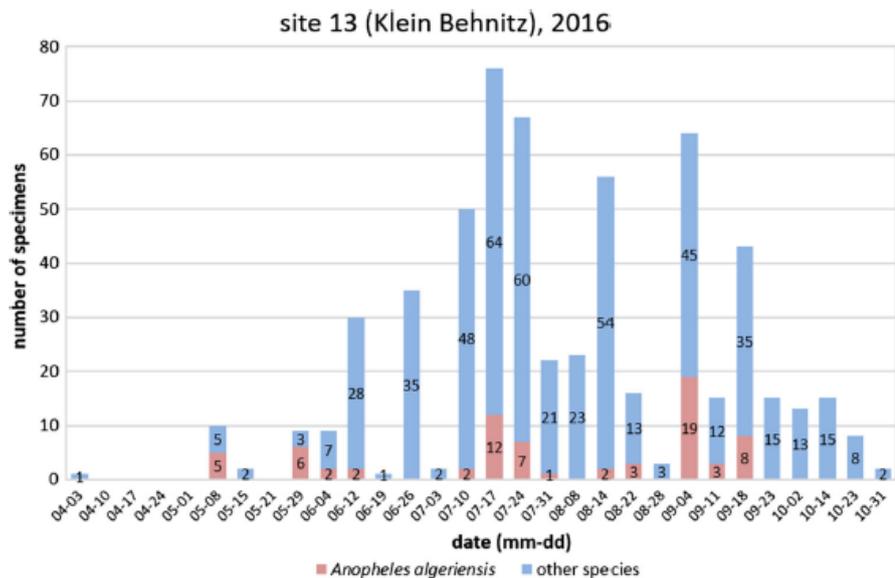


in 1955. This collection place is most likely identical with ‘Serrahn’ (site 6 in Table 1), where 48 specimens in the Peus collection originate from. According to the label, these specimens were collected in June 1955 in a stable (probably of that forester’s house). Another region of *An. algeriensis* occurrence, ‘Müritz’, as mentioned by Weyer (1939), could be delimited to the surroundings of the city of Waren based on a reference by Rioux et al. (1966) to a written note by Weyer. In the same piece of literature by Weyer (1939), the ‘Spreewald’, a huge lowland area composed of wetlands and moorlands in the German federal state of Brandenburg, was listed as a collection area. No further details on the specific site could be identified.

Further localities referred to in the Peus collection bring the number of *An. algeriensis* collection sites in Germany until 1970 to nine (Table 1 and Fig. 1). Sites 8 and 9 in Fig. 1 are geographically close together and belong to the same large landscape mosaic made of swamps, bogs and marshes.

Whilst there were no additional reports on *An. algeriensis* for the next 40 years, probably not least owing to the negligence of mosquito research in Germany, the species was re-discovered after half a century in the German federal state of Schleswig-Holstein, north of Hamburg, in 2013, in the form of two larvae (Krüger & Tannich 2014). Figure 1 provides an overview of all German *An. algeriensis* collection sites and areas that came

Fig. 4 Numbers of specimens of *An. algeriensis* and other species collected at site 13, 2016



to our attention by extensive literature research and inspection of the Peus collection, in addition to the new sites presented in this contribution.

Although we were not able to identify larval breeding sites and, after several years of extensive mosquito monitoring, agree with Scholte et al. (2011) that “most probably, *An. algeriensis* is one of the rarest mosquito species in central Europe” in terms of area colonised, our collection data confirm findings by Edwards (1932), Enikolopov (1944) and Rioux et al. (1966) that this species may locally occur at high population densities. We also could show that *An. algeriensis* adults may be active in a central European climate at least from early May until mid-September with an abundance peak in August and early September, which agrees with observations on larval occurrence made by Aitken (1953) under Mediterranean conditions on Sardinia.

Due to the predominant belief that *An. algeriensis* does not enter human dwellings and occurs at low abundances, there has been common agreement that—despite its apparent vector competence—the species is of negligible importance as a malaria vector (Kligler 1930; Weyer 1939; Russell et al. 1943; Bates et al. 1949; Postiglione et al. 1973; Bruce-Chwatt & de Zulueta 1980; Trari et al. 2004). According to our literature analysis and own data, this argumentation is not always applicable, so the vector role of *An. algeriensis* should be re-evaluated at least for times and places with high population densities (cf. Hedeon 1957). Although no studies exist on the vector competence for pathogens other than malaria parasites, such as viruses or filarial worms, this cannot be excluded considering the vector competence of closely related *Anopheles* species (Kampen & Walther 2018). We did not find any mosquito-borne pathogens in our *An. algeriensis* specimens, and for the time being, chances to find any in Germany are vanishingly small (e.g. Jöst et al. 2010, 2011a, b; Kronefeld et al., 2014a, b). In Spain, Vázquez et al. (2011) found neither West Nile virus nor Usutu virus in 102 examined specimens. The situation in central Europe may change, however, with continuing climate warming, and, by all means, *An. algeriensis* should not principally be excluded as a vector of disease agents in its natural distribution range in the Mediterranean.

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3.3 Tolerance of three *Aedes albopictus* strains from different geographical origins towards winter temperatures under field conditions in northern Germany

Lisa Tippelt, Doreen Werner, Helge Kampen

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Tolerance of three *Aedes albopictus* strains (Diptera: Culicidae) from different geographical origins towards winter temperatures under field conditions in northern Germany

Lisa Tippelt^{1*}, Doreen Werner², Helge Kampen¹

1 Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald–Insel Riems, Germany, **2** Leibniz Centre for Agricultural Landscape Research, Muencheberg, Germany

* lisa.tippelt@fli.de



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Abstract

The continuing spread of the Asian tiger mosquito *Aedes albopictus*, a vector of many arboviruses and some dirofilarial worms, in Europe calls for advanced investigations on its ecological ability to establish and overwinter in temperate, more northern geographic regions. To meet this purpose, eggs of *Ae. albopictus* laboratory strains of tropical, subtropical and temperate origin were exposed to field conditions during one or two winter seasons in north-eastern Germany. After 1 to 16 weeks of outdoor exposure, eggs were flooded in the laboratory, and the hatching rates were determined. During the winter season 2015/2016, when temperatures reached -10°C , the subtropical strain showed hatching after all time periods while the tropical strain displayed hatching only until two weeks of cold exposure. In the winter season 2016/2017, with temperatures as low as -6°C , all three strains produced hatching larvae after all time periods. Both the hatching rates and the hatching behaviour differed between the strains. Larvae of the subtropical and temperate strains hatched in installments over a period of four weeks while the larvae of the tropical strain hatched within a short time period, often one week. The results of the study demonstrate that *Ae. albopictus* strains of different, even tropical, origin might be able to survive a central European winter, although this is likely to depend on the specific course of the temperatures. Further studies with different temperature regimes and different mosquito strains are needed to specify these findings.

Introduction

Aedes albopictus (Skuse, 1894), commonly known as the Asian tiger mosquito, is a relatively thermophilic mosquito species with an Asian-Pacific origin [1]. It is not only restricted to tropical areas but also occurs in more temperate regions of eastern Asia [2]. Within a few decades, this species expanded its distribution area to North and South America [3, 4], Africa [5] and

Europe [6]. In Europe, it was found for the first time in Albania in 1979 [7], but only spread over the Mediterranean after additional introductions to Italy a decade later [8]. It is now established in at least 19 European countries [6]. The international trade with used tyres and lucky bamboo has played a major role in its global displacement [7, 9, 10].

Due to its aggressive biting behaviour during the day, *Ae. albopictus* is a severe nuisance [11]. It uses a broad range of vertebrate hosts as blood sources but preferentially feeds on humans [12, 13]. As it is a competent vector of dirofilarial worms and more than 20 arboviruses [14, 15], it has a high medical and veterinary importance. Recent cases and outbreaks of dengue and chikungunya in Italy, Croatia and France mediated by *Ae. albopictus* [16–21] demonstrate that the potential of this species of spreading diseases in Europe is extraordinary.

In Germany, *Ae. albopictus* was first found in 2007 in the form of its eggs while monitoring the southwestern federal state of Baden-Württemberg [22]. Later on, adult specimens were regularly trapped in the same region [23–25]. All places where specimens were initially found were closely located to motorways, suggesting an importation of adults from southern Europe by vehicles to Germany [25].

In 2014, larvae and pupae of *Ae. albopictus* were found for the first time over an extended period of almost three months at a southern German locality [26]. Meanwhile, there is evidence for overwintering [27–29].

Aedes albopictus is known to have a high ecological plasticity. Individuals of different geographic origin can react very differently to ecological parameters, including temperature [15]. In tropical areas, for example, the species shows continuous reproduction [30] while it overwinters by diapausing in the egg stage in more temperate areas [31]. Diapausing is triggered by a decrease in temperature and day length [32]. Thus, under certain conditions, females start producing dormant eggs that are better adapted to cold temperatures than non-diapausing eggs [33]. Eggs of individuals from tropical regions are usually not able to diapause as demonstrated for populations from South America [34]. The ability of overwintering is therefore an important trait for establishment of *Ae. albopictus* in central Europe.

The present study aims to add further experimental evidence to elucidate the cold tolerance of *Ae. albopictus* and its ability to overwinter in central Europe under field conditions. The primary purpose of the study was not to generate high hatching rates as necessary for efficient laboratory rearing but to demonstrate whether hatching after exposure to wintry conditions is possible or not. Even low hatching rates after the winter must be supposed to produce sufficient individuals to build up a population during the next vegetative season and to guarantee local maintenance through the years.

Material and methods

Ethics statement

According to EU regulations and national law, no permissions were necessary for conducting the experiments. No endangered or protected species were involved in this study.

Mosquito rearing

The study was conducted with three laboratory strains of *Ae. albopictus*, one from Mauritius, hereafter referred to as 'tropical strain', one from Rimini, Italy, hereafter referred to as 'sub-tropical strain', and one from Freiburg, Germany, hereafter referred to as 'temperate strain'. The tropical and subtropical strains had been kept in the laboratory for numerous generations (about two years) at $25 \pm 1^\circ\text{C}$, $70 \pm 5\%$ relative humidity and a light:darkness regime of 12:12 hours. The temperate strain was reared from larval collections made in summer 2015 in

Freiburg, southern Germany, and has been held in an insectary at the same conditions for more than a year. For the experiments described, generation F8 was used.

To produce eggs, the mosquitoes were fed on bovine EDTA blood via a membrane feeding system (Hemotek, UK). Wooden spatulas, with one end submerged in stale tap water, or moist filter paper were offered as oviposition supports. All experiments were started with non-diapausing and not cold-acclimated eggs.

Experimental setup

Spatulas loaded with *Ae. albopictus* eggs were transferred into plastic boxes. The boxes were closed but holes were drilled in their lids and sides for ventilation. Several of these boxes were put in a larger plastic container covered with mesh which was positioned on the institute's premises on the Isle of Riems in the Baltic Sea (Greifswald, Germany, 54.182268, 13.369745) under a bush where it was sheltered from strong winds. A data logger (HOBO Pro v2 temp/RH, Onset Computer Corporation, USA), programmed to measure the temperature and relative humidity every two hours, was also placed in the secondary container. Further spatulas with eggs, stored in plastic beakers in the insectary of the institute at above laboratory rearing conditions, were used as controls.

The eggs were to remain on the spatulas, both outdoors and indoors, for various time periods during wintertime after which they were flooded in the laboratory and checked for larval hatching. Only eggs not older than two weeks and produced in the same blood feeding cycle were used for the study.

Winter 2015/2016. In the winter 2015/2016, only the tropical and the subtropical strains were tested. As the tropical strain was newly introduced to the institute and needed some time for producing sufficient numbers of eggs, the study was started at different time points for each of the strains: eggs of the subtropical strain (laid 4–8 December) were brought outdoors 16 December 2015, while the tropical strain eggs (produced 8–11 January) followed 13 January 2016.

For statistical reasons, at least 30 eggs were meant to be processed per spatula in the winter 2015/2016. This minimum number of eggs would allow the detection of a minimum hatching rate of at least 10% with a 95% confidence interval under the assumption of a bimodal distribution. The number of eggs actually used ranged from a minimum of 34 (tropical strain) to a maximum of 1,340 eggs per spatula (subtropical strain) (Table 1).

After 1, 2, 4, 8, 12, 14 and 16 weeks of being outside, the spatulas were brought into the laboratory and flooded for larval hatching. Before flooding, eggs were counted. If the spatulas were covered with high numbers of eggs, pictures were taken and eggs were counted with the software NIS Elements (Nikon, Japan) by clicking on the picture. Empty egg shells, possibly caused by hatching in reaction to intruding precipitation water during the experiment, were excluded from the total score of eggs. Flooding was done by submerging the spatulas in containers with stale tap water. Hatched larvae were also counted and removed from the flooding containers during the process of counting. Larvae found dead after successful hatching were evaluated like living ones.

Table 1. Number of eggs used in the winter study 2015/2016.

Mosquito strain	Experimental group	Mean number of eggs per spatula (min.–max.)
tropical strain	controls	77 (50–144)
	treatments	57 (34–110)
subtropical strain	controls	661 (146–1,340)
	treatments	730 (376–1,191)

<https://doi.org/10.1371/journal.pone.0219553.t001>

Flooding of samples was performed in two steps. First, eggs were flooded for four weeks in the same water. After that, the spatulas were removed from the water and dried for one week in the laboratory. The flooding water was filtrated through common laboratory filter paper for retaining non-hatched eggs potentially detached and floating in the water. With a brush, these eggs were re-attached onto the spatulas. Second, all eggs hitherto non-hatched were flooded again in stale tap water for another two weeks after the drying period. Unhatched eggs were not checked for being viable or not.

Winter 2016/2017. In the winter season 2016/2017, all three *Ae. albopictus* strains were used. The methodology was slightly changed: instead of wooden spatulas, the blood-fed mosquito females were offered moist filter paper for oviposition as this substrate turned out to be favoured and, thus, to be more effective in producing high numbers of eggs. Furthermore, the number of eggs used in the experiment was standardised: some 30 of the produced eggs were transferred from the filter paper onto a wooden spatula by means of a paint brush. Per time period, two of these spatulas from the same oviposition event were tested under exactly the same conditions, resulting in one replication per time period and temperature. Thus, the number of eggs per treatments or controls tested at the same time was increased to about 60 as compared to the winter 2015/2016 study, allowing the detection of a minimum hatching rate of 5% with a 95% confidence interval under the assumption of a bimodal distribution.

Furthermore, the experiments started at the same time (7 December 2016) for all three mosquito strains involved. Eggs used had been laid between 20 November and 1 December 2016. Time periods tested and the flooding regime were also changed: eggs were flooded after 2, 5, 8, 12 and 16 weeks for two weeks, dried for one week and flooded for another week. For both floodings, stale tap water was used as hatching medium.

Statistical analysis

For evaluating the hatching success, relative proportions of hatched larvae were calculated per study period and strain. A factorial design was chosen for the experiments as three different factors would influence the response variable, the hatching rate. The first factor is the 'strain' with two different levels (tropical, subtropical) in the winter 2015/2016 and three levels (tropical, subtropical, temperate) in the winter 2016/2017. The second factor ('exposure') is the time period the eggs were exposed to low temperatures. The first winter comprised six and the second winter five time period levels. The last factor is the 'group' and defines the assignment of eggs to the levels 'treatment' and 'control'.

For the winter 2015/2016, we decided to only compare hatching results within a strain as the experiments with the two strains could not be started contemporaneously and no replicates were run. Hence, hatching results of all exposure times were summarised and compared using a Kruskal-Wallis test. In the winter 2016/2017, a direct comparison between the strains was possible. Therefore, all factors and their impacts on the hatching rate could be tested applying a multifactorial ANOVA. For meeting the assumptions of the ANOVA, data were square root-transformed. Subsequently, a Tukey's honestly significant different (HSD) post hoc test was applied for revealing the significant levels within the various factors. All statistical tests were made with the software R (version 3.4.1.) without using additional packages.

Results

The 2015/2016 experiment started at temperatures of 11°C (16 December 2015, subtropical strain) and -2°C (13 January 2016, tropical strain), respectively, as measured by the data logger in the secondary container. Two cold periods occurred in early and late January, respectively, with the first lying before the outdoor exposure of the tropical strain: this first cold period

lasted from 2 to 7 January with temperatures of almost -10°C for 2 hours, each, at two days and a temperature maximum of -3°C (Fig 1A, S1 Table). The second cold period lasted from 18 to 23 January with a temperature decrease to -8°C for 4 hours and a maximum temperature of -3°C . At the end of the experiment, April 6 (subtropical strain) and April 20 (tropical strain), temperatures measured 13°C and 22°C , respectively. Relative humidity fluctuated between 37% (minimum) and 100% (maximum), with higher variation from early March onwards, but mostly remaining between 70 and 100% (Fig 1A).

The temperature at the beginning of the winter 2016/2017 study was 3°C . It decreased to a minimum of almost -6°C and again showed two colder periods, one in early January and one by mid-February (Fig 2A, S1 Table). The first cold period lasted from 5 to 7 January and was characterised by temperatures as low as -5°C for 4 hours, whereas the second cold spell lasted from 8 to 14 February with a minimum temperature of almost -6°C for 4 hours and a maximum temperature of -1°C . At the end of the experiment (29 March), the temperature was measured as 10°C . Relative humidity varied between 56% (minimum) and 100% (maximum), with values close to 100% during the whole winter season and greater fluctuations starting only in early March (Fig 2A).

Regarding the tropical strain, larvae hatched from eggs kept outside only after one and two weeks of exposure in the winter 2015/2016 (Fig 1B). Interestingly, the two weeks' exposure sample produced larvae only after the drying period, but not during the four weeks' flooding period. Hatching rates of controls were usually around 50% and almost 90% in one case. In the winter 2016/2017, this strain showed hatching in all treatments, although with very low hatching rates after 12 weeks of cold exposure (Fig 2B). Hatching results of controls fluctuated between 70% and nearly 90% (S3 Table).

The cohort treatment eggs of the subtropical strain showed hatching after all time periods in both winters. However, the longer the eggs had been in the field, the lower the hatching rates got (Fig 1C). After 16 weeks outside in the winter 2015/2016, larvae hatched from only 10% of the eggs. Such a pattern was not observable in the winter 2016/2017 when hatching rates of treatments did not decrease with time of cold temperature exposure (Fig 2B). Hatching rates in the controls peaked at ca. 76% in the winter 2015/2016 and almost 90% in the winter 2016/2017 (Fig 1C, S2 and S3 Tables).

The *Ae. albopictus* strain from Freiburg, Germany, which was included in the study only in the winter 2016/2017, showed larval hatching in all treatment groups, although rates were much lower than in the other two strains (Fig 2B). However, also the controls presented with much lower hatching rates than the controls of the other strains (Fig 2B).

The statistical comparison of treatments and controls of the winter 2015/2016 showed significantly different values for the tropical strain ($\chi^2 = 8.61$, $p < 0.01$), but not for the subtropical strain ($\chi^2 = 1.64$, $p > 0.05$). The results of the ANOVA suggests that all studied factors ('strain', 'exposure' and 'group') as well as their interactions had a significant influence on the hatching rate (Table 2).

The Tukey's HSD post hoc test indicates that the temperate strain is responsible for the significant result within the factor 'strain' ($p < 0.001$), while the pairwise comparison between the exposure time of 2 and 12 weeks produces the significance within the factor 'exposure' ($p < 0.001$). If the pairwise comparisons of the treatments and controls of the same strain are considered, it can be shown that the low hatching result of the treatments of the tropical strain exposed for 12 weeks outside produced the significant result. Pairwise comparisons between the controls of different exposure times were insignificant for all strains.

In addition to the hatching rates, the hatching pattern differed between the two *Ae. albopictus* strains used in the winter season 2015/2016. Larvae of the tropical strain often hatched within a short time window after flooding (Fig 3A, S4 Table). Consequently, hatching events

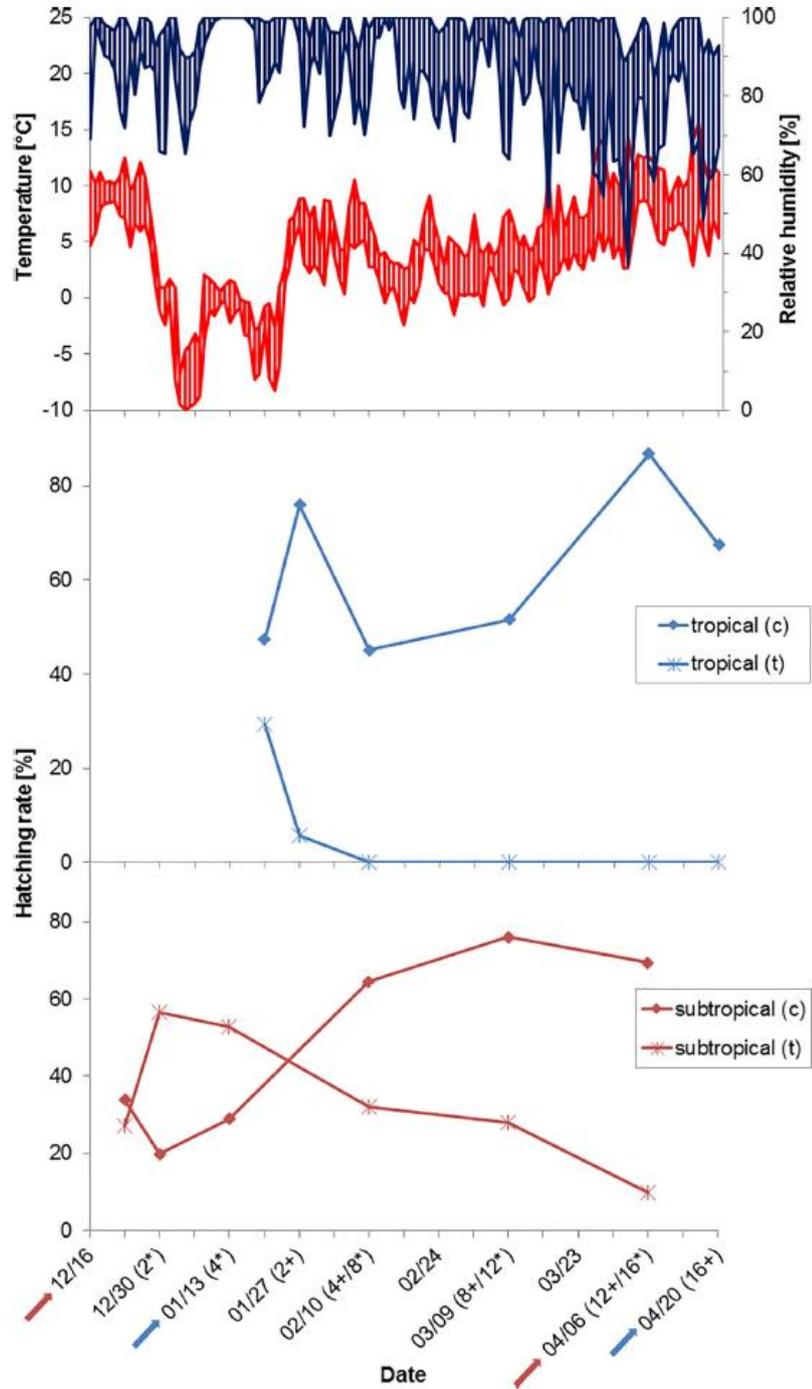


Fig 1. Temperature (red) and humidity profiles (blue) at the study site on the Isle of Riems in the winter 2015/2016 (top) with hatching rates of the tropical (middle, blue lines) and subtropical strain (bottom, red lines); c = controls, t = treatments, no replicates run. Arrows mark the start/end of the experiments with the two mosquito strains. The numbers in parentheses, representing dates of withdrawal of a subsample for flooding, indicate the number of weeks elapsed since the start of the experiment (* = subtropical strain, + = tropical strain).

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often only occurred at the beginning of the four weeks' and the two weeks' flooding periods. The only exception was registered at the end of the two weeks' flooding period of the last treatment when one late larva hatched. By contrast, larvae of the subtropical strain hatched in installments, i.e. hatching occurred over an extended time period. Most samples of this strain showed a hatching peak at the beginning and after three weeks of the four weeks' flooding period (Fig 3B, S4 Table). Furthermore, eggs of both strains showed a higher hatching response after the drying period than during the four weeks' flooding period.

Increased hatching at the beginning of the flooding periods was also noticed in the winter season 2016/2017 (S5 Table). However, a clear difference in hatching behaviour between the tropical and the subtropical strains, as observed in the winter season 2015/2016, was hardly noticeable, probably due to the shorter initial flooding period.

Discussion

The results of the study support the assumption that the cold hardiness of *Ae. albopictus* eggs is dependent on the geographic origin of the strain. Specifically, *Ae. albopictus* strains originating from a warmer climate have a lower tolerance against low temperatures than those from regions with a cooler climate [33, 35, 36].

Not surprisingly, the tropical strain used in this study had a worse performance in the winter 2015/2016 than the subtropical strain. However, hatching of the tropical strain occurred in all treatments in the winter 2016/2017, although temperatures had temporarily gone down to -6°C and were only slightly above those of the winter 2015/2016. These completely different hatching results cannot only be linked to the innate temperature tolerance of the strain but seems rather related to the phenomenon of cold acclimation. Cold tolerance can artificially and naturally be achieved by exposing eggs to low temperatures above freezing point and has a major effect on hatching rates, as demonstrated by Hanson & Craig [37]. These authors also showed that the eggs of temperate *Ae. albopictus* that had been acclimated for a long time had higher hatching rates than those acclimated to the same temperature for a short time. This did not account for eggs of the used tropical strain. However, the apparent absence of cold-acclimation in the tropical strain could also have been caused by exposing the eggs to a temperature of -10°C which is lethal to non-acclimated eggs of tropical *Ae. albopictus* even after short exposure [36]. Cold acclimation could also not be shown after exposure to -2°C [38]. As stressed in the methodological description of the experiments, eggs had not artificially been acclimated to low temperatures prior to the study as done in other experiments [37, 39]. The available time period in which eggs could natural cold acclimation greatly differed between the winters 2015/2016 and 2016/2017. In the winter 2015/2016, eggs of the tropical strain were transferred from laboratory conditions to an outside temperature of -2°C in mid-January. Therefore, practically no cold acclimation was possible. By contrast, temperatures were unusually high in December 2015, when eggs of the subtropical strain were brought outside, and started dropping below 5°C not before the end of December. These divergent conditions might have led to the huge differences in hatching rates of the tropical and subtropical strains.

As opposed to the winter 2015/2016, the time period available for cold acclimation was considerably longer for the tropical strain in the winter 2016/2017 when the temperature measured 3°C at the beginning of the experiment and went down to subzero values not before

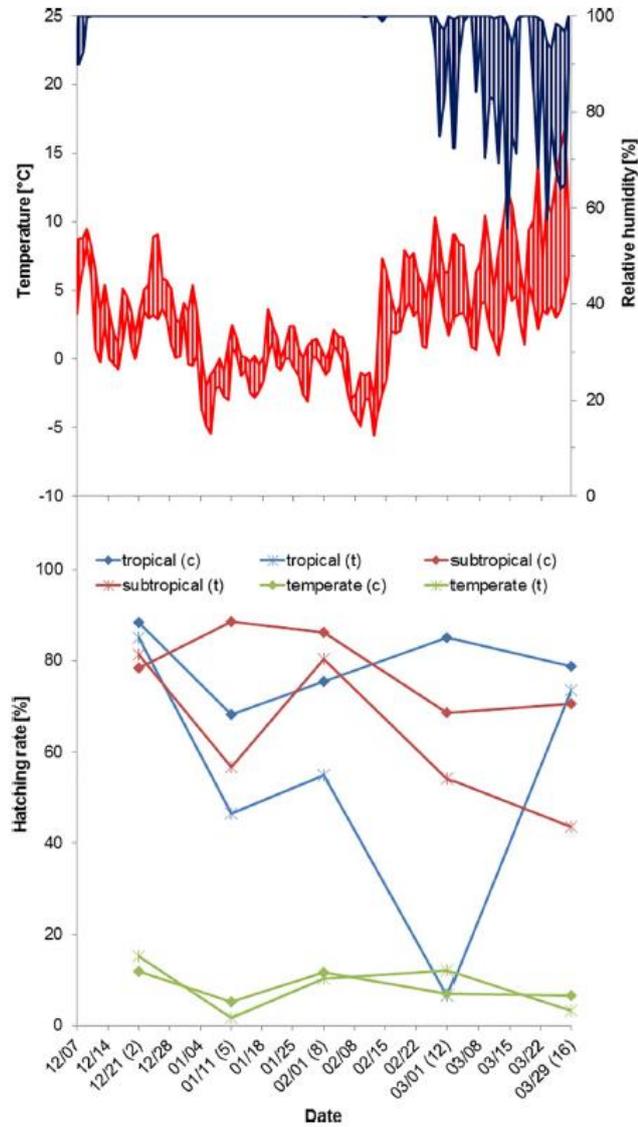


Fig 2. Temperature (red) and humidity profiles (blue) at the study site on the Isle of Riems in the winter 2016/2017 (top) with mean hatching rates of all strains (c = controls, t = treatments); one replicate run. The numbers in parentheses, representing dates of withdrawal of subsamples for flooding, indicate the number of weeks elapsed since the start of the experiment.

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early January, about one month after the start of the experiment. Consequently, hatching rates were much higher and more similar to the subtropical strain than in the winter before. To our knowledge, this is the first description of cold acclimation in a tropical strain of *Ae. albopictus*.

Table 2. Result of the ANOVA relating hatching results to the factors 'strain', 'exposure' and 'group'.

Factor	F-value	p-value
strain	358.57	< 0.001*
group	31.99	< 0.001*
exposure	10.98	< 0.001*
strain : group	6.15	< 0.01*
strain : exposure	5.35	< 0.001*
exposure : group	4.57	< 0.01*
strain : exposure : group	5.88	< 0.001*

* significant

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In addition, the measured minimum temperature had an influence on the hatching rates of the tropical strain, as shown by the results of the statistical tests in both winters. Naturally, the tropical strain is not exposed to low temperatures, so no selection pressure exists in favour of the development of cold-resistant eggs. Thomas et al. [36], who used the same *Ae. albopictus* tropical and subtropical strains experimentally, had previously shown that the tropical strain produced hatching results more similar to another tropical mosquito species, *Ae. aegypti* (Linnaeus, 1762), than to the subtropical *Ae. albopictus* strain. They depicted that hatching of the tropical strain did not occur anymore at -7°C and -10°C , when eggs were exposed to these temperatures for longer than one hour.

In the winter 2015/2016, the tropical strain was exposed to a minimum temperature of -8°C for four hours. Nevertheless, two larvae succeeded in hatching from 35 eggs. Thomas et al. [36] described such a surprising hatching event in the same mosquito strain, with one hatched larva out of 20 eggs after exposure to -10°C for one hour, and Hanson [40] reported hatching rates lower than 14% for a tropical strain from Malaysia exposed to a minimum temperature of -10°C for an unknown duration during a field experiment in Japan. In the present study, the minimum temperature of -6°C , measured for less than two hours in the winter 2016/2017, also caused a considerable decrease in the hatching rate of the tropical strain in the 12 weeks' treatments followed by a certain recovery. This conspicuous hatching rate was in stark contrast to all other hatching rates of the strain, causing a huge variance with significant divergences within this strain. This is contrary to findings of other studies showing only insignificant variation of the hatching rates, followed by cessation of hatching [36, 38, 40, 41]. Therefore, a random effect caused by the overall small sample size cannot be excluded, although the effect occurred in both parallel treatments. Further studies could show if this effect can be replicated on a large scale or had only randomly been produced.

Despite the exceptional low hatching rate of the 12 weeks' treatment, the present study clearly shows that the lethal temperature or the lethal exposure time was not reached for the eggs of the tropical strain in the winter 2016/2017, in contrast to the winter 2015/2016. In summary, it is very likely that the interaction of temperature minimum and duration, or a putative cold acclimation, had contributed to the hatching results of the tropical strain in the present study.

With high hatching rates, the subtropical strain presented surprisingly robust in a realistic central European winter scenario with fluctuating temperatures and humidity, although temperatures went down to -10°C in the winter 2015/2016. Laboratory experiments have shown that eggs of the same laboratory strain could endure temperatures of -10°C for at least 24 hours and still exhibited hatching after exposure to temperatures as low as -12°C for four hours [36]. Interestingly, only the treatments in the winter 2016/2017 were significantly

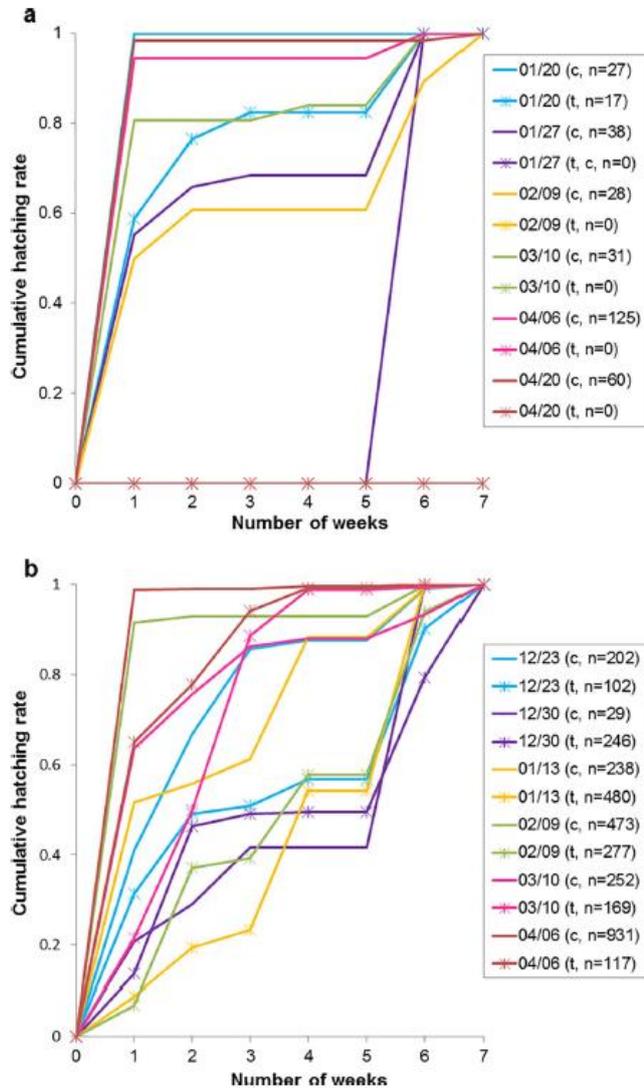


Fig 3. Cumulative hatching rates of the tropical strain (a) and of the subtropical strain (b) in the winter 2015/2016 (c = controls, t = treatments). The numbers in parentheses indicate the total number of hatched larvae. The cumulative hatching rates add up to the value '1' which represents the final hatching rates as presented in Fig 1B and 1C, respectively.

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different from their controls while the differences between treatments and controls were not significant in the winter 2015/2016 (Table 2). Yet, this is no evidence for different temperature tolerances in the two winter seasons but might be generated by the different hatching rates of the controls in the winter 2015/2016: Controls flooded after the first three time intervals in the winter 2015/2016 had much lower hatching rates as compared to the high hatching rates of the

controls flooded later (Fig 1C). Such restriction caused by an imbalance of hatching rates did not occur in the winter 2016/2017. The data lead to the conclusion that below survival temperatures were not reached for the subtropical strain at the site of exposure in any of the two winters.

The temperate strain showed a poor hatching performance throughout the experiments. Neither the treatments nor the controls reached hatching rates higher than 18%. A possible explanation for this observation could be the incomplete adaptation of this strain to laboratory conditions as it had only recently been acquired from the field, with only few generations passed in the laboratory environment before the start of the experiments. This phenomenon of low winter hatching rates in newly established laboratory strains is also known from other laboratories. Due to this unusual hatching behaviour, the explanatory power of the comparisons of treatments and controls both within this strain and with other strains is limited. However, despite the low hatching rates, the temperate strain showed hatching after all treatments, suggesting a certain survival potential of the population under low temperatures. Interestingly, the statistical analysis of the hatching results of the temperate strain only leads to insignificant influences of exposure time and assignment to control or treatment group. This could be interpreted as a consequence of the small differences between the hatching rates of treatments and controls but also as a limitation of the study due to the small overall sample sizes. The treatments of the temperate strain showed no significant cold-related decrease in the hatching rate irrespective of exposure time to low temperatures. Notwithstanding, a higher tolerance of the temperate strain towards cold temperatures is just speculative and possibly obscured by the overall low hatching rates of this strain.

Due to the local experimental conditions, the present study has two major limitations with respect to generalisation. First, the temperature tolerance of the strains was tested at one particular location only. Hawley et al. [39], for example, could show that the hatching results differed at different localities in one and the same state of the USA, and that the location with the lowest temperature minimum did not necessarily produce the lowest hatching rates. A comparison with regions with harsher winter conditions would therefore be interesting to conduct in the future. In the present study, eggs were kept in boxes at a location protected from wind but not from temperature. These conditions of egg storage may be comparable to the overwintering of eggs attached to the inner walls of tyres. It could be shown that temperatures only slightly differed inside and outside the tyres [33]. However, *Ae. albopictus* eggs can be found in a huge variety of natural and artificial containers [35, 42], the latter including underground constructions such as sewage systems and cesspits [43, 44]. These must be considered to provide significantly more shelter from adverse winter conditions than our experimental setup, which gives reason to assume that eggs surviving our scenario would have also survived in sheltered places under natural conditions.

Second, under natural conditions, eggs of *Ae. albopictus* from non-tropical regions endure low temperatures through diapausing eggs. In this study, low temperature tolerance was only tested for non-diapausing eggs. It can be expected that hatching results of diapausing eggs would be even higher, as indicated for field conditions by Hanson & Craig [37] and for laboratory conditions by Thomas et al. [36]. In summary, the second point rather emphasises the relevance of our results: even non-diapausing eggs of certain strains of *Ae. albopictus* are able to survive low temperature periods.

Our experiments suggest that age had no impact on the hatching results as no significant decrease became evident in all controls of all three strains. However, depending on the hatching conditions, egg age might affect hatching rates. Mogi [41] could show for *Ae. albopictus* strains from different eastern Asian and Pacific islands that hatching rates of some strains flooded in water supplemented by bacterial broth were as high as 80 to 90% after 60 to 90 days

after oviposition but dropped to 20 to 30% after 120 days after oviposition. Zheng et al. [45] who flooded eggs in bacterial broth found that eggs of three months' age had a higher average hatching rate than eggs flooded after one or two months. Later than three months, the hatching rates gradually decreased until zero in week 24 which was much later than observed by Mogi [41]. In contrast to that, Gubler [46] determined 243 days as the maximum survival time of *Ae. albopictus* eggs continuously flooded with tap water. In the present study, the high hatching rates observed in the controls of the tropical and subtropical strains flooded after four months in both winters suggest that this dropping point was not yet reached, and eggs remained viable until the end of the experiment.

The first overwintering of *Ae. albopictus* in Germany is assumed to have occurred from 2014 to 2015, based on the finding of *Ae. albopictus* developmental stages in Freiburg, federal state of Baden-Wuerttemberg, early in the season 2015 at the very same location as they had occurred the year before [44]. Recently, Pluskota et al. [27] demonstrated overwintering of *Ae. albopictus* in Germany by showing that eggs collected in summer were able to produce larvae after winter field exposure in the Black Forest Mountains in southwestern Germany. The authors missed presenting data on the hatching rate, making a comparison with our data impossible. Notably, the winter season 2015/2016 in Germany and particularly in its southwestern part was characterised by extremely mild temperatures and only short periods below freezing point [47]. Instead of winter temperatures at the very location of egg exposure, Pluskota et al. [26] presented mean temperatures of January measured by a meteorological station about 37 km away. However, when considering survival or extinction, the microclimate, i.e. the temperature and humidity at the exact position of egg location, is decisive. Furthermore, minimum temperatures are possibly even more important for assessing cold tolerance than mean temperatures, as also stressed by Thomas et al. [36]. Single events of extreme temperatures could have a crucial impact on overwintering because they can exceed physiological limits of the species and therefore cause irreversible damage to the eggs.

Aedes albopictus appears to be able to adapt to a changing environment very quickly [15]. Its drought-resistant eggs have facilitated its displacement over long distances, and its preference for artificial containers have enabled conquering urban and suburban areas providing optimal feeding and developmental conditions. Globalisation as well as climate change favour the spread of this species [15], although still in 2007, Benedict et al. [48] considered the risk low. By contrast, more recent models suggest a high risk of establishment of this species in western and southern parts of Germany [49–51]. One of them even predicts almost the whole of Germany as suitable for *Ae. albopictus* [52]. However, the binary presentation of the predictions (differentiated into unsuitable and suitable areas only) do not allow any statement about the gradual suitability of areas and their various probabilities of being colonized after introduction.

Conclusions

Although, as opposed to the winter study 2016/2017, the winter study 2015/2016 was not standardised and therefore not statistically analysable, both studies provide evidence of *Ae. albopictus* egg survival under natural winter conditions in parts of Germany, although shown for relatively mild winter climates only. With respect to the recorded minimum temperature of -10°C , the hatching results of the winter 2015/16 may, however, allow a transfer of results to wider parts of Germany.

Despite the differences in hatching rates, our results show that all three *Ae. albopictus* strains tested are principally able to survive ordinary German winters, i.e. winters without prolonged severe cold periods, albeit the tropical one only survived one complete winter season.

Aedes albopictus occurrence has not been shown so far at the selected study location, but is conceivable under present climatic conditions as its future spread over the whole of Germany is considered possible given progressing climate warming [52]. Consequently, this invasive vector species must be expected to have survival potential in a much wider area than its current distribution range.

It can be assumed that most of the *Ae. albopictus* specimens found in the field in Germany have been introduced from southern Europe or are descendants from those [24–26]. The Italian population used in this study and possibly representative for other southern European strains, performed particularly well in both winters and appears to have a high cold tolerance. Future studies in the laboratory and in the field will show if *Ae. albopictus* is able to cope with central European winter temperatures in the long term and to become permanently established in Germany and other more northern European countries. Practically, this will hopefully also be hampered by control actions which have locally been implemented already in Germany [27].

Supporting information

S1 Table. Temperatures and relative humidities recorded during the winters 2015/2016 and 2016/2017.

(XLSX)

S2 Table. Total numbers of eggs, hatched larvae and hatching rates obtained for the tropical and subtropical strains in the winter 2015/2016.

(XLSX)

S3 Table. Total numbers of eggs, hatched larvae and hatching rates for the tropical, subtropical and temperate strains obtained in the winter 2016/2017.

(XLSX)

S4 Table. Cumulative numbers and proportions of hatched larvae per week obtained for the tropical and subtropical strains in the winter 2015/2016 (c = controls, t = treatments).

(XLSX)

S5 Table. Cumulative numbers and proportions of hatched larvae per week obtained for the tropical, subtropical and temperate strains in the winter 2016/2017 (c = controls, t = treatments).

(XLSX)

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Author Contributions

Conceptualization: Lisa Tippelt, Doreen Werner, Helge Kampen.

Data curation: Lisa Tippelt.

Formal analysis: Lisa Tippelt.

Investigation: Lisa Tippelt.

Methodology: Lisa Tippelt, Helge Kampen.

Project administration: Doreen Werner, Helge Kampen.

Resources: Doreen Werner, Helge Kampen.

Supervision: Helge Kampen.

Validation: Lisa Tippelt.

Visualization: Lisa Tippelt.

Writing – original draft: Lisa Tippelt.

Writing – review & editing: Doreen Werner, Helge Kampen.

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3.4 Low temperature tolerance of three *Aedes albopictus* strains
(Diptera: Culicidae) under constant and fluctuating
temperature scenarios

Lisa Tippelt, Doreen Werner, Helge Kampen

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RESEARCH

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Low temperature tolerance of three *Aedes albopictus* strains (Diptera: Culicidae) under constant and fluctuating temperature scenarios

Lisa Tippelt^{1*}, Doreen Werner² and Helge Kampen¹**Abstract**

Background: *Aedes albopictus*, a vector of numerous viruses and filarial worms, has already established in 20 countries in Europe, mainly colonising subtropical regions. Continuing adaptation to climatic conditions in temperate areas would probably result in a spread to more northern European countries, producing an increasing risk of mosquito-borne pathogen transmission over a much greater area. Based on previous studies showing that *Ae. albopictus* is able to overwinter in Germany, this study aims to determine more exactly its ecological limits of enduring low temperatures.

Methods: Non-diapausing and experimentally induced diapausing eggs of three different *Ae. albopictus* strains (tropical, subtropical and temperate origins) were exposed to four different regimes with constant temperatures and three different regimes with fluctuating temperatures in a course of a day for a minimum of 2 and a maximum of 30 days. The hatching rate of larvae after cold exposure of the eggs was taken as a measure of cold tolerance.

Results: The experiments showed that the tropical *Ae. albopictus* strain had a lower cold tolerance than the subtropical and the temperate strains. The eggs of all used strains were able to survive constant temperatures as low as -5°C for an exposure period of 30 days, while constant temperatures as low as -10°C were endured for 2 days by the tropical strain and for 10 and 20 days by the subtropical and temperate strains, respectively. At fluctuating temperatures, both the subtropical and the temperate strains exhibited hatching under all temperature regimes, even with a minimum temperature of -10°C , whereas the tropical strain ceased hatching after an exposure period of 30 days under the temperature regime with a minimum temperature of -10°C . The analyses showed that the temperature played the major role in interpreting the hatching rates of the eggs. The condition, whether the eggs were diapausing or not, had no significant influence, although results indicated a slightly higher cold tolerance of diapausing eggs at -10°C .

Conclusions: It must be expected that subtropical and temperate strains of *Ae. albopictus* are able to withstand common central European winters and are able to establish in considerable parts of the continent.

Keywords: Asian tiger mosquito, Cold acclimation, Cold hardiness, Constant temperatures, Diapause, Fluctuating temperatures, Hatching, Overwintering, Temperature tolerance

*Correspondence: lisa.tippelt@fli.de

¹ Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald, Insel Riems, Germany

Full list of author information is available at the end of the article



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Background

The Asian tiger mosquito, *Aedes albopictus* (Skuse, 1894), is a thermophilic invasive mosquito species originating from tropical and temperate regions in southeastern Asia [1]. It is active during the day and feeds on both animals and humans, with a preference for human hosts [2]. The species is a feared vector as it is able to transmit more than 20 viruses and filarial worms pathogenic to animals and humans [3–5]. Since the beginning of this millennium, cases of autochthonous mosquito-borne diseases like chikungunya and dengue have substantially increased in southern Europe (e.g. in Italy, France and Croatia), probably mediated by established *Ae. albopictus* populations [6–11]. In 2018, the first autochthonous cases of dengue occurred in Spain [12] which is not surprising as infected *Ae. albopictus* had been collected in the field already in 2015 [13].

Within the last 30 years, *Ae. albopictus* has managed to spread from its native distribution area to all continents except Antarctica [5]. The trade in used tyres and ornamental plants such as ‘lucky bamboo’ (*Dracaena* spp.) has played an important role in its global dispersal [14]. In Europe, the species was first detected in 1979 in Albania [15], but only a further introduction event to Genoa, Italy, in 1990 led to its rapid spread across southern Europe, probably facilitated by favourable climatic conditions [16]. At present, *Ae. albopictus* has been found in 28 European countries and is established in 20 of them [17, 18]. The recent detections in Germany, England and Portugal demonstrate its continuing spread throughout Europe [19–21].

The first detection of *Ae. albopictus* in Germany occurred in 2007 in the form of eggs [22], followed by findings of adults in 2011 and 2012 [23–25]. For several years, all females caught in Germany were assumed to have been introduced *via* motorways from southern Europe [22, 24], and local reproduction was regarded highly unlikely due to unsuitable climatic conditions. Only in 2014, larvae and pupae were collected in southern Germany over several months, demonstrating reproductive potential [26]. Repeated overwintering of *Ae. albopictus* has later been demonstrated in Germany, and several populations are now considered established [27–29]. Modelling approaches based on climatic parameters suggest that a spread to other regions of Germany is possible [30–33].

The ability of *Ae. albopictus* to survive winter temperatures is crucial for its establishment in temperate regions [34]. *Aedes albopictus* is known to overwinter as a pharate larva in the egg stage *via* diapausing [35]. Diapause is a form of dormancy linked to a strongly reduced metabolic activity as a result of seasonal adaptation, although diapause is not strictly linked to the

winter season. In contrast to quiescence, diapause is not reversible by flooding as a hatching stimulus once initiated [36]. Pharate larvae will only get susceptible to this stimulus as soon as diapause is terminated by an increase in temperature and a prolongation of the daily photoperiod [37]. Only *Ae. albopictus* from subtropical and temperate regions are able to overwinter by producing diapausing eggs while tropical strains reproduce continuously during all seasons [38]. Naturally, diapause is induced in the pupa or adult of the female parent under autumnal conditions, characterized by a progressive decrease in photoperiod length and temperature [39]. Laboratory experiments demonstrated that the photoperiod plays a more decisive role than the temperature. In fact, temperatures as high as 25–27 °C together with a short photoperiod were able to induce a diapause response but this response almost or even totally diminished in mosquitoes reared at 29 °C [40]. A temperature of 21 °C has proven most reliable for inducing diapause [40].

It is well known that diapausing eggs have a higher tolerance against low temperatures than non-diapausing eggs [41, 42]. Also, both laboratory and field experiments have shown that tropical *Ae. albopictus* strains have a lower tolerance against low temperatures than temperate strains [42–44]. Furthermore, it could be demonstrated that strains originating from subtropical regions exhibit a tolerance against low temperatures that is intermediate between those of temperate and tropical strains exposed to low temperatures for 6 days [41]. Thomas et al. [42] examined the cold tolerance of a tropical and a subtropical strain and could demonstrate that non-diapausing eggs of the tropical strain withstand –10 °C and diapausing eggs of the subtropical strain even survive exposure to –12 °C for one hour. However, the authors only considered short-term exposure to constant low temperatures with a maximum exposure period of 24 h. Results indicate that hatching could be possible for exposure periods longer than 24 h at temperatures higher than –10 °C.

This study aimed at finding the limits of egg survivability after long-term cold exposure of up to 30 days under constant and fluctuating temperature scenarios. Thus, the intention of the study was not to produce high experimental hatching rates but to check whether hatching is possible at all and to elucidate ecological limits.

Methods

Origin and keeping of mosquitoes

Three *Ae. albopictus* laboratory strains were used: one originating from Mauritius, which will be called ‘tropical strain’ for the purpose of this paper; one from Rimini, Italy, called ‘subtropical strain’; and one from Freiburg,

Germany, called 'temperate strain'. The first two strains were long-maintained laboratory colonies with more than 70 generations, whereas the temperate strain was collected in the field in August 2015 and at the time of the study had developed 20 generations in the laboratory. All strains were reared according to a standard protocol also used by Thomas et al. [42] under constant temperature (25 ± 1 °C), relative humidity (RH) ($70 \pm 5\%$) and light regime (12 h light:12 h darkness). Experiments with tropical *Ae. albopictus* strains are commonly conducted at a temperature of 27 °C [45], whereas experiments with strains of different origins, including temperate ones, are mostly done at lower temperatures, such as 25 °C [39, 44]. In our experiments, we applied this lower rearing temperature to provide better conditions for the subtropical and temperate strain.

Larvae were reared in basins filled with stale tap water and fed with ground fish food (TetraMin; Tetra, Melle, Germany). Pupae were transferred to beakers filled with up to 250 ml of stale tap water, which were placed in cages ($30 \times 30 \times 30$ cm³; Watkins & Doncaster, Leominster, UK) for adult emergence. Adults were provided with a sugar solution (8% fructose) *ad libitum*. Once a week, females were membrane-fed with bovine EDTA-blood warmed to 37 °C *via* an artificial feeding system (Hemotek, Blackburn, UK). Beakers filled with stale tap water and equipped with standard filter paper that protruded from the water were offered for oviposition. After drying the filter papers with attached eggs in Petri dishes for 3 to 4 days, the dishes with the still moist filter papers were sealed with parafilm (Bemis Company, Neenah, Wisconsin, USA) to minimise further desiccation. Dishes were stored in the insectary until further processing.

For the experiments, non-diapausing eggs of the tropical, subtropical and temperate strain as well as diapausing eggs of the subtropical and temperate strains were used. Before starting the experiments, it was checked if laboratory conditions had affected the diapause response of the tropical strain. It could be confirmed that the tropical strain was still not susceptible to diapausing conditions. For producing diapausing eggs, larvae of the subtropical and the temperate strains not older than L3 stage were transferred in beakers to a climate chamber with a photoperiod of 8 h light:16 h darkness, a constant air temperature of 20 ± 1 °C and a relative humidity of $70 \pm 5\%$. Procedures for larval feeding, blood-feeding, oviposition and egg storage were not changed.

Preparation of eggs

To avoid age further influencing analyses as a covariate, the non-diapausing eggs used in this study had a similar age of about 3 (minimum 2.5, maximum 4) months. This is the age at which the diapause state is terminated, and

eggs are susceptible to hatching stimuli [41]. Around 60 (56–69) eggs from the same oviposition cycle were split into two subsets of around 30 eggs transferred to separate wooden spatulas with the aid of a brush, leading to two treatments and controls each for the same strain and the same rearing conditions (routine laboratory rearing conditions and diapause-inducing conditions), temperature treatment and exposure period. Hence, two spatulas with *c.* 30 eggs each were exposed simultaneously to exactly the same conditions. By means of a stereomicroscope, it was verified that no eggs showing signs of desiccation or empty egg-shells produced by spontaneous hatching were used for the experiments. Each spatula was placed into a separate Petri dish sealed with adhesive tape. The Petri dishes were kept in an upright position in open plastic boxes, which were transferred into a climate chamber (KBWF 720; Binder, Tuttlingen, Germany; temperature variation: ± 0.5 °C) programmed to run the desired temperature regime.

As the diapausing populations did not reproduce as efficiently as the non-diapausing populations, diapausing eggs were of limited availability, especially in the temperate strain. Therefore, analysis of diapausing populations was not possible for all exposure periods, and treatments at higher temperatures had to be cut. By all means, at least the minimum and the maximum exposure periods of 2 and 30 days, respectively, were considered.

Cold exposure

Two experimental setups were followed, one with exposure of eggs to constant temperatures and one with exposure of eggs to fluctuating temperatures. The first study was carried out using four different temperatures (0, -5, -10 and -15 °C) and five different exposure periods at each of these temperatures (2, 5, 10, 20 and 30 days). In the second setup, three different cooling-warming cycles were applied, with a maximum temperature of 5 °C in all treatments and different minimum temperatures. The minimum temperatures were set to 0, -5 or -10 °C, respectively, leading to a temperature difference of 5, 10 and 15 K. Accordingly, these different treatments will be called '5K group', '10K group' and '15K group' hereafter. All fluctuating temperature treatments underwent a standardised day-night temperature cycle with an exposure period of 8 h at the maximum temperature of 5 °C, an exposure period of 8 h at the minimum temperature (0, -5 or -10 °C) and 4 h, each, for reaching the respective minimum and maximum temperatures (Fig. 1). For the experiments, it was important to choose a maximum temperature above, but close to, 0 °C because of two reasons. First, a maximum temperature slightly above the freezing point is

common in many areas in Germany during day time in winter and was given in field studies with *Ae. albopictus* in northeastern and central Germany [29, 46]. Secondly, the chosen temperature threshold allows testing three different temperature amplitudes traversing the freezing point twice in every temperature cycle.

Eggs were exposed to these fluctuating temperature cycles for the same time periods as those exposed to constant temperatures, i.e. 2 to 30 days. For comparison, two sets of controls of the same populations were exposed to routine insectary temperature (25 ± 1 °C) or to a constant temperature of 5 °C, respectively. Temperature was measured in the insectary or the climate chamber, respectively. The first control was used as a comparison to optimal developmental summer conditions and is called 'summer control'. By contrast, the second control, called 'winter control', was only applied for the fluctuating temperature scenario and should demonstrate the hatching rate for the maximum temperature of this temperature scenario. Hence, it serves as an internal control to test if the maximum temperature itself, without any fluctuations, could influence the hatching of the eggs. During all treatments (5 to -10 °C), eggs were kept in the dark.

Procedure after cold treatment

After having passed the set temperature regimes, eggs were counted again and flooded by transferring the spatulas into beakers filled with 300 ml stale tap water, according to previous studies [47, 48]. The number of hatched larvae was recorded during the whole flooding process, with larvae found dead treated just as living ones since both obviously succeeded in hatching. After two weeks of flooding, the spatulas were removed from the beakers and dried for one week. By filtrating the water of each beaker with the aid of common filter paper, detached eggs were retained and re-attached onto the spatulas using a brush. After drying for one week, the spatulas were flooded for another week. Former experiments with the same strains had shown that this procedure will lead to hatching rates of 60–90% in the controls of the tropical and subtropical strains kept at 25 °C [46]. Hatching was taken as a measure for the eggs to survive treatment. Non-hatched eggs were not checked for larvae being viable or not and were considered dead. According to studies with other insects, low temperatures do not only affect mortality of eggs but of later life stages as well, denoted as delayed mortality [49, 50]. However, such a phenomenon did not play a crucial role in *Ae. albopictus* [41]. Therefore, this study only aimed for the egg stage, i.e. the hatching rates, and did not track the impact of low temperatures during the egg stage on later life stages.

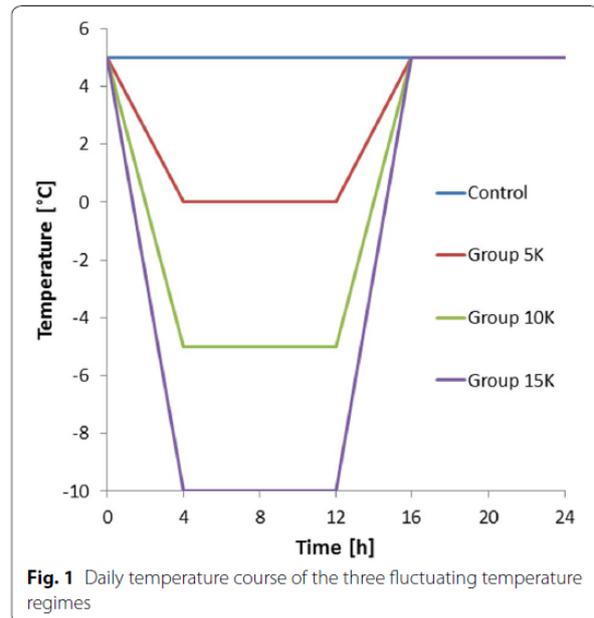


Fig. 1 Daily temperature course of the three fluctuating temperature regimes

Statistical analysis

For statistical analysis, calculated hatching rates were compared. The influence of five different factors on the response variable, the hatching rate, was examined: (i) strain: three levels (tropical, subtropical and temperate strains); (ii) condition: two levels (no diapause and diapause); (iii) temperature: six levels (25 °C, 5 °C, 0 °C, -5 °C, -10 °C and -15 °C). For the fluctuating temperature scenario, the minimum temperature was used in the analyses. The factor 'temperature' is also suitable for differentiating between treatments and controls as only controls were exposed to 25 °C or 5 °C, respectively; (iv) exposure: nine levels (16 h, 40 h, 48 h (2 days), 80 h, 120 h (5 days), 160 h, 240 h (10 days), 480 h (20 days), 720 h (30 days)). For better comparing treatments with constant and fluctuating temperatures, the exposure periods at the minimum temperature were used for the analyses in the case of the fluctuating temperature scenario, whereas the whole exposure period was considered in the case of the constant temperature scenario; (v) scenario: two levels (constant and fluctuating)

Data were square root-transformed for reaching normality followed by multifactorial ANOVA. After conducting an ANOVA, a Tukey's honestly significant different (HSD) *post-hoc* test was applied for identifying the decisive levels of the significant factors. For examining the hatching pattern, the hatching results were split into three categories, 'normal hatching' (without delay), 'delayed hatching' and 'no hatching', followed by calculating Cramér's V for estimating the effect size of each

factor. For all statistical tests as well as for the preparation of figures, the software R (version 3.5.2.) was employed, supported by the packages *lsr* [51], *ggplot2* [52], *ggpubr* [53] and *vcd* [54].

Results

Constant temperature scenario

All three mosquito strains showed hatching after exposure to 0 °C and -5 °C for up to 30 days (Fig. 2). The -10 °C temperature approach revealed great differences between the strains (Fig. 2c) and produced hatching rates with a maximum of about 25% (2–20 days exposure, depending on the strain) and samples with no hatching at all (5–30 days exposure, depending on the strain). No hatching at all occurred after exposure to -15 °C for 2 and 5 days (Fig. 2d). Because of the latter, no treatments with longer exposure periods were tested.

The hatching rates of the controls of the tropical strain were generally very high and varied between 80–100%. After exposure to 0 °C, no significant decrease of the hatching rates was observed (all $P > 0.05$, Fig. 2a). By comparison, treatments showed a decrease in hatching rates after exposure to temperatures of -5 and -10 °C, and hatching finally ceased altogether after exposure to -10 °C for longer than 2 days (Fig. 2c). A significant decrease of the hatching rate was observed after exposure periods of 10 and 30 days at -5 °C ($P < 0.01$), but not after an exposure period of 20 days at the same temperature ($P > 0.05$, Fig. 2b).

The controls of the subtropical strain reached hatching rates ranging from 43–87%, which were generally lower than those of the tropical strain. Treatments exhibited no conspicuous decrease of hatching rates after exposure to constant temperatures of 0 and -5 °C in both non-diapausing and diapausing eggs (Fig. 2a, b). However, after exposure to -10 °C, hatching rates strongly decreased, and hatching ceased after exposure periods of 10 and 20 days, respectively (Fig. 2c). In this temperature regime, hatching rates of the non-diapausing eggs greatly varied from those of the diapausing ones, with the latter being able to endure the temperature twice as long (10 vs 5 days). After an exposure to -10 °C for 20 days, neither non-diapausing nor diapausing eggs showed hatching (Fig. 2c). As related to the controls, there was a significant decrease in hatching rates of non-diapausing eggs after an exposure to -10 °C (all $P < 0.001$) and in the diapausing eggs after exposure periods longer than 2 days (all $P < 0.05$).

Of all examined strains, the controls of the temperate strain were the ones with the lowest hatching rates which rarely exceeded values of 50% in both non-diapausing and diapausing eggs. Only three controls of the diapausing eggs reached higher values (69 and 90% in the

two-day-controls and 59% in one 30-day-control, respectively). Like the subtropical strain, treatments of the temperate strain showed no significant decrease of hatching rates as temperatures went down to -5 °C, albeit generally presenting much lower hatching rates (Fig. 2a, b). Hatching rates of the treatments of both non-diapausing and diapausing eggs of the temperate strain also generally remained below 50%, but some exceptions occurred in the diapausing eggs (Fig. 2a). At -10 °C, hatching of non-diapausing and diapausing eggs still occurred after 20 days representing the longest exposure period with hatching of all tested strains and conditions (non-diapausing and diapausing eggs). There were significant decreases in hatching rates of the non-diapausing eggs after exposure periods of 5, 20 and 30 days (all $P < 0.01$) but of the diapausing eggs only after an exposure period of 30 days ($P < 0.01$).

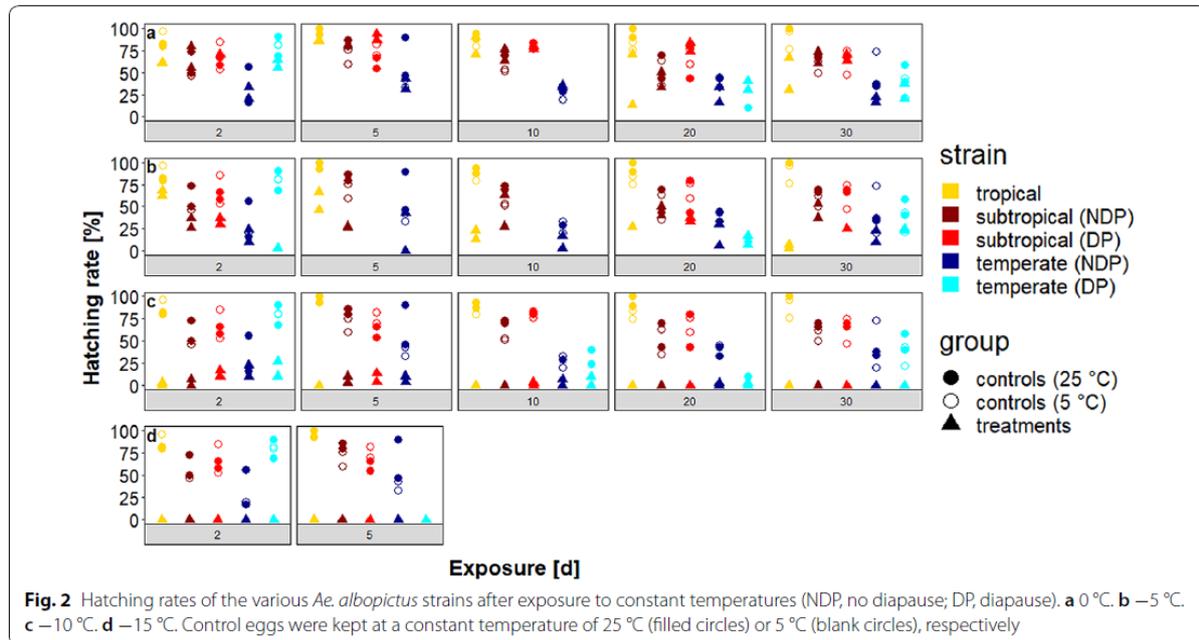
Fluctuating temperature scenario

Under fluctuating temperatures, hatching rates of the controls were generally slightly lower than those obtained under the constant temperature scenario (when referring to controls in the fluctuating temperature scenario, summer controls are meant). The tropical strain had hatching rates between 47–100% in the controls, whereas the treatments exhibited hatching in almost all temperature regimes with a conspicuous decrease in hatching in the 10K group (Fig. 3a, b).

Hatching rates in the 15K group did not exceed values of 10% after all exposure periods and had a complete shortfall of hatching after an exposure period of 30 days (all $P < 0.001$, Fig. 3c). Hence, the treatments exposed for 20 days were the last ones with hatching. Similar to the treatments at constant temperatures, the ones at fluctuating temperatures showed no significant decrease in hatching rates in the 5K group, while in the 10K group distinct decreases in hatching were observable after exposure periods of 20 and 30 days, with significance in the latter ($P < 0.01$, Fig. 3b).

The hatching rates of the controls of the subtropical strains varied between 47–85% in both non-diapausing and diapausing eggs. Treatments showed hatching in all three groups for both physiological egg conditions, without any significant decreases at minimum temperatures of 0, -5 and -10 °C (Fig. 3). This was also the case for the maximum exposure period of 30 days (Fig. 3c).

As in the constant temperature scenario, the controls of the temperate strain had the lowest hatching rates of all controls, mostly exhibiting values below 50%, but in two cases above 80%. Treatments of this strain showed hatching in all groups and after all exposure periods without any significant decrease in hatching rates (Fig. 3).



Influencing factors

The statistical analysis demonstrated that the factor ‘temperature’ had the greatest effect on the hatching rate ($F_{(5, 368)} = 164.86, P < 0.001$), followed by the factors ‘strain’ ($F_{(2, 368)} = 40.08, P < 0.001$), ‘scenario’ ($F_{(1, 368)} = 19.41, P < 0.001$), ‘exposure’ ($F_{(8, 368)} = 10.48, P < 0.001$) and ‘condition’ ($F_{(1, 368)} = 2.26, P = 0.134$). Hence, all examined factors except ‘condition’ had a significant influence on the hatching rate. The Tukey’s HSD *post-hoc* test could specify that the significance of the factor ‘strain’ was related to the temperate strain. Furthermore, there were significant interactions between the temperatures 25 °C and –5, –10 and –15 °C (all $P < 0.001$), leading to a significant, probably cold-related, decrease in the hatching rates.

Hatching pattern

The eggs in the experiments were flooded for a total period of three weeks with an intermediate break of one week. All strains showed a hatching peak at the beginning of both flooding periods. However, in the treatments exposed to constant temperatures of –5 and –10 °C, strikingly many treatments exhibited hatching only during the second flooding period but not during the first. A mosaic plot depicts the dependency of the temperature on the hatching pattern for all treatments at the different temperatures (Fig. 4).

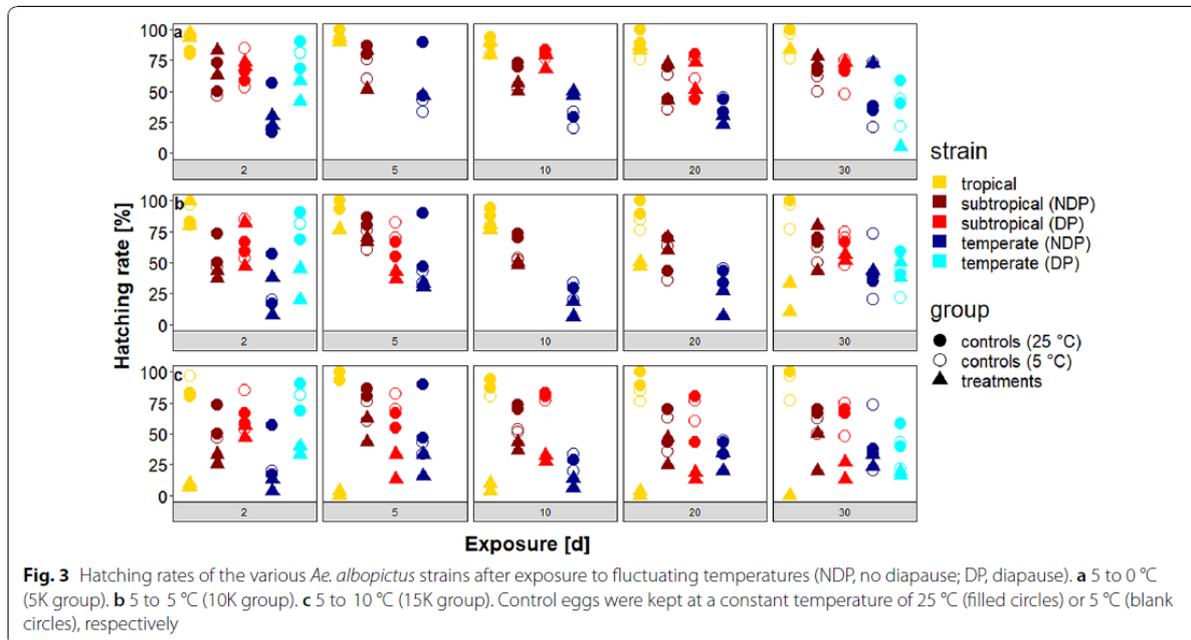
Hatching in both flooding periods (normal hatching) predominated after exposure to temperatures of 0 and –5 °C. Delayed and no hatching did not play any role after exposure to 0 °C. However, delayed hatching

occurred more frequently after exposure to –5 and, particularly, –10 °C in comparison to 0 °C. After exposure to a temperature of –5 °C, the tropical strain and non-diapausing eggs of the temperate strain showed hatching only in the second flooding period (delayed hatching), whereas after exposure to –10 °C, treatments of diapausing and non-diapausing eggs of all strains displayed delayed hatching, most frequently in the previously mentioned strains. This translates to the conclusion that right before the complete lack of hatching, i.e. after the preceding exposure period, delayed hatching or single shortfalls were present in all strains. After exposure to –15 °C, exclusively ‘no hatching’ results occurred in all strains (data not shown).

Statistical analyses resulted in highly significant P -values for each factor ($P < 0.05$). The calculation of Cramér’s V produced the largest value for ‘temperature’ ($CV = 0.60$), followed by ‘scenario’ ($CV = 0.22$). The other factors had CV s between 0.19–0.12.

Discussion

The data obtained in this study clearly demonstrate that the ability of hatching after cold exposure and, thus, the tolerance against low temperatures is dependent on the origin of the strain, the specific exposure temperature and the duration of exposure to minimum temperatures. In the present study, it could be shown that both minimum temperature and exposure period to minimum temperatures play significant roles for the outcome of the



hatching results. The importance of temperature was also stressed in other experiments [42, 43, 55]. In agreement with the findings of Thomas et al. [42], our results further suggest that the exposure period becomes highly important at thermal limits of survival, i.e. at -10 °C.

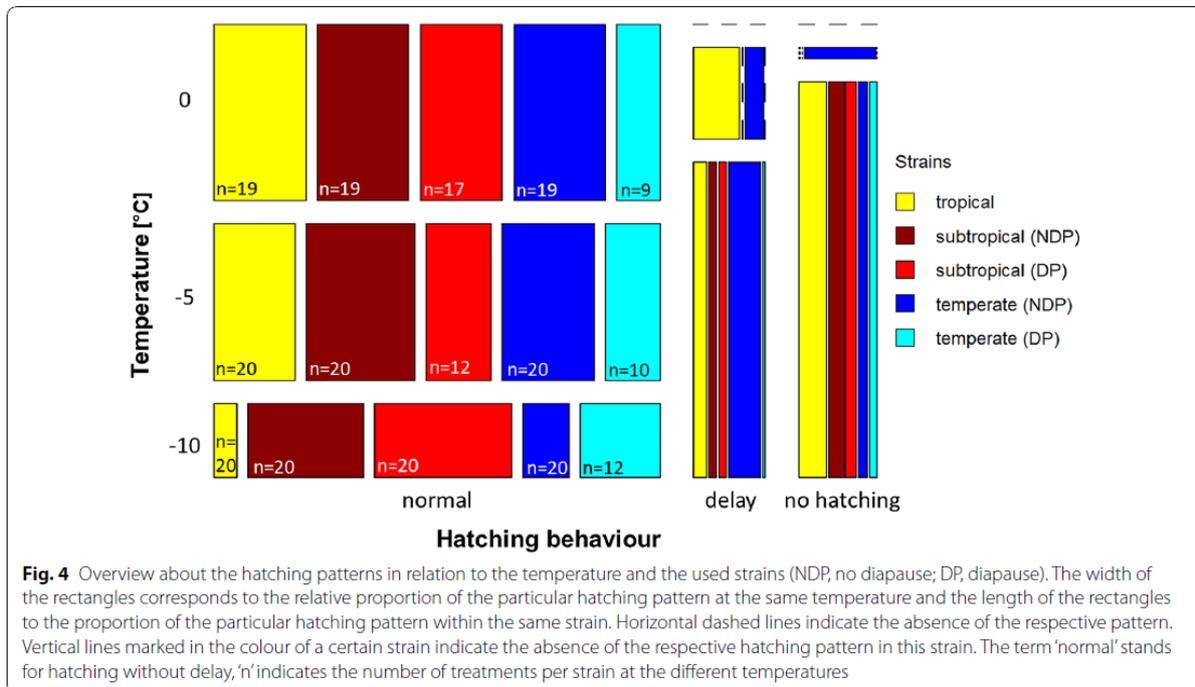
The statistical analysis of the hatching rates of the different *Ae. albopictus* strains used in this study also indicate that their origins have an impact on their cold hardiness. Despite the limited sample size, the results allow a precise differentiation between the tropical *Ae. albopictus* strain on the one side, and the subtropical and the temperate strains on the other side due to the remarkably high capability of resisting low temperatures in the latter two. This finding is in agreement with other laboratory and field studies with North American, Asian and European *Ae. albopictus* strains [42–44, 46, 55, 56].

Interestingly, the subtropical and tropical strains did not differ significantly regarding hatching rates, but their hatching rates were quite different from those of the temperate strain. However, if the longest exposure periods with hatching are considered, the subtropical and temperate strains were more similar and differed tremendously from the tropical strain, which becomes most obvious by the hatching results at a constant temperature of -10 °C. The statistical significance between the temperate strain on the one side and the tropical and subtropical strains on the other might be caused by the overall low hatching rates in both controls and treatments of the temperate strain. This phenomenon

also occurred in previous field experiments with the same strains and was thought to be attributed to incomplete adaptation to laboratory conditions [46]. Consequently, a field vs laboratory effect cannot be ruled out and can only be verified or falsified by testing the cold tolerance of the temperate strain again when adapted to laboratory conditions for a much longer time. In spite of this, the temperate strain was the only strain exhibiting hatching after an exposure period of 20 days at -10 °C. Thus, the temperate strain appears to be somewhat more cold-hardy, possibly caused by having gone through a bottleneck during its first winters in Germany, as has been described for populations in North America [57]. It is assumed that the German *Ae. albopictus* population (temperate strain) originates from Italy [24, 25], where the subtropical strain comes from. Genetic analyses in fact suggest that the population of Freiburg is closely related to one from northern Italy [27].

In addition to laboratory effects, several other factors, not considered in the present study, might influence the cold hardiness of *Ae. albopictus* such as maternal effects [39], genetic drift [58] or rapid adaptation [59], which occurred during adaptation to conditions in Germany. Hence, these factors also have to be taken into account when interpreting the different cold hardiness of the temperate and subtropical strains.

The tropical and subtropical strains used in this study were the same as examined by Thomas et al. [42],



allowing direct comparisons of the hatching results. Based on these, tolerance of the tropical strain against low temperatures is higher in the present study. Thomas et al. [42] demonstrated that eggs exposed to a constant temperature of $-10\text{ }^{\circ}\text{C}$ for longer than one hour did not produce larvae. By contrast, the experiments conducted in this study still demonstrated hatching after exposure periods of 30 days at $-5\text{ }^{\circ}\text{C}$ and of 2 days at $-10\text{ }^{\circ}\text{C}$. For the subtropical strain, Thomas et al. [42] showed endurance for four hours at $-10\text{ }^{\circ}\text{C}$, as compared to 5 days for non-diapausing eggs and 10 days for diapausing eggs of the same strain in this study. One main reason for these divergent results could be physiological adaptation to the long rearing in the laboratory. Both strains used in the present study have passed more generations in the laboratory and therefore have a longer rearing history than the ones used by Thomas et al. [42]. A well-known problem in rearing insects and their adaptation to laboratory conditions is the genetic selection due to artificial and isolated keeping [60]. However, the hatching results obtained after low temperature exposure in both the tropical and subtropical strains demonstrated that rearing conditions apparently had not substantially reduced the capability of the laboratory strains to withstand low temperatures.

In a former field study using the same tropical, subtropical and temperate strains, eggs were able to hatch after short-term exposure to a minimum temperature of

$-6\text{ }^{\circ}\text{C}$. Furthermore, eggs of the subtropical strain were able to hatch after an exposure period of two hours at $-10\text{ }^{\circ}\text{C}$ [46]. Additionally, field data demonstrated that an *Ae. albopictus* strain from Jena in central Germany can withstand -10 to $-11\text{ }^{\circ}\text{C}$ for four hours [29]. The results of the laboratory experiments carried out in this study are in agreement with those findings as laboratory experiments showed that hatching also occurred after exposure to $-10\text{ }^{\circ}\text{C}$. In contrast to the previously discussed factors, the factor 'condition', i.e. using non-diapausing or diapausing eggs, did not significantly influence the hatching rates in the experiments. These findings fit very well to those produced by Hanson & Craig [41] who described diapause as having a lower effect on the tolerance against low temperatures in *Ae. albopictus* than cold acclimation. However, if the thermal limits are considered, especially the treatments of eggs exposed to a constant temperature of $-10\text{ }^{\circ}\text{C}$, diapausing eggs seemed to have an advantage, although this could be demonstrated for the subtropical strain only (Fig. 2c). The experiments conducted by Thomas et al. [42] provided an equivalent indication, thus supporting the findings of our study. Although short-term exposure (up to four hours) of non-diapausing eggs could endure $-12\text{ }^{\circ}\text{C}$ for a longer time than the diapausing ones in that study, diapausing eggs were able to withstand $-10\text{ }^{\circ}\text{C}$ for a longer exposure period than their non-diapausing counterparts. As in our study, those experiments were not able to find a significant

difference between survival temperatures of the different 'conditions'.

As diapause is a complex process mainly influenced by a gradual decrease of photoperiod and temperature, it is questionable if larvae and pupae held under constant conditions of low photoperiod and temperature instead of a gradual decrease will give rise to females that behave in a similar way as their natural populations.

In the experiments applying a fluctuating temperature scenario, the hatching rates of the treatments were higher than under a constant temperature scenario. This is quite logical, as the net exposure time to sub-zero temperatures under a fluctuating temperature scenario was only one-third of that of the exposure time to the same temperature under a constant temperature scenario. However, this difference is also present when comparing the absolute exposure time to minimum temperatures. This could be shown best for the hatching rates of the tropical and subtropical strains under a constant temperature of -10°C as compared to a fluctuating temperature regime with minimum temperatures of -10°C . At a constant temperature of -10°C , no hatching occurred after exposure periods of 5 days and more in the tropical strain. However, under a fluctuating temperature regime with a minimum temperature of -10°C (15K group) hatching even occurred after an exposure period of 20 days, which corresponds to a total net exposure time of about 5 days at -10°C . A possible reason for this could be that cold acclimation occurred. All eggs used for the treatments had not been cold-acclimated prior to the experiments, but treatments exposed to a fluctuating temperature scenario had the chance of acclimation to low temperatures during the experiment as they experienced a daily period of 5°C for eight hours. As mentioned before, Hanson & Craig [41] demonstrated a significant influence of cold acclimation on the tolerance of *Ae. albopictus* against low temperatures by keeping eggs for 60 days at 10°C prior to the cold experiments. The authors conceded that an optimal temperature for cold acclimation could not be found. Therefore, it is not clear how long a duration at 5°C would be necessary to cause such an acclimation effect.

Interestingly, both the eggs of the subtropical and of the tropical strains seemed to have undergone some kind of cold acclimation. That came unexpected in the case of the tropical strain, as cold acclimation could not be shown in other studies using tropical non-diapausing *Ae. albopictus* strains [41, 61]. However, in a field study with the same tropical mosquito strain as in this study, cold acclimation was supposed to have contributed to the striking difference between the hatching rates of the tropical strain in two consecutive winter seasons in Germany [46]. Besides, cold acclimation could be demonstrated for larvae of *Ae. aegypti* exposed to temperatures as low

as -6°C quite recently [62]. Therefore, it is feasible to assume that cold acclimation also occurs in tropical *Ae. albopictus* strains. Consequently, the distinct natural day-night cycle of the temperature, which typically appears in temperate geographical zones, might enhance the tolerance against low temperatures due to the occurrence of cold acclimation on a small scale.

It can be stated that the controls exposed to a constant temperature of 5°C do not always exhibit a higher hatching rate as the treatments. However, there was no significant influence between the hatching rates of the winter control and the ones of the treatments. There are two possible explanations for this phenomenon. First, the winter controls were not exposed to optimal developmental temperatures like the summer controls kept at 25°C . Consequently, the hatching rates were lower than in the summer controls, and in several cases even lower than those of the respective treatments. Secondly, the insignificant hatching rates of the winter control and the treatments demonstrate that a minimum temperature as low as -5°C is not relevant for the resulting hatching rate. However, this is not the case for the tropical strain for longer exposure periods, which perfectly corresponds to the lower tolerance of this strain against low temperatures.

When considering hatching in *Ae. albopictus*, a variety of other factors than temperature and duration of cold exposure like humidity, the age of the eggs or the hatching medium play a role [44, 63]. The present study primarily concentrates on hatching after egg exposure to low temperature conditions depending on exposure times. Furthermore, the artificial standardisation of freeze-thaw cycles to a constant time period of eight hours caused differences in chilling, varying from $1.25^{\circ}\text{C}/\text{h}$ in the 5K group to $3.75^{\circ}\text{C}/\text{h}$ in the 15K group. It cannot be excluded that different degrees of acclimation resulted and had an influence on the hatching results. However, this factor has to be considered as a cofactor in these experiments as it is linked to a certain temperature regime and cannot be regarded or analysed separately. Future experiments could show how important the speed of chilling is for the hatching rate.

When analysing the hatching patterns, the factor 'temperature' had the strongest impact of all examined factors, based on its Cramér's value (CV). According to benchmarks for the CV proposed by Cohen [64], the temperature has a large effect, whereas the scenario has a medium and the other factors have a small effect on the hatching pattern. The occurrence of delayed hatching at temperatures as low as -10°C could be interpreted as a warning signal that physiological limits of the strain are approaching. Unfortunately, the analysis of such hatching patterns has not yet been addressed in ecological

experiments with *Ae. albopictus*. Our results suggest that this aspect, especially the consideration of delayed hatching, might be worthwhile in future examinations.

For a further spread of *Ae. albopictus* in temperate climate zones, the mean January temperature is often quoted as an important feature for its survival and, thus, its ability of overwintering [16, 31]. For modelling climatic suitability of certain areas for overwintering, a mean January temperature of 0 °C is a common threshold. However, Waldock et al. [65] argued for an adaptation of this threshold to -4 °C as this value leads to a better match of mean temperature with demonstrated species occurrence. This is further supported by studies demonstrating overwintering at temperatures lower than 0 °C. The mean January temperature can actually vary between -2 °C for Japan [66], -3 °C for populations in China and South Korea and even -5 °C for those in the USA [1]. However, permanent populations are also found at Chinese locations with a mean January temperature of -5 °C [67]. Our study suggests that the mean temperature at which survival is possible is lower than -2 °C, which would be in agreement with findings linked to an *Ae. albopictus* population established in central Germany [29]. Mean temperatures give a good estimate for the suitability of certain areas on larger scales for mosquito species in general but minimum temperatures are more important on smaller scales as these define the survival with respect to microclimate [65]. The laboratory experiments showed that exposure to -10 °C for longer than 10 days resulted in survival of the subtropical and the temperate strains which could similarly be shown for a temperate strain in the USA [43]. Hence, our findings confirm those of Thomas et al. [42] and emphasise the importance of minimum temperatures, in addition to mean temperatures, for estimating *Ae. albopictus* survival during cold spells. Furthermore, as temperate regions are affected by a daily temperature cycle, the temperature range also plays a role in interpreting the survival potential in winter. However, this factor often remains obscure as most laboratory experiments concentrated on constant temperatures. Our experiments indicate that fluctuating temperatures increase the hatching rate, possibly due to cold acclimation. The average temperature in Germany was 0.6 °C in January 2019 and was only slightly warmer than the long-term trend [68]. Thus, the 10K group reflects the winter conditions in Germany best. It has to be considered, however, that maximum and minimum temperatures may vary considerably in different areas of Germany. For instance, there are relatively warm regions in western Germany that exhibit daily temperature ranges similar to the 5K group regime and relatively cold regions in the eastern part of the country where daily temperature variations resemble the 15K group regime.

However, it is not clear, if daily temperature ranges larger than 15K could lead to adverse results. Furthermore, it has to be noted that winter temperatures do not define the presence and absence of *Ae. albopictus* alone. Other climatic factors such as summer temperature and precipitation, but also ecological ones like the availability of breeding sites, have to be taken into account when modeling the potential spread of this species on a regional or even global scale [5, 16, 65]. Despite this, the ability to survive the winter due to its physiological plasticity is one of the main driving factors for the northward spread of *Ae. albopictus* [5].

Correspondingly, another important aspect influencing the spread of *Ae. albopictus* is global warming. Rising mean temperatures which can be observed globally will also lead to an increase of mean temperatures in winter seasons. Thus, the last five years (2015–2019), which were the warmest ever recorded in Germany, were characterised by exceptionally mild winters [69]. However, climatic projections do not only predict that the annual number of frost days will decrease in the future throughout Europe, but also the persistence of extreme cold weather events [70, 71]. Although these cold extremes will be less frequent [71], they will have an influence on the overwintering capability of *Ae. albopictus* in temperate regions. Actually, models considering a variety of factors, in addition to winter temperatures, suggest an increased climatic suitability for *Ae. albopictus* in many regions of Germany due to climate change. In particular, western and southern Germany will be at high risk for future establishment of this mosquito species [30–33]. In light of these studies, it can be assumed that suitable areas for a successful overwintering and thus the distribution range of *Ae. albopictus* will further increase rather than decrease in the future. Since the tiger mosquito is an efficient vector of numerous pathogens, including dengue and chikungunya viruses, its spread and establishment is in the long term assumed to be followed by mosquito-borne disease cases or even outbreaks.

Conclusions

Our results reveal that the eggs of *Ae. albopictus* are able to survive low temperatures for a much longer exposure period than 24 hours. Low temperature tolerance in the tropical and subtropical strains seems to be much higher as previously indicated [42]. In contrast to field experiments carried out formerly [46], much longer exposure periods could be tested in the laboratory experiments presented here. Therefore, this study could elucidate physiological limits at which hatching is possible as well as critical exposure periods much better than field experiments. Recent models could show that there is a high probability of spread of *Ae. albopictus* especially

in western and southern parts of Germany usually characterised by mild winters [30–33]. Our results strongly attest the capability of *Ae. albopictus* to overwinter in regions with winter minimum temperatures of $-10\text{ }^{\circ}\text{C}$, being in agreement with findings in the field [29, 46, 72]. These results together with the indication of short-term tolerance of this species against temperatures as low as $-12\text{ }^{\circ}\text{C}$ [42] should be considered in future projections and justify comprehensive mosquito monitoring programmes, also including the public [19].

Abbreviations

ANOVA: analysis of variance; CV: Cramér's Value; EDTA: Ethylenediaminetetraacetic acid; HSD: honestly significant different.

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Authors' contributions

LT, DW and HK designed the study. LT performed the experiments and the statistical analyses. LT, DW and HK wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹ Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald, Insel Riems, Germany. ² Leibniz Centre for Agricultural Landscape Research, Muencheberg, Germany.

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4 Own contributions

- (I) Tippelt, L., Walther, D., & Kampen, H. (2017). The thermophilic mosquito species *Uranotaenia unguiculata* Edwards, 1913 (Diptera: Culicidae) moves north in Germany. *Parasitology Research*, 116(12), 3437-3440. doi: 10.1007/s00436-017-5652-2.

Lisa Tippelt: Organisation of trapping, morphological and genetic identification of mosquitoes, data analysis and interpretation, manuscript preparation and editing

Doreen Walther: Overall monitoring design, data interpretation, manuscript preparation and editing

Helge Kampen: Overall monitoring design, supervision, data interpretation, manuscript preparation and editing

- (II) Tippelt, L., Walther, D., Scheuch, D. E., Schäfer, M., & Kampen, H. (2018). Further reports of *Anopheles algeriensis* Theobald, 1903 (Diptera: Culicidae) in Germany, with evidence of local mass development. *Parasitology Research*, 117(8), 2689-2696. doi: 10.1007/s00436-018-5938-z.

Lisa Tippelt: Organisation of trapping, morphological and genetic identification of mosquitoes, data analysis and interpretation, manuscript preparation and editing

Doreen Walther: Overall monitoring design, data interpretation, manuscript preparation and editing

Mandy Schäfer: Screening for pathogens (Filarioidea), manuscript editing

Dorothee E. Scheuch: Screening for pathogens (arboviruses), manuscript editing

Helge Kampen: Overall monitoring design, supervision, data interpretation, manuscript preparation and editing

Own contributions

- (III) Tippelt, L., Werner, D., & Kampen, H. (2019). Tolerance of three *Aedes albopictus* strains (Diptera: Culicidae) from different geographical origins towards winter temperatures under field conditions in northern Germany. *PLoS One*, 14(7), e0219553. doi: 10.1371/journal.pone.0219553.

Lisa Tippelt: Conceptualisation and study design, coordination and conduction of experiments, statistical analysis and data interpretation and visualisation, manuscript preparation and editing

Doreen Werner: Conceptualisation and study design, provision of mosquitoes, data interpretation, manuscript preparation and editing

Helge Kampen: Conceptualisation and study design, provision of mosquitoes and equipment, coordination of experiments, supervision, data interpretation, manuscript preparation and editing

- (IV) Tippelt, L., Werner, D., & Kampen, H. (2020). Low temperature tolerance of three *Aedes albopictus* strains (Diptera: Culicidae) under constant and fluctuating temperature scenarios. *Parasites & Vectors*, 13(1), 587. doi: 10.1186/s13071-020-04386-7.

Lisa Tippelt: Conceptualisation and study design, coordination and conduction of experiments, statistical analysis, data interpretation and visualisation, manuscript preparation and editing

Doreen Werner: Conceptualisation and study design, provision of mosquitoes, data interpretation, manuscript preparation and editing

Helge Kampen: Conceptualisation and study design, provision of mosquitoes and equipment, coordination of experiments, supervision, data interpretation, manuscript preparation and editing

In agreement:

PD Dr. Helge Kampen

Lisa Tippelt

5 General discussion

5.1 Detection of *Uranotaenia unguiculata* and *Anopheles algeriensis*

The two caught *Ur. unguiculata* females are the first findings of the species in northeastern Germany. Furthermore, the sampling site in Saxony-Anhalt represents the most northern location in Germany as well as in Europe as a whole (Ramsdale & Snow, 2001; Filatov, 2017). However, as this evidence only comprised one individual per monitoring site in each case, it could be argued that these detections were a result of accidental displacement; i.e. cannot be considered a proof of a locally reproducing population. Two arguments disprove this assumption.

First, *Ur. unguiculata* is a species which is not known to be susceptible to an accidental displacement neither of the adults nor their preimaginal states, such as eggs or larvae. This can be better clarified in comparison to a species which is well known for been accidentally displaced over long distances: *Ae. albopictus*. One possible route of its introduction is the motorway as adult mosquitoes tend to enter cars by mistake at stop-overs at motorway service stations (Eritja et al., 2017). This possibility can be almost certainly excluded for the two mentioned sample sites because both locations are far away from any motorway. Two other proven ways of dispersal of *Ae. albopictus* are the displacement of eggs via shipping of used tires and of larvae as well as pupae via the trade with ornamental plants like the lucky bamboo (Reiter, 1998; Hofhuis et al., 2009). In contrast to *Ae. albopictus*, the ecology of preimaginal states of *Ur. unguiculata* strongly contradicts these possibilities. *Uranotaenia unguiculata* produce non-dormant eggs (Becker et al., 2010), i.e. which hatch soon after embryonic development is completed, and larvae are not known to be associated to those plant species involved in international trade. Consequently, both the ecology of the adults and the preimaginal states makes an accidental displacement very unlikely.

Second, the confirmation of one sampling site showing reproduction in the subsequent year suggests that the female caught at this site was one of several or many of an established population. It is rather probable that the population also existed in the previous year and hibernated. It may also be assumed that a locally reproducing population also exists at the location in Brandenburg because that one provides more suitable local climatic conditions than

the site in Saxony-Anhalt. However, it remains unclear how the species managed to overcome such great distances from the only previously known detection site in the Upper Rhine Valley (Becker & Kaiser, 1995). No reports exist about occurrences of this species at locations between that site and the new ones before 2016. Still, it is rather likely that the species is present in other parts of Germany between the sample site in the Upper Rhine Valley and the new ones because many regions in between provide more suitable climatic conditions than the two in the northeastern part of Germany. The assumption of firm establishment of *Ur. unguiculata* in northeastern Germany is further supported by findings of adults at the very same place in Thuringia, Central Germany, in 2017 and 2018 (Kuhlisch, pers. communication).

There are two factors possibly preventing the detection of further specimens and sampling sites, respectively. First, the collections made in the framework of this thesis and from Thuringia comprised only two individuals each. This would indicate a generally small population size and a rather low abundance of *Ur. unguiculata* in more northern situated European regions as supported by only one single larva found in southern Germany in 1994 by Becker & Kaiser (1995). In order to detect more individuals, the sampling effort may have to be increased, especially in late summer and early autumn as these are the months when *Ur. unguiculata* populations peak (Becker et al., 2010; Rudolf et al., 2015). Second, not finding the species at other sampling sites could not only be due to insufficient sampling effort but also to the sampling method. Like every method for collecting mosquitoes, the BG sentinel trap (Biogents, Germany) used in this study has its advantages and drawbacks. On the one hand, the trap can be operated easily, even by non-professional instructed users, as no dry ice as for other CO₂-baited mosquito traps is needed and the sample effort is rather low in comparison to netting or dipping. The person in charge only has to switch on the ventilator and to open the carbon dioxide supply. The BG sentinel trap can catch a variety of different mosquito species. A field study conducted by Lühken et al. (2014) demonstrated that the number of different species that can be found in BG sentinel traps throughout the trapping season surmount the ones collected by other traps. This is supported by the outcome of the monitoring project 2015-2017 in northeastern Germany, which reached the number of 30 species and two species groups, a result coming close to the number of species expected in the sampled area. On the other hand, mosquito collections using BG sentinel traps lead to a biased catch in that *Cx. pipiens* complex specimens are overrepresented (Lühken et al., 2014), which could be confirmed, for instance, by the observations made during monitoring activities in eastern Germany in 2015 (Tippelt et al., 2018). Hence, there are certain species which are underrepresented because they are not

caught or in only small proportions. It might be possible that *Ur. unguiculata* belong to these species with a low catchability by using BG sentinel traps. Therefore, other collection methods have to be considered for getting a better estimate of the existent population of this species, like dipping for larvae and pupae or netting and other trapping methods for catching adult specimens. For instance, observations showed that *Ur. unguiculata* can be caught with the application of black or fluorescent light alone or even with sound traps using frog calls (Camp et al., 2018). Altogether, this leads to the conclusion that the trapping method applied in the monitoring programme is appropriate for determining a high species diversity, but not necessarily for the detection of *Ur. unguiculata*. Nevertheless, the latest evidence in Thuringia indicates that there are further suitable habitats for this species in Germany. Model predictions showed that there is a high suitability for an occurrence along the Upper Rhine valley as well as in a small area in the eastern part of Germany, near the border to Poland (Filatov, 2017). Interestingly, neither the two described sampling sites from this thesis nor the third in Thuringia are located in the projected suitable areas.

With regard to *An. algeriensis*, the case is rather different. First, the presence at two sampling sites in northeastern Germany is not an evidence for a northward shift as this species had been detected in that region in the past (Weyer, 1939; Baer, 1960). Second, the number of caught individuals was much higher than for *Ur. unguiculata* and even exceeded 200 specimens at one location, leading to the predominant species at this sampling site. This demonstrates that this rarely encountered species is able to achieve quite high abundances, as particularly shown for the location in Wustrow. The findings of *An. algeriensis* at three sampling sites represent the first detection of adults after nearly half a century. Interestingly, most of the historical sampling sites were located in the eastern part of Germany. However, it is not clear if the distribution of the positive sampling sites resembles an actual concentration of *An. algeriensis* in eastern Germany or if this circumstance has to be attributed to a more precise monitoring of that region. Despite tremendous sampling efforts throughout the vegetative season of the subsequent year, it was not possible to detect the breeding habitats of *An. algeriensis*, although the abundances at two of the three sampling sites do not allow any other conclusion than the existence of a locally reproducing population. The monitoring of the site in Wustrow showed that the high population densities present in 2015 were reconfirmed two years later, even though with a smaller proportion in relation to the other caught mosquito species than in 2015. Thus, it is highly probable that *An. algeriensis* was also present in 2016. The findings clearly point out the

persistence and dominance of *An. algeriensis* in a number of samples, although two vegetative seasons are not representative for the assessment of a population. Consequently, long-term monitoring should be carried out, the more so as the sampling site in Wustrow could be identified as a rather suitable habitat for one of the rarest central European mosquito species, according to Scholte et al. (2011). Further examinations will show if this species will hold its status or if there are other hotspots elsewhere in or outside of Germany.

Both the detections of *Ur. unguiculata* and of *An. algeriensis* are highly valuable for the mosquito monitoring scheme as they are evidence for these species to continue existing in Germany. Interestingly, there was one sampling site inhabited by both *An. algeriensis* and *Ur. unguiculata*, which might be worth further monitoring efforts. Finally, these findings demonstrate that thermophilic mosquito species tend to spread in Germany or reach higher abundances than expected. This is further supported by the detection of *Cs. longiareolata* and *An. petragani*, other thermophilic mosquito species recently established in Germany (Becker & Hoffmann, 2011; Becker et al., 2016; Kampen et al., 2017).

5.2 Temperature experiments with *Aedes albopictus*

Crucial for the establishment of thermophilic species in Germany are not only suitable temperatures in summer but also the ability of the mosquitoes to cope with winter temperatures. The field experiments demonstrate that every *Ae. albopictus* strain tested survived at least one winter season in the field. All strains were capable of withstanding wintry conditions with a minimum temperature of -8°C . The tropical strain was able to withstand the winter 2016/2017, but not the winter 2015/2016. It has to remain unanswered whether this failure is predominantly attributed to a generally lower cold tolerance, or if it is the result of an incomplete cold acclimation. The subtropical strain was the only strain which was able to overwinter 2015/2016 and 2016/2017. By contrast, the temperate strain was analysed only in the winter 2016/2017 and survived.

The laboratory experiments confirmed the findings from the field and added further evidence for the physiological limits in terms of minimum temperature and maximum exposure time. The treatments exposed to -10°C were particularly appropriate for distinguishing the different strains according to their tolerance against low temperatures with the lowest tolerance in the

tropical and the highest tolerance in the temperate strain. These findings verified the extraordinary ecological plasticity of *Ae. albopictus* as described by other experimenters (Hanson & Craig, 1994, 1995b; Mogi, 2011; Thomas et al., 2012). Furthermore, the experiments could demonstrate that diapausing eggs had only an advantage at physiological limits and that fluctuating temperatures could enhance low temperature tolerance. Both outcomes are especially relevant for temperate areas as those regions are characterised by seasonal changes in photoperiod and diurnal temperatures.

Altogether, the field and especially the laboratory studies conducted in this thesis demonstrate that the tolerance of *Ae. albopictus* against low temperatures is well developed with respect to both the absolute minimum temperature and the exposure periods at that temperature some strains could endure. That makes it rather unlikely that this invasive mosquito species will disappear again due to unsuitable conditions once introduced. The temperature studies also suggest a high potential for *Ae. albopictus* to become established in further areas in Germany. Although there have been no autochthonous cases of chikungunya, dengue or Zika in Germany so far, the circulation of the viruses proven by several disease cases and outbreaks in European countries in the past (e.g. Gjenero-Margan et al., 2011; Venturi et al., 2017; Giron et al., 2019), the existence of established competent vectors together with the implications of global change will increase the risk of their emergence and circulation in Germany, as predicted for chikungunya and dengue (Thomas et al., 2018).

5.3 Synthesis and outlook

The results of the four studies contributing to this thesis demonstrate that thermophilic species tend to spread in Germany and that they are able to overwinter, facilitating the development of stable populations in the following year. The ongoing process of global warming as well as the increase in globalisation, including mass transport of animals and humans, has an influence on the presence and distribution of species and can lead to a shift of distribution areas and the introduction of non-native species (Tatem et al., 2006; Gould & Higgs, 2009; Tatem et al., 2012). Invasive species like *Ae. japonicus* and *Ae. albopictus*, which are already established in Germany, probably will continue to spread in Germany.

Together with invasive species, viruses and other mosquito-borne pathogens can be introduced and gain a foothold in Europe, resulting in regular outbreaks of mosquito-borne diseases when

suitable conditions are met. The progressive dispersal of mosquito-borne diseases since the beginning of this century and their trend for spreading northwards is expected to continue. Especially the arrival and putative overwintering of West Nile virus in Germany have proved that conditions in Germany are becoming more and more suitable for the replication and circulation of mosquito-borne viruses in their vectors.

Climate change will not only affect the biology of the mosquito itself and its distribution but also the replication of pathogens, which can result in the occurrence and spread of mosquito-borne diseases in hitherto unaffected regions (Reiter, 2008; Weaver & Reisen, 2010; Sassnau et al., 2014; Thomas et al., 2018). This development is not only restricted to mosquitoes and mosquito-borne diseases but can be transferred to other arthropod vectors, like biting midges or ticks, and the disease agents they transmit (Gould & Higgs, 2009; Dantas-Torres et al., 2012). However, it has to be taken into account that the presence of a competent vector alone does not inevitably lead to the outbreak of an infectious disease (Randolph & Rogers, 2010) but adds a further prerequisite necessary for the transmission and circulation of a pathogen.

The monitoring of mosquitoes plays an important role in the surveillance of mosquito-borne diseases. First, monitoring programmes provide data about the inventory and the phenology of the existing species and thus enable the identification of competent vectors in the case of a disease outbreak. Second, the introduction of non-indigenous species, which pose a threat for human and animal health, can be detected at an early point in time, facilitating control measures before these species have reached high population densities. And third, the detection of associated pathogens in the mosquitoes prior to the first animal or human cases can serve as an early warning system projecting an imminent possible threat to susceptible vertebrate hosts near future, as shown with the mass dying event caused by the Usutu virus which had been predicted after findings of the virus in mosquitoes one year before (Jöst et al., 2011a; Becker et al., 2012). In this regard, the continued monitoring of mosquitoes and their associated pathogens poses a crucial tool for surveillance and facilitates a quick and adequate assessment of, and response to, novel vectors and health risks for animals and humans. This should be linked to raising awareness for these risks and for the role of haematophagous arthropods as vectors, both in the responsible health authorities and the public. Mosquitoes are no longer only a nuisance for animals and humans in Germany but can also be vectors of the agents of serious infectious diseases.

6 Summary

Culicidae or mosquitoes are medically important dipterans which can mediate a variety of infectious diseases in animals and humans. This thesis deals with the distribution of two rare mosquito species caught in the framework of a monitoring project in northeastern Germany and the ecology of the Asian tiger mosquito. These species are examples for thermophilic mosquito species which are assumed to be favoured by climate change and thus are spreading northwards. This could especially be shown for *Uranotaenia unguiculata*, which was found at two locations far away from its only known detection site. Although only one adult was caught at each location, larval evidence in the subsequent year account for an established population rather than the introduction of single individuals. Another rare mosquito species found is *Anopheles algeriensis*. This species was detected at three different sampling sites, and reached at two of them a much higher abundance than *Ur. unguiculata*.

The ecological part of this thesis includes field and laboratory experiments for studying the low temperature tolerance of eggs of the Asian tiger mosquito, *Aedes albopictus*, a competent vector of numerous viruses and other pathogens. Three different strains from a tropical, subtropical and temperate origin were exposed to low winter temperatures, both in the field and in a controlled laboratory environment. The field experiments demonstrate that all used strains were capable of withstanding wintry conditions with a minimum temperature of -8°C . The laboratory experiments pointed out that all strains could endure temperatures of -10°C for a certain maximum exposure time. This survival exposure time highly depended on the origin of the strain and varied between 2 and 20 days. The temperate strain had a slightly higher tolerance against low temperatures than the subtropical strain. The tropical strain exhibited the lowest performance both in the field and the laboratory experiments. Diapausing eggs had an advantage in regards of cold tolerance only at the physiological limits of the strains. Furthermore, eggs were able to endure minimum temperatures for a longer time under a fluctuating rather than under a constant temperature scenario.

The results of both experiments show that there are strains of *Ae. albopictus* with a quite high ability to endure low temperatures. This makes it rather probable that these invasive species will have no or only minor problems coping with winters in Germany in the future and will continue to spread, which is connected with epidemiological implications for public and veterinary health.

7 Zusammenfassung

Culicidae, auch bekannt als Stechmücken, sind medizinisch bedeutsame Zweiflügler, die als Vektoren eine Vielzahl von Krankheitserregern auf Menschen und Tiere übertragen können. Die vorliegende Arbeit beschäftigt sich einerseits mit der Verbreitung von zwei seltenen Stechmückenarten, welche im Rahmen eines Monitoringprojekts gefangen wurden, und andererseits mit der Ökologie der Asiatischen Tigermücke. Bei allen Arten handelt es sich um thermophile Arten, bei denen angenommen wird, dass der Klimawandel ihre Verbreitung Richtung Norden begünstigt. Dies konnte vor allem für die Art *Uranotaenia unguiculata* gezeigt werden, da sie an zwei Orten gefunden wurde, die ausgesprochen weit entfernt waren von der einzigen jemals zuvor beschriebenen Nachweisstelle in Deutschland. Obwohl an beiden Fundorten jeweils nur ein einziges adultes Individuum gefangen werden konnte, ergab eine Beprobung von potenziellen Bruthabitaten im darauffolgenden Jahr, dass lokale Reproduktion stattfand. Somit konnte eine ausschließliche Verschleppung von adulten Einzelindividuen weitgehend ausgeschlossen werden. Eine weitere seltene Stechmückenart, die im Zuge des Monitorings nachgewiesen werden konnte, ist *Anopheles algeriensis*. Diese Art wurde an drei Standorten gefangen, an zwei von ihnen mit einer viel höheren Abundanz als *Ur. unguiculata*. Den ökologischen Teil dieser Arbeit machen Feld- und Laborexperimente mit *Aedes albopictus* aus. Sie dienten der Ermittlung der Kältetoleranz der Eier dieser Spezies, die als Vektor für eine Vielzahl von Viren und andere Pathogene gilt. Drei verschiedene Stämme, die aus tropischen, subtropischen und gemäßigten Breiten stammen, wurden niedrigen Temperaturen, welche typischerweise im Winter herrschen, unter Feld- und Laborbedingungen ausgesetzt. Die Experimente belegen, dass alle untersuchten Stämme prinzipiell einen Winter mit einem Temperaturminimum von -8 °C im Feld überleben konnten. Die Laborexperimente konnten hingegen zeigen, dass alle Stämme in der Lage waren, Temperaturen von -10 °C für eine gewisse Zeit zu ertragen. Die Überlebensfähigkeit schwankte je nach Stamm zwischen 2 und 20 Tagen. Dabei hatte der Stamm aus den gemäßigten Breiten eine Kältetoleranz, die nur wenig höher lag als die des subtropischen Stammes. Der tropische Stamm hingegen besaß die geringste Toleranz gegenüber niedrigen Temperaturen, sowohl in den Freiland- als auch in den Laborexperimenten. Diapausierende Eier zeigen nur eine höhere Kältetoleranz nahe den physiologischen Grenzen der jeweiligen Stämme. Weiterhin konnte festgestellt werden, dass Eier unter fluktuierenden Temperaturen eine bestimmte Minimaltemperatur länger aushalten

Zusammenfassung

konnten als unter konstanten Temperaturen. Somit zeigen beide Experimente, dass gewisse Stämme von *Ae. albopictus* eine sehr hohe Toleranz gegenüber niedrigen Temperaturen haben. Das macht es sehr wahrscheinlich, dass diese invasive Art Winter in Deutschland oder anderen mitteleuropäischen Ländern überleben und ihre Ausbreitung weiter fortschreiten werden. Diese Entwicklung wird epidemiologische Auswirkungen auf die Human- und Veterinärmedizin haben.

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I. Eigenständigkeitserklärung

Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Lisa Tippelt

II. Publications and talks

II.1 Publications

*Tippelt, L., Walther, D., & Kampen, H. (2017). The thermophilic mosquito species *Uranotaenia unguiculata* Edwards, 1913 (Diptera: Culicidae) moves north in Germany. *Parasitology Research*, *116*(12), 3437-3440. doi: 10.1007/s00436-017-5652-2.

*Tippelt, L., Walther, D., Scheuch, D. E., Schäfer, M., & Kampen, H. (2018). Further reports of *Anopheles algeriensis* Theobald, 1903 (Diptera: Culicidae) in Germany, with evidence of local mass development. *Parasitology Research*, *117*(8), 2689-2696. doi: 10.1007/s00436-018-5938-z.

Tippelt, L., Kuhlisch, C., Schuhbauer, A., Schleip, I., Kampen, H. & Walther, D. (2018): Mosquito monitoring 2015 in eastern Germany. *Mitteilung der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie* *21*, 207-213.

*Tippelt, L., Werner, D., & Kampen, H. (2019). Tolerance of three *Aedes albopictus* strains (Diptera: Culicidae) from different geographical origins towards winter temperatures under field conditions in northern Germany. *PLoS One*, *14*(7), e0219553. doi: 10.1371/journal.pone.0219553.

*Tippelt, L., Werner, D., & Kampen, H. (2020). Low temperature tolerance of three *Aedes albopictus* strains (Diptera: Culicidae) under constant and fluctuating temperature scenarios. *Parasites & Vectors*, *13*(1), 587. doi: 10.1186/s13071-020-04386-7.

* part of this thesis

II.2 Oral presentations

“Spatiotemporal monitoring of mosquitoes in northeastern Germany” – Meeting of the German Society for Medical Entomology and Acarology, 12-14 September 2016, Halle

“Studies on low temperature tolerance of *Aedes albopictus* (Diptera: Culicidae)” – FLI Junior Scientists Meeting, 21-23 September 2016, Jena

“Mosquito monitoring 2015 in eastern Germany” – Meeting of the German Society for General and Applied Entomology, 13-16 March 2017, Freising

“Studien zur Temperaturtoleranz von *Aedes albopictus* (Culicidae: Diptera)” – Meeting of the German Society for Medical Entomology and Acarology, 18-20 September 2017, Leipzig

“Laborversuche zur Toleranz von *Aedes albopictus* (Culicidae: Diptera) gegenüber niedrigen Temperaturen” – Meeting of the German Society for Medical Entomology and Acarology, 20-22 September 2018, Frankfurt/Main

"Knowing the vector – mosquito monitoring and ecological examinations in the light of emerging mosquito-borne diseases" – talk during study visit at the European Centre for Disease Prevention and Control (ECDC), 25-29 June 2018, Stockholm