# MOSQUITOES AND BITING MIDGES: DATA COLLECTION, IDENTIFICATION, SPECIES DISTRIBUTION, AND BREEDING ECOLOGY

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# Abstract

This PhD thesis summarises thirteen stand-alone publications on mosquitoes (Diptera: Culicidae) and *Culicoides* biting midges (Diptera: Ceratopogonidae). Although species of both groups are potential vectors for a variety of pathogens, these insects received little attention in scientific work in Germany until the beginning of the 21st century. However, with the outbreak of the bluetongue disease and the introduction and spread of exotic mosquito species, the situation changed abruptly and scientific research increased in both research groups.

This thesis, first of all, is focused on different scientific questions regarding the species distribution for two vector groups: biting midges and mosquitoes. Most of the questions this research dealt with, apply to both vectors groups. However, this thesis also gives attention to some aspects specific to one or the other group. The conclusions drawn from this research outlined here not only add to the fundamental research but will directly or indirectly add knowledge to the field of applied vector ecology, which will be also helpful for future monitoring concepts. The work covers the total gain of new scientific insight into vector ecology, starting with new results on sampling (1) and species identification (2), and continuing with correlative studies (3) and experimental studies on species distribution (4). One chapter is devoted to each of these four topics:

The first chapter of this thesis presents two methodological studies. Both investigate the efficacy of (light-)suction traps in dependence of the type of trap for mosquitoes and the position of the trap relative to potential hosts for biting midges. The two

studies demonstrated that the different traps or the position of the traps significantly affects the trapping efficacy. Hence, the type of trap and their positioning need to be selected with caution when planning to study the distribution of both vector groups.

In the second chapter, new alternative methods for the identification of cryptic vector species were evaluated. Two studies explored the differentiation of female *Culex pipiens* s.s. and *Cx. torrentium* with morphometric wing characters and the four German members of the *Anopheles maculipennis* group with a real-time PCR assay. Both tools proofed to be useful for a fast and reliable identification, which can be used to understand the environmental factors affecting these species, which is, at least in Germany, a highly neglected field of research.

The studies in the third chapter evaluated the species distribution of immature mosquitoes and biting midges on the local scale (i.e. breeding sites) and analyses the distribution of adults on larger scales (i.e. landscape):

First, breeding sites were studied to evaluate the correlations of physico-chemical data with the presence-absence of *Cx. pipiens* s.l. and *Cx. torrentium*, the impact of edaphic factors on the density of dung-breeding *Culicoides* biting midges, and the biting midge density in relation to the position and substrate temperature in a cattle dung heap. The results of the first study revealed that the *Cx. pipiens* s.l. and *Cx. torrentium* often occur in sympatry at the studied breeding sites and there were no substantial differences concerning presence-absence of the two species with regard to the various environmental parameters investigated. The second study highlights the importance of soil moisture, organic substance, and soil texture on the number of emerging *Culicoides chiopterus* and *C. dewulfi*. Increasing soil moisture positively correlated with the number of emerging adults for both species of biting midges, while the variable organic substance was only positively correlated with the abundance of *C. chiopterus*. In contrast, soil textures showed conflicting results, i.e., a positive and negative relationship with the same variables. Furthermore, in the third study, the larval density of *C. obsoletus* s.s. was negatively correlated with increasing substrate temperature in the dung heap.

Secondly, cooperating with the research group Environmental Informatics of the University of Oldenburg, a prototypic software tool was developed to predict the local spread of exotic mosquito species. This tool offers extensive application possibilities, e.g. an exact geographic definition for the design of mosquito surveillance or control programs.

Thirdly, the distribution of the various *An. maculipennis* group members from Germany were studied. Most remarkable was the decline of *An. atroparvus* compared to historic data, which might be explained by the differences in the overwintering strategy compared the other *Anopheles* species. *An. atroparvus* is expected to be more dependent on shelter sites in the immediate vicinity to cattle hosts that were lost as a result of a changed farm building design. Moreover, an elaborated study evaluated which landscape scale, or even multi-scale model, might be suitable to model the distribution of different biting midge species in Germany. The study demonstrated that multi-scale modelling is a promising approach to predict the distribution of *Culicoides* species. It offers the opportunity to include a diverse set of variables from different scales. This is especially important for hematophagous insects, e.g. when breeding sites, resting sites, or host density have to be taken into account for modelling, which are generally distributed across several scales.

The fourth chapter of this thesis focuses on the breeding ecology of dung-breeding *Culicoides* (*C. chiopterus* and *C. dewulfi*). These experimental studies explicitly evaluated causal relationships between emergence rates and environmental parameters. These studies cover three different topics: 1) Effects of mechanical disturbance of breeding sites. With regard to the development of juvenile biting midges, disturbance should increase the mortality and, thus, was assumed to be an alternative control method. However, no significant reduction of emerging adults was recorded by application of this approach. 2) Effects of disturbance by flooding on the survival of immature biting midges. In this experiments, no individuals emerged from the "permanently flooded" treatment, hence, clearly indicated that long-termed flooding caused a significant increase in mortality. 3) The third study in Chapter 4 is dedicated to seasonal effects and winter development. Effects of temperature and photoperiod on the development of the overwintering population are analysed in order to judge on regulation factors and man-made effects which might uncouple these regulations. The results highlighted the importance of temperature on the spring emergence of dung-breeding *Culicoides* in Northern Germany.

Any observation has a blind spot. This applies with regard to different aspects of vector ecology, e.g. the knowledge on the advantages and disadvantages of a sampling or identification method, the significance of variables not included in species distribution models, or the ecological interpretation of those variables being included. The different studies in this thesis filled several of these blind spots by a critical evaluation of trapping and identification methods, and the examination of the correlative or causal relationship of the factors affecting the species distribution.

# Zusammenfassung

Diese Doktorarbeit fasst dreizehn eigenständige Publikationen über Stechmücken (Diptera: Culicidae) und Gnitzen (Diptera: Ceratopogonidae) zusammen. Obwohl die Arten beider Gruppen als potenzielle Vektoren für eine Reihe von Pathogenen bekannt sind, haben sie bis Anfang des 21. Jahrhunderts nur sehr wenig Aufmerksamkeit in Deutschland erhalten. Dies änderte sich jedoch schlagartig als es zu dem Ausbruch der Blauzungenkrankheit und der Einschleppung und Ausbreitung von exotischen Stechmückenarten kam.

Diese Arbeit behandelt in erster Linie unterschiedliche Forschungsfragen hinsichtlich der Artverbreitung von zwei Gruppen von Vektoren: Gnitzen und Stechmücken. Die meisten der hier behandelten Fragen sind für beide Gruppen relevant, stellen aber teilweise auch gruppenspezifische Themen dar. Die Arbeit umfasst sowohl Aspekte der Grundlagenforschung als auch der angewandten Vektorökologie, die z.B. auch für die Entwicklung von Monitoringsprogrammen relevant sind. Diese Arbeit behandelt den vollständigen wissenschaftlichen Arbeitsprozess, der in vektorökologischen Studien stattfindet: Datensammlung (1), Artbestimmung (2), Studien zu korrelativen (3) und kausalen Zusammenhängen von Umweltfaktoren mit der jeweiligen Artverbreitung (4). Ein Kapitel behandelt einen dieser Arbeitsprozesse:

Das erste Kapitel der Arbeit stellt zwei methodische Studien vor. Diese beschäftigten sich mit der Effizienz von (Licht-)Saugfallen: zum einen die Wahl des Fallentyps für Stechmücken und zum anderen die Position der Falle relativ zu potenziellen Wirten für Gnitzen. Beide Studien zeigten, dass unterschiedliche Fallen bzw. die Position der

Fallen einen signifikanten Einfluss auf die Fangeffizienz haben. Daher ist es wichtig den Fallentyp und die Fallenposition explizit zu wählen.

Im zweiten Kapitel werden alternative Methoden für die Bestimmung von kryptischen Vektorarten evaluiert. Zwei Studien testeten die Differenzierung von weiblichen *Culex pipiens* s.s. und *Cx. torrentium* mit morphometrischen Flügelmerkmalen und die Bestimmung der vier deutschen Arten der *Anopheles maculipennis* Gruppe mit einem real-time PCR Verfahren. Diese Methoden erlauben eine schnelle und korrekte Bestimmung und sind aufgrund ihrer Zuverlässigkeit insbesondere gut dafür geeignet die Beziehung dieser Arten mit Umweltfaktoren zu verstehen, was besonders in Deutschland ein stark vernachlässigtes Forschungsfeld darstellt.

Die Publikationen des dritten Kapitels haben die Artverbreitung von immaturen Stechmücken und Gnitzen auf der lokalen Skala (d.h. Brutplätze) und die Analyse der Verbreitung von Imagines auf größeren Skalenebenen (d.h. Landschaften) zum Thema.

In den ersten Studien wurden Brutplätze bearbeitet, um den Zusammenhang zwischen physico-chemischen Parametern und der Präsenz-Absenz der Arten *Culex pipiens* s.l. und *Cx. torrentium* zu bestimmen, den Zusammenhang von Bodenparametern mit der Dichte von Gnitzen in Kuhfladen und die Dichte von Gnitzen in der Relation zur Position und der Substrattemperatur in einem Misthaufen zu ermitteln. Die Ergebnisse der ersten Studien zeigten, dass *Cx. pipiens* s.l. und *Cx. torrentium* in der Regel zusammen in denselben Brutplätzen auftraten und es keine substanziellen Unterschiede zwischen der Präsenz-Absenz beider Arten in Abhängigkeit von unterschiedlichen Umweltparametern gab. Die zweite Studie zeigte die Bedeutung von Bodenfeuchte, organischer Substanz und Bodentextur für die Emergenz von *Culicoides chiopterus* und *C. dewulfi* aus Kuhfladen. Steigende Bodenfeuchte korrelierte positiv mit der Anzahl schlüpfender Gnitzen, wohingegen die organische Substanz nur einen positiv Zusammenhang für *C. chiopterus* zeigte. Schlussendlich demonstrierte die dritte Studie einen negativen Zusammenhang der Larvendichte von *C. obsoletus* s.s. mit ansteigender Temperatur im Misthaufen.

Als zweites haben wir mit der Forschungsgruppe Umweltinformatik der Universität Oldenburg einen Softwareprototypen entwickelt, welcher zur Vorhersage der lokalen Ausbreitung exotischer Stechmücken genutzt werden kann. Dieses Werkzeug bietet eine Vielzahl von Anwendungsmöglichkeiten: z.B. die exakte geographische Eingrenzung von Probenahme- oder Bekämpfungsgebieten.

Als drittes wurde die Verbreitung der unterschiedlichen deutschen Arten der *An. maculipennis* Gruppe untersucht. Bemerkenswert war die Abnahme von *An. atroparvus* im Vergleich mit dem historischen Auftreten der Art. Dies kann vielleicht durch die Überwinterungsstrategien der *Anopheles* Arten erklärt werden. Es wird davon ausgegangen, dass *An. atroparvus* eine stärkere Abhängigkeit von Überwinterungsplätzen in der Nähe von Blutswirten (z.B. Rindern) hat, die im Zuge eines veränderten Baustils der Ställe verloren gingen. Des Weiteren wurde untersucht, welche Landschaftsskala oder ob sogar multiskalige statistische Modelle für die Verbreitungsmodellierung von Gnitzen in Deutschland geeignet sind. Die Studie zeigte, dass multiskalige Modelle einen vielversprechenden Ansatz bieten, um die Verbreitung von Gnitzen zu modellieren. Diese Methode erlaubt die Integration mehrerer Variablen unterschiedlicher Skalen. Dies ist insbesondere für blutsaugende Insekten wichtig, da z.B. Brutplätze, Rastplätze oder die Vektordichte in der Modellierung berücksichtigt werden können, die in der Regel über mehrere Skalen verteilt sind.

Das vierte Kapitel dieser Abschlussarbeit behandelt die Brutökologie von Gnitzen, die ausschließlich in Dung brüten (*C. chiopterus* und *C. dewulfi*). Diese experimentellen Arbeiten betrachteten explizit die kausalen Zusammenhänge zwischen dem Gnitzenschlupf und Umweltparametern. Diese Studien behandeln drei Themen: 1) Den Effekt von mechanischer Störung der Brutplätze: Es wurde erwartet, dass Störung die Mortalität juveniler Gnitzen erhöht und somit als alternative Bekämpfungsmethode genutzt werden könnte. Jedoch konnte keine signifikante Reduktion der Emergenz beobachtet werden. 2) Die Effekte von Störung durch Überflutung: In diesem Experiment schlüpften keine Imagines von den permanent überfluteten Kuhfladen, was deutlich zeigt, dass eine dauerhafte Überflutung die Mortalität signifikant erhöht. 3)

Die dritte Studie des letzten Kapitels behandelt die saisonalen Effekte auf die Winterentwicklung: Die Effekte der Temperatur und der Tageslänge auf die überwinternde Population wurden untersucht, um die regulierenden Faktoren und anthropogenen Effekte dieser Regulation zu bestimmen. Die Ergebnisse zeigten, dass die Temperatur einen wichtigen Faktor für die Frühjahrsemergenz von Gnitzen in Norddeutschland darstellt.

Jede Untersuchung hat einen blinden Fleck, was auch für die unterschiedlichen Aspekte von vektorökologischen Studien gilt: z.B. das Wissen über die Vor- und Nachteile von Probenahme- oder Bestimmungsmethoden; die Signifikanz von Variablen, die nicht in Habitatmodellen integriert wurden; oder die ökologische Interpretation der Variablen, die integriert wurden. Durch eine kritische Evaluation von Fang- und Bestimmungsmethoden und die Bestimmung von korrelativen oder kausalen Zusammenhängen von Umweltfaktoren und der Artverbreitung schließt diese Arbeit wichtige Wissenslücken für das Forschungsgebiet der Vektorökologie.

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# Preface

Motivated by field studies during my Bachelor study program, I planned to conduct my Bachelor thesis on the central theme of temporary water bodies. At the same time, massive mosquito plagues occurred in the Wadden Sea National Park in Lower Saxony (Northern Germany), and thus I conducted a basic study on temporary water bodies to identify the plague species and their breeding sites. This work initiated my interest in the topic of vector ecology. Around the same time, the bluetongue virus spread all over Europe and the exotic Asian Tiger mosquito was first detected in Germany, resulting in an increasing public, scientific interest and research activities. That made it possible to conduct this PhD thesis covering different thematic aspects of vector ecology from the evaluation of data collection methods to experimental studies on the basic ecology of mosquitoes and biting midges.

# Papers included in this thesis

## Part of the chapters "Introduction" and "Breeding ecology of *Culicoides*"

Paper 0 **Lühken, R.**, Steinke, S., & Kiel, E. (2014). Potential impact of climate and landscape change on mosquitoes and biting midges in Germany. In: Lozán, J. L., Grassl, H., Karbe, L. & G. Jendritzky (Eds.), *Warnsignal Klima: Gefahren für Pflanzen, Tiere und Menschen*. 2. Auflage. Elektron. Veröffent. (Kap. 3.2.17), [German].

## Data collection

Paper A.1 **Lühken, R.**, Pfitzner, W. P., Börstler, J., Garms, R., Huber, K., Schork, N., Steinke, S., Kiel, E., Becker, N., Tannich, E., & Krüger, A. (2014). Field evaluation of four widely used mosquito traps in Central Europe. *Parasites & Vectors*, *7*, 268.

Paper A.2 **Lühken, R.**, & Kiel, E. (2012). Distance from the stable affects trapping of biting midges (Diptera, Ceratopogonidae). *Journal of Vector Ecology*, *37*, 453–457.

## Identification

Paper B.1 Börstler*, J., **Lühken*, R.**, Rudolf, M., Steinke, S., Melaun, C., Becker, S., Garms, R., & Krüger, A. (2014). The use of morphometric wing characters to discriminate female *Culex pipiens* and *Culex torrentium*. *Journal of Vector Ecology*, *39*, 204–212. *contributed equally to this paper

Paper B.2 **Lühken, R.**, Czajka, C., Steinke, S., Jöst, H., Schmidt-Chanasit, J., Kiel, E., Krüger, A., & Tannich, E. (in preperation for *Parasites & Vectors*). Distribution of the various *Anopheles maculipennis* group members from Germany identified by newly developed real-time PCR assays. *this manuscript is also partly included in the Chapter "Species distribution"

## Species distribution

Paper C.1 **Lühken, R.**, Steinke, S., Leggewie, M., Becker, S., Krüger, A., Tannich, E., & Kiel, E. (submitted to the *Journal of Medical Entomology*): Physico-chemical characteristics of *Culex pipiens* s.l. and *Culex torrentium* breeding sites in Germany.

Paper C.2 **Lühken, R.**, Kiel, E., & Steinke, S., & Fladung, R. (submitted to *Parasitology Research*). Impact of edaphic factors on the presence of dung breeding *Culicoides*.

Paper C.3 **Lühken, R.**, Kiel, E., & Steinke, S. (2014). *Culicoides* biting midge density in relation to the position and substrate temperature in a cattle dung heap. *Parasitology Research*, *113*, 4659–4662.

Paper C.4 Vogel, U., **Lühken, R.**, & Kiel, E. (2014). A tool for simulating the spread of invasive mosquitoes. In: Gomez, J. M. et al. (Eds.), *Proceedings of the 28th International Conference on Informatics for Environmental Protection – Enviroinfo Oldenburg 2014* (pp. 279–286).

Paper B.2 **Lühken, R.**, Czajka, C., Steinke, S., Jöst, H., Schmidt-Chanasit, J., Kiel, E., Krüger, A., & Tannich, E. (in preperation for *Parasites & Vectors*). Distribution of the various *Anopheles maculipennis* group members from Germany identified by newly developed real-time PCR assays. *this manuscript is also partly included in the Chapter "Identification"

Paper C.5 **Lühken, R.**, Kiel, E., Gethmann, J., Staubach, C., Conraths, F. J., & Kranz, P. (in preperation). Comparison of single- and multi-scale models for predicting the distribution of *Culicoides* biting midges.

## Breeding ecology of *Culicoides*

Paper D.1 **Lühken, R.**, Kiel, E., & Steinke, S. (2014). Impact of mechanical disturbance on the emergence of *Culicoides* from cowpats. *Parasitology Research*, *113*, 1283–1287.

Paper D.2 **Lühken, R.**, Steinke, S., Wittmann, A., & Kiel, E. (2014). Impact of flooding on the immature stages of dung-breeding *Culicoides* in Northern Europe. *Veterinary Parasitology*, *205*, 289–294.

Paper D.3 **Lühken, R.**, Steinke, S., Hoppe, N., & Kiel, E. (submitted to *Veterinary Parasitology*). Effects of temperature and photoperiod on the development of overwintering immature *Culicoides chiopterus* and *Culicoides dewulfi*.

# Contents

# Chapter 1

# Introduction

partly published: **Lühken, R.**, Steinke, S., Kiel, E. (2014). Potential impact of climate and landscape change on mosquitoes and biting midges in Germany. In: Lozán, J. L., Grassl, H., Karbe, L. & G. Jendritzky (Eds.), *Warnsignal Klima: Gefahren für Pflanzen, Tiere und Menschen*. 2. Auflage. Elektron. Veröffent. (Kap. 3.2.17), [German].

## 1.1 Background to this thesis

Although several species of mosquitoes (Diptera: Culicidae) and *Culicoides* biting midges (Diptera: Ceratopogonidae) are potential vectors for several pathogens, these insect families have not received research focus in Germany for several decades. While studies on biting midges were predominantly restricted to freshwater species (Havelka, 1976), which are probably not included in pathogen transmission (Purse et al., 2015), intensive studies on the distribution and ecology of mosquitoes were being conducted until the middle of the 20th century. This research predominantly focused on malaria vectors of the *Anopheles maculipennis* group (Weyer, 1938). However, with the eradication of malaria, this field of research collapsed (Weyer, 1956). It is presumed that the intensive drainage of marshes, swamps, and fens was responsible for the elimination of malaria (reviewed by Maier, 2004). Additionally, housing and sanitation were improved, and advances in the diagnostic methods and in the treatment of malaria were achieved (e.g. Bruce-Chwatt & de Zulueta, 1980). With regards to biting midges, knowledge gaps were even larger because researchers had not considered these insects as being vectors of pathogens in Germany. However, with the outbreak of the bluetongue disease and the introduction and spread of exotic mosquito species, the situation abruptly changed at the beginning of the 21st century.

## *Culicoides* transmitted viruses

In 2006, bluetongue disease was detected for the first time in Germany, Belgium, and the Netherlands (Conraths et al., 2012). The bluetongue virus is transmitted by different *Culicoides* species (Meiswinkel et al., 2007; Dijkstra et al., 2008) and the disease predominantly affects domestic and wild ruminants, but can also affect camelids (Conraths et al., 2012). Clinical signs are, among several other characteristics, birth failure, reduced milk donation, or even death (in particular in sheep and goats). In the month after being detected for the first time, the virus rapidly spread all over Europe. The costs for compensatory payments, vaccination, etc. amounted to more than 250 million Euros in Germany (Conraths et al., 2012) and 200 million Euros in the Netherlands (Velthuis et al., 2010). Shortly after this epidemic, the Schmallenberg virus was detected for the first time in 2011 (Hoffmann et al., 2012). This virus is also transmitted by biting midges (De Regge et al., 2012; Rasmussen et al., 2012). This pathogen, in particular, caused the deformed premature birth and stillbirth of lambs and calves (Garigliany et al., 2012), and spread very quickly through Europe (Gubbins et al., 2014).

## Mosquito transmitted pathogens

The first intensive studies on pathogens transmitted by mosquitoes in Germany detected several viruses in different mosquito species (Usutu virus, Batai virus, and Sindbis virus) (Jöst et al., 11a,b,c). Due to the observed mass mortalities of birds (especially blackbirds) in southern Germany, the Usutu virus attracted the most attention from the population among the three viruses (Becker et al., 2012; Bosch et al., 2012). A single human case of Usutu virus infection occurred in southern Germany (Allering et al., 2012).

Additionally, parasitic nematodes transmitted by mosquitoes were considered. *Dirofilaria repens* and *D. immitis* were recorded in different mosquito species (Czajka et al., 2014; Kronefeld et al., 14a), as well as in dogs (Sassnau et al., 2013). The first autochnous case of a human *D. repens* in Germany was reported in 2014 (Tappe et al., 2014).

## Exotic-species

Currently, the German mosquito fauna changes remarkably. Pluskota et al. (2008) detected eggs of the exotic Asian tiger mosquito (*Aedes albopictus*) for the first time in 2008. Intensified studies demonstrated several events related to the introduction of the species during the following year (Kampen et al., 2012; Werner et al., 2012; Becker et al., 2013). Nevertheless, also larvae and pupae of the species were detected in Southern Germany (Werner & Kampen, 2014), it is not currently considered to be an established species in Germany. In contrast, the Asian bush mosquito (*Ochlerotatus japonicus japonicus*) was found in southern Germany for the first time in 2008 (Schaffner et al., 2009), but spread quickly and reached northwestern Germany within a few years (Becker et al., 2011; Huber et al., 2012; Kampen et al., 2012; Werner et al., 2012; Werner & Kampen, 2013). Genetic studies highlighted the fact that that *Oc. j. japonicus* was most likely introduced from Japanese populations (Huber et al., 2014), while the recent dispersal in Germany was strongly driven by punctual introductions, e.g. by human-mediated transport (Huber et al., 2014; Zielke et al., 2014). Research pays particular attention to these exotic species(e.g. Gratz, 2004), which have been proven to, or were expected to, be competent vectors for different pathogens, e.g. individuals of German *Oc. j. japonicus* populations, known to be susceptible for the Japanese encephalitis virus (Huber et al., 2014).

## 1.2 Outline to this thesis

This thesis primarily focuses on the different aspects of species distribution in biting midges and mosquitoes. It will cover the total gain of new scientific insight into vector ecology, starting with new results on sampling (Chapter 2) and species identification (Chapter 3), and continuing with correlative studies on species distribution (Chapter 4). Chapter 4 focuses on the analysis of correlations between vector distribution/density and environmental parameters, while an identification of the causal connection was out of the scope of these studies. However, knowing the causality is most important when analysing species distribution, which is the focus of Chapter 5. While the basic factors affecting the distribution of mosquitoes are comparatively well understood (e.g. Spencer et al., 1999; Juliano, 2009), the ecology of biting midges has hardly been studied at all in detail and therefore should be the subject of this chapter. One chapter is devoted to each of these topics (Chapter 2-5) linking together different stand–alone publications.

However, why is it sensible to look at *Culicoides* biting midges and mosquitoes in the same thesis? At first glance, both groups appear to be quite different: *Culicoides* biting midges are tiny insects that are primarily expected to breed in moist substrates (Kettle & Lawson, 1952), while mosquitoes are comparatively large, solely breeding in water bodies (Becker et al., 2010). Nevertheless, besides these differences, the most important similarity is that the females of most species in both groups are anautogenous, requiring frequent blood meals to produce eggs, thus making them potential vectors of pathogens. Furthermore, due to the previously described research deficits regarding the role of landscape and ecological parameters in mosquito and biting midge ecology, there are many basic and unanswered questions, e.g. inquiries regarding data collection or species distribution. Many of these aspects apply to both groups or, at least, are comparable between the groups. Therefore, studies on these fundamental aspects in mosquitoes are also helpful with understanding the ecology of biting midges, and vice versa.

This transferability of knowledge is considered to have a particular importance in the context of vector ecology, which is strongly influenced by short–term developments (e.g. disease outbreaks), and generally require short–term strategies (e.g. implementation of a monitoring programme). During the bluetongue epidemic in Germany, this necessity was evident: there was an urgent need to rapidly implement a biting midge monitoring programme in order to identify the native biting midge vectors. Unfortunately, the required quantity of *Culicoides* standard traps (Onderstepoort trap) was not available at that time. Forced to promptly decide on alternatives, scientists and stakeholders had to rely on the modified Biogents Sentinel trap. This trap had previously been developed to sample the yellow fever mosquitoes (*Aedes aegypti*), but had not been evaluated for trapping of *Culicoides* and the study aims (species present/absent, abundances, proportion of males/females, etc.).

Similarities between both groups match all four topics covered by this thesis:

- They are collected with (light–)suction traps, thus there are similar questions regarding the standardisation and performance of trapping.
- They include cryptic and sibling species, which cannot be differentiated by morphological characteristics, thus alternative identification methods (e.g. molecular identification) have to be developed and applied.
- They are widely distributed in Germany, while the correlation or even causal factors with environmental parameters are mostly unknown.

Hence, this thesis will not focus on a specific topic within the areas of biology and ecology for both vector groups. Instead, the recurrent themes are those scientific questions that apply to both biting midges and mosquitoes. These questions are important for improving our knowledge on species distribution and planning effective monitoring strategies.

## Data collection

Following the research process, the next chapter will outline the data collection procedures (Chapter 2). Due to their potential as pest species and vectors of pathogens, many sampling tools have been developed to collect mosquitoes and biting midges. The striking diversity of the methods is obvious, especially in Silver's (2008) book "Mosquito Ecology – Field Sampling Methods". It required as many as 1470 pages to describe all of the methods of data collection for the different life stages, focusing solely on the family Culicidae. Nevertheless, different types of (light-)suction traps are the most common tools used for the monitoring of both mosquitoes and biting midges (Dyce et al., 1972; Silver, 2008). These traps have the advantage of being autonomous sampling devices, i.e. the person in charge of installing will not directly affect the catch and does not even have to attend the trapping process. Nevertheless, these sampling results indirectly depend on the sampling person, because standardisation of the trapping parameters (e.g. type of trap or position to potential hosts) is essential in order to achieve comparable trapping results. Therefore, two papers in this thesis evaluated the trapping efficacy of four widely-used mosquito traps in Central Europe (Paper A.1), as well as the effect of distance on the stabilisation of trapping biting midges (Paper A.2).

## Identification

The species identification step follows the data collection procedures (Chapter 3). Not surprisingly, studies on the ecology of the vectors depend on the reliable identification of the species. At the same time, many vector species are considered to be sibling or cryptic species, as they can hardly be, or in some cases, they cannot at all be, identified based on classical morphological characters. In *An. daciae*/*An. messeae*, e.g., only the eggs are considered to be distinguishable (Nicolescu et al., 2004), and in *Culex pipiens*/*Cx. torrentium*, only the identification of males is considered to be reliable (summarised by Weitzel et al., 2014). However, these morphologically similar species sometimes have remarkable differences regarding their vector competence (e.g. female *Culex pipiens*/*Cx. torrentium*, Lundström, 1994) or insecticide resistance (e.g. members of the *An. gambiae* complex, Ranson et al., 2011), which makes differentiation of these species important. Because classical morphological techniques are not applicable, alternative methods of species identification were developed. The Chapter "Species identification" thus includes two publications, both applying relatively new methods: geometric morphometrics to differentiate female *Cx. pipiens* s.s./*Cx. torrentium* (Paper B.1) and a real-time polymerase chain reaction assays to differentiate the four German members of the *An. maculipennis* group (Paper B.2)

## Species distribution

When the data are compiled, the analyses of the species distribution can be carried out. Species distribution analysis can be conducted on different scales. This thesis both considers the distribution of immature stages on the local scale (i.e. breeding sites) and analyses the distribution of adults on larger scales (i.e. landscape).

First, three papers are included in this chapter. These papers focus on the factors affecting the distribution of mosquitoes and biting midges on the local scale. There is hardly any up-to-date information on the breeding ecology of German mosquitoes, and even less ecological knowledge exists for biting species. Therefore, breeding sites were analysed to evaluate the correlations of physico-chemical data with the presence-absence of *Cx. pipiens* s.l. and *Cx. torrentium* (Paper C.1), the impact of edaphic factors on the density of dung-breeding *Culicoides* biting midges (Paper C.2), and the biting midge density in relation to the position and substrate temperature in a cattle dung heap (Paper C.3).

Second, cooperating with the research group Environmental Informatics of the University of Oldenburg, Paper C.4 presents a prototypic tool, developed to predict the local spread of exotic mosquito species. The introduction and spread of these species in Germany has recently become the most important topic in vector ecology (Becker et al., 2013). However, previous studies did not focus on the spread through human traffic by cars and through autonomous spread on a local scale (e.g. Tatem et al., 2006). The theoretical and practical background for the modelling of this dynamic has been tackled here for the first time.

Third, with the description of a new member of the *An. maculipennis* group (*An. daciae*) in 2004 (Nicolescu et al., 2004), research interests in *Anopheles* mosquitoes in Germany increased again and, thus, Paper B.2 analysed the recent distribution of the various *An. maculipennis* group members. Moreover, the environmental land cover parameters affecting the distribution of *Culicoides* adults in Germany still need to be studied. Predictors on different scales can be used for species distribution modelling. However, because little is known regarding the ecology and flight range of *Culicoides* biting midges (reviewed by Kirkeby et al., 2013), an a priori selection of the appropriate scale for modelling the species distribution was not possible. The Paper C.5, therefore, evaluated which landscape scale, or even multi-scale model, might be suitable to model the distribution of different biting midge species in Germany.

## Breeding ecology of *Culicoides*

Previous studies on the breeding ecology of biting midges in Central Europe were solely descriptive, sampling different potential breeding sites on farms (e.g. Ninio et al., 2011; González et al., 2013). Only a few studies focused on the correlative connection between physical-chemical parameters and species distribution (Foxi & Delrio, 2010; Zimmer et al., 2010; Harrup et al., 2013; Zimmer et al., 2014). Experimental approaches to study the causal association of environmental factors and immature development of biting midges were mostly conducted with species that were not native to Central Europe (e.g. Bishop et al., 96a,b, 2005; Veronesi et al., 2009). Hence, it is unclear if these results are transferable to our native species. Therefore, in intending to analyse basic environmental factors in more detail, three experiments with immature dung breeding *Culicoides* were conducted. These studies cover three different topics: 1) an alternative control method that attempts mechanical disturbance (Paper D.1), 2) the impact of flooding on the survival of immature biting midges (Paper D.2), and 3) the effects of temperature and photoperiod on the development of the overwintering population (Paper D.3).

# Chapter 2

# Data collection

Data collection is the first step in ecological studies. It is accurate in saying that any sampling method has its own advantages and disadvantages (Sutherland, 2006). The knowledge of the pros and cons of a sampling method is important in planning data collection, analysing the resulting data, and allowing for a sensible use of the data in further analyses (e.g. species distribution modelling). Suction traps are most commonly used in the monitoring of mosquitoes and biting midges (Dyce et al., 1972; Silver, 2008). Besides acquisition costs or user-friendliness, there are two important parameters that must be considered: the trapping sensitivity and the trapping efficacy. In this thesis, “trapping sensitivity” is interpreted as the probability of catching a vector species and “trapping efficacy” is defined as the capability to catch high individual numbers.

Recently, there were increasing attempts at using vector data from different sources for large-scaled predictions of species distribution (e.g. Fischer et al., 2014). The most well known vector maps on the European-level are the mosquito, tick, and phlebotomine maps, available from the website hosting the European-wide vector distribution maps from the European Centre for Disease Prevention and Control (http://www.ecdc.europa.eu/). These maps rely on presence-absence data from surveillance activities in the different European countries. However, different monitoring programmes completely, or partly, use different trapping methods, with different sensitivities for the targeted species. Knowing the certainty of the method would help correctly interpret existing distribution maps.

The problems regarding different trapping techniques might even increase in the context of the modelling of the spread of vector-borne diseases, which commonly includes vector abundance as an important parameter (Græsbøll et al., 2012; Bessell et al., 2013; Brugger & Rubel, 2013). Therefore, differences in the trapping efficacy of the different included traps might also affect the result of the spread modelling. Nevertheless, if scientists are aware, that, e.g., trap X has a tenfold higher efficacy in the trapping of a mosquito species in focus compared to trap Y, vector abundance data can be standardised by this factor or the type of trap can be included as a variable in the analyses of the vector abundance.

Results of the sampling and trapping protocols could be significantly affected by environmental factors, like wind and temperature, or even the phase of the moon. These factors cannot be, or are not easy to, control (Bishop et al., 2000; Carpenter et al., 2008). However, these are natural differences between trapping sites, which should be compensated for by using several trapping periods, or such variables (e.g. temperature) should be included in the analysis to understand the differences of the distribution and phenology between the sites. In contrast, further factors can be controlled and should thus be standardised in order to achieve comparable results from entomological surveillance programmes:

- First, the trapping method can be standardised, which could require a different efficacy for abundance, taxa, sex, or feeding stadium (Holbrook, 1985; Anderson & Linhares, 1989; Venter et al., 2009; Viennet et al., 2011).
- Second, some authors have mentioned that the positions of the traps, such as the height of the traps (Venter et al., 2009) or the surrounding habitat of the traps (Bishop et al., 1994, 1995), can have a significant impact on the sampling results.

Several of these factors can be standardised quite easily. However, there is generally a need to identify the effect direction and the effect magnitude of these factors influencing vector sampling.

## 2.1 Paper A.1: Field evaluation of four widely used mosquito traps in Central Europe

Mosquito monitoring and surveillance programmes in Europe use various types of mosquito traps, but only a few studies have compared the different mosquito traps. Therefore, trapping efficacy of the four trap types primarily used in Europe [the Biogents Sentinel (BG) trap, the Heavy Duty Encephalitis Vector Survey (EVS) trap, the Centers for Disease Control (CDC) miniature light trap, and the Mosquito Magnet Patriot Mosquito (MM) trap] was compared. Seventy 24-hour trap comparisons were conducted at 10 different locations in northern and southern Germany, representing urban, forest and floodplain biotopes.

The trapping performance between the four traps were significantly different. Summarising the paper, it can be concluded that the BG trap showed a better or similar performance compared to the CDC, EVS, or MM trap with regard to trapping efficacy for most common mosquito species in Germany, including diversity of mosquito species and number of mosquitoes per trapping period (Fig. 2.1). Thus, the BG trap is probably the best solution for general monitoring or surveillance programmes of adult mosquitoes in Central Europe. However, depending on the study aims, it might be necessary to select another trap, e.g., the CDC trap if the monitoring of *Aedes vexans* is intended (Fig. 2.1).

The objective of this study was to compare the traps when they are being used the way the manufacturers recommend and how the scientists would operate them. However, the four trapping devices used in this study differ in construction and in the mechanisms to lure and trap mosquitoes. Except for the addition of a dry ice bucket

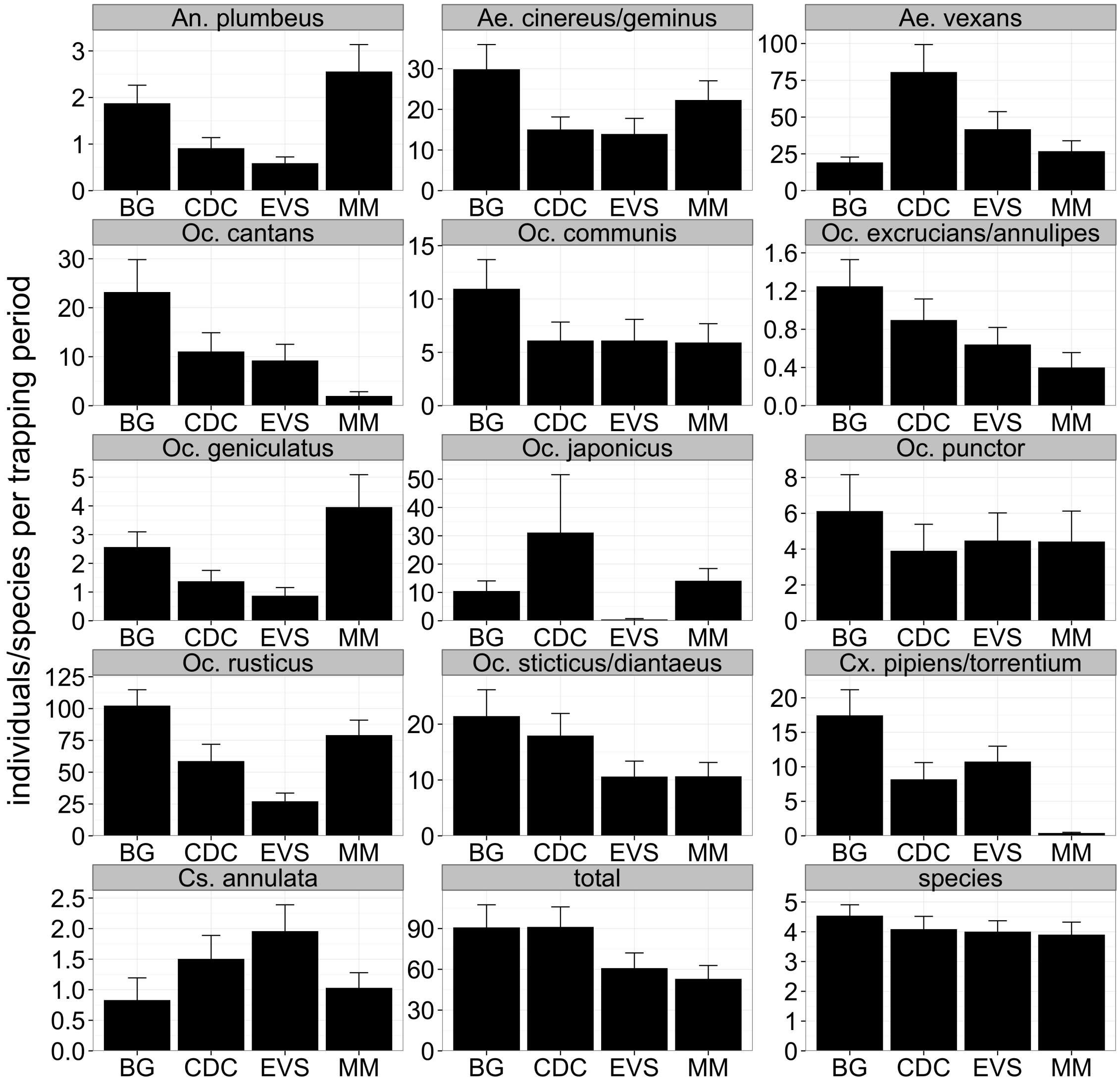


Figure 2.1: Mean ±SE number of trapped individuals per trapping period for each species and the total number of individuals and the mean ±SE number of species among the four trapping devices. Only mosquito species caught with more than 100 individuals are shown and trapping periods were only included if the species was detected with at least one individual in the corresponding trapping period at the sampling location.

to the CDC trap, we did not interfere with recommended trap configurations (e.g. with lure, without lure, or placing the traps at different heights). Therefore, an identification of the factors resulting in differences in the performances was not possible. However, changes of configurations would probably result in a different performance of the various traps. A comprehensive understanding of the trap differences requires standardisation, e.g. using the same amount of $CO_2$ , the same lure for all traps, or placing the traps at the same height.

## 2.2 Paper A.2: Distance from the stable affects trapping of biting midges

Different studies demonstrated positive correlations between biting midge abundance and the presence and abundance of hosts (Bellis & Reid, 1996; Baylis et al., 2010; Garcia-Saenz et al., 2011; Viennet et al., 2011). Therefore, the number of trapped biting midges should decrease with increasing distance to the stable. This study, thus, compared the number of trapped *Culicoides* with Biogents Sentinel (BG) traps at distances of 50 m, 100 m, and 200 m to the stable.

The number of trapped females from the Obsoletus group (Fig. 2.2) and the females from the Pulicaris group significantly decreased with increasing distance to the stable. Because of a significant decrease of collected biting midges, even at small distances from the stable, sampling in the immediate vicinity of stables ($<$ 25 m) probably promises the highest trapping success. However, this also suggests that comparative studies on biting midges should be interpreted carefully if trap positions relative to the stable differ. These results stress the necessity of standardising trap position as far as possible in order to achieve comparable data.

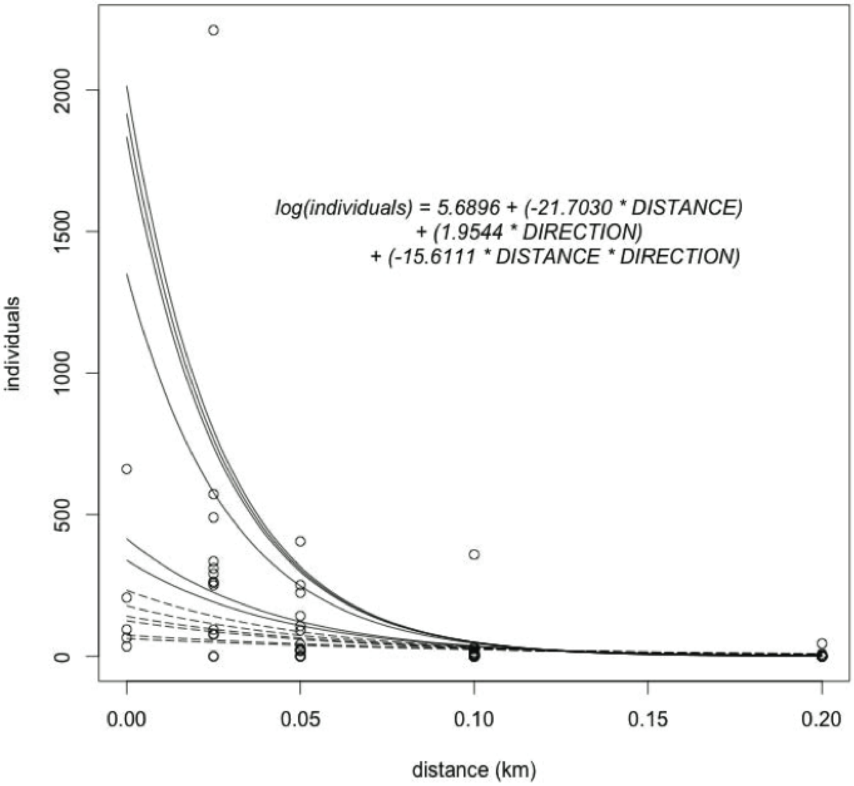


Figure 2.2: Number of females of the Obsoletus group caught with light suction traps positioned in five different distances from the stable on one farm in northern Germany. The best fit regression lines are shown. Solid line: DIRECTION above zero; dashed line: value below zero.

Additional studies have demonstrated a decreasing trapping success of *Culicoides* with increasing distance to the stable (Rigot et al., 2012). However, spatial interference between UV-light suction traps might be a problem, yet the spatial dependence of the BG samples is largely unknown. Rigot & Gilbert (2012) and Kirkeby et al. (2013) demonstrated a significant spatial interaction for the Onderstepoort trap and the Centers for Disease Control miniature light trap. A similar effect has to be expected for the BG traps. The impact on the study results presented here is not predictable because we do not know to what extent the traps were affected by interference. Nevertheless, due to the strong decrease of collected females with increasing distances to the stable, at least for the Obsoletus group (Fig. 2.2), there is probably also a significant impact under the presumption of trap interference. Further studies should investigate the spatial interaction for BG trap in order to improve study designs and allow for spatial-independent samples.

## 2.3 Conclusions

The two studies demonstrated that the different traps and the position of the traps significantly affect the trapping efficacy of the traps for mosquitoes or biting midges. Therefore, the positioning and the type of trap have to be selected with caution when planning to study the distribution of both vector groups. Modelling studies should either include the trap or the distance to the potential hosts as variables, or the data have to be corrected for these variables. The two studies presented here only focused on the trapping efficacy in relation to the type of trap and the distance to potential hosts. However, there might also be a strong impact of these variables on the sensitivity of the traps, which should be evaluated to get a more complete impression of the advantages and disadvantages of the tested traps.

# Chapter 3

# Identification

The most important requirement for ecological studies is a correct identification of the species under study. At the same time, researchers in the field of vector ecology commonly have to deal with cryptic species, which cannot currently be differentiated morphologically, at least not until differentiating morphological characteristics are identified. Even if there is no interest in understanding the ecology of the vectors, the reliable differentiation of the species is especially important if species differ in vector competence, which was, e.g., demonstrated in *Culex pipiens* vs. *Cx. torrentium* (Lundström, 1994). In contrast, female members of the morphologically-similar Obsoletus group are frequently not differentiated further, because there is no knowledge on the vector competence differences between the species (Purse et al., 2015), e.g., in disease spread modelling (Græsbøll et al., 2012; Bessell et al., 2013; Brugger & Rubel, 2013).

As with other species, mosquitoes, and biting midges used to be, and still are, identified using morphological characteristics (Becker et al., 2010; Mathieu et al., 2012). Hence, several of these species cannot be distinguished, e.g., while the males of the Obsoletus group can be easily differentiated using the morphology of the hypopygia, females show partly overlapping characteristics (Garros et al., 2014). Due to problems regarding missing morphological differences within different life stages (e.g. larvae) or between males and females, further methods have been applied to differentiate the species. Two identification assays using these methods were applied in the course of this thesis:

1. **Geometric morphometrics:** This is a relatively new tool for the differentiation of species. The approach uses landmarks (points with an x-, y-, and z-coordinates), semi-landmarks (specified number of points using an algorithm, e.g., between two defined points), outlines, or surfaces to analyse shape differences between species. These shape differences can be used for the identification of species (Muñoz-Muñoz et al., 2011; Lorenz et al., 2012), population structure (Wilke et al., 2014), or the identification of an impact of different environmental parameters on the development processes (Stephens & Juliano, 2012). The method was successfully used to identify different medical and veterinary important phlebotomines (Godoy et al., 2014), mosquitoes (Lorenz et al., 2012), or *Culicoides* (Muñoz-Muñoz et al., 2011).
2. **Real-time PCR-identification:** PCR-based (Polymerase Chain Reaction) molecular assays for the differentiation of multiple, closely related sibling species are common techniques applied to different vector groups, including mosquitoes (Danabalan et al., 2013; Kronefeld et al., 14b) and biting midges (Garros et al., 2014). Real-time PCR is still considered to be in its infancy (Lau et al., 2013). Nevertheless, the method 1) is faster and 2) can minimise the risk of laboratory contamination (Fotedar et al., 2007; Parija, 2011), because the method allows for the single PCR reaction in a closed tube format, i.e., it does not require post-PCR gel electrophoretic analyses.

A current example for the need of new methods to analyse the distribution of vectors is the distribution of *Cx. torrentium.* Martini described the species quite late in 1921. Nevertheless, subsequent research highlighted that the species must be considered rare in Europe (Mattingly, 1951; Service, 1968; Struppe, 1989). Only the development of high-throughput molecular tools allowed for the screening of a large number of samples, e.g. generated during large-scaled monitoring programmes, which demonstrated that the species is, at least recently, quite common (Rudolf et al., 2013).

## 3.1 Paper B.1: The use of morphometric wing characters to discriminate female *Culex pipiens* and *Cx. torrentium*

Female *Cx. pipiens* s.s. and *Cx. torrentium* cannot be differentiated reliably by classical morphological characteristics (e.g. scales or colour) (summarized by Weitzel et al., 2014). In previous studies, some authors proposed that wing characteristics, and especially the ratios of wing veins, might be a reliable characteristic for the differentiation of these species (Natvig, 1948; Mohrig, 1969). This study re-evaluated the quality of wing characteristics. We used a recently-developed PCR protocol (Rudolf et al., 2013) to correlate molecular typing with multi-, bi-, and univariate wing morphometry to differentiate female specimens of *Cx. pipiens* s.s. and *Cx. torrentium.* The analysis of the $r_{2/3}$ / $r_3$ indices resulted in a correct classification of more than 91% of all tested specimens. Using the absolute length of vein $r_{2/3}$ alone still allowed for more than 90% accurate discrimination. Furthermore, the classification accuracy of the linear discriminant analysis exceeded 97%. Thin-plate spline grids indicated that the differences between the species were predominantly expressed in the shrinkage of the $r_{2/3}$ vein (Fig. 3.1).

It remains unclear why other studies from Britain and Russia that used wing morphology did not find a difference in the populations of *Cx. pipiens* s.s. and *Cx. torrentium* (Service, 1968; Fedorova & Shaikevich, 2007). Therefore, future studies should especially focus on the transferability of our results to other locations. Furthermore, experimental studies should evaluate the stability of the wing characteristics under different breeding conditions, e.g. physico-chemical parameters or competition (e.g. Stephens & Juliano, 2012).

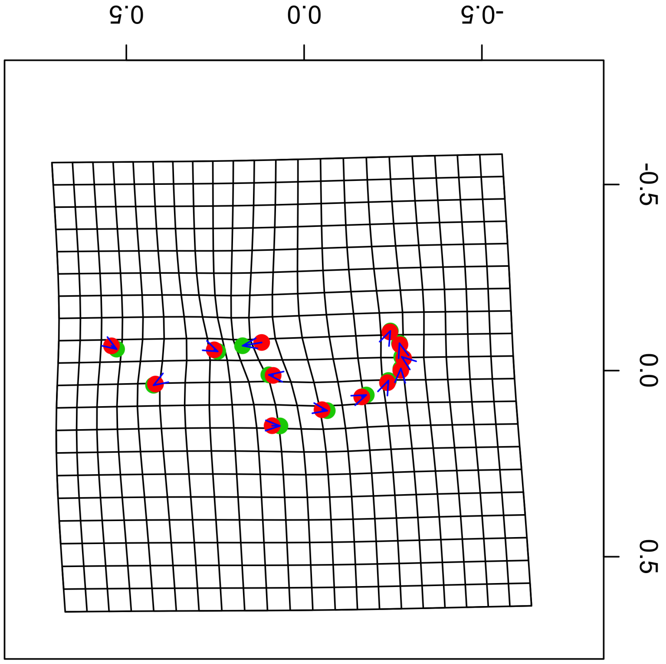


Figure 3.1: The thin-plate spline grid shows the variation in shape of the female right wing (species differences; *Cx. torrentium* - *Cx. pipiens* s.s.). Reference shape is represented by *Cx. torrentium* (red circles), target shape by *Cx. pipiens* s.s. (green circles), blue arrows indicate direction of change. Where regions relatively elongate, the grid look stretched and where those regions are relatively shortened, the grid look compressed.

## 3.2 Paper B.2: Distribution of the various *Anopheles maculipennis* group members from Germany identified by newly developed real-time PCR assays

There are four different members of the *Anopheles maculipennis* group known to be present in Germany: *An. atroparvus*, *An. messeae*, *An. maculipennis* s.s., and the recently-detected *An. daciae* (Talbalaghi & Shaikevich, 2011; Kronefeld et al., 2012;

Weitzel et al., 2012). Morphological species identification is restricted to eggs, while no reliable morphological characteristics are known to differentiate larvae, pupae and adults of the four species (Becker et al., 2010). Until now, only polymerase chain reaction (PCR) methods using agarose gel electrophoresis techniques can be used to identify non-egg stages (Danabalan et al., 2013; Kronefeld et al., 14b). However, real-time PCR (RT PCR) is a faster alternative, which especially reduces the problem of laboratory contamination (Fotedar et al., 2007; Parija, 2011).

Different RT PCR assays were developed targeting nucleotide differences in the internal transcribed spacer 2 (ITS2) gene. First, a multiplex RT PCR assay was established, which is able to identify single individuals of *An. atroparvus*, *An. maculipennis* s.s., and *An. daciae/An. messeae*. Single *An. daciae/An. messeae* specimens are further differentiated using a FRET PCR (Fluorescent Resonance Energy Transfer PCR) (Fig. 3.2). As both assays use primer sequences that are conserved between the different members of the An. maculipennis group, they are not applicable to pools of mosquitoes. Additionally, three singleplex RT-PCRs were developed to identify single individuals of *An. atroparvus*, *An. maculipennis* s.s., and *An. daciae/An. messeae* in *An. maculipennis* group pools of up to 25 individuals, which are commonly used in surveillance programmes for mosquito-borne pathogens to reduce the number of samples to be analysed.

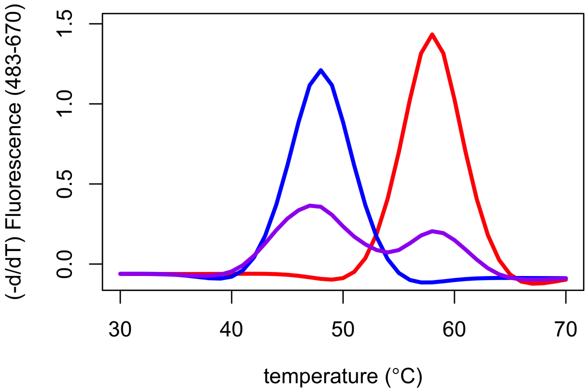


Figure 3.2: Negative derivative melting curves of the real-time PCR for the differentiation of *Anopheles daciae*/*Anopheles messeae* (blue=*An. messeae*, red=*An. daciae*, purple=suspected hybrid of *An. daciae* and *An. messeae*).

## 3.3 Conclusions

Morphometric and molecular techniques are useful for differentiating female *Cx. pipiens* s.s./*Cx. torrentium* and *An. maculipennis* group members, respectively. Such tools can be used to further understand the distribution of these vector species (e.g. Rudolf et al., 2013). Furthermore, both techniques can also be used to understand the environmental factors affecting these species, which is, at least in Germany, a highly-neglected field of research (e.g. see Paper C.1).

# Chapter 4

# Species distribution

In the field of vector ecology, a lot of species distribution modelling is conducted (e.g. Fischer et al., 2014). At the same time, distribution modelling is a very wide field of research with numerous available tools, techniques, and much more published research (e.g. Elith & Leathwick, 2009). Nevertheless, regarding vector ecology, there are still many open research questions in Germany. In particular, the recent species distribution and the underlying environmental factors are hardly studied for mosquitoes and biting midges.

An important decision before starting the modelling is the decision on the scales. This decision should be based on the research question (e.g. breeding ecology of a vector species), but is very often influenced by the availability of environmental data provided on a specific scale (e.g. land cover data only on a 10 km scale). However, in both cases, the selected scale does not need to be appropriate in order to model or even understand the species distribution. As previously shown by Hamer & Hill (2000), the selection of the spatial scale affects the outcome of the modelling, e.g. decreases the variance explained or bias regression coefficients, which might result in wrong conclusions and interpretations (reviewed by Bradter et al., 2013). This chapter summarises papers on different scales, which were carried out on the local scale, i.e. breeding sites (Paper C.1, Paper C.2, and Paper C.3), and the large scale, i.e. landscapes (Paper B.2, Paper C.4, and Paper C.5).

Solely focusing on large-scaled variables in ecological studies on mosquitoes and biting midges could result in an interpretational problem. For example, an increasing probability of a biting midge species occurrence with an increase of the land cover parameter "forest" around the trapping sites might be only explainable, when it is known that fallen leaves characterise the species breeding sites. Additionally, analysing the distribution of the species on the local scale might also work as a ground truth for large-scaled models, i.e. give indications if the correlations with large-scale variables are causal or correlative. In the past, such studies were partly hampered by the inability to identify the immature stages (e.g. *Culex pipiens* s.l./*Cx. torrentium*) or by a general missing interest due to missing relevance as a vector (e.g. *Culicoides* species). This chapter includes three different studies conducted to achieve more detailed information on the breeding ecology of mosquitoes and biting midges: 1) correlation of the presence-absence of *Cx. pipiens* s.l./*Cx. torrentium* with physical-chemical parameters (Paper C.1), 2) the abundance of *Culicoides* in cowpats and dung heaps with edaphic parameters (Paper C.2), and 3) with temperature/height (Paper C.3).

Most predictive models only focus on the static prediction of the potential distribution of vector species. However, the distribution of exotic species which were recently found in Germany (Becker et al., 2013; Kampen & Werner, 2014), is in particular affected by dynamic processes, such as autonomous and human mediated dispersal (Huber et al., 2014; Zielke et al., 2014). These dynamics have to be processed with dynamic models, e.g. a cellular automaton, which is described in Paper C.4.

Due to their role as vectors of malaria, the *Anopheles maculipennis* group members were intensively studied in the past (e.g. Martini et al., 1931; Weyer, 1938, 1941), but an updated overview of the species distribution of all four *Anopheles maculipennis* members known for Germany is still missing. Paper B.2 analysed the recent distribution of the various members with a distribution modelling method using random forest variable selection with twelve environmental variables: bioclimatic (4), altitude (1), and land cover (7).

The final paper (Paper C.5.) analysed land cover variables and tries to determine if the presence-absence of German *Culicoides* species is correlated to some of these variables. As little is known about the ecology and flight range of biting midges, a selection of the appropriate scale for the modelling of species distribution a priori was not possible. Therefore, the same landscape variables were extracted from different spatial scales, which were used separately for single-scale models or all together for multi-scale modelling in order to predict species distribution.

## 4.1 Paper C.1: Physico-chemical characteristics of *Culex pipiens* s.l. and *Culex torrentium* breeding sites in Germany

As already mentioned before (Paper B.1), morphological characters fail to distinguish the females as well as the immature stages of *Cx. pipiens* s.l. and *Cx. torrentium* (summarised by Weitzel et al., 2014). Hesson et al. (2011) already highlighted that the information on the breeding ecology of *Cx. pipiens* s.l. and *Cx. torrentium* is, at best, fragmentary, and a comprehensive study on the breeding ecology of both species is still needed. Therefore, this study aimed to examine the correlation between physico-chemical parameters of water bodies and the presence-absence of immature *Cx. pipiens* s.l. and *Cx. torrentium*, which had been precisely differentiated by molecular methods. As these species differ in vector competence (Lundström, 1994), the knowledge on their breeding site preferences is assumed to help to understand the distribution of both species and associated pathogens.

However, Medlock et al. (2005) summarised that there are no bionomic differences between the species. At least on the level of breeding sites and based on the environmental parameters included, this study does not allow another conclusion. The results revealed that the two species often occur in sympatry at the studied breeding sites, and there were no substantial differences concerning presence-absence of the two species with regard to the various environmental parameters investigated.

As highlighted by Silvertown (2004), there is a general problem of identifying the appropriate niche axes, i.e. environmental parameters, prior to analysis. Accordingly, it might be possible that additional factors which were not included into this study, such as dietary niche separation, might be responsible for a partitioning within the breeding sites along some other ecological axis. Dahl et al. (1993) demonstrated selective particle uptake by larvae of different mosquito larvae, and Gilbreath et al. (2013) found resource partitioning between the larvae of two regularly sympatric mosquito species. Nevertheless, it can be concluded that both species probably have a strong overlap in their realised ecological niche, but there might be a divergence in respect to other breeding site parameters not studied in detail so far. Therefore, future research on the differences in the breeding ecology of both species should focus on the evaluation of relative species abundances and experimental studies to test competition theory (e.g. see Silvertown, 2004).

## 4.2 Paper C.2: Impact of edaphic factors on the presence of dung breeding *Culicoides*

*Culicoides chiopterus* and *C. dewulfi* are expected to exclusively breed in dung (Kettle & Lawson, 1952). Therefore, both species should be commonly present on livestock farms, as it was also proposed for the dung-breeding *C. brevitarsis* (Cannon & Reye, 1966). Nevertheless, these species do not necessarily show an equal distribution, and abundance and environmental factors appear to be important. Scolamacchia et al. (2013) emphasised that soil parameters were correlated with the abundance of both species, which had been recorded by light trap samples. However, species specific differences characterised the statistical response. While the abundances of *C. dewulfi* correlated negatively with moisture-retentive soils and positively with organic content of the soil, *C. chiopterus* responded in an opposite way. However, due to missing information on the breeding sites and comparable modelling studies, a comprehensive explanation of these modelling results was not possible. It remained to be resolved if these results actually reflect the impact of soil conditions or if these are only proxies for other environmental variables not included. The objective of this study therefore was to compare the colonisation densities of *Culicoides* biting midges in cowpats in relation the edaphic parameters of the corresponding topsoil. A better knowledge of this aspect of *Culicoides* breeding ecology will help to understand the distribution of both species, e.g. the interpretation of modelling results using soil information.

The results of the study highlight the importance of soil moisture, organic substance, and soil texture on the number of emerging *C. chiopterus* and *C. dewulfi*. Increasing soil moisture positively correlated with the number of emerging adults for both, while the variable organic substance was only positively correlated with the abundance of *C. chiopterus*.

Indeed, physical-chemical parameters of the cowpat itself might have an important impact on the distribution and abundance of dung breeding *Culicoides*. However, large-scaled information on cowpats is, of course, not available, and therefore also could not be used for large-scaled species distribution models. In contrast, soil parameters are available and already applied for *Culicoides* species distribution modelling, e.g. the Harmonised World Soil Database (Scolamacchia et al., 2013).

The models in this paper showed conflicting correlations between *C. chiopterus* and the soil texture variables. The species showed a positive correlation with increasing proportion of substrate with larger grain sizes, and at the same time a positive correlation with the proportion of substrate with smaller grain sizes. According to Scolamacchia et al. (2013), *C. chiopterus* prefer moisture-retentive soils, i.e. soils with a higher proportion of smaller grain sizes. However, the conflict in the results of this study might be caused by the usage of several highly correlated variables in the models, which make it difficult to identify the concrete correlation.

## 4.3 Paper C.3: *Culicoides* biting midge density in relation to the position and substrate temperature in a cattle dung heap

At least in Northern Europe, biting midges need an overwintering strategy to resist harsh climate conditions with low winter temperatures. However, at the same time, vector surveillance studies in Northern Europe indicated very low but regular activity of adult *Culicoides* also in winter (Losson et al., 2007; Bauer et al., 2009; Clausen et al., 2009; Hoffmann et al., 2009; Kiel et al., 2009; Mehlhorn et al., 2009). These findings raised a discussion about whether biting midges caught in winter times represent an overwintering adult population or were recently emerged imagines (Losson et al., 2007). The hibernation strategy of biting midges probably is an important factor influencing the overwintering of the Bluetongue virus or Schmallenberg virus (Koenraadt et al., 2014). Dung heaps offer warm breeding sites (Husted, 1994), which might be suitable for a continuing development or even emergence at low air temperatures in winter. This study recorded the density of immature *Culicoides* in dung heap material and started to describe substrate temperature at the sites where immature stages where found.

Depending on the amount of decaying organic material, it seems reasonable that the temperature differs within a dung heap (Husted, 1994). While the density of larvae was negatively correlated with increasing substrate temperatures, *Culicoides* larvae were found in a temperature range between 7.9 and 38.0 °C (mean 16.6 °C) (Fig. 4.1). It can be concluded that dung heaps offer temperature conditions which allow the survival and probably also the development to adults for immature *Culicoides* also under harsh climate conditions in winter.

Future research regarding the ecology of biting midges should focus on the life-history parameters of *Culicoides* species and determine temperature thresholds and time of development under different temperature regimes, which was already done for *C. imicola* (Veronesi et al., 2009). This information can be used in mechanistic models

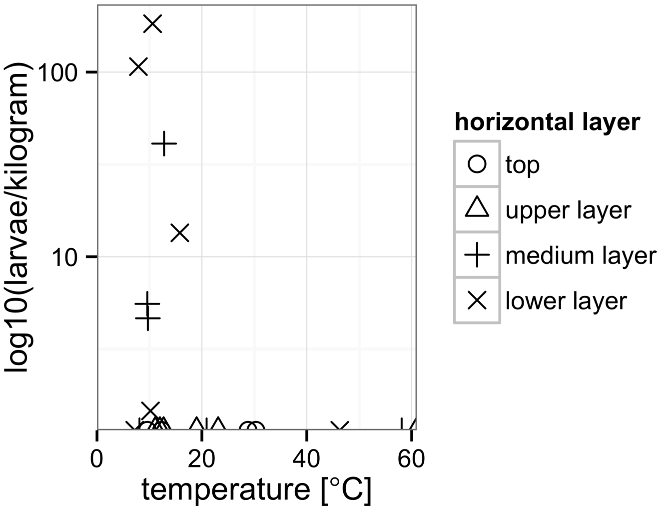


Figure 4.1: Immature *Culicoides* densities per kilogram recorded in the outer layer of a dung heap in response to sampling spot temperatures. Further differentiated according to the four horizontal layers: top (N=4), upper layer (N=6), medium layer (N=6), and lower layer (N=6)

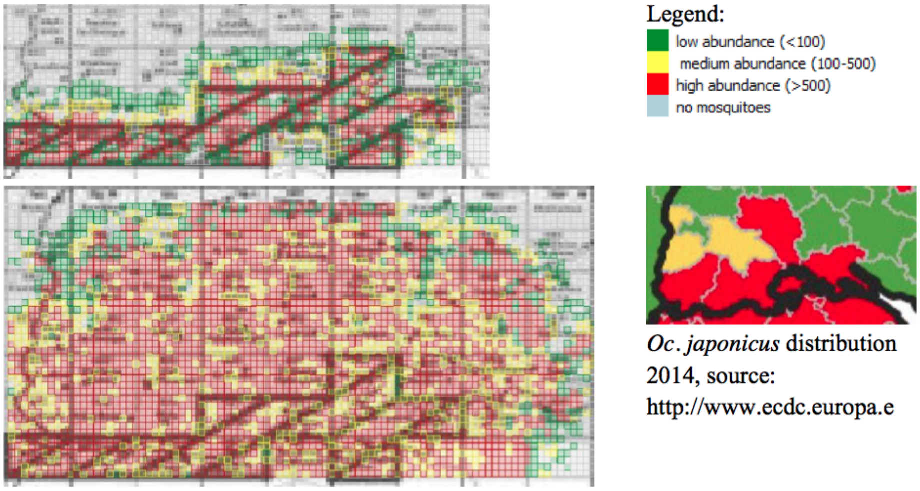
to predict *Culicoides* phenology, e.g. probability of winter emergence from dung heaps. Furthermore, it is just as important that future breeding site studies do not omit the winter period, which has previously not been included in most of studies.

## 4.4 Paper C.4: A tool for simulating the spread of invasive mosquitoes

At least for the Asian tiger mosquito (*Aedes albopictus*), there are several habitat models on the basis of climate data and landscape parameters, which give information on the actual and potential distribution in Europe (e.g. Fischer et al., 2014). However, there are only a few studies on the spatial-temporal spread of invasive mosquito species via traffic (Tatem et al., 2006). This applies in particular to the small-scaled, regional spread through natural dispersal and human transport (e.g. cars). Information on the potential spread of invasive species especially can help to develop an adequate surveillance program and control strategies. Therefore, the aim of this study was to develop a software tool that is able to model the local spatial-temporal spread of invasive mosquitoes through natural dispersal and their transportation through human transport via cars.

The modelling results revealed a much faster spread of the invasive *Ochlerotatus japonicus* compared to the observed spread in the real world (Fig. 4.2). This result might be explained by different reasons: 1) the input data do not have a very good quality and are only based on a very broad meshed mosquito survey. Accordingly, colonised areas with very low densities of *Oc. japonicus* might not be detected. Hence, the currently published distribution maps might not reflect the actual distribution of the species. 2) Furthermore, the model uses several assumptions and simplifications, e.g. each cell has the same, comparatively high temperature (20 °C) and there are no temperature variations in space and time, which significantly accelerate the spread in the model. Therefore, the incorporation of upper and lower development temperature thresholds might give a more realistic picture.

However, the ultimate objective of this project is to establish a tool which allows for predicting the future spread and distribution of the exotic mosquito species. This tool offers extensive application possibilities, e.g. an exact geographic definition for the design of mosquito surveillance or control programmes. The next steps in this ongoing project are the evaluation of the system by real-world scenarios as well as the enhancement of the layers by a refined concept of time.



*Oc. japonicus* distribution 2014, source: http://www.ecdc.europa.e

Figure 4.2: Simulated mosquito occurrences after pre-simulation (left, upper) and after 120 timesteps (left, lower). Positive *Oc. japonicus* raster grids in 2011 (Huber et al., 2012) in a resolution of a topographic map (1:25.000, 18km x 10km) are edged dark gray (left, upper). The small sketch in the right shows the observation from *Oc. japonicus* in this region from 2014.

## 4.5 Paper B.2: Distribution of the various *Anopheles maculipennis* group members from Germany identified by newly developed real-time PCR assays

Between 2011 and 2013, a total of 722 individuals and 95 pools of the *An. maculipennis* group were collected from 72 different sites in Germany. These samples were analysed with a newly developed RT PCR assay for the identification of the four species using nucleotide differences in the internal transcribed spacer 2 (ITS2) gene (see Paper B.2 in Chapter 3). All gathered data were used for species distribution modelling using random forest variable selection with twelve environmental variables.

Species distribution models demonstrated that bioclimatic variables, as well as altitude and land cover are useful variables for the prediction of the species distributions in Germany. *Anopheles atroparvus* was restricted to the coastal regions and northwestern Germany. In contrast, *An. maculipennis* s.s. had a wide distribution, with a lower occurrence probability in eastern and southern Germany. Finally, *An. messeae* showed a decreasing occurrence probability from the north to the south, whereas *An. daciae* did not occur in western and southern Germany.

Most remarkable was the detection of suspected hybrids between *An. daciae*/*An. messeae* (Fig. 3.2) and the lower prevalence of *An. atroparvus* compared to historic data (Fig. 4.3). The decline of *An. atroparvus* might be explained by the differences in the overwintering strategy compared the other *Anopheles* species. *Anopheles atroparvus* is expected to be more dependent on shelter sites in the immediate vicinity to cattle hosts that were lost as a result of a changed farm building design.

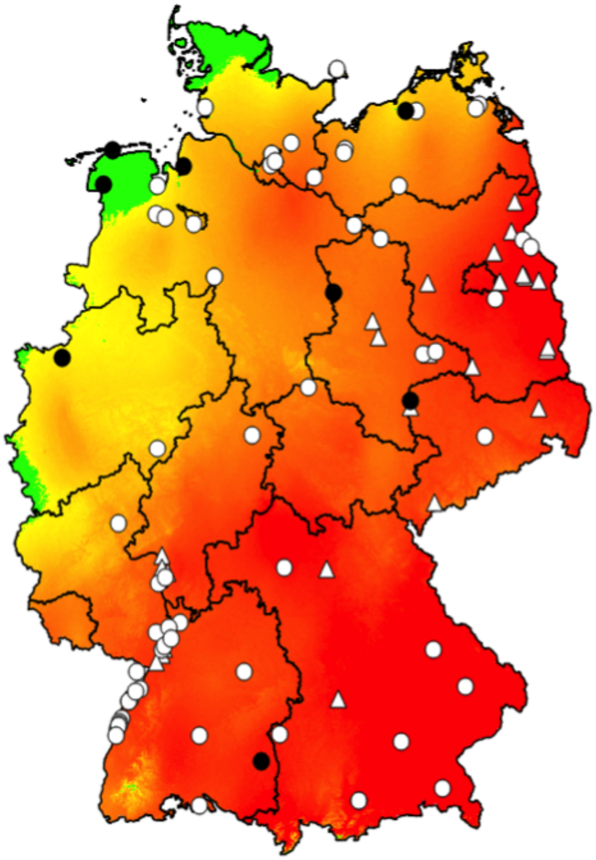


Figure 4.3: Distribution of *An. atroparvus* in Germany. Distribution data: dots=data gathered in this study, triangles=data from other studies (Weitzel et al., 2012; Kronefeld et al., 14b); black=presence, white=absence. Prediction of species distribution: probability of occurrence increase from red over orange to yellow, green indicate presence according the selected threshold.

## 4.6 Paper C.5: Comparison of single- and multi-scale models for predicting the distribution of *Culicoides* biting midges

This study analysed *Culicoides* presence-absence data from 46 sampling sites in Germany, where monitoring had been carried out from April 2007 until May 2008. *Culicoides* presence-absence data were analysed in relation to data from the Automatic Topographic Cartographic Information System (ATKIS® ) in order to prove whether the prevalence of biting midges is correlated to land cover data around the trapping sites. For the analysis eight scales were differentiated, i.e. buffer zones with the radii 0.5, 1, 2, 3, 4, 5, 7.5, and 10 km around each site were differentiated. Several land cover variables (e.g. arable land) were selected in order to characterise the environment around the traps for each buffer zone. Eight single-scale models based on averaged generalised linear models and two multi-scale models (averaged generalised linear models and generalised linear models with random forest variable selection) were tried to build for each species.

The study demonstrated that multi-scale modelling is a promising approach to model the distribution of *Culicoides* species. A multi-scale approach offers the opportunity to include a diverse set of variables from different scales. This is especially important for hematophagous insects, e.g. when breeding sites, resting sites, or host density have to be taken into account for modelling, which are generally distributed across several scales.

The most important problem of this study is the interpretation of the final models. Several species were influenced by different land cover variables on different scales or the same variables have a different algebraic sign (positive or negative) on different scales (Fig. 4.4), e.g. a negative correlation with forest on the local scale and a positive correlation with forest on a larger scale. This interpretational problem even increases in multi-scale models in which one final model can include the same variable on different scales with different algebraic signs.

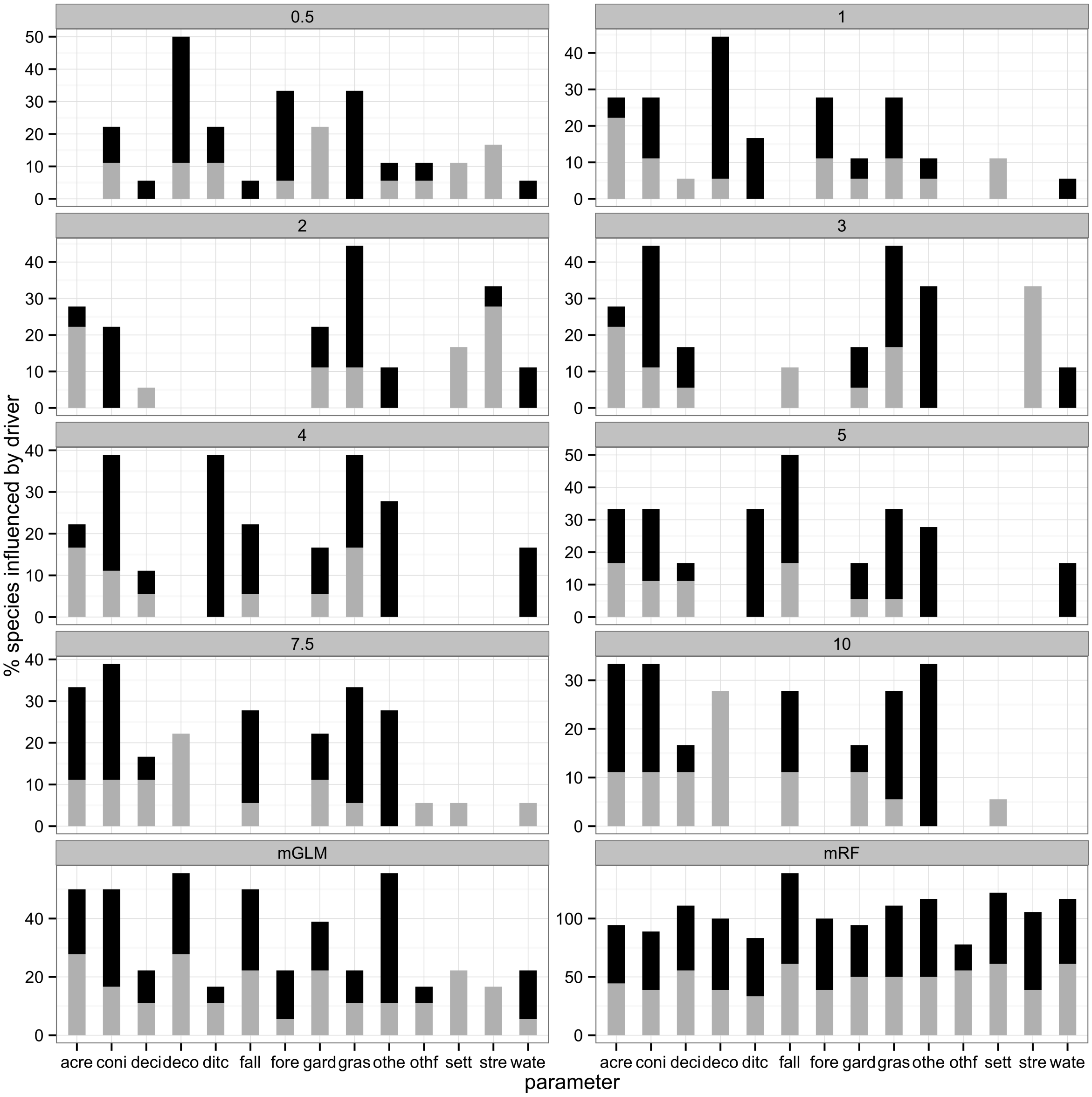


Figure 4.4: Percentage of species influenced by each variable in the different models (gray = positive coefficient, black = negative coefficient). Single-scale models on the eight different scales and multi-scale models (multi-scale model built with model-averaging = mGLM and random forest variable selection = mRF) are shown. For the abbreviations of the coefficients, please refer to Table C.5 in the Appendix C.

## 4.7 Conclusions

The papers presented in this chapter answered many questions regarding the ecology and distribution of mosquitoes and biting midges in Germany, but even more new questions follow, e.g.:

- Is there a difference in the breeding ecology of *Cx. pipiens* s.l. and *Cx. torrentium*?
- Do any physico-chemical parameters of the cowpats affect the distribution of *C. dewulfi* and *C. chiopterus*?
- What are the temperature thresholds and time of development under different temperature regimes for native *Culicoides* species?
- Is it possible to adapt the simulation tool in an appropriate way to correctly predict the spread of *Oc. japonicus* in Germany?
- Which factor is actually responsible for the decline of *An. atroparvus*?
- At which scales are the different resources (breeding sites, hosts, resting sites) for *Culicoides* biting midges distributed, and therefore on which scales should we extract environmental parameters for species distribution modelling?

Clearly, all six papers indicated a lack of knowledge about the causal connections between the species distribution and the species density with environmental parameters. Therefore, besides the evaluation of different modelling techniques and the implementation of further parameters, there is an urgent need for experimental studies on these relationships. The next chapter gives the results of three experimental studies conducted to analyse the causal relationships in more detail.

# Chapter 5

# Breeding ecology of *Culicoides*

partly published: **Lühken, R.**, Steinke, S., Kiel, E. (2014). Potential impact of climate and landscape change on mosquitoes and biting midges in Germany. In: Lozán, J. L., Grassl, H., Karbe, L. & G. Jendritzky (Eds.), *Warnsignal Klima: Gefahren für Pflanzen, Tiere und Menschen.* 2. Auflage. Elektron. Veröffent. (Kap. 3.2.17), [German].

The previous chapter covered different studies which used large- or small-scaled environmental data to model the distribution of mosquitoes and biting midges. However, most of these studies do not provide more than a description of correlation. These models give important information, but of course also have limits. The data basis for mosquito and biting midge models is mostly based on (light-)suction trap data. The different trap types (e.g. Biogents Sentinel trap or Centers for Disease Control miniature light trap) used in the various monitorings in Europe do not have the same efficacy to trap different species (Lühken et al., 2014). Furthermore, they usually do not allow an interpretation of the real population size in the field, because most of them target a specific subgroup of the total population (Viennet et al., 2011), e.g. $CO_2$ -traps predominantly trap host-seeking females. Besides this general problem with the reliability of the data, Reiter (2001) demonstrated that the impact of climate conditions are, at least for mosquitoes, not the most important factor for the distribution of mosquito-borne pathogens. Direct human activities (e.g. the provision of breeding sites through drinking-water-tanks), however, can change the distribution and spread of vector-associated pathogens much stronger.

There is an incomplete picture of the breeding site preference and the biotic and abiotic parameters affecting the colonisation and population dynamic of native mosquitoes and biting midges in Germany. Different studies successfully demonstrated that this knowledge could be helpful to predict the species distributions. Modelling strategies for the Asian tiger mosquito (*Aedes albopictus*), for example, successfully implemented professional expertise on the autecology of the species. The included data, here the tolerated, minimal temperature (Fischer et al., 2014), were deduced from sampling data, because there are only few laboratory studies giving information on this topic. However, the data from the field studies were acceptable to get relative good distribution maps and predictions of the current distribution (Caminade et al., 2012). At the same time, much information is missing for this species in Europe, e.g. information on the speed of local, ecological adaptation or information on the number of individuals, which are needed for the establishment of a population in a non-colonised area (Fischer et al., 2014). A bigger knowledge gap existed regarding the ecology of the Asian bush mosquito (*Ochlerotatus japonicus japonicus*). Several studies documented a fast spatial and temporal spread of the species in Germany (Becker et al., 2011; Huber et al., 2012; Kampen et al., 2012; Werner et al., 2012; Werner & Kampen, 2013). At the same time, there is no information on the biotic and abiotic conditions in the breeding sites.

Most European studies on the breeding sites of biting midges were conducted at agricultural farms analysing the prevalence of the species in different substrates (e.g. cowpats, dung heaps, etc.) (Foxi & Delrio, 2010; González et al., 2013), but only few studies analysed the underlying biotic and abiotic factors. Zimmer et al. (2013) found a negative correlation between the number of *Culicoides obsoletus*/*C. scoticus* larvae and pupae and the magnesium and calcium content in the silage residues, but a positive correlation with the content of acid detergent fibre and acid detergent lignin. Another study in England found a positive impact of soil moisture and pH on the distribution of *C. obsoletus* in breeding sites (Harrup et al., 2013). In contrast, Zimmer et al. (2014) found no significant correlation between physico-chemical parameters and the presence or abundance of different *Culicoides* species.

As for mosquitoes, several studies for biting midges demonstrated that information on the autecology of the species could help to interpret results from modelling studies. Purse et al. (2011) highlighted conflicting modelling results for *C. impunctatus*, with a negative correlation of cattle density and a positive correlation with the surrounding land cover of pasture. However, previous studies indicated that the species has a preference for high densities of rush (Blackwell et al., 1994, 1999). Therefore, it was concluded that the species prefers pastures, which have a high percentage of rush due to high soil moisture and moderate grazing (Purse et al., 2011). Another example is *C. imicola*. A modelling study by Wittmann et al. (2001) revealed that the species does not occur in areas with a yearly precipitation higher than 1,000 mm. However, a sensible interpretation of these results only allows the knowledge on the ecology of the species with pupae, which cannot float and will die if the breeding sites are flooded (Nevill et al., 2007).

## 5.1 General comments on the method

The three publications presented below all studied *Culicoides* species breeding in cow dung. Therefore, this section presents a short discussion of the study methods. Already in the year 1966, Cannon & Reye emphasised that cowpats provide the possibility to conduct laboratory studies in order to understand the biology of dung breeding *Culicoides*. Later, Campbell (1974) proposed a similar idea and stated that a comparison of emergence from *Culicoides* adults is the best way to estimate the density of juveniles, because extraction methods mostly underestimate juvenile density. However, this was not supported by a recent comparative study (Steinke et al., 2014). Cowpats, in particular, provide the advantage that they can be taken to the laboratory as a whole, while the *Culicoides* stay in their natural breeding site. This is especially an advantage for developmental stages, which are difficult to extract (e.g. immotile eggs) (Allingham, 1991). Furthermore, they are known breeding sites for potential vectors of the Bluetongue virus in Northern Europe: *C. chiopterus* and *C. dewulfi* (Kettle & Lawson, 1952; Dijkstra et al., 2008).

On the other hand, there are some problems regarding the use of cowpats for the analysis of *Culicoides* ecology. Cowpats have the advantage of being predominantly colonised by only two biting midge species (*C. dewulfi* and *C. chiopterus*); this fact might turn into a disadvantage if the study aims to draw conclusions on the general ecology of biting midges. Furthermore, different studies detected strong colonisation difference between the cowpats (Zimmer et al., 2014). Although scientists can compensate for this problem by separating the cowpats in quarters and apply a treatment for each, the distribution of biting midges breeding in cowpats is unknown. Therefore, further studies should focus on this topic, e.g. the position of immature *Culicoides* in cowpats (summarised by Bishop et al., 96b). Another problem inheres in the collection of colonised cowpats, if aiming to study different life stages of *Culicoides*, i.e. eggs and different larval stages. Until today, the species of the Obsoletus group cannot be kept in laboratory cultures, which would allow the gain of each life stage. Additionally, the phenology of the species is not known well enough to allow exact samplings of specific life stages from the field.

## 5.2 Paper D.1: Impact of mechanical disturbance on the emergence of *Culicoides* from cowpats

Recently, the most frequently used method in order to control biting midges is the application of insecticides to habitats where larvae develop, resting sites, or host animals, while these were generally not helpful to reduce pathogen transmission (reviewed by Carpenter et al., 2008). On the other hand, data on *C. brevitarsis* recorded by Bishop et al. (2005) in Northern Australia revealed that emergence from cowpats decreased when this substrate had been naturally disturbed by dung beetles. The authors attributed this decrease in emergence to alterations of the dung texture (e.g. thickness of crust), causing changes in physico-chemical processes (e.g. drying). Inspired by these scientific publication, a study was conducted to evaluate the impact of mechanical disturbance on the emergence of *Culicoides* from cowpats, representing important breeding sites for *C. chiopterus* and *C. dewulfi* in Northern Europe (Kettle & Lawson, 1952; Dijkstra et al., 2008). The compact structure of cowpats was destroyed with a trash rake and the dung spread over the ground. However, there were no significant differences between the emergence of *Culicoides* from disturbed and control cowpats (Fig. 5.1).

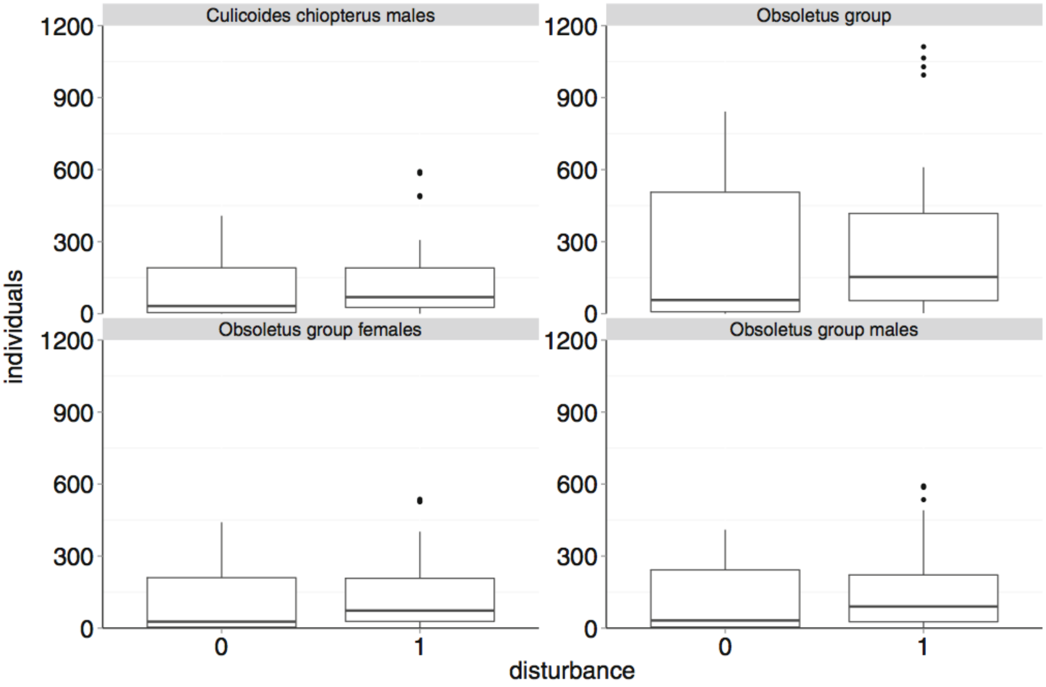


Figure 5.1: Number of emerged biting midges from disturbed (disturbance=1, n=23) and control (disturbance=0, n=23) cowpats between April 4, 2011 and May 2, 2011.

Concluding from the results published by Bishop et al. (2005), the numbers of emerging adults were assumed to be significantly reduced when cowpats had been mechanically disturbed. Surprisingly, the study results did not confirm this hypothesis, i.e. no significant reduction of emerging *Culicoides* biting midges by disturbance was recorded. For the interpretation of this result, some framework conditions of our study have to be considered. First of all, the time period of the experiment could be important. *Culicoides* larvae overwinter in the third or fourth larval stage (Mellor, 1990), which might be relatively tolerant against the kind of applied mechanical disturbance. Additionally, larvae and pupae breeding in cowpats are generally exposed to harsh environmental parameters during the winter period (e.g. sunlight, temperature, snow, or rain) for a comparatively long time. Hence, compared to the natural disturbance during the winter, the mechanical disturbance applied might have been only of low impact.

## 5.3 Paper D.2: Impact of flooding on the immature stages of dung-breeding *Culicoides* in Northern Europe

Soil wetness is known to be important affecting the distribution of several *Culicoides* species (e.g. Peters et al., 2013). Furthermore, flooding is known to be one of the most important factors for the mortality of immature *C. imicola* because the pupae cannot float (Nevill, 1967). Members of the Obsoletus group are also considered to avoid waterlogged habitats (González et al., 2013). However, this was not deduced from experiments on the impact of flooding on immature stages, but from studies on the breeding habitats (Hill, 1947; Zimmer et al., 2008). Therefore, this study aimed to evaluate the impact of flooding on the development of immature *C. chiopterus* and *C. dewulfi* breeding in cowpats.

The emergence of Obsoletus group individuals were compared for cowpats kept at four different moisture regimes in a greenhouse: (1) “dry” (no water added), (2) “control” (regularly moistened), (3) “alternately flooded”, and (4) “permanently flooded” to compare *Culicoides* emergence. Flooding had a significant negative impact on the emergence of *Culicoides* (Fig. 5.2). No individuals emerged from the “permanently flooded” treatment, and only two individuals were sampled from the “alternately flooded” treatment. The results highlight the importance of soil moisture on the distribution of *C. chiopterus* and *C. dewulfi*.

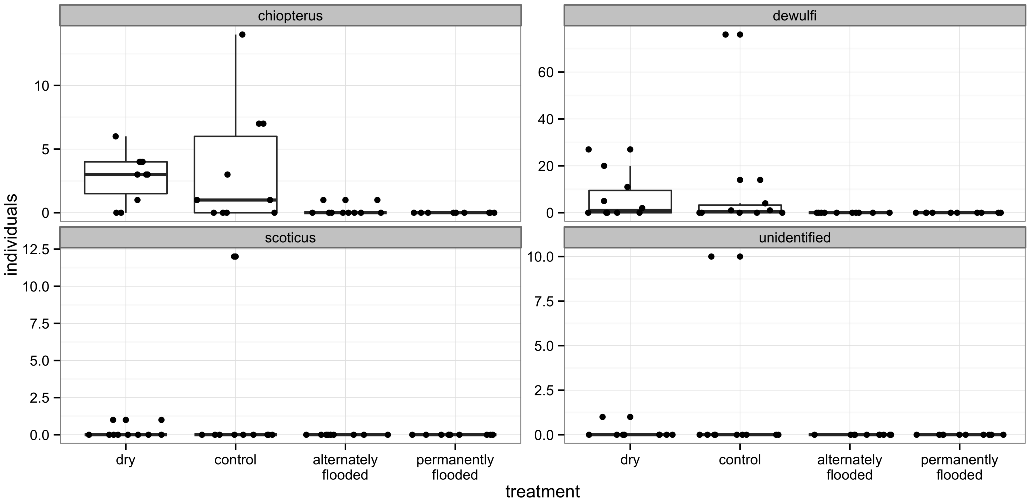


Figure 5.2: The number of individuals of *Culicoides* species emerging from samples in the four treatments. A random jitter was added to visualise the raw data.

Concluding from physiological and behavioural adaptations of other *Culicoides* species, it can be argued that pupae of *C. chiopterus* and *C. dewulfi* are in danger of drowning when breeding sites are flooded as they cannot float. However, it cannot be concluded from these experiments whether the strong reduction in emergence is caused by a high mortality of larvae, pupae, or both, which should be evaluated in further studies.

## 5.4 Paper D.3: Effect of temperature and photoperiod on the development of overwintering immature *Culicoides chiopterus* and *Culicoides dewulfi*

At least in the temperate climate zone, there is a recurrent phenological pattern characterised low numbers or a total lack of *Culicoides* adults during the winter period followed by a population peak in spring (Hoffmann et al., 2009). However, the factors stimulating the development of overwintering biting midges and causing the spring emergence are unknown. Therefore, this experimental study addressed the question whether temperature, photoperiod, or the combination of temperature and photoperiod affect the development of overwintering immature dung breeding *Culicoides* resulting in this peak.

The experiment highlights the importance of temperature on the spring emergence of *C. chiopterus* and *C. dewulfi* (Fig. 5.3), both of which did not respond differently to the four temperature-photoperiod regimes

The development of immature biting midges under different photoperiod regimes has hardly been investigated experimentally. In this study we compared two different photoperiod regimes for overwintering immature *Culicoides* collected from the field on the same date. The results from the experiment presented in this paper can be brought into alignment with data collected by Searle et al. (2012), who revealed juvenile development to correlate with day length. If it is assumed that overwintering biting midges are in diapause and if a critical photoperiod of around eight hours daylight is supposed to be the relevant stimulus, day lengths shorter than the critical photoperiod should maintain diapause, while day lengths longer than the critical photoperiod should terminate diapause (Tauber & Tauber, 1976).

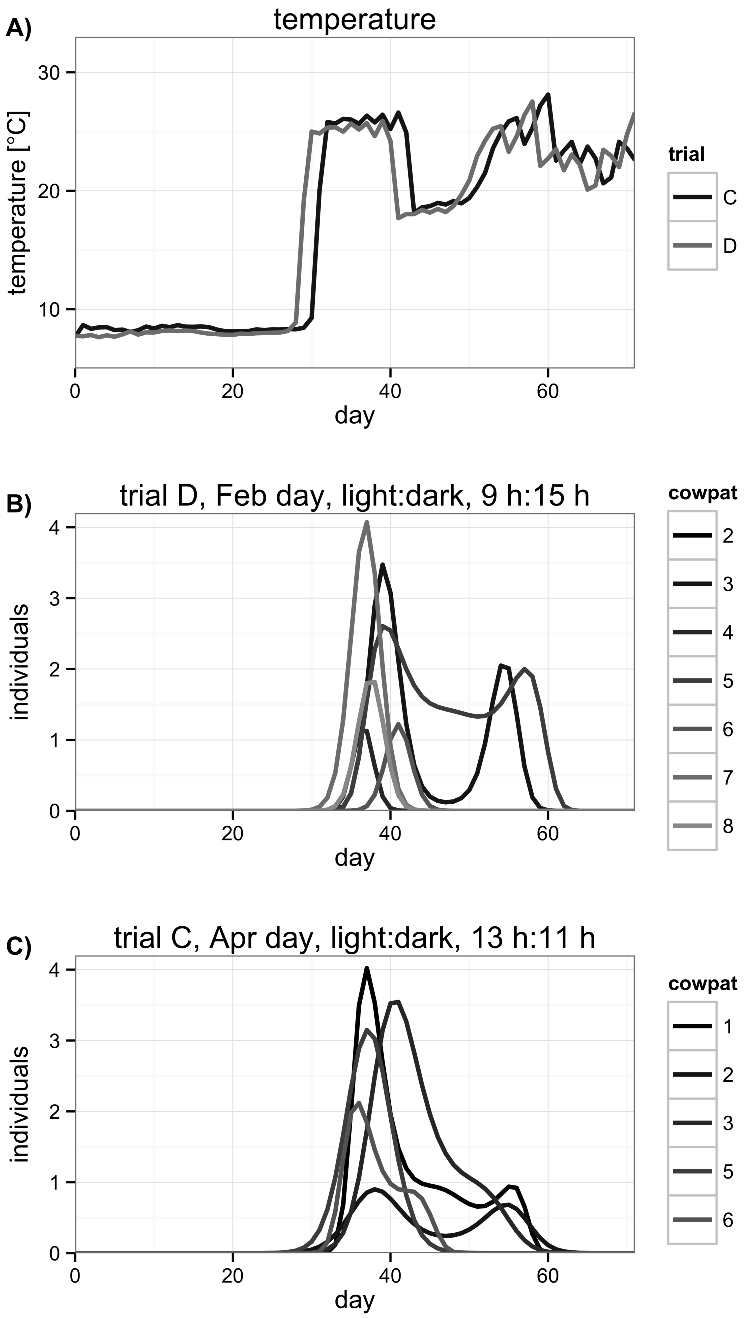


Figure 5.3: **A:** Mean temperature in the wooden boxes of trials C and D; **B:** Fitted generalised additive models for the emergence of *C. chiopterus* females from the cowpats in the temperature-photoperiod trial D; **C:** Fitted generalised additive models for the emergence of *C. chiopterus* females from the cowpats in the temperature-photoperiod trial C.

The emergence in both experimental regimes decreased when the temperatures dropped and, vice versa, increased again for some of the samples when temperatures increased. However, based on this experiment, scientists can neither judge on the lower or upper temperature threshold for dormancy nor conclude whether larval *Culicoides* are capable to continue development but slow down the development at low temperatures.

Although the data indicated a clear impact of temperature but no effect of the two different photoperiods studied, further questions remain to be answered, e.g. the diapause mechanism allowing overwintering larvae of *Culicoides* to withstand harsh winter conditions. As in other taxa (Belozerov, 2008), eco-physiological information on overwintering *Culicoides* is missing. Thus, it is not known if the immature biting midges we collected in February were in diapause (controlled endogenously) or in quiescence (controlled exogenously). Such detailed information would allow a more accurate understanding of the *Culicoides* spring phenology.

## 5.5 Conclusions

An understanding and the analyses of the local factors affecting the distribution and phenology of mosquitoes and biting midges can improve specific predictions and interpretations of species distribution models. Most modelling studies use a huge number of large-scale data (soil, temperature, etc.) (Purse et al., 2011; Rigot et al., 2012; Scolamacchia et al., 2013), resulting in a restricted interpretability of relationships with environmental parameters. These data are very helpful for large-scaled predictions, and the shifting of distribution under climate change can be quite easily included. However, for several vector species, changes of local factors other than temperature might be much more important, e.g. times of drought which reduce the breeding period of mosquitoes. At least for the application of control measurements, an exact knowledge of the breeding sites and the ecology of vectors is important (Carpenter et al., 2008).

# Chapter 6

# Conclusions and future research

## 6.1 Blind spots in vector ecology and some filled knowledge gaps

The basis of all research is observation (e.g. Penck, 1960). Needless to say, this of course also matches the field of vector ecology, where scientists aim to explain the distribution of vectors and their associated pathogens. However, according to observation theory, a fundamental problem of all observation is that "We do not see that we do not see" (von Foerster, 2006, p. 26), i.e. each observation has a blind spot. This applies with regard to different aspects of vector ecology, e.g. the knowledge of the advantages or/and disadvantages of a sampling or identification of a method, the significance of variables not included in species distribution models, or the ecological interpretation of those variables being included. However, as stated by Luhmann (1990), this observational deficit can be solved by observing the studied system externally, here defined as sampling method, species identification, or species distribution models. This external observation, which is conducted during different studies in this thesis, consisted of a critical evaluation of trapping and identification methods and the examination of the correlative or causal relationship of the factors affecting the species distribution.

## Data collection

Different trapping systems [(light-)suction traps meant here] differ according to trapping sensitivity and efficacy (Silver, 2008). If solely looking on the samples from these traps without any further information, one could only express the number of specimens for each collected species. There is no information about the missing species or the trapping efficacy compared to other methods. Therefore, experimental studies are necessary to evaluate the trapping performance:

Paper A.1 The trapping performance between the four evaluated traps was significantly different. Summarising the paper, the Biogents Sentinel (BG) trap is probably the best solution for general monitoring or surveillance programmes of adult mosquitoes in Central Europe. However, depending on the study aims, it might be necessary to select another trap, e.g. the Centers for Disease Control miniature light trap trap for the monitoring of *Aedes vexans*.

Paper A.2 The number of trapped females from the Obsoletus group and females from the Pulicaris group with BG traps significantly decreased with increasing distance to the stable. Therefore, sampling in the direct surroundings of stables ($< 25$ m) probably promises the highest trapping success. However, this also suggests that comparative studies on biting midges should pay attention to the trap positioning.

## Identification

Vector ecology commonly studies the distribution of species and their respective frequency. However, this kind of investigations is strongly affected by the definition of the observational units, i.e. the definition of species. Therefore, it is at least as important to develop applicable methods for their identification. Species which cannot be identified by morphological characters are very common within different vector groups, including mosquitoes, e.g. only the eggs of *Anopheles daciae*/*An. messeae* are distinguishable (Nicolescu et al., 2004) or only the males of *Culex pipiens* s.l./*Cx. torrentium* are distinguishable (summarised by Weitzel et al., 2014). The reliable differentiation of the species is especially important if they differ in vector competence, which was demonstrated, for example, in *Cx. pipiens* and *Cx. torrentium* (Lundström, 1994).

Paper B.1 For *Cx. pipiens* s.s. and *Cx. torrentium*, the analysis of the $r_{2/3}$ / $r_3$ indices resulted in a correct classification of more than 91% of all tested specimens. Using the absolute length of vein $r_{2/3}$ alone still allowed for more than 90% accurate discrimination. Furthermore, classification accuracy of linear discriminant analysis exceeded 97%. Thin-plate spline grids indicated that the differences between the species are predominantly expressed in the shrinkage of the $r_{2/3}$ vein.

Paper B.2 Different real-time Polymerase chain reaction (RT PCR) assays were developed targeting nucleotide differences in the internal transcribed spacer 2 (ITS2) gene of *An. maculipennis* group members. First, a multiplex RT PCR assay was established, which is able to identify single individuals of *An. atroparvus*, *An. maculipennis* s.s., and *An. daciae*/*An. messeae*. Subsequently, single individuals of *An. daciae*/*messeae* are further differentiated using a FRET PCR (Fluorescent Resonance Energy Transfer PCR). Additionally, three singleplex RT PCRs were developed to identify single individuals of *An. atroparvus*, *An. maculipennis* s.s., and *An. daciae*/*An. messeae* in *An. maculipennis* group pools of up to 25 individuals, which are commonly used in surveillance programmes for mosquito-borne pathogens to reduce the number of samples to be analysed.

## Species distribution

At least since the availability of large-scaled environmental data there have also been increasing attempts to model the distribution of vector species on large scales (e.g.

Fischer et al., 2014). However, although the connection is usually interpreted causally, a general problem of these modelling approaches is that the results are only correlative associations of the included variables and the species distribution or abundance (reviewed by Dormann et al., 2012), but no causal relationships can be inferred. Nevertheless, these studies provide valuable insight into the distribution of species, thus allowing more precise predictions, which, in turn, may constitute new hypotheses. This thesis included six papers on the correlation of environmental parameters with the distribution/density of mosquitoes or biting midges:

| | |
|---|---|
| Paper C.1 | *Culex pipiens* s.l./*Cx. torrentium* often occur in sympatry at the studied breeding sites, but there were no substantial differences concerning presence-absence of the two species with regard to the various environmental parameters investigated. |
| Paper C.2 | Soil moisture, organic substance, and soil texture correlated with the number of emerging *Culicoides chiopterus* and *C. dewulfi*. With increasing soil moisture the number of emerging adults increased for both, while the variable organic substance was only significant for *C. chiopterus* showing a positive correlation. |
| Paper C.3 | The density of *Culicoides* larvae in a dung heap was negatively correlated with increasing substrate temperatures. *Culicoides obsoletus* larvae were found in a temperature range between 7.9 and 38.0 °C (mean 16.6 °C). It can be concluded that dung heaps offer temperature conditions which allow the survival and probably also the development to adults for immature *Culicoides* also under harsh climate conditions in winter. |
| Paper C.4 | A software tool was developed which is capable of modelling the local spatial-temporal spread of invasive mosquitoes through natural dispersal and their transportation through human transport via cars. |
| Paper B.2 | Most remarkable was the lower prevalence of *An. atroparvus* compared to historic data. The decline of An. atroparvus might be explained by the |

differences in the overwintering strategy compared the other *Anopheles* species. *Anopheles atroparvus* is expected to be more dependent on shelter sites in the immediate vicinity to cattle hosts that were lost as a result of a changed farm building design.

Paper C.5 Model performance indicators demonstrated that ATKIS® land cover data can be successfully used to build species distribution models for *Culicoides* biting midges. There were no significant differences between performance indicators of models built with land cover data from different buffer zones around the trapping sites. The same applies for multi-scale models, which mostly did not show significant differences compared to single-scale models. However, the overall performance of these models was higher. Furthermore, multi-scale models mostly fulfilled the best performance for the different species using the index “area under the receiver operating characteristic curve”.

## Breeding ecology of *Culicoides*

However, if the causal nexus of a variable and a species is not known in detail, important variables might be not included. Therefore, because of the lack of basic knowledge concerning the ecology of the vectors (e.g. breeding site parameters), a comprehensive interpretation of the modelling results is often subject to uncertainties. Thus, besides further modelling studies on the distribution of our native vector species, experiments within a controlled framework are needed to understand the causal relationships between explaining variables and vector distribution:

Paper D.1 There was no significant differences between the emergence of *Culicoides* from mechanically disturbed and control cowpats.

Paper D.2 No dung-breeding *Culicoides* emerged from the “permanently flooded” treatment, and only two individuals were sampled from the “alternately flooded" treatment. This result highlights the importance of soil moisture on the distribution of *C. chiopterus* and *C. dewulfi*.

Paper D.3 The paper indicated the importance of temperature on the spring emergence of *C. chiopterus* and *C. dewulfi*, both of which did not respond differently to the four temperature-photoperiod regimes.

## 6.2 Future research

### Data collection

As expected from many other studies (Farajollahi et al., 2009; Hoel et al., 2007), the trap comparison in this thesis indicated significant differences of trapping efficacy between the four evaluated mosquito traps (Paper A.1). However, the underlying factors resulting in these differences are mostly unknown. Therefore, further studies aiming to understand the differences are required, both from a behaviour ecological perspective as well as a more technical point of view, i.e. for future construction of suction traps.

Furthermore, there is an ongoing discussion about how the data from different trapping systems refer to the real world. Several studies highlighted that there could be a strong disparity between the number of vectors collected with traps and the abundance of the vectors or their biting rate (Viennet et al., 2011; Elbers & Meiswinkel, 2014). However, vector abundance and biting rate are also important variables included in spread models for vector-borne diseases (Græsbøll et al., 2012; Brugger & Rubel, 2013). Therefore, further studies should focus on the correlation between these variables and trapping results.

## Identification

As demonstrated, morphometric techniques are useful for the reliable identification of vector species (Paper B.1, Paper B.2) which cannot be differentiated by classical morphological characteristics. Therefore, morphological morphometrics might be also a promising tool to identify larvae and pupae of mosquitoes and biting midges. Immature stages of several species are hardly or not at all differentiable (e.g. *Cx. pipiens* s.l./*Cx. torrentium* larvae). However, such identification techniques are needed to understand the vector distribution on the local scale or the relationship with environmental parameters of the breeding sites.

Furthermore, as demonstrated for different species, besides for identification, morphometric tools can also be used to quantify the impact of biotic or abiotic parameters (e.g. competition or the presence of predators) on the fitness of the specimens (e.g. Stephens & Juliano, 2012). Thus, it might be an interesting tool to link the different life stages of mosquitoes and biting midges, e.g. breeding site conditions and adult survival rate.

## Species distribution

The distribution of the morphologically very similar species *C. chiopterus*/ *C. dewulfi* and *Cx. pipiens* s.l./*Cx. torrentium* is not yet understood. Both species are regularly found sympatric in the same breeding site (Paper C.1, Paper C.1, Paper D.1, Paper D.2, and Paper D.3), but the exact ecological segregation of the species pairs is not known. Therefore, future activities should focus on experimental designs appropriate to reveal existing differences, e.g. comparison of inter- and intraspecific competition of the larvae (e.g. see Silvertown, 2004).

## Breeding ecology of *Culicoides*

There is still a huge lack of knowledge on the basic ecology of the native *Culicoides* biting midge species in Central Europe. Several studies increased our knowledge, which helps to understand the distribution and phenology of the species (e.g. Rigot et al., 2012). However, most of these studies are predominantly looking for a correlative relationship, and hardly any study focused on the causal relationship with environmental parameters. Concurrently, there is a huge lack for these data, which are needed to interpret the results from currently available distribution and phenology models.

Future research should especially intend to gather basic life-history parameters for our native *Culicoides* species; there is a special lack of data on survival rates or developmental rates under different abiotic parameters, e.g. temperature regimes. The need for such data becomes particularly compelling if spread models for *Culicoides*-borne diseases (e.g. bluetongue disease (Græsbøll et al., 2012) are examined. In these models, the survival rate of the *Culicoides* vectors is mostly derived from a paper on the exotic species *C. soronenis* (Gerry & Mullens, 2000). However, it is not known to which extend these data also apply to our native species.

# Appendix A

# Data collection

## A.1 Field evaluation of four widely used mosquito traps in Central Europe

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### Abstract

#### Background

To monitor adult mosquitoes several trapping devices are available. These are differently constructed and use various mechanisms for mosquito attraction, thus resulting

in different trapping sensitivities and efficacies for the various species. Mosquito monitoring and surveillance programs in Europe use various types of mosquito traps, but only a few comparisons have been conducted so far. This study compared the performance of four commercial trapping devices, which are commonly used in Europe.

### Methods

Four different traps, Biogents Sentinel trap (BG trap), Heavy Duty Encephalitis Vector Survey trap (EVS trap), Centres for Disease Control miniature light trap (CDC trap) and Mosquito Magnet Patriot Mosquito trap (MM trap) were compared in a 4x4 latin square study. In the years 2012 and 2013, more than seventy 24-hour trap comparisons were conducted at ten different locations in northern and southern Germany, representing urban, forest and floodplain biotopes.

### Results

Per 24-hour trapping period, the BG trap caught the widest range of mosquito species, the highest number of individuals of the genus *Culex* as well as the highest number of individuals of the species *Ochlerotatus cantans*, *Aedes cinereus/geminus*, *Oc. communis* and *Culex pipiens/torrentium.* The CDC trap revealed best performance for *Aedes vexans* whereas the MM trap was most efficient for mosquitoes of the genus *Anopheles* and the species *Oc. geniculatus.* The EVS trap did not catch more individuals of any genus or species compared to the other three trapping devices. The BG trap caught the highest number of individuals per trapping period in urban environment as well as in wet forest, while the CDC trap caught the highest number of individuals in the floodplain biotopes. Additionally, the BG trap was most efficient for the number of mosquito species in urban locations.

### Conclusion

The BG trap showed a significantly better or similar performance compared to the CDC, EVS or MM trap with regard to trapping efficacy for most common mosquito species in Germany, including diversity of mosquito species and number of mosquitoes per trapping period. Thus, the BG trap is probably the best solution for general monitoring or surveillance programs of adult mosquitoes in Central Europe.

## Background

Most mosquito monitoring and surveillance programs include the monitoring of adults using different types of trapping devices. Due to automatic trapping by aspiration, mosquito traps have the advantage of relative low costs for data collection in combination with a constant effort independently of the operator, resulting in comparable samples from different trapping sites. Therefore, adult traps are commonly used for the inventory of mosquito biodiversity (Versteirt et al., 2012), surveillance of invasive mosquitoes at potential introduction sites (Becker et al., 2013), monitoring of mosquito-borne pathogens (Jöst et al., 11a), or the reduction of mosquito nuisance (Jackson et al., 2012). However, in the course of an increasing attention for mosquitoes due to the worldwide spread of invasive mosquitoes (Bonizzoni et al., 2013; Kampen & Werner, 2014; Kaufman & Fonseca, 2014) and mosquito-borne pathogens (Gubler, 2007) also the number of commercially available traps increased, which are distributed as tools for scientific studies or for mosquito control (Jackson et al., 2012; Kröckel et al., 2006; Drago et al., 2012). These trapping devices use various cues for mosquito attraction (e.g. carbon dioxide, heat, water vapour, olfactory lures, or visual cues), which may influence trapping efficacies for the different genera or species (Silver, 2008).

Previous studies on the comparison of mosquito traps were predominantly conducted in North and South America (Dennett et al., 2004; Irish et al., 2008; Farajollahi et al., 2009). Many of these studies focused primarily on the effectiveness of the traps to catch invasive and/or highly vector-competent species (e.g. *Aedes albopictus*) (Farajollahi et al., 2009; Hoel et al., 2007). Due to the spread of invasive mosquitoes (Medlock et al., 2012) and mosquito-borne pathogens (e.g. West Nile virus Sambri et al., 2013) in Europe, mosquito monitoring activities have substantially increased during recent years (Becker et al., 2013; Engler et al., 2013), but only a few studies have compared the efficacy of different mosquito traps for this region. The Mosquito Magnet Commercial Pro caught more mosquito individuals and a wider range of species than the Centres for Disease Control miniature light trap (CDC trap) in Great Britain (Hutchinson et al., 2007). In contrast, Reusken et al. (2011) found that the CDC trap performed better than the Mosquito Magnet Liberty in the Netherlands. A limited study in Germany compared the Bidirectional Fay-Prince Trap, Biogents Sentinel (BG trap) and Mosquito Magnet Liberty, but did not find significant differences (Rose et al., 2006). The most comprehensive comparison of mosquito traps was conducted in northern Italy with the experimental Biogents BG Eisenhans de Luxe, CDC trap and two mosquito traps for the reduction of mosquito nuisance (Acti Power Trap PV 440 and Acti Power Trap MT 250 Plus) (Drago et al., 2012). For the collection of *Aedes albopictus*, a better trapping efficacy was found for the Biogents BG Eisenhans de Luxe compared to the other three trapping devices. Differences between the BG and CDC traps were reported only for *Anopheles atroparvus* during a trap comparison in Spanish wetlands (Roiz et al., 2012).

Previous nationwide monitoring programs of mosquito species in Europe used different trapping devices, e.g. Mosquito Magnet Liberty Plus in Switzerland (Versteirt et al., 2012), the CDC trap and Mosquito Magnet counter-flow trap in Sweden (Lundström et al., 2013), Mosquito Magnet Liberty Plus in Belgium, Netherlands and Luxembourg (Versteirt et al., 2012, 2009), or Heavy Duty Encephalitis Vector Survey trap and BG traps in Germany (Rudolf et al., 2013). However, a comprehensive comparison of trapping efficacies of these adult mosquito traps commonly used in Central Europe has not been conducted and the choice between the different trapping devices is based on expert judgment or studies from other regions (Versteirt et al., 2012). Therefore, the present study aimed to compare four trapping devices for mosquito adults. Our objectives were (i) to compare the efficacies of traps concerning the variety of mosquito species and the overall number of mosquitoes, as well as (ii) to identify the most efficient trap for different biotopes.

## Methods

Trap comparisons were conducted in the years 2012 and 2013 during 19 sampling periods in ten different locations in northern (3 locations) and southern Germany (7 locations) (Table A.1, Figure A.1). Locations in northern Germany included gardens in urban areas and a cattle farm, and in southern Germany floodplain areas, a wet forest, a cemetery in an urban environment, and the edge of a wood in an urban environment.

Four different traps were compared, which all have been developed to collect host-seeking mosquitoes by aspiration, but differ in their mechanisms of attraction and trapping: (1) Biogents Sentinel trap (BG trap) (BioGents, Regensburg, Germany, http://www.biogents.com/) with BG Lure sachets (BioGents, GmbH, Regensburg, Germany, http://www.biogents.com/) and $CO_2$ from a gas cylinder, (2) Heavy Duty Encephalitis Vector Survey trap (EVS trap) (BioQuip Products, Rancho Dominguez, California, USA; http://www.bioquip.com/) with $CO_2$ from dry ice (2.5 kg per 24 hours) and without EVS trap lamp, (3) Centres for Disease Control miniature light trap (CDC trap) (BioQuip Products, Rancho Dominguez, California, USA; http://www.bioquip.com/) with CDC bulb and with $CO_2$ from dry ice (2.5 kg per 24 hours), which was also put in EVS dry ice containers above the trap, and (4) the Mosquito Magnet Patriot Mosquito trap (MM trap) (MosquitoMagnet, Lititz, Pennsylvania, USA; http://www.mosquitomagnet.com/) with R-Octenol (Mosquito-Magnet, Lititz, Pennsylvania, USA; http://www.mosquitomagnet.com/). The MM trap converts propane into $CO_2$. EVS and CDC traps were hung on low trees or wooden posts (trap opening approximately at 1 m height), whereas the BG and MM traps were placed on the ground following manufacturers instructions.

A 4x4 latin square experimental design was applied. At each location, all traps were placed approximately 50 m from each other at four different sampling points. Every 24 hours, all traps were rotated to the next position to reduce sampling point specific differences. One complete trapping cycle per latin square consisted of four 24-hour trapping periods. Mosquitoes were collected every 24 hours in the late afternoon, killed in a freezer and morphologically identified in the laboratory (Mohrig, 1969; Becker et al., 2010). Four morphologically very similar species were summarized as species pairs (*Aedes cinereus/geminus*, *Ochlerotatus excrucians/annulipes*, *Ochlerotatus sticticus/diantaeus* and *Culex pipiens/torrentium*), because a morphological differentiation is not possible or doubtful in cases where the material is in poor condition. In terms of the taxonomy of Aedini species, the generic names used here follow the system of Becker et al. (2010) and are not adopted from the revisions of Reinert et al. (2009).

Generalized linear mixed models (GLMM) were used to analyse the effect of different trapping devices on the number of caught individuals for all species/genera per trapping period, total number of individuals/species per trapping period and total number of caught individuals/species per trapping period differentiated for aggregated biotopes. GLMMs allow dependent variables to be modelled while controlling for independent random variables (in this case the latin square number) to test the statistical significance of a fixed independent variable (type of trapping device). Mean and standard errors of differences in least squares means associated with a mixed linear model were calculated. Furthermore, Simpson's diversity index per trapping period was caculated to compare the recorded species diversity among the four trapping devices. Data preparation, visualization and statistical analyses were conducted with R (R Core Team, 2014) using functions from the packages ggplot2 (Wickham, 2009), lm4 (Bates et al., 2014), lmerTest (Kuznetsova et al., 2013), plyr (Wickham, 2011), sp (Roger, Bivand & Pebesma, Roger et al.; Pebesma & Bivand, 2005), and vegan (Oksanen et al., 2013).

## Results

A total of 83 trap comparisons were conducted. However, due to organisational and technical issues, nine trapping periods comprised only three different trapping devices (BG trap, CDC trap, and EVS trap), thus resulting in 323 24-hour sampling periods (83 x BG trap, 83 x CDC trap, 83 x EVS trap, 74 x MM trap).

During the study 24,094 mosquitoes were caught, belonging to 21 species or morphologically indistinguishable pairs of species (Table A.2) and comprising 43% of the established 49 mosquito species in Germany (Table A.2, Additional file A.7) . All species known to be abundant in Germany and to occur in high density were detected (Table A.2) (Becker & Hoffmann, 2011). Most abundant species were *Aedes vexans* (30.0%), *Aedes cinereus/geminus* (17.0%), *Culex pipiens/torrentium* (12.2%), *Ochlerotatus sticticus/diantaeus* (9.9%) and *Ochlerotatus cantans* (9.7%). *Culex hortensis*, *Culex territans*, and *Culiseta morsitans* were only caught with one individual. Undetected species are predominantly classified as less common in Germany (Additional file A.7). The BG trap showed the best performance for individuals of the genus *Culex* and the MM trap for the genus *Anopheles* (Figure A.2, Table A.3). During the entire study the highest number of species was caught with the CDC trap followed by the BG trap, EVS trap, and MM trap, but the total number of species detected was quite similar between the four trapping devices (Figure A.3). However, the BG trap caught significantly more species per trapping period compared to CDC trap, EVS trap, and MM trap, while there were no significant differences between the latter three traps (Figure A.4, Table A.4). This was also supported by slightly higher species diversity indices for the BG trap (Figure A.5).

BG and CDC traps caught significantly more mosquitoes per trapping period compared to EVS and MM traps (Figure A.4, Table A.4). The four trapping devices differed in performance regarding their efficacy to trap individual mosquito species. The BG trap caught significantly more individuals of the species *Oc. cantans*, *Ae. cinereus/geminus*, *Oc. communis*, and *Cx. pipiens/torrentium* per trapping period. The CDC trap outcompeted the other devices by trapping significantly more *Ae.*

*vexans* individuals per trapping period and the MM trap caught significantly more individuals of *Oc. geniculatus* per trapping period. In contrast, the EVS trap did not outperform for any species. The MM trap caught the smallest number of individuals of the species *Cx. pipiens/torrentium* and *Oc. cantans* per trapping period. Additionally, the CDC trap with light outcompeted the EVS trap without light for *Aedes vexans* and *Ochlerotatus sticticus/diantaeus*.

The four traps showed differences in their suitability for the three aggregated biotopes investigated. The BG trap caught significantly more individuals per trapping period in the urban environment as well as in wet forest (Figure A.6, Table A.5), while the CDC trap caught most individuals per trapping period in the floodplain. Moreover, the BG trap was most efficient for the trapping of the variety of mosquito species per trapping period in an urban environment (Figure A.7, Table A.6).

## Discussion

Several commercial trapping devices for mosquitoes are available, which are used for nuisance reduction, mosquito monitoring, or surveillance. This study compared the performance of four aspiration traps in Germany (Biogents Sentinel trap (BG trap), Heavy Duty Encephalitis Vector Survey trap (EVS trap), Centres for Disease Control miniature light trap (CDC trap), and Mosquito Magnet Patriot Mosquito trap (MM trap)), which are commonly used in Central Europe. During the study period we found all mosquito species, which are known to be abundant in Germany. The four traps detected a similar number of species. However, the BG trap was found to trap the largest diversity of mosquito species per trapping period, while there were no differences between the other three traps. Additionally, the BG and CDC traps caught more mosquito individuals per trapping period than the EVS and MM traps. This matches a trap comparison from the Netherlands, which found the CDC trap catching more individuals than the MM trap (Reusken et al., 2011). In the same study, the CDC trap was found to catch more species than the MM trap, a result that is not supported by our study. Our results are also in contrast to a trap comparison from Great Britain, which identified the MM trap to catch more species and individuals than the CDC trap (Hutchinson et al., 2007).

Contrary to a Spanish study (Roiz et al., 2012), which did not find differences between CDC and BG traps in collecting *Cx. pipiens*, our study indicated that the BG trap caught significantly more *Cx. pipiens/torrentium* per trapping period compared to the other three trapping devices. Our findings are in agreement with results of Reusken et al. (Reusken et al., 2011), who showed a very low trapping efficacy of the MM trap for *Cx. pipiens/torrentium*, while Drago et al. (Drago et al., 2012) did not find significant differences between BG and CDC traps for these species. Furthermore, the BG trap outperformed the other three traps for the three floodwater species *Oc. cantans*, *Ae. cinereus/geminus* and *Oc. communis*. A high trapping efficacy of the BG trap for members of the genera *Aedes* and *Ochlerotatus* was supported by several studies (reviewed by Drago et al., 2012). The MM trap also had the lowest performance for *Oc. cantans*.

The CDC and MM traps performed better than the other three traps for one particular species each. The CDC trap caught the highest number of *Ae. vexans* per trapping period, which matches the results of a study from the U.S. (Becker & Hoffmann, 2011), in which the CDC trap performed better than the BG trap. In contrast, another U.S. study did not find clear differences between the BG and CDC trap (Farajollahi et al., 2009). Our study showed that the MM trap caught the highest number of *Oc. geniculatus*, which is in agreement with a study from the U.K., in which the MM trap caught more individuals of this species compared to the CDC trap (Hutchinson et al., 2007).

The four trapping devices used in this study differ in constructions and mechanisms to lure and trap mosquitoes. Except for the addition of a dry ice bucket to the CDC trap, we used the traps according to the manufacturers' instructions and did not interfere with recommended trap configurations (e.g. with lure vs. without lure or different heights). However, changes of configurations might result in a different performance of the various traps.

Although the $CO_2$-effusion rate from dry ice (CDC and EVS traps) is probably more temperature-dependent than from gas cylinders (BG trap), this probably does not have a strong impact on the trapping efficacy. The same probably applies to the amount of $CO_2$ and the type of $CO_2$ dispersal from small holes on the EVS dry ice bucket (EVS and CDC traps) or tubes (BG and MM traps) (Irish et al., 2008), which should not cause profound differences between the traps. However, it is surprising that the CDC trap revealed significantly higher trapping efficacy for the species *Ae. vexans* and *Oc. sticticus/diantaeus* compared to the rather similar EVS trap. The trap cover of the CDC trap (33 cm in diameter) has a positive impact on the diffusion range of the $CO_2$ (Bhalala & Arias, 2009), which may result in significant trapping differences for this very abundant species. Another explanation could be the secondary attractant of light, which is only used with the CDC trap. However, the study by Becker et al. (Becker et al., 1995) did not reveal a significant impact of light on the trapping efficacy of the CDC trap for *Aedes*.

The BG and MM traps use different chemical lures additional to carbon dioxide to increase their trapping efficacy by imitating the olfactory cues of potential hosts (e.g. octenol for ruminant breaths or lactic acid as component of sweat) (Hoel et al., 2007). Such lures can have significant influences on the trapping efficacy for particular mosquito species, but do not necessarily cause differences (Hoel et al., 2007; Roiz et al., 2012; Rose et al., 2006). Only for *An. plumbeus* we found a significantly better performance for the two lure-containing traps (BG and MM traps) compared to those without lure (CDC and EVS traps). However, with the exception of *Oc. geniculatus*, the BG trap performed similar or even better compared to the MM trap. Although the BG Lure used for the BG traps might explain the better performance for some of the species, it probably does not explain the differences for all of them, as there were no significant differences for the trapping of *Cx. pipiens* with and without BG Lure in a previous German study (Rose et al., 2006).

We conducted our trap comparison at ten sampling locations distributed in northern and southern Germany, which were analysed as aggregated biotopes for floodplain, urban and wet forest, respectively. According to its trapping performance for *Culex* species, we found the BG trap to be superior in an urban environment. The CDC trap was the most efficient trapping device for *Aedes vexans* and therefore should be the first choice for the floodplain environment and the BG trap showed an outstanding performance for some of the snow-melt mosquito species and therefore trapped most mosquito individuals in the wet forest.

## Conclusion

This study compared four adult mosquito traps (BG trap, EVS trap, CDC trap, and MM trap) under different environmental conditions in Germany with a total of 323 24-hour sampling periods (83 x BG trap, 83 x CDC trap, 83 x EVS trap, 74 x MM trap) and the analysis of more than 24,000 mosquitoes from 21 species most common in Central Europe. The BG trap showed the best performance regarding the number of mosquitoes and the number of mosquito species per trapping period and outperformed the other three traps for the genus *Culex* and for four species (*Oc. cantans*, *Ae. cinereus/geminus*, *Oc. communis* and *Cx. pipiens/torrentium*). The CDC trap was the most efficient trap for *Ae. vexans* and the MM trap for the genus *Anopheles* and the species *Oc. geniculatus*. The EVS trap did not show advantages for any species or genus compared to the other three traps. Additionally, the MM trap had a very low efficacy for *Cx. pipiens/torrentium* and *Oc. cantans*. According to its efficacy for the number of mosquitoes and the range of species at various environments, the BG trap is recommended as the general monitoring trapping device for common mosquito species in Central Europe, while the CDC trap is the best choice to trap large numbers of mosquitoes particularly in floodplain biotopes.

## Competing interests

Over the last three years, the company Biogents, which is the producer of the BG mosquito traps included in this study, was a research partner of the authors NB, KH, EK, AK, RL, WPP and ET in a surveillance study for invasive mosquitoes in Germany. Biogents had no role in study design, data collection and analysis, preparation of the manuscript or decision on publication.

## Authors contribution's

Designed the study: AK, RL, WPP, EK. Performed the data collection: JB, RG, KH, AK, RL, NS, SS, NB, ET, WPP. Analysed the data: RL. Wrote the paper: RL. Contributed to the manuscript drafting: AK, ET, WPP, RG, EK, NS, SS.

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## Tables

Table A.1: Characterisation of the sampling locations and sampling periods. The temperature and precipitation during the sampling period were derived from the nearest weather station (DWD, 2014).

| **ID** | **Description** | **Aggregated biotop** | **Sampling period** | **Temperature during sampling period [°C] (mean, minimum-maximum range)** | **Precipitation during sampling period [mm] (mean, minimum-maximum range)** |
|---|---|---|---|---|---|
| **1** | Garden in an urban area | Urban | 05.09.-09.09.2012 | 16.9 (8.4-28.4) | 0.0 (0.0-0.0) |
| | | | 10.06.-14.06.2013 | 15.4 (5.1-24.3) | 2.1 (0.0-8.5) |
| | | | 30.07.-03.08.2013 | 22.2 (13.9-34.9) | 1.0 (0.0-4.6) |
| **2** | Cattle farm within a suburban environment | Urban | 19.08.-23.08.2013 | 16.9 (9.2-24.1) | 3.6 (0.0-18.0) |
| | | | 26.08.-30.08.2013 | 17.3 (7.5-24.3) | 0.0 (0.0-0.0) |
| **3** | Garden in an urban area | Urban | 03.06.-06.06.2012 | 10.2 (4.9-15.1) | 1.4 (0.0-3.9) |
| | | | 09.07.-13.07.2012 | 16.0 (10.4-22.8) | 5.2 (0.0-8.2) |

Table A.1: Characterisation of the sampling locations and sampling periods. The temperature and precipitation during the sampling period were derived from the nearest weather station (DWD, 2014).

| **ID** | **Description** | **Aggregated biotop** | **Sampling period** | **Temperature during sampling period [°C] (mean, minimum-maximum range)** | **Precipitation during sampling period [mm] (mean, minimum-maximum range)** |
|---|---|---|---|---|---|
| | | | 28.08.-01.09.2012 | 16.2 (8.8-24.9) | 0.2 (0.0-0.6) |
| | | | 10.06.-14.06.2013 | 15.6 (4.9-24.3) | 3.8 (0.0-14.8) |
| | | | 08.07.-12.07.2013 | 17.2 (10.3-25.4) | 0.0 (0.0-0.0) |
| **4** | Forest in river inundation area | Floodplain | 17.07.-21.07.2012 | 18.0 (10.0-27.2) | 1.9 (0.0-5.3) |
| | | | 24.07.-28.07.2012 | 22.6 (2.1-33.3) | 6.5 (0.0-16.8) |
| | | | 03.08.-07.08.2012 | 20.4 (11.4-32.3) | 0.1 (0.0-0.7) |
| | | | 13.08.-17.08.2012 | 21.2 (9.4-33.6) | 1.9 (0.0-9.6) |
| **5a** | Mixed forest | Wet forest | 04.07.-08.07.2013 | 20.8 (12.1-28.8) | 0.1 (0.0-0.5) |

Table A.1: Characterisation of the sampling locations and sampling periods. The temperature and precipitation during the sampling period were derived from the nearest weather station (DWD, 2014).

| **ID** | **Description** | **Aggregated biotop** | **Sampling period** | **Temperature during sampling period [°C] (mean, minimum-maximum range)** | **Precipitation during sampling period [mm] (mean, minimum-maximum range)** |
|---|---|---|---|---|---|
| **5b** | Mixed forest | Wet forest | 04.07.-08.07.2013 | 20.8 (12.1-28.8) | 0.1 (0.0-0.5) |
| **6a** | Cemetery within a urban environment | Urban | 26.08.-30.08.2013 | 17.4 (9.2-26.1) | 0.0 (0.0-0.0) |
| **6b** | Edge of a wood within a urban environment | Urban | 26.08.-30.08.2013 | 17.4 (9.2-26.1) | 0.0 (0.0-0.0) |
| **7** | Forest in river inundation area | Floodplain | 19.08.-23.08.2013 | 23.6 (10.5-35.2) | 0.3 (0.0-1.3) |
| | | | 07.09.-11.09.2012 | 18.4 (5.4-30.3) | 3.2 (0.0-15.9) |
| **8** | Forest in river inundation area | Floodplain | 02.09.-06.09.2012 | 17.9 (7.6-26.7) | 0.0 (0.0-0.0) |

Table A.2: Number and percentage of trapped individuals for the mosquito species caught with the four different trapping devices. Occurrence in Germany classified after Becker & Hoffmann (2011) (occurrence: ++++ = massive; +++ = abundant; ++ = frequent; + = regularly; (+) = rare; - = not classified; * species complex includes *Anopheles atroparvus*, *An. daciae*, *An. maculipennis*, *An. messeae*).

| **Species** | **BG** | **%** | **CDC** | **%** | **EVS** | **%** | **MM** | **%** | **Total** | **Occurence in Germany** |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Anopheles maculipennis* s.l.*** | 0 | 0.0 | 18 | 33.3 | 18 | 33.3 | 18 | 33.3 | 54 | +++ |
| ***Anopheles claviger*** | 2 | 3.1 | 33 | 50.8 | 9 | 13.8 | 21 | 32.3 | 65 | ++ |
| ***Anopheles plumbeus*** | 105 | 33.1 | 51 | 16.1 | 33 | 10.4 | 128 | 40.4 | 317 | ++ |
| ***Aedes cinereus/geminus*** | 1,552 | 38.0 | 783 | 19.2 | 725 | 17.7 | 1,027 | 25.1 | 4,087 | ++/proven |
| ***Aedes rossicus*** | 6 | 66.7 | 3 | 33.3 | 0 | 0.0 | 0 | 0.0 | 9 | ++ |
| ***Aedes vexans*** | 841 | 11.6 | 3,544 | 49.0 | 1,837 | 25.4 | 1,016 | 14.0 | 7,238 | ++++ |
| ***Ochlerotatus cantans*** | 1,206 | 51.9 | 565 | 24.3 | 470 | 20.2 | 84 | 3.6 | 2,325 | ++ |
| ***Ochlerotatus caspius*** | 1 | 8.3 | 10 | 83.3 | 1 | 8.3 | 0 | 0.0 | 12 | (+) |
| ***Ochlerotatus communis*** | 208 | 39.8 | 116 | 22.2 | 116 | 22.2 | 83 | 15.9 | 523 | + |

Table A.2: Number and percentage of trapped individuals for the mosquito species caught with the four different trapping devices. Occurrence in Germany classified after Becker & Hoffmann (2011) (occurrence: ++++ = massive; +++ = abundant; ++ = frequent; + = regularly; (+) = rare; - = not classified; * species complex includes *Anopheles atroparvus*, *An. daciae*, *An. maculipennis*, *An. messeae*).

| **Species** | **BG** | **%** | **CDC** | **%** | **EVS** | **%** | **MM** | **%** | **Total** | **Occurence in Germany** |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Ochlerotatus excrucians/ annulipes*** | 50 | 41.0 | 35 | 28.7 | 25 | 20.5 | 12 | 9.8 | 122 | (+)/++ |
| ***Ochlerotatus geniculatus*** | 144 | 29.8 | 77 | 15.9 | 49 | 10.1 | 214 | 44.2 | 484 | (+) |
| ***Ochlerotatus japonicus*** | 84 | 18.7 | 249 | 55.3 | 4 | 0.9 | 113 | 25.1 | 450 | + |
| ***Ochlerotatus punctor*** | 141 | 35.6 | 90 | 22.7 | 103 | 26.0 | 62 | 15.7 | 396 | + |
| ***Ochlerotatus rusticus*** | 818 | 38.3 | 470 | 22.0 | 217 | 10.1 | 633 | 29.6 | 2,138 | ++ |
| ***Ochlerotatus sticticus/ diantaeus*** | 857 | 36.0 | 718 | 30.1 | 424 | 17.8 | 384 | 16.1 | 2,383 | +++/(+) |
| ***Ochlerotatus* spec.** | 30 | 68.2 | 6 | 13.6 | 3 | 6.8 | 5 | 11.4 | 44 | |
| ***Culex hortensis*** | 0 | 0.0 | 1 | 100.0 | 0 | 0.0 | 0 | 0.0 | 1 | - |
| ***Culex pipiens/ torrentium*** | 1,398 | 47.5 | 655 | 22.3 | 861 | 29.3 | 29 | 1.0 | 2,943 | ++++/++++ |

Table A.2: Number and percentage of trapped individuals for the mosquito species caught with the four different trapping devices. Occurrence in Germany classified after Becker & Hoffmann (2011) (occurrence: ++++ = massive; +++ = abundant; ++ = frequent; + = regularly; (+) = rare; - = not classified; * species complex includes *Anopheles atroparvus*, *An. daciae*, *An. maculipennis*, *An. messeae*).

| **Species** | **BG** | **%** | **CDC** | **%** | **EVS** | **%** | **MM** | **%** | **Total** | **Occurence in Germany** |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Culex territans*** | 0 | 0.0 | 1 | 100.0 | 0 | 0.0 | 0 | 0.0 | 1 | ++ |
| ***Culiseta annulata*** | 59 | 16.0 | 107 | 29.0 | 139 | 37.7 | 64 | 17.3 | 369 | ++ |
| ***Culiseta morsitans*** | 1 | 100.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 1 | + |
| ***Culiseta* spec.** | 2 | 66.7 | 0 | 0.0 | 0 | 0.0 | 1 | 33.3 | 3 | |
| ***Coquillettidia richiardii*** | 17 | 21.8 | 14 | 17.9 | 20 | 25.6 | 27 | 34.6 | 78 | + |
| **Unidentified Culicidae** | 18 | 35.3 | 27 | 52.9 | 5 | 9.8 | 1 | 2.0 | 51 | |
| **Total** | 7,540 | 31.3 | 7,573 | 31.4 | 5,059 | 21.0 | 3,922 | 16.3 | 24,094 | |

Table A.3: Mean ±SE differences in least squares means associated with the mixed linear models for the number of individuals per trapping period among the four trapping devices. Only mosquito genera caught with more than 100 individuals are shown and trapping periods were only included if the genus was detected with at least one individual in the corresponding trapping period at the sampling location (only significant differences shown). BG: Biogents Sentinel trap, EVS: Heavy Duty Encephalitis Vector Survey trap, CDC: Centres for Disease Control miniature light trap, MM: Mosquito Magnet Patriot Mosquito trap, Estimate: differences in least squares means, SE: standard error, DF: degrees of freedom, t: t-value, p: p value.

| **Response variable** | **Traps** | **Estimate** | **SE** | **DF** | **t** | **p** |
|---|---|---|---|---|---|---|
| ***Anopheles*** | BG vs. MM | -0.905 | 0.349 | 300.7 | -2.59 | 0.010 |
| | CDC vs. MM | -0.950 | 0.349 | 300.6 | -2.72 | 0.007 |
| | EVS vs. MM | -1.456 | 0.349 | 300.6 | -4.17 | <0.001 |
| ***Aedes/Ochlerotatus*** | BG vs. EVS | 23.570 | 10.264 | 299.1 | 2.30 | 0.022 |
| | BG vs. MM | 23.796 | 10.654 | 299.5 | 2.23 | 0.026 |
| | CDC vs. EVS | 32.398 | 10.260 | 299.0 | 3.16 | 0.002 |
| | CDC vs. MM | 32.624 | 10.650 | 299.5 | 3.06 | 0.002 |
| ***Culex*** | BG vs. CDC | 8.933 | 2.807 | 299.2 | 3.18 | 0.002 |
| | BG vs. EVS | 6.475 | 2.807 | 299.2 | 2.31 | 0.022 |
| | BG vs. MM | 16.962 | 2.911 | 300.5 | 5.83 | <0.001 |
| | CDC vs. MM | 8.029 | 2.910 | 300.5 | 2.76 | 0.006 |
| | EVS vs. MM | 10.486 | 2.910 | 300.5 | 3.60 | 0.000 |
| ***Culiseta*** | BG vs. EVS | -0.963 | 0.328 | 298.5 | -2.93 | 0.004 |

Table A.4: Mean ±SE differences in least squares means associated with the mixed linear models for the number of trapped individuals per trapping period for each species and the total number of individuals and the mean ±SE number of species among the four trapping devices. Only mosquito species caught with more than 100 individuals are shown and trapping periods were only included if the species was detected with at least one individual in the corresponding trapping period at the sampling location (only significant differences shown). BG: Biogents Sentinel trap, EVS: Heavy Duty Encephalitis Vector Survey trap, CDC: Centres for Disease Control miniature light trap, MM: Mosquito Magnet Patriot Mosquito trap, Estimate: differences in least squares means, SE: standard error, DF: degrees of freedom, t: t-value, p: p value.

| **Response variable** | **Traps** | **Estimate** | **SE** | **DF** | **t** | **p** |
|---|---|---|---|---|---|---|
| ***Anopheles plumbeus*** | BG vs. CDC | 0.641 | 0.299 | 301.2 | 2.14 | 0.033 |
| | BG vs. EVS | 0.858 | 0.299 | 301.2 | 2.87 | 0.004 |
| | CDC vs. MM | -1.032 | 0.307 | 302.1 | -3.36 | 0.001 |
| | EVS vs. MM | -1.249 | 0.307 | 302.1 | -4.07 | <0.001 |
| ***Aedes cinereus/geminus*** | BG vs. CDC | 9.304 | 2.354 | 301 | 3.95 | <0.001 |
| | BG vs. EVS | 10.002 | 2.354 | 301 | 4.25 | <0.001 |
| | BG vs. MM | 6.052 | 2.418 | 301.3 | 2.5 | 0.013 |
| ***Aedes vexans*** | BG vs. CDC | -32.42 | 7.296 | 301.1 | -4.44 | <0.001 |
| | CDC vs. EVS | 20.566 | 7.293 | 301.1 | 2.82 | 0.005 |
| | CDC vs. MM | 28.547 | 7.49 | 301.7 | 3.81 | <0.001 |
| ***Ochlerotatus cantans*** | BG vs. CDC | 7.732 | 2.456 | 301.1 | 3.15 | 0.002 |
| | BG vs. EVS | 8.877 | 2.456 | 301.1 | 3.61 | <0.001 |
| | BG vs. MM | 13.929 | 2.524 | 301.4 | 5.52 | <0.001 |

Table A.4: Mean ±SE differences in least squares means associated with the mixed linear models for the number of trapped individuals per trapping period for each species and the total number of individuals and the mean ±SE number of species among the four trapping devices. Only mosquito species caught with more than 100 individuals are shown and trapping periods were only included if the species was detected with at least one individual in the corresponding trapping period at the sampling location (only significant differences shown). BG: Biogents Sentinel trap, EVS: Heavy Duty Encephalitis Vector Survey trap, CDC: Centres for Disease Control miniature light trap, MM: Mosquito Magnet Patriot Mosquito trap, Estimate: differences in least squares means, SE: standard error, DF: degrees of freedom, t: t-value, p: p value.

| **Response variable** | **Traps** | **Estimate** | **SE** | **DF** | **t** | **p** |
|---|---|---|---|---|---|---|
| | CDC vs. MM | 6.197 | 2.523 | 301.4 | 2.46 | 0.015 |
| | EVS vs. MM | 5.052 | 2.523 | 301.4 | 2 | 0.046 |
| ***Ochlerotatus communis*** | BG vs. CDC | 1.11 | 0.427 | 301 | 2.6 | 0.01 |
| | BG vs. EVS | 1.11 | 0.427 | 301 | 2.6 | 0.01 |
| | BG vs. MM | 1.497 | 0.439 | 301.2 | 3.41 | 0.001 |
| ***Ochlerotatus excrucians/annulipes*** | BG vs. EVS | 0.299 | 0.138 | 301.3 | 2.17 | 0.031 |
| | BG vs. MM | 0.418 | 0.142 | 302.6 | 2.95 | 0.003 |
| ***Ochlerotatus geniculatus*** | BG vs. EVS | 1.144 | 0.466 | 301.1 | 2.45 | 0.015 |
| | BG vs. MM | -1.039 | 0.479 | 301.6 | -2.17 | 0.031 |
| | CDC vs. MM | -1.845 | 0.479 | 301.6 | -3.85 | <0.001 |
| | EVS vs. MM | -2.183 | 0.479 | 301.6 | -4.56 | <0.001 |
| ***Ochlerotatus punctor*** | BG vs. MM | 0.976 | 0.334 | 301.2 | 2.92 | 0.004 |
| ***Ochlerotatus rusticus*** | BG vs. CDC | 4.198 | 1.764 | 301 | 2.38 | 0.018 |

Table A.4: Mean ±SE differences in least squares means associated with the mixed linear models for the number of trapped individuals per trapping period for each species and the total number of individuals and the mean ±SE number of species among the four trapping devices. Only mosquito species caught with more than 100 individuals are shown and trapping periods were only included if the species was detected with at least one individual in the corresponding trapping period at the sampling location (only significant differences shown). BG: Biogents Sentinel trap, EVS: Heavy Duty Encephalitis Vector Survey trap, CDC: Centres for Disease Control miniature light trap, MM: Mosquito Magnet Patriot Mosquito trap, Estimate: differences in least squares means, SE: standard error, DF: degrees of freedom, t: t-value, p: p value.

| **Response variable** | **Traps** | **Estimate** | **SE** | **DF** | **t** | **p** |
|---|---|---|---|---|---|---|
| | BG vs. EVS | 7.246 | 1.764 | 301 | 4.11 | <0.001 |
| | EVS vs. MM | -5.159 | 1.812 | 301.2 | -2.85 | 0.005 |
| ***Ochlerotatus sticticus/diantaeus*** | BG vs. EVS | 5.248 | 1.373 | 301 | 3.82 | <0.001 |
| | BG vs. MM | 5.675 | 1.411 | 301.2 | 4.02 | <0.001 |
| | CDC vs. EVS | 3.542 | 1.373 | 301 | 2.58 | 0.01 |
| | CDC vs. MM | 3.969 | 1.411 | 301.2 | 2.81 | 0.005 |
| ***Culex pipiens/torrentium*** | BG vs. CDC | 8.957 | 2.797 | 301.1 | 3.2 | 0.002 |
| | BG vs. EVS | 6.475 | 2.797 | 301.1 | 2.31 | 0.021 |
| | BG vs. MM | 17.026 | 2.873 | 302 | 5.93 | <0.001 |
| | CDC vs. MM | 8.069 | 2.872 | 301.9 | 2.81 | 0.005 |
| | EVS vs. MM | 10.55 | 2.872 | 301.9 | 3.67 | <0.001 |
| ***Culiseta annulata*** | BG vs. EVS | -0.975 | 0.347 | 300.8 | -2.81 | 0.005 |
| | EVS vs. MM | 0.703 | 0.357 | 301.4 | 1.97 | 0.05 |

Table A.4: Mean ±SE differences in least squares means associated with the mixed linear models for the number of trapped individuals per trapping period for each species and the total number of individuals and the mean ±SE number of species among the four trapping devices. Only mosquito species caught with more than 100 individuals are shown and trapping periods were only included if the species was detected with at least one individual in the corresponding trapping period at the sampling location (only significant differences shown). BG: Biogents Sentinel trap, EVS: Heavy Duty Encephalitis Vector Survey trap, CDC: Centres for Disease Control miniature light trap, MM: Mosquito Magnet Patriot Mosquito trap, Estimate: differences in least squares means, SE: standard error, DF: degrees of freedom, t: t-value, p: p value.

| **Response variable** | **Traps** | **Estimate** | **SE** | **DF** | **t** | **p** |
|---|---|---|---|---|---|---|
| **Total** | BG vs. EVS | 30.106 | 10.598 | 301 | 2.84 | 0.005 |
| | BG vs. MM | 41.781 | 10.889 | 301.3 | 3.84 | <0.001 |
| | CDC vs. EVS | 30.289 | 10.595 | 301 | 2.86 | 0.005 |
| | CDC vs. MM | 41.965 | 10.885 | 301.3 | 3.86 | <0.001 |
| **Species** | BG vs. CDC | 0.446 | 0.215 | 301 | 2.08 | 0.039 |
| | BG vs. EVS | 0.531 | 0.215 | 301 | 2.47 | 0.014 |
| | BG vs. MM | 0.619 | 0.221 | 301.1 | 2.8 | 0.005 |

Table A.5: Mean ±SE differences in least squares means associated with the mixed linear models for the number of trapped individuals per trapping period among the four trapping devices and the three aggregated biotopes (only significant differences shown). BG: Biogents Sentinel trap, EVS: Heavy Duty Encephalitis Vector Survey trap, CDC: Centres for Disease Control miniature light trap, MM: Mosquito Magnet Patriot Mosquito trap, Estimate: differences in least squares means, SE: standard error, DF: degrees of freedom, t: t-value, p: p value.

| **Response variable** | **Biotope** | **Traps** | **Estimate** | **SE** | **DF** | **t** | **p** |
|---|---|---|---|---|---|---|---|
| **Total** | Floodplain | BG vs. CDC | -81.304 | 22.617 | 98.2 | -3.59 | 0.001 |
| | Floodplain | CDC vs. EVS | 77.536 | 22.597 | 98.1 | 3.43 | 0.001 |
| | Floodplain | CDC vs. MM | 82.400 | 23.742 | 99.2 | 3.47 | 0.001 |
| | Urban | BG vs. CDC | 11.106 | 5.247 | 168.1 | 2.12 | 0.036 |
| | Urban | BG vs. EVS | 13.851 | 5.247 | 168.1 | 2.64 | 0.009 |
| | Urban | BG vs. MM | 20.203 | 5.430 | 169.1 | 3.72 | <0.001 |
| | Wet forest | BG vs. CDC | 216.625 | 38.277 | 27.0 | 5.66 | <0.001 |
| | Wet forest | BG vs. EVS | 243.375 | 38.277 | 27.0 | 6.36 | <0.001 |
| | Wet forest | BG vs. MM | 281.750 | 38.277 | 27.0 | 7.36 | <0.001 |

Table A.6: Mean ±SE differences in least squares means associated with the mixed linear models for the number of trapped species per trapping period among the four trapping devices and the three aggregated biotopes (only significant differences shown). BG: Biogents Sentinel trap, EVS: Heavy Duty Encephalitis Vector Survey trap, CDC: Centres for Disease Control miniature light trap, MM: Mosquito Magnet Patriot Mosquito trap, Estimate: differences in least squares means, SE: standard error, DF: degrees of freedom, t: t-value, p: p value.

| **Response variable** | **Biotope** | **Traps** | **Estimate** | **SE** | **DF** | **t** | **p** |
|---|---|---|---|---|---|---|---|
| **Species** | Urban | BG vs. CDC | 1.106 | 0.249 | 168.0 | 4.44 | <0.001 |
| | Urban | BG vs. EVS | 0.660 | 0.249 | 168.0 | 2.65 | 0.009 |
| | Urban | BG vs. MM | 0.981 | 0.258 | 168.5 | 3.80 | <0.001 |

## Figures

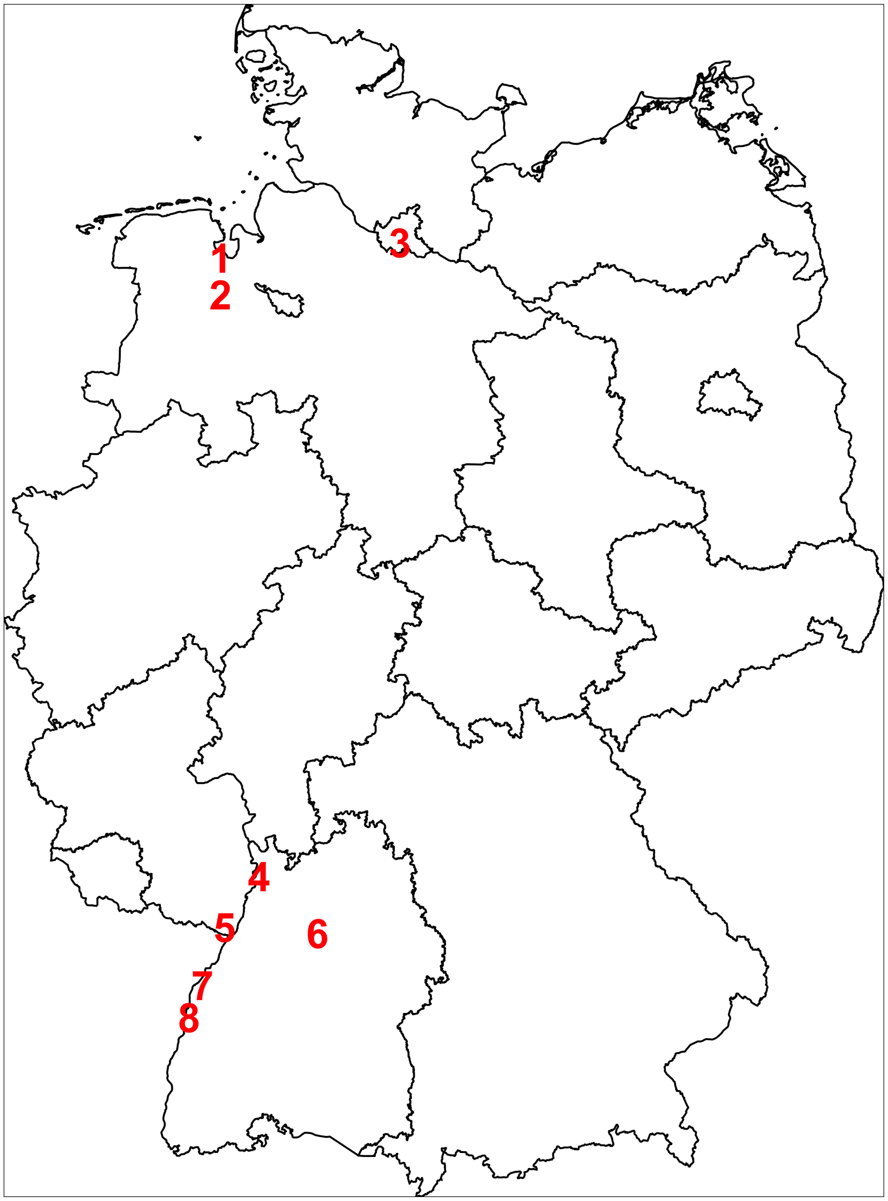

Figure A.1: Sampling locations of the trap comparisons in Germany. Numbers correspond to the IDs in Table A.1.

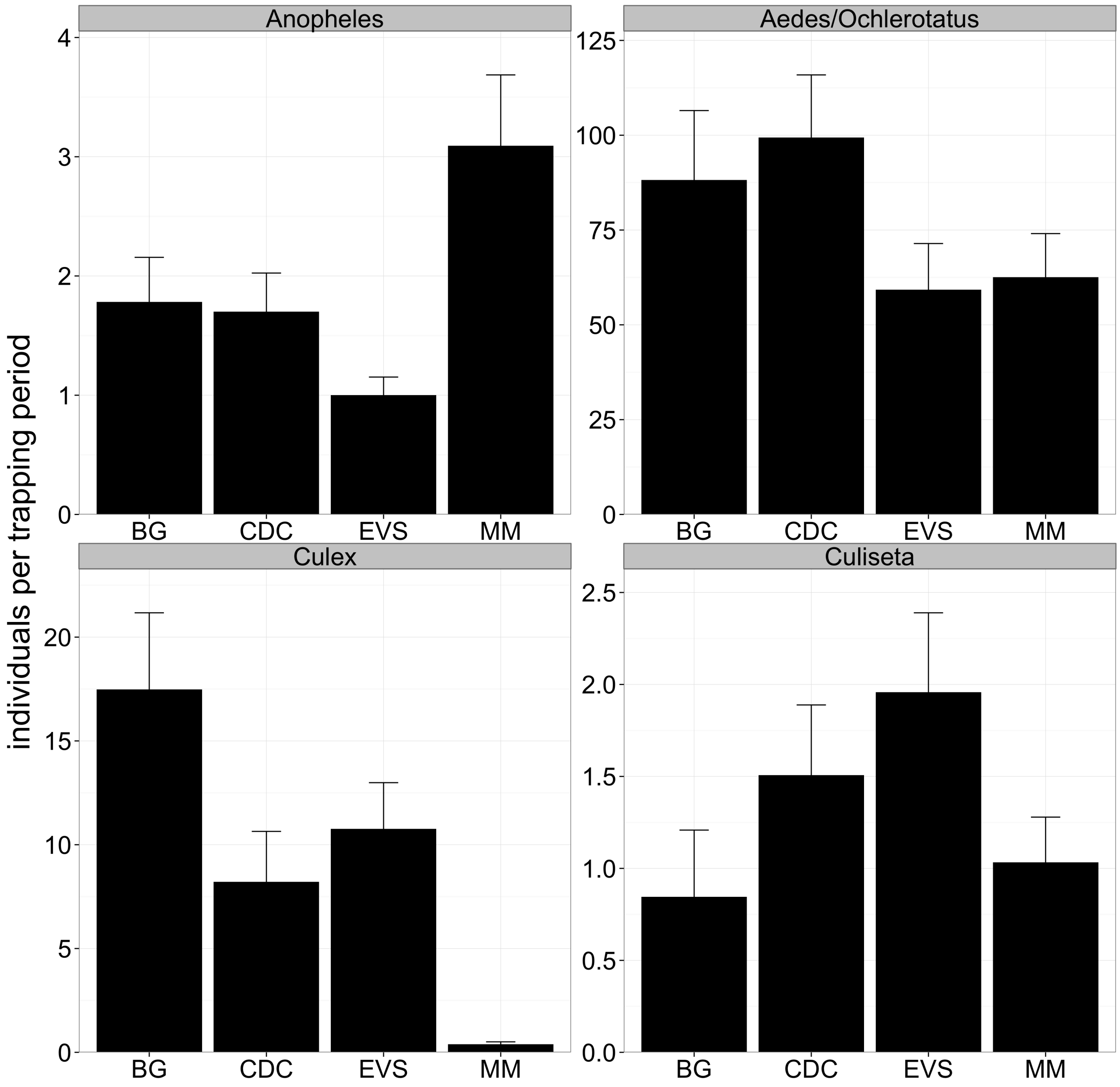

Figure A.2: Mean ±SE number of trapped individuals per trapping period among the four trapping devices. Only mosquito genera caught with more than 100 individuals are shown and trapping periods were only included if the genus was detected with at least one individual in the corresponding trapping period at the sampling location.

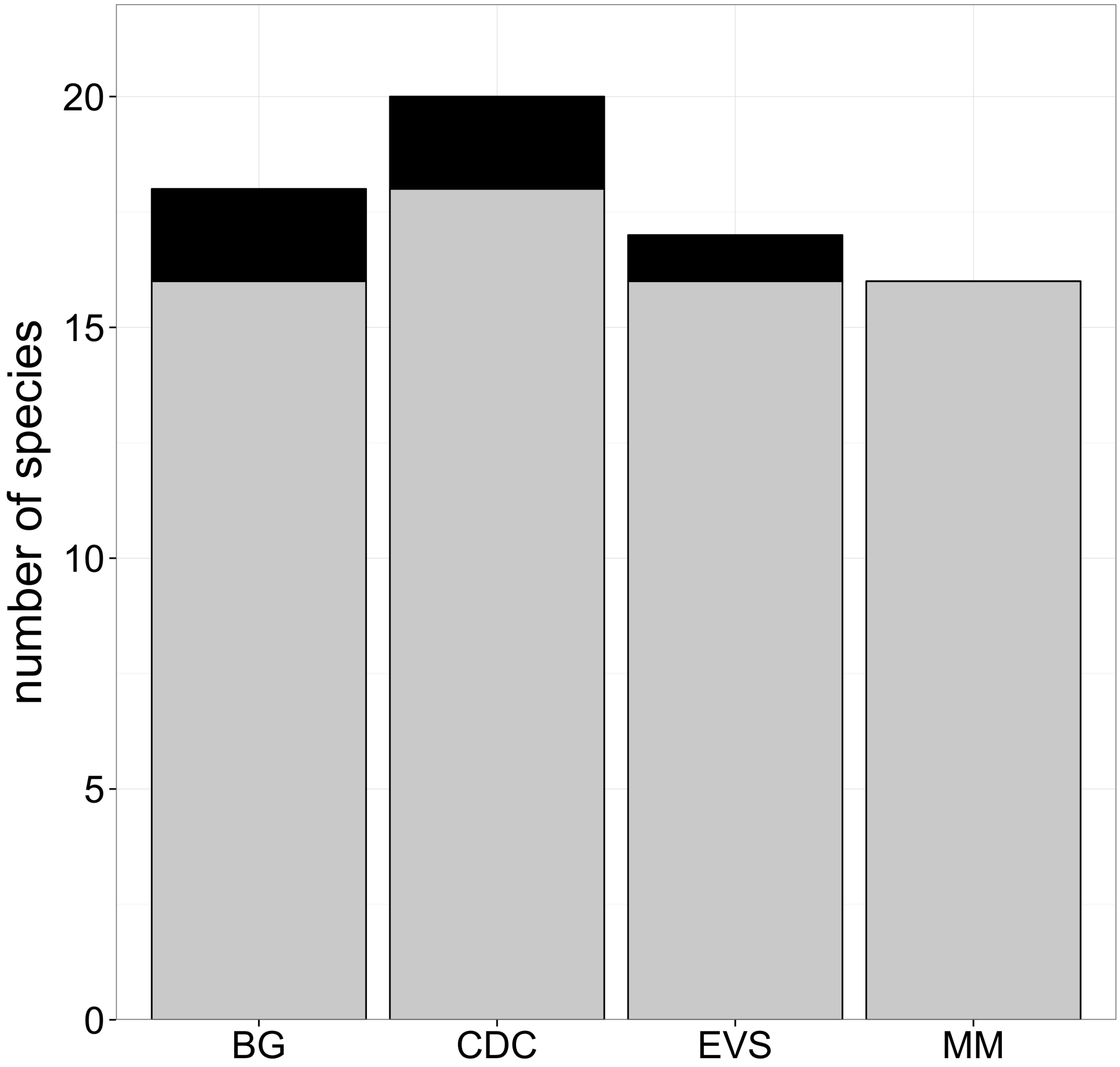

Figure A.3: Total number of species caught among the four trapping devices (grey = number of species without singletons, black = singletons).

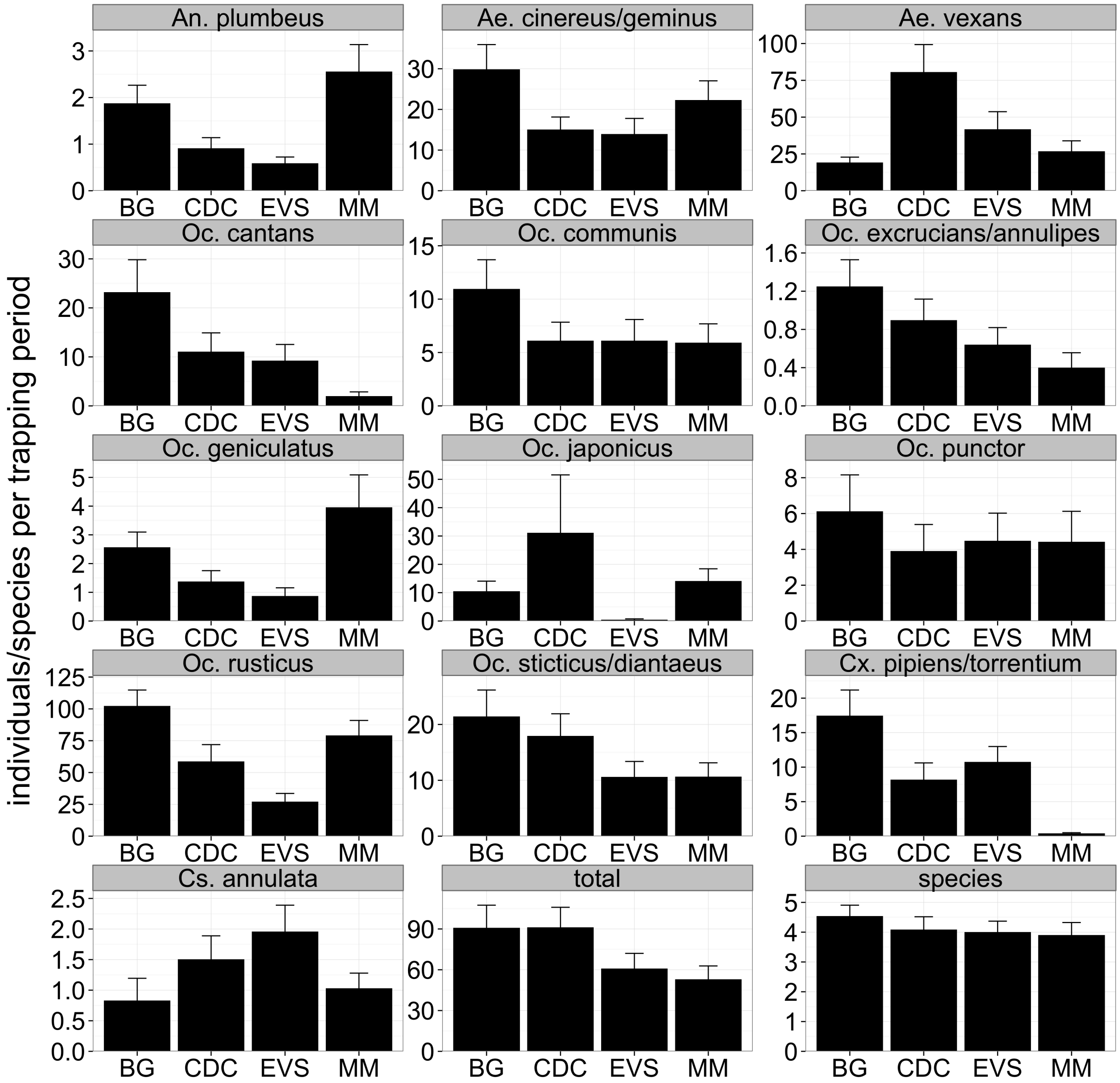

Figure A.4: Mean ±SE number of trapped individuals per trapping period for each species and the total number of individuals and the mean ±SE number of species among the four trapping devices. Only mosquito species caught with more than 100 individuals are shown and trapping periods were only included if the species was detected with at least one individual in the corresponding trapping period at the sampling location.

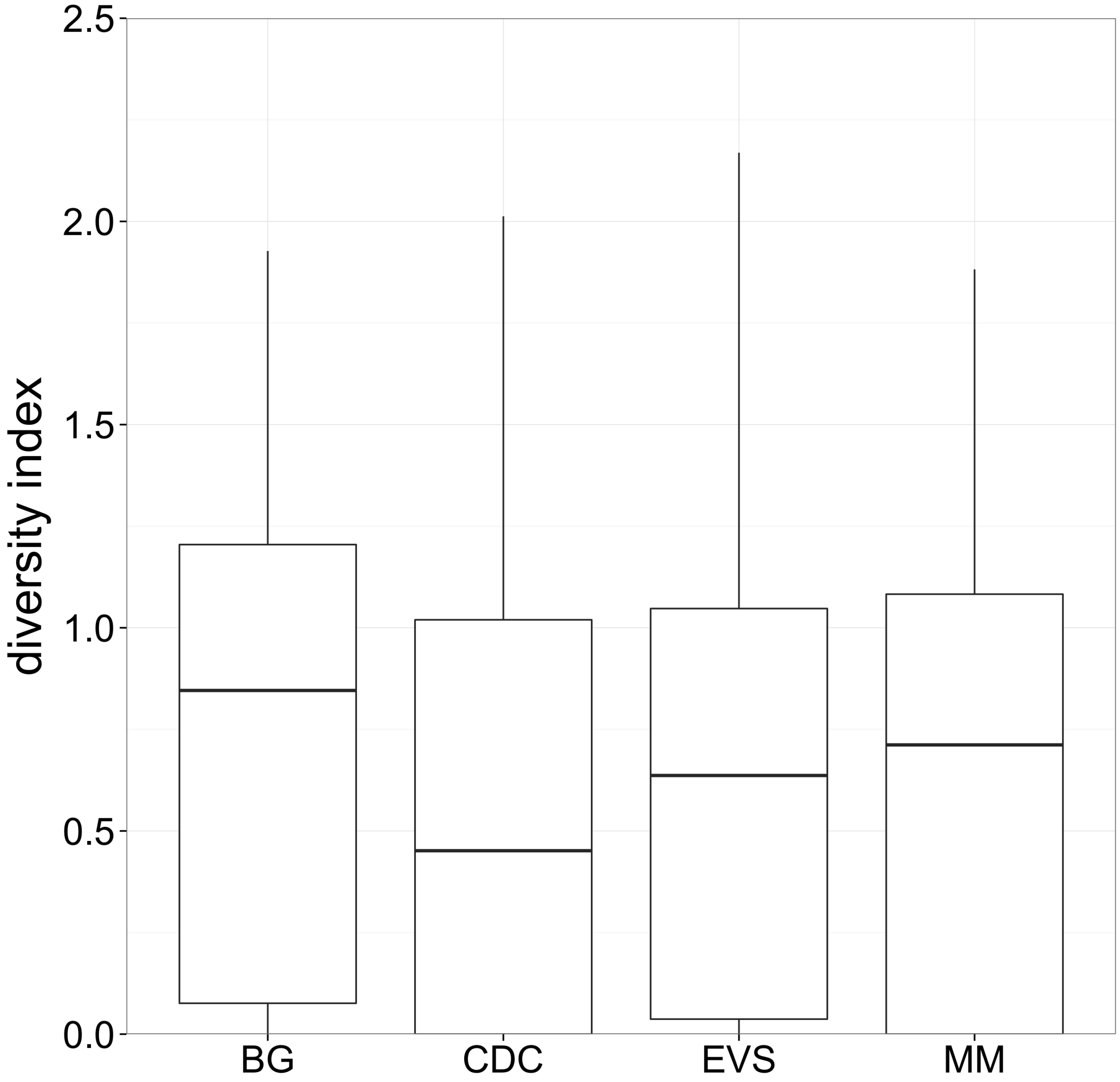

Figure A.5: Boxplots of Simpson's diversity indices per trapping period among the four trapping devices.

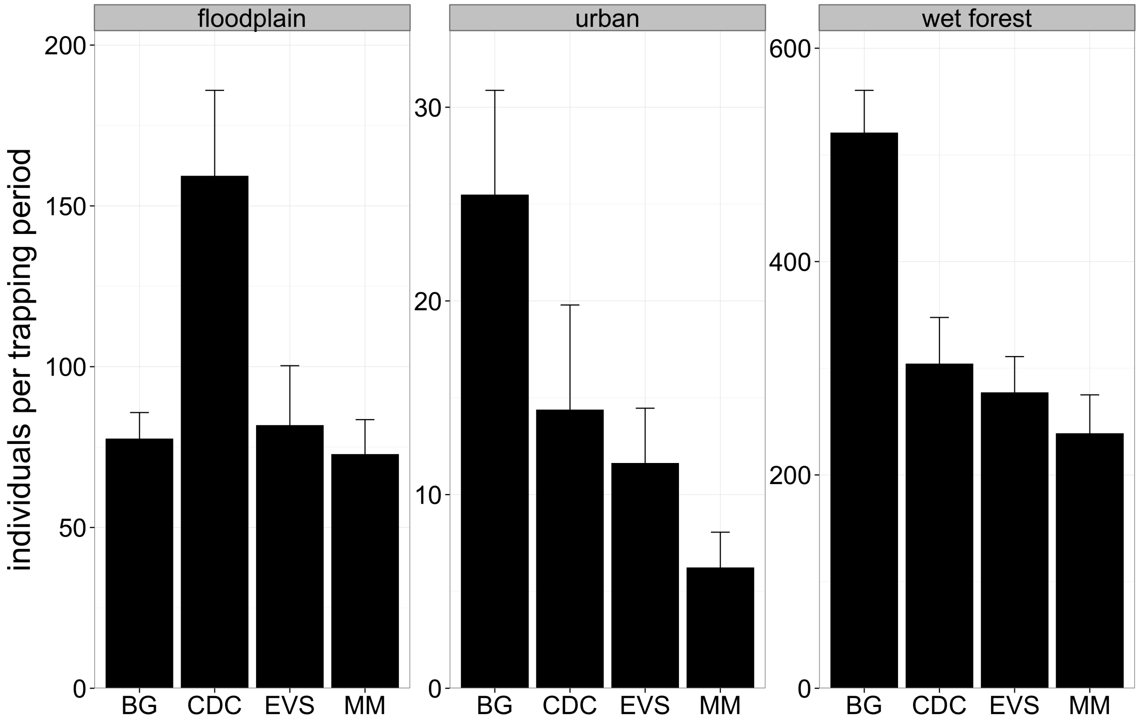

Figure A.6: Mean ±SE number of trapped individuals per trapping period among the four trapping devices and the three aggregated biotopes.

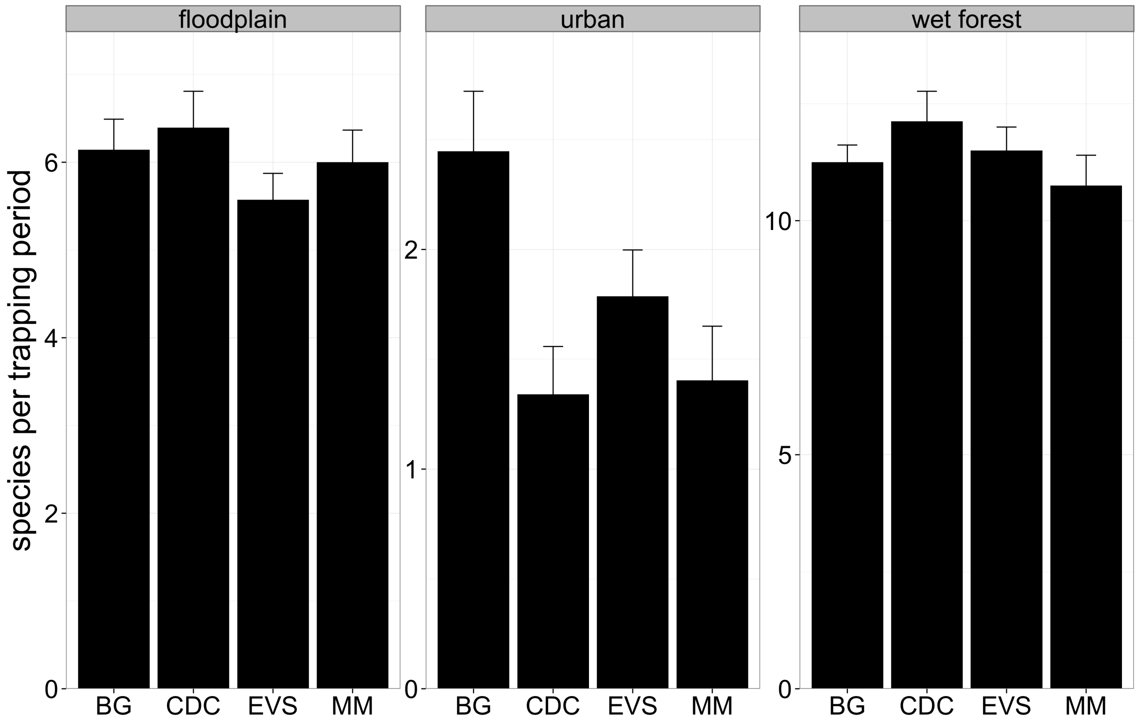

Figure A.7: Mean ±SE number of trapped species per trapping period among the four trapping devices and the three aggregated biotopes.

## Supplementary material

Table A.7: Mosquito species not caught with the four different trapping devices. Occurrence in Germany classified after Becker & Hoffmann (2011) (occurrence: ++ = frequent; + = regularly; (+) = rare; - = not classified; * species is not established; [ ] = not counted in the species lists).

| **Species** | **Occurence in Germany** |
|---|---|
| ***An. algeriensis*** | - |
| ***Ae. albopictus**** | [(+)] |
| ***Oc. cataphylla*** | (+) |
| ***Oc. cyprius*** | - |
| ***Oc. dorsalis*** | (+) |
| ***Oc. detritus*** | (+) |
| ***Oc. flavescens*** | (+) |
| ***Oc. intrudens*** | - |
| ***Oc. leucomelas*** | + |
| ***Oc. nigrinus*** | (+) |
| ***Oc. refiki*** | - |
| ***Oc. riparius*** | - |
| ***Oc. pullatus*** | (+) |
| ***Cx. martinii*** | - |
| ***Cx. modestus*** | ++ |
| ***Cs. alascaensis*** | (+) |
| ***Cs. fumipennis*** | - |
| ***Cs. glaphyroptera*** | (+) |
| ***Cs. longiareolata*** | (+) |
| ***Cs. subochrea*** | (+) |
| ***Cs. ochroptera*** | + |

Table A.7: Mosquito species not caught with the four different trapping devices. Occurrence in Germany classified after Becker & Hoffmann (2011) (occurrence: ++ = frequent; + = regularly; (+) = rare; - = not classified; * species is not established; [ ] = not counted in the species lists).

| **Species** | **Occurence in Germany** |
|---|---|
| ***Ur. unguiculata*** | (+) |

## A.2 Distance from the stable affects trapping of biting midges (Diptera, Ceratopogonidae)

published: **Lühken, R.** & Kiel, E. (2012). Distance from the stable affects trapping of biting midges (Diptera, Ceratopogonidae). *Journal of Vector Ecology*, *37*, 453-457.

### Introduction

Several species of *Culicoides* biting midges (Diptera, Ceratopogonidae) are competent vectors of important veterinary pathogens in Europe. Bluetongue virus (BTV) has emerged in Europe since the late 1990s and is transmitted by several species of the genus *Culicoides* (Caracappa et al., 2003; De Liberato et al., 2005). African horse sickness (AHS) was observed at the end of the last century on the Iberian Peninsula (Mellor & Hamblin, 2004) and since the end of 2011 there has been evidence that biting midges are important vectors of the Schmallenberg Virus (SBV) emerging in Central Europe (Hoffmann et al., 2012). SBV causes considerable congenital damage, premature births, and reproductive disorders in ruminants.

Monitoring programs for biting midges are generally based on UV-light/suction trap sampling (Mehlhorn et al., 2009; Nielsen et al., 2010). Results of the sampling and trapping protocols could be significantly affected by uncontrollable environmental factors like wind and temperature (Bishop et al., 2000) and/or even the phase of the moon (Bishop et al., 2000; Carpenter et al., 2008). In contrast, there are controllable factors that could be standardized and would result in more comparable results from entomological surveillance programs. Firstly, there is the standardization of the trapping method, which could have different specificity for abundance, taxa, sex, or feeding stadium (Holbrook, 1985; Anderson & Linhares, 1989; Venter et al., 2009; Viennet et al., 2011). Secondly, some authors mentioned that the positions of the traps have a significant impact on the sampling results, such as height (Venter et al., 2009) or habitat (Bishop et al., 1995, 1994). In this study, we were interested in the impact of the distance to the stable on the trapping result. Due to a positive correlation between biting midge abundance and the presence and abundance of hosts (Bellis & Reid, 1996; Baylis et al., 2010; Viennet et al., 2011; Garcia-Saenz et al., 2011), we expected a decreasing number of biting midges to occur in traps with increasing distance to the stable.

## Methods

The study was conducted between May and June, 2009 at one livestock farm that is surrounded by meadows in northern Germany. This site represents a typical dairy farm with 220 dairy cattle and is located in a typical agricultural region in the marshland of Lower Saxony. The grazing density of cattle was approximately 7.3 individuals per hectare, but the cattle were in the stables during the night.

Sampling of *Culicoides* spp. was conducted with BG- Sentinel$^{TM}$ light traps fitted with ultraviolet light. Each of the three traps was placed in concentric circles in distances of 25, 50, 100, and 200 m (variable DISTANCE) around one trap in direct proximity to the stable (Figure A.8a). All traps were installed at 1.5 m above the ground and operated with one car battery each per sampling period. A photo sensor was installed in the immediate vicinity of each trap. The photo sensor was adjusted to illuminate the ultraviolet light from sunset to dawn, because the activity of *Culicoides* spp. was expected to be highest in this time period (Kettle et al., 1998). The traps were sampled five times, every seventh day (variable SAMPLINGPERIOD). Insects were collected in 70% ethanol and stored in separate containers. The samples were presorted in order to separate biting midges from other insects and to differentiate members of the *C. obsoletus* and *C. pulicaris* group (also *C. obsoletus* or *C. pulicaris* in the subsequent text) from other Ceratopogonidae.

All data analyses and graphs were made with the program R (R Core Team, 2014). Due to overdispersion in the data, collection data of females of the *Culicoides obsoletus* and *Culicoides pulicaris* group were analyzed with negative binomial generalized linear models through the function glm. nb from the package MASS (Venables & Ripley, 2002). The variables DISTANCE, DIRECTION, SAMPLINGPERIOD, and all interactions between the variables were implemented in the model. The value of the variable DIRECTION for each trap *tr* was calculated as: $\mathrm{a}_{tr} = \pi\alpha_{tr}/180$ $\mathrm{DIRECTION}_{tr} = \sin(\mathrm{a}_{tr} + 5\pi/4)$ where $\alpha$tr is the compass direction in degree for each trap tr measured from the center of the stable (Figure A.8b). We used a phase shift of $5\pi/4$ in the calculation of DIRECTION because the prevailing wind direction in northern Germany is southwest. The value of the variable DIRECTION is close to 1 if the direction of the trap is southwest relative to the stable and close to -1 if the direction is northeast. DIRECTION is close to zero if the position of the trap relative to the stable is either northwest or southeast.

According to recommendations by Zuur et al. (2009), hypothesis testing for significance of the variables and their interactions were conducted using the likelihood ratio test (function lrtest) from the lmtest package (Zeileis & Hothorn, 2002), dropping each term in turn and comparing the full model with a nested model. The non-significant variables with the lowest impact on the deviance of the model were excluded and then the model was refitted with the remaining terms until all terms were significant. Z-statistics and graphs of the final models are presented. Correlation between the number of females of the *Culicoides obsoletus* and *Culicoides pulicaris* groups were analyzed using the Spearman's rank correlation test (function cor.test) from the stats package. Additionally, non-parametric chi-square tests for trend were applied to evaluate the proportion of samples positive for females of the *Culicoides obsoletus* or *Culicoides pulicaris* group using the function prop.trend.test from the stats package.

## Results

A total of 21,436 biting midges was collected in addition to 297,918 of other arthropods. *C. pulicaris* accounted for 20% with 75.6% females (3,253 females/4,301 ind.), *C. obsoletus* for 53.6% with 76.4% females (8,778 females/11,486 ind.) and other

Ceratopogonidae for 26.4% with 66.6% females (3,761 females/5,649 ind.) of the samples.

The number of collected females of *C. pulicaris* was significantly affected by the variable DISTANCE ($\chi^2{}_1 = 8.9$, $P < 0.01$; Table A.8 and Figure A.9), while the number of *C. obsoletus* was additionally affected by the variable DIRECTION as we found a significant interaction for the variables DISTANCE and DIRECTION ($\chi^2{}_1 = 5.2$, $P < 0.05$; Table A.8 and Figure A.10). The number of trapped females significantly decreased with increasing distance to the stable for both groups. The *C. obsoletus* group only had higher numbers in the traps southwestwards from the stable relative to the other traps (Figure A.10).

Neither the interaction DISTANCE and DIRECTION nor the single variable DIRECTION were significant for *C. pulicaris* ($\chi^2{}_1 = 1.2$, $P > 0.05$ and $\chi^2{}_1 = 0.8$, $P > 0.05$). The variable SAMPLINGPERIOD was not significant for both groups (*C. obsoletus*: $\chi^2{}_1 = 0.3$, $P > 0.05$; *C. pulicaris*: $\chi^2{}_1 = 0.8$,$P > 0.05$). Furthermore, no significant interaction was found for the three-way interaction among SAMPLINGPERIOD, DISTANCE, and DIRECTION (*C. obsoletus*: $\chi^2{}_1 = 0.1$, $P > 0.05$; *C. pulicaris*: $\chi^2{}_1 = 0.5$, $P > 0.05$) or the two-way interactions SAMPLINGPERIOD and DISTANCE (*C. obsoletus*: $\chi^2{}_1 = 0.002$, $P > 0.05$; *C. pulicaris*: $\chi^2{}_1 = 1.1$, $P > 0.05$) or SAMPLINGPERIOD and DIRECTION (*C. obsoletus*: $\chi^2{}_1 = 3.3$, $P > 0.05$; *C. pulicaris*: $\chi^2{}_1 = 0.4$, $P > 0.05$).

There was a highly significant, positive correlation between the number of collected females of both groups (Spearman rho = 0.75, $P < 0.001$). The number of samples with females of the *Culicoides obsoletus* group significantly decreased with increasing distance of the traps to the stable (chi-square test for trend, $\chi^2{}_1 = 5.7$, $P < 0.05$; Figure A.11), but no significant effect was found for the *Culicoides pulicaris* group (chi-square test for trend, $\chi^2{}_1 = 0.4$, $P > 0.05$; Figure A.11).

## Discussion

This study demonstrated a significant impact of the trap position on the results of light suction trap sampling. Although the farm was surrounded by meadows rotationally used for diary cattle, the number of females of the *C. obsoletus* and the *C. pulicaris* groups significantly decreased with increasing distance to the stable. Additionally, we found a lower proportion of samples positive for females of the *C. obsoletus* group but not for the *C. pulicaris* group. This matches the results from other studies, which found a positive correlation between biting midge abundance and the presence and abundance of hosts (Bellis & Reid, 1996; Baylis et al., 2010; Viennet et al., 2011; Garcia-Saenz et al., 2011).

We found a significant correlation between the number of female *C. obsoletus* and *C. pulicaris*. Nevertheless, the number *C. obsoletus* females showed a stronger decrease with increasing distance to the stable than *C. pulicaris*, and the number of samples with females present significantly decreased with increasing distance but did not show a significant trend for *C. pulicaris*. These differences might be caused by different breeding site preferences, but a general knowledge of the breeding sites of most important vector biting midges is still missing (Nielsen et al., 2010). However, surroundings of livestock farms provide an abundance of suitable habitats (Zimmer et al., 2008). Pre-adult stages of *C. obsoletus* are expected to be closely connected to anthropogenic livestock farming substrates (Nielsen et al., 2010). Species were confirmed for dried dung, animal litter, and maize silage (Zimmer et al., 2010), which generally occur close to the farms. With preferences for wet soils and bogs (Purse et al., 2003), *C. pulicaris* is generally expected to be more euryoeciously distributed (Nielsen et al., 2010).

The impact of the variable direction on the number of *C. obsoletus* females has to be evaluated with caution because the position of traps was not uniformly distributed in respect to the compass direction. The main wind direction in northern Germany is southwest, which perhaps resulted in higher values in the traps positioned southwestward from the stable but, as reviewed by (Sedda et al., 2012), at present there are no studies about the small-scaled impact of wind on *Culicoides* flight. A much more important factor could be the position of the traps relative to productive breeding sites that cannot be evaluated from our study.

Further studies need to evaluate whether a decreasing trapping success with increasing distance to the stable is a general pattern for other farms and for all species of biting midges. Additionally, we are concerned about the spatial interaction between UV-light/suction traps, as the spatial dependence of the samples is largely unknown. Rigot & Gilbert (2012) demonstrated a significant spatial interaction for Onderstepoort-type blacklight traps in a distance of 50 m. A similar effect may be expected for the BG-Sentinel$^{TM}$ light traps. The impact on our study results is hardly predictable, because we do not know to what extent the traps were affected by interference. Nevertheless, due to the strong decrease of collected females with increasing distance to the stable, at least for *C. obsoletus*, there is probably also a significant impact under the presumption of trap interference. Further studies should study the spatial interaction for BG-Sentinel$^{TM}$ light traps to improve study designs and allow spatial independent samples.

Because of a significant decrease of collected biting midges even in small distances from the stable, sampling in the direct surroundings of stables ($< 25$ m) probably promises the highest trapping success. However, this suggests that comparative studies on biting midges should be interpreted carefully if trap positions relative to the stable differ. Moreover, it is necessary to standardize trap position as far as possible in order to achieve comparable data.

## Acknowledgment

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# Table

Table A.8: Results of negative binomial generalized linear models in R. Negative estimate values indicate that the abundance of females of the *Culicoides obsoletus* and *Culicoides pulicaris* group decreased with increasing value of the corresponding coefficient.

| ***Culicoides* females** | **Coefficients** | **Estimate ± SE** | **z value** | **Pr(>\|z\|)** |
|---|---|---|---|---|
| ***Culicoides obsoletus*** | Intercept | 5.690 ± 0.333 | 17.099 | <0.001 |
| | Distance | -21.703 ± 3.253 | -6.671 | <0.001 |
| | Direction | 1.954 ± 0.618 | 3.160 | <0.01 |
| | Distance:Direction | -15.611 ± 5.922 | -2.636 | <0.01 |
| | Intercept | 4.589 ± 0.307 | 14.929 | <0.001 |
| ***Culicoides pulicaris*** | Distance | -9.128 ± 2.980 | -3.064 | <0.01 |

## Figures

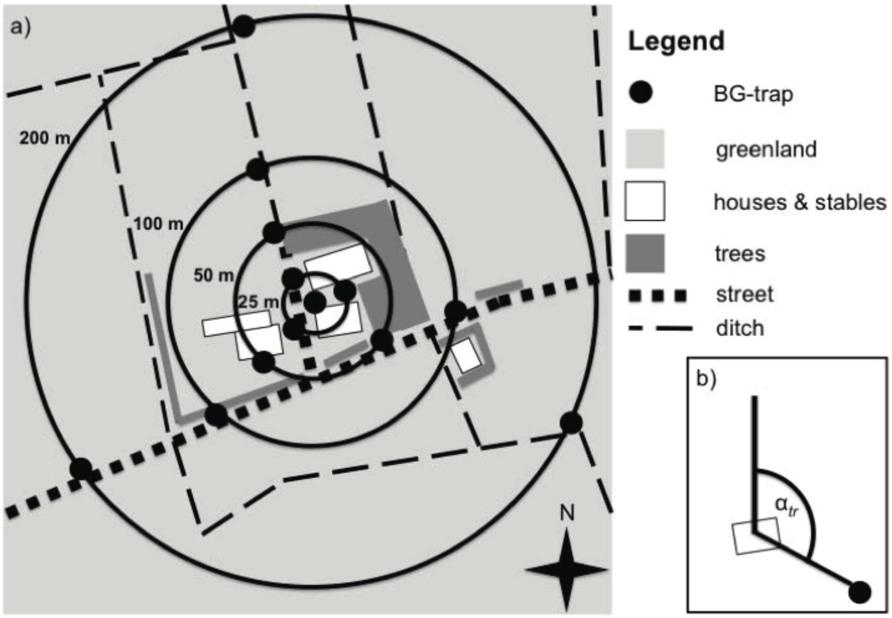

Figure A.8: (a) Map of the study site in northern Germany. (b) Sketch of the determination of α.

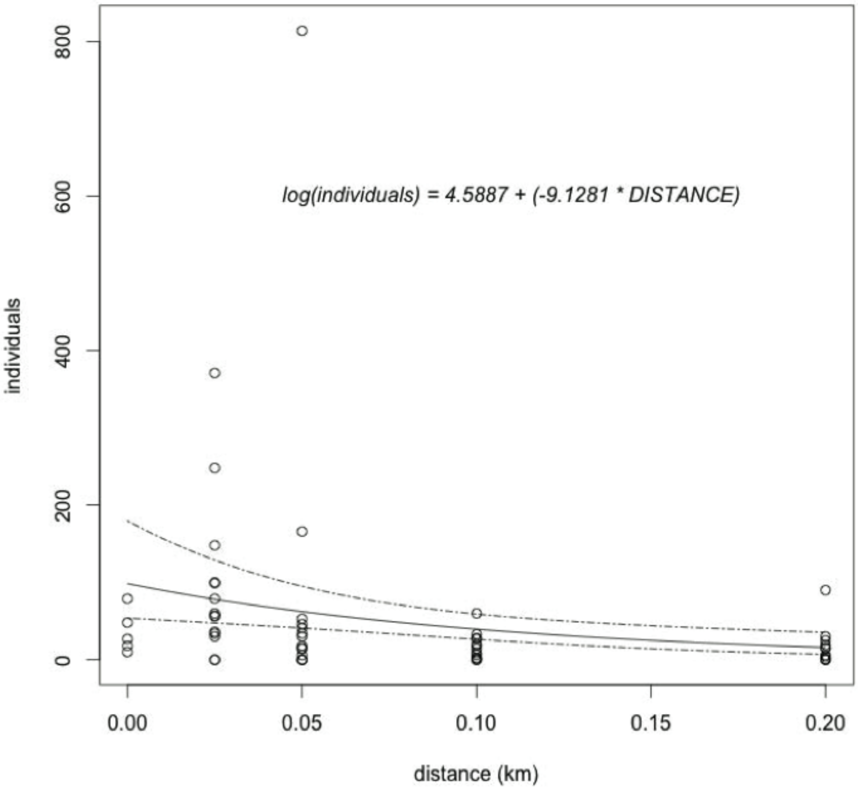

Figure A.9: Number of females of the *Culicoides pulicaris* group caught with light suction traps positioned in five different distances from the stable on one farm in northern Germany. The best fit regression line with confidence intervals is shown.

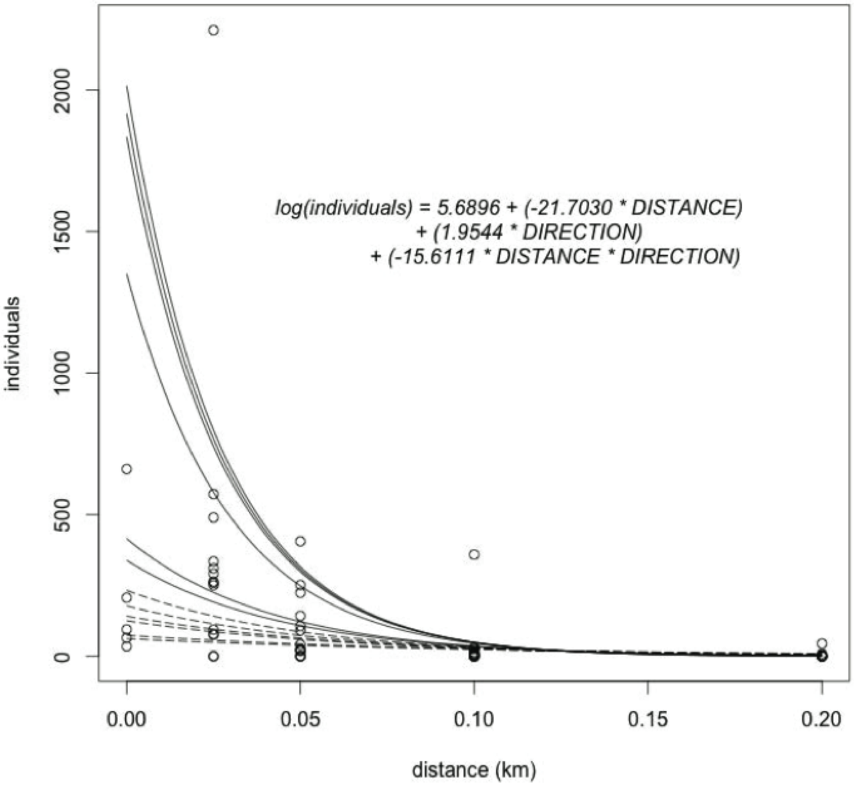

Figure A.10: Number of females of the *Culicoides obsoletus* group caught with light suction traps positioned in five different distances from the stable on one farm in northern Germany. The best fit regression lines are shown. Solid line: DIRECTION above zero; dashed line: value below zero.

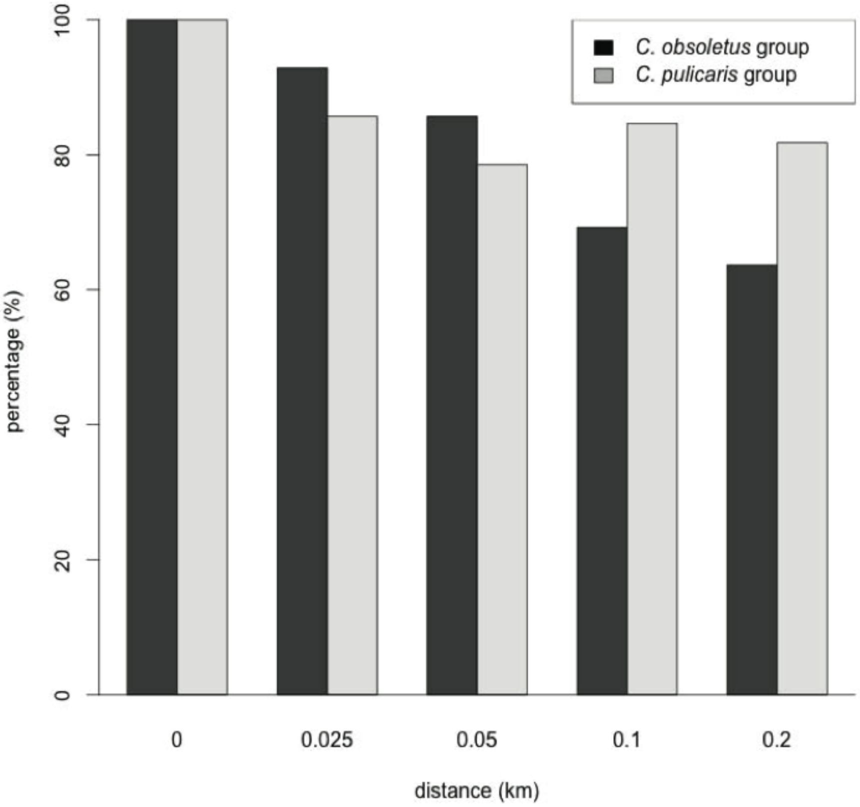

Figure A.11: Percentage of samples with females of the *Culicoides obsoletus* and *C. pulicaris* group present, caught with light suction traps positioned at five distances from the stable on one farm in northern Germany.

# Appendix B

# Identification

## B.1 The use of morphometric wing characters to discriminate female *Culex pipiens* and *Culex torrentium*

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### Abstract

The reliability of the length of wing radial vein $r_{2/3}$ as a character for the morphological discrimination of the two potential arbovirus vectors *Culex pipiens* s.s. and *Cx. torrentium* from Germany was reassessed, after this character had been neglected for

more than 40 years. Additionally, multivariate morphometric analyses were applied to evaluate wing shape variation between both species. Although high-throughput molecular tools are now available to differentiate the two species, a simple, low-cost routine alternative may be useful in the absence of a molecular laboratory, such as under semi-field conditions. A thin-plate splines transformation confirmed that primarily the shrinkage of vein $r_{2/3}$ is responsible for the wing differences between the two species. In the bivariate analysis, the $r_{2/3}$/ $r_3$ indices of *Cx. pipiens* s.s. and *Cx. torrentium* were 0.185 and 0.289, respectively, resulting in a correct classification of more than 91% of all tested specimens. Using the absolute length of vein $r_{2/3}$ alone still allowed for more than 90% accurate discrimination. Furthermore, classification accuracy of linear discriminant analysis exceeded 97%.

## Introduction

The northern house mosquito, *Culex* (*Culex*) *pipiens* Linnaeus, is one of the most common and widespread mosquito species throughout urban and suburban areas in many faunal regions worldwide (Weitzel et al., 2009). It was therefore not surprising when it became evident that this taxon comprises a complex of several sibling species, namely *Cx. australicus*, *Cx. pipiens* sensu stricto and *Cx. quinquefasciatus*, with geographic restrictions to some degree (Harbach, 2011, 2012). As a consequence, the potential role of the various complex members as vectors of diseases had to be correlated to their respective geographic distribution and behavioral traits (e.g., host preference). In central and southern Europe, *Cx. pipiens* has been reported to be infected with West Nile virus (WNV) (Parreira et al., 2007; Papa et al., 2013; Radrova et al., 2013), and it has been shown experimentally to be capable of transmission (Balenghien et al., 2008). The enzootic transmission of WNV between birds is facilitated by the mainly ornithophagic behavior of the biotype Pipiens of *Cx. pipiens* (here referred to as *Cx. pipiens* s.s.). Nevertheless, mammalophagy is also possible and indicates that the species is also a potential bridge-vector for avian-to-mammal or avian-to-human transmission (Radrova et al., 2013).

Martini (1924) described *Cx.* (*Culex*) *torrentium* from Germany, which was considered a rare species in Europe for the following decades (Struppe, 1989). More recently, it has been shown to be widespread and common in central Europe (Rudolf et al., 2013; Hesson et al., 2014) and predominate in Sweden (Hesson et al., 2011). It belongs to the Pipiens group of the subgenus *Culex* but not to the *Cx. pipiens* complex (Harbach, 2011), although some authors regard it as such (e.g. Becker et al., 2010). *Culex torrentium* closely resembles *Cx. pipiens* and can only be distinguished with certainty by the male genitalia, whereas the females are morphologically almost identical (Service, 1968; Becker et al., 2010). According to Weitzel et al. (2011), discrimination between *Cx. torrentium* and *Cx. pipiens* was neglected until differential vector capacities were discovered. *Culex torrentium* is experimentally highly susceptible to Sindbis virus in Sweden, Norway, and Russia (Lundström, 1994; Lundström et al., 99a) and turned out to be the main enzootic vector for Sindbis in Sweden (Hesson et al., 2011).

Natvig (1948) and Mohrig (1969) described a difference in the wing venation as a morphological character that could be used for species discrimination. According to (Mohrig, 1969) the wing vein $r_{2/3}$ of *Cx. torrentium* females measures about one-third, but at least one-quarter of vein $r_2$, whereas in *Cx. pipiens* it measures only about one-sixth and rarely one-fifth of $r_2$ (Grodnitsky, 1999). Service (1968) rejected the reliability of this character for specimens from Great Britain, as did (Fedorova & Shaikevich, 2007) for Muscovite specimens.

Only recently, genetic tools based on various analyses of COI and ace-2 genes (Smith & Fonseca, 2004; Shaikevich, 2007; Hesson et al., 2010, 2011; Engdahl et al., 2014) and of allozymes (Weitzel et al., 2011) have been applied to address the taxonomic problem, particularly in view of the emerging risks of disease transmission in temperate regions of central and northern Europe. These methods, however, are rather time and labor-intensive when it comes to the processing of thousands of specimens, e.g., for mosquito surveillance programs. Furthermore, not all of the above-mentioned studies were able to distinguish the biotypes within *Cx. pipiens*. An advanced method is a multiplex real-time PCR to distinguish various species and biotypes of *Culex* from both single and pooled specimens (Rudolf et al., 2013). In order to clarify the reliability of the wing morphology, which would allow for a quick species separation of the two potential vectors even under semi-field conditions without a molecular laboratory, we used the new PCR protocol to correlate molecular typing with multi-, bi-, and univariate wing morphometry of mainly German specimens of *Cx. pipiens* s.s. and *Cx. torrentium.*

## Methods

### Mosquito sampling and identification

Collection details of all 390 specimens are listed in Table B.1. The samples consisted of 228 female *Cx. pipiens* s.s. and 162 *Cx. torrentium.* In order to obtain a priori identified samples we used two strategies. (i) Control group (n=52 *Cx. pipiens* s.s., 48 *Cx. torrentium*): For most of the control group samples (n=55), we reared single egg batches and used the resulting adult males for assessing their genitalia and thus revealing the identity of each egg batch, i.e., *Cx. pipiens* and *Cx. torrentium* (Mohrig, 1969; Becker et al., 2010). Similarly, three samples (n=16) were obtained from rearing larvae and they turned out to be monospecific for either *Cx. pipiens* or *Cx. torrentium. Culex* egg batches and larval samples were collected from natural breeding sites such as water ponds or rain barrels. Each egg batch or larval sample was kept in a 100 ml plastic cup filled with a suspension of distilled water and 5 g of dry hay under ambient temperature (approximately 18 to 25° C) until adult emergence. The male genitalia were usually scored on freshly killed specimens under the dissection microscope with 40x magnification, for the shape of the ventral paraproct arm: either very prominent, strongly sclerotized (*Cx. torrentium*), or minute and transparent (*Cx. pipiens*). The identity of 29 specimens in the control group without corresponding males was assumed to be *Cx. pipiens* according to (Rudolf et al., 2013; Hesson et al., 2014) and field observations (A. Krüger, R. Garms, unpublished) who found virtually no *Cx. torrentium* in the North Sea coastal area.

(ii) Test group: The majority of the test group specimens (251 for bi- and multivariate analyses and 14 additional for multivariate analyses) were identified using a multiplex real-time PCR according to Rudolf et al. (2013) or a conventional PCR followed by sequencing a portion of the ace2 gene (Smith & Fonseca, 2004).

Adult mosquito females were either collected indoors, using aspirators, or trapped with CO2-baited, odor-baited, or gravid traps (Table B.1). The following commercial traps were used: BG- Sentinel (Biogents, Regensburg, Germany), and EVS (Bioquip, Rancho Dominguez, CA, U.S.A.).

### Morphometric data collection

One wing was cut off from each female, briefly submerged in absolute ethanol, and mounted under a cover slip in Euparal (Euparal 3C 239, Waldeck GmbH & Co. KG, Münster, Germany). In order to make the respective veins more transparent for easier microscopic assessment, the attached wing scales were scratched off very carefully using a needle prior to mounting.

The mounted wings were photographed under a stereomicroscope. For each specimen 13 raw landmark coordinates (Grodnitsky, 1999) were collected with the program CLIC (http:// mome-clic.com/) for a total of 122 individual mosquitoes: only for the right wing (43 individuals), only for the left wing (40 individuals) and for both the left and right wings (39 individuals) (Figure 1). In our case, landmarks are intersections of wing veins with other wing veins and the wing margin. Non-shape variation such as individual size differences had to be excluded from the morphometric dataset, because we were only interested in shape differences between the wings. Therefore, generalized procrustes analyses in the program CLIC were applied. All raw landmarks were translated, re-scaled and rotated to produce the procrustes residuals.

To obtain an initial sample pool in a similar manner as Mohrig (1969), we generated by manual scoring by eye a control group consisting of specimens without molecular typing.

### Multivariate analyses

Because from the older literature (Natvig, 1948; Mohrig, 1969) it cannot be reconstructed whether $r_{2/3}$ was the only or the most variable vein, we conducted a multivariate approach. Principal component analysis (PCA) was used to analyze the shape differences of the wings between 42 *Cx. torrentium* and 40 *Cx. pipiens* s.s. PCA on the procrustes residuals was sensitive for shape variation, because non-shape variation was eliminated prior to this. Shape differences between the specimens were visualized by PCA scatterplots. The mean shape of all analyzed individuals of *Cx. torrentium* and *Cx. pipiens* was situated where the principal components crossed. PCA was conducted with the function rda from the vegan package (Oksanen et al., 2013) of the program R (R Core Team, 2014). The original variance of the dataset consisting of 13 landmarks each with an x and y coordinate for each individual was summarized by the principal components. The number of principal components, which together gave rise to 95% of the original variance of the dataset, was used for multivariate analysis of variance (MANOVA) to test for significant shape differences between both species.

Inter- and intra-observer variation of landmark collection can affect the precision of measurements and hence the results of PCA and species classification. Therefore, for five randomly selected specimens we repeated the collection of raw landmark coordinates from the right wing four times (Bigoni et al., 2010). A single observer did three repeats and another observer did the fourth. Between each collection of landmarks of the five specimens, we waited at least one week, in order to minimize an over optimistic observer bias by memorizing the wings. The generalized procrustes analyses of the raw landmarks from the precision measurement were done together with the total sample. Procrustes residuals of the precision of measurement and the total sample were together analyzed with PCA.

Thin-plate splines (TPS) transformation was used to show the variation of wing shape between the two species. TPS visualize the differences of the raw landmark configuration of *Cx. torrentium* (left wing: n=43, right wing: n=42) onto the raw landmark configuration of *Cx. pipiens* s.s. (left wing: n=35, right wing: n=40) by smooth interpolation of the interlandmark space. Thin-plate spline grids can be used to identify the responsible landmarks for the differences between the species, e.g., the differences of wing shapes shown in the PCA scatterplots. TPS grids were produced with the function tpsgrid from the Shapes package (Dryden, 2003).

Linear discriminant analysis (LDA) with the procrustes residuals was performed for assessment of species classification. LDA describes intergroup differences by linear combinations of all variables. Each specimen was classified as *Cx. torrentium* or *Cx. pipiens* by its scoring based on the discriminant function. The analysis was conducted with the function LDA in the MASS package (Venables & Ripley, 2002).

Species classification accuracy was measured as the percentage of correctly classified specimens. Because classification accuracies measured on non-independent data, i.e., the same dataset for the execution of the LDA and its validation, probably give an over optimistic assessment of the discriminant power, we conducted internal validation of the morphometric species differentiation of *Cx. torrentium* and *Cx. pipiens* because there was no independent dataset available. Species classification accuracy was assessed using bootstrapping. Ten thousand randomly stratified (50% *Cx. pipiens* s.s./ 50% *Cx. torrentium*) bootstrap samples were taken from the procrustes residuals from the left (n=78) and right wings (n=82) and a LDA performed. Bootstrap percentile confidence intervals of the overall classification accuracies and those of *Cx. pipiens* s.s. and of *Cx. torrentium* were constructed.

### Bivariate analyses

For this part of the study radial veins (Grodnitsky, 1999) $r_{2/3}$ and $r_3$ (landmarks 12-13 and 13-4, respectively), not $r_2$ (landmarks 13-3) as in Mohrig (1969), were analyzed (Figure B.1). Veins $r_2$ and $r_3$ are of almost equal length leading to a similar relative length to $r_{2/3}$ in both cases. Natvig (1948) and Mohrig (1969) found the relative length of stem vein $r_{2/3}$ for *Cx. torrentium* to be about one third (0.33), but at least one quarter (0.25) of vein $r_2$, in *Cx. pipiens* s.s. about one sixth (0.167), rarely one fifth (0.2) of vein $r_2$. The mounted wings were scored directly by eye under 40x magnification with a stereomicroscope and a unit scale ocular. The arithmetic means with standard deviations of the wing vein length $r_{2/3}$ and the index $r_{2/3}/r_3$ were separately calculated for the control group of the two species as well as for the test group. Furthermore, normal distribution of the indices and the corresponding t-tests and Mann Whitney U-Tests were calculated using SOFA package (www.sofastistics.com), again separately for the control and test groups. The results were used to assemble a simple classification matrix.

## Results

### Multivariate analyses

The repeated precision measurements for each of the five randomly selected specimens (three *Cx. torrentium*, two *Cx. pipiens*) are clustered close together in the PCA scatterplot (Figure B.2). This indicates that the inter- and intra-observer variation is relatively small compared to the sample variability between the different specimens, and therefore does not have a strong impact on the classification accuracy. The PCA of the right wing showed that PC1 represented the part of shape variability that is most responsible for species differences (Figure B.2). PCA on the left wing showed similar results (data not shown). The first 10 principal components of the PCA gave rise to more than 95% of the original variance for the left and right wing datasets and were used as new variables to carry out MANOVAs to assess the significance of species differences in the samples. MANOVAs indicated significant differences between *Cx. torrentium* and *Cx. pipiens* using the landmarks from the left ($p < 0.01$) and right wing ($p < 0.01$). TPS grid of the right wings shows the shape changes visualized by morphing from 42 *Cx. torrentium* to 40 *Cx. pipiens* s.s. (Figure B.3). The differences are predominantly expressed in the shrinkage of the $r_{2/3}$ vein.

### Bivariate analyses

Since the multivariate analyses using digital tools confirmed the interspecific difference of vein $r_{2/3}$, our next aim was to generate a bivariate control group by means of "manual" scoring by eye of veins $r_{2/3}$ and $r_3$. This group consisted of 52 female *Cx. pipiens* and 48 *Cx. torrentium* with confirmed identity. The scatterplot of the two vein measures (Figure B.4) indicates that vein $r_3$ (x- axis) does not differ between the two species whereas $r_{2/3}$ is longer in *Cx. torrentium* (plotted are microscopic ocular scale units, not theabsolute measures). The mean absolute lengths of $r_{2/3}$ were 283 ± 43 µm (mean ± SD) for *Cx. pipiens* and 429 ± 30 µm for *Cx. torrentium*, respectively. This resulted in a mean $r_{2/3}/r_3$ index in *Cx. pipiens* s.s. controls of 0.185 ± 0.028, and in *Cx. torrentium* of 0.289 ± 0.029. In both species normal distribution of the indices was observed, and the corresponding t-test and Mann Whitney U-Test revealed significant differences between the indices of the two species ($p < 0.001$). The boxplots of the indices of the control specimens summarize these differences, including the medians (0.185 for *Cx. pipiens* and 0.283 for *Cx. torrentium*), quartiles and percentiles (Figure B.5).

The next step was to test the reliability of the $r_{2/3}/r_3$ vein index to identify a greater number of females, in particular wild-caught females from trap catches. We identified 162 *Cx. pipiens* (biotype pipiens) and 89 *Cx. torrentium* by PCR, which were used for the bivariate analyses. The respective mean $r_{2/3}/r_3$ index in the *Cx. pipiens* test group was 0.185 ± 0.028, and that in the *Cx. torrentium* test group was 0.289 ± 0.029. In both species, a normal distributionof the indices was observed (Figure B.6), and the corresponding t-test and Mann Whitney U-Test revealed a significant difference between the indices of the two species ($p < 0.001$).

The boxplots of the indices of the two species are shown in Figure B.7, including the medians (0.194 for *Cx. pipiens* and 0.278 for *Cx. torrentium*), quartiles, percentiles and outliers. In contrast to the control group, the *Cx. pipiens* test group exhibited 10 outliers at higher indices.

### Accuracy of the morphometric classification

Classification accuracy of the discriminant analysis was high (>97%) for the original dataset (Table B.2, Figure B.8) and in the validation by bootstrapping for both species (Table B.2). This indicates that LDA was able to accurately classify *Cx. torrentium* and *Cx. pipiens* independently from the selected specimens and performs well in classifying both species.

The arithmetic mean of the vein indices $r_2/r_{2/3}$ of the combined datasets from the control group and the test group was calculated with 0.24, the mean length of vein $r_{2/3}$ of both groups was 357.5 µm. These values were subsequently applied as discriminant values to estimate the accuracy of the morphometric classification vs egg batch identity (control group) or PCR typing (test group). As shown in Table B.3, correct classification by the indices in the control group reached 98% and in the test group still more than 91%. Using the univariate approach with vein $r_{2/3}$ only, the correctness of *Cx. pipiens* classifications decreased to 96% in the control and 90% in the test groups, but remained between 97 and 100% for *Cx. torrentium.*

## Discussion

This study aimed at the evaluation of multi-, bi- and univariate wing morphometry for the identification of female *Cx. pipiens* s.s. and *Cx. torrentium.* Firstly, we studied wing shape differences between both species. A multivariate morphometric analysis of insect species with several wing landmarks is commonly used to analyze within-population morphometric variation or to differentiate species (Calle et al. 2002). Our analyses demonstrated significant wing shape differences between *Cx. pipiens* and *Cx. torrentium.* Linear discriminant analysis showed high classification accuracy. Secondly, we reassessed the taxonomic value of a seemingly neglected character, the differential length of wing vein $r_{2/3}$ in female *Cx. pipiens* and *Cx. torrentium.* This character has first been described for Scandinavian populations of both species (Natvig, 1948) and subsequently for German populations (Mohrig, 1969). However, both authors used vein r2 as standard whereas we used $r_3$. Nevertheless, although our values for the $r_{2/3}/r_3$ indices are closer to each other (*Cx. pipiens*, 0.185 vs *Cx. torrentium*, 0.289) than the $r_{2/3}/r_2$ indices of Mohrig (1969) (0.167 vs 0.33), our mean values fall into the ranges given by Mohrig. Hence, we could show that even the univariate measure of $r_{2/3}$ alone allows for the correct identification of more than 90% of mainly German specimens, despite intraspecific body size variation, without allometric scaling. Uni- and bivariate analyses are in agreement with our multivariate analyses, which indicated that the differences between the species are predominantly expressed in the shrinkage of the $r_{2/3}$ vein. It remains unclear why Service (1968) and Fedorova & Shaikevich (2007) did not find a difference in populations from Britain and Russia respectively.

An interesting by-product that has already been discussed by Rudolf et al. (2013) is the apparent increase in *Cx. torrentium* abundance during the last 40 years. During a two-year survey in Hamburg in 1967-68, Zielke (1970) detected only a single male. We could partially reproduce his results by re-examining 61 females from his original collection, of which 59 showed $r_{2/3}/\ r_2$ indices typical for *Cx. pipiens.* The remaining two individuals with indices typical for *Cx. torrentium* were PCR-typed *Cx. pipiens* s.s. This development of *Cx. torrentium*, turning from a relatively rare species 40-60 years ago, into one of the most abundant and widespread nowadays, applies to the rest of Germany and probably to many European countries (Mattingly, 1951; Service, 1968; Struppe, 1989; Becker et al., 2010; Hesson et al., 2014). For example, Snow & Ramsdale (1999) cited references from between 1925 and 1991 regarding the absence of *Cx. torrentium* from Belgium and the Netherlands, from where the species is only recently reported (Hesson et al., 2014). To further elucidate the issue, old museum specimens labelled *Culex pipiens* should be analyzed retrospectively.

In the course of our study, we also examined the wings of 1,095 *Culex* females without corresponding PCR typing (data not shown), collected in domestic overwintering shelters (cellars, garages) in northern and southern Germany during two consecutive winters from 2011 to 2013. All but eight showed $r_{2/3}/r_2$ indices typical for *Cx. pipiens* s.s. Out of these eight individuals with indices typical for *Cx. torrentium*, only four were subsequently PCR-typed *Cx. torrentium*, the other four were *Cx. pipiens* s.s., thus representing morphometric outliers. The fact that the collection sites were in areas of known sympatry of both species in trap collections (Rudolf et al., 2013) may suggest that *Cx. torrentium* avoids cellars or other peridomestic overwintering shelters. It also confirms the results from the test group analyses with seven out of 10 outliers from overwintering specimens, which turned out to be aberrant *Cx. pipiens* s.s. The wing characters of *Cx. pipiens* s.s. appear to be more variable, which is in accordance with lower classification accuracy for *Cx. pipiens.*

In conclusion, the linear discriminant analyses, $r_{2/3}/r_2$ indices and even the $r_{2/3}$ vein length alone allow for a >90% accurate species identification and quick separation at least of Central European populations of the two potential vectors. Vector and pathogen surveillance usually rely on adult trap catches that have to be sorted and identified manually to species level where possible (so far only as "pipiens and/or torrentium"), using a dissecting microscope, meaning that each specimen must be handled individually anyway. In a second step, a wing measurement of each identified *Cx. pipiens*/*torrentium* female can be conducted within a few minutes to separate the two species, just requiring a unit scale ocular and ethanol, but without extra costs for consumables. Specimens with indecisive wing indices could be excluded immediately. In contrast, genetic tools for mass screening and identification require DNA/RNA extraction, followed by PCR amplification and product analyses (e.g., restriction enzyme digestion, electrophoresis or sequencing), all of which takes more than one hour. In addition, these techniques depend on molecular laboratory equipment and costly consumables (e.g., enzymes, primers) and are therefore often conducted with pooled mosquitoes in order to reduce the costs. This procedure hampers the exact identification of each specimen in case of sympatry and the determination of the respective infection rates. The method described herein represents a low-cost routine alternative in the absence of a molecular laboratory.

## Acknowledgments

We are grateful to Thomas Kruppa, Christian Timmann, Monica Hagedorn, Tim Layer, Tobi Spielmann, Egbert Tannich, Bernd Noack, and Hanna Jöst for mosquito collections. We thank Ellen Kiel and Marlis Badusche for logistic and technical assistance. This work was financially supported by the Leibniz Association, grant number SAW-2011-BNI-3, the German Federal Ministry for Environment, Nature Conservation and Nuclear Safety (BMU) through the Federal Environment Agency (UBA), grant number FKZ371148404, the Biodiversity and Climate Research Centre (BiK-F, www.bik-f.de), Frankfurt/Main, the research funding programme "LOEWE – Landes-Offensive zur Entwicklung Wissenschaftlich- OEkonomischer Exzellenz" of Hesse's Ministry of Higher Education, Research, and the Arts as well as the German Research Council (grant no. DFG BiodivERsA KL 2087/6- 1). We also thank the management of the Hamburg-Ohlsdorf cemetery and the Hamburg authority for urban development and environment for permissions to carry out mosquito collections.

# Tables

Table B.1: Collection and identification details of samples used in this study. Abbreviations: nd=not detemined; Pip=*Cx. pipiens* s.s.; Torr=*Cx. torrentium.* * Identity assumed according to Rudolf et al. (2013) ; ** Identity derived from only one PCR ID.

| **Group** | **Federal State/ Country** | **Locality** | **Coll. Date** | **Coll. method/ Stage** | **Male ID** | **PCR-ID** | **No. of indiv. Bivariate analyses** | **Multivar. Analysis** |
|---|---|---|---|---|---|---|---|---|
| Control Pip | Brandenburg | Wildau, rain barrel | 19.7.2008 | larval breed | Pip | nd | 5 | |
| | Lower Saxony | East Friesia, West-overledingen-Völlen, cow stable | 14.10.2008 | indoor resting | nd | nd* | 29 | |
| | Hamburg | St. Pauli, batch8 | 1.8.2011 | egg batch breed | Pip | nd | 14 | |
| | Hesse | Heubach | 13.8.2011 | larval breed | Pip | nd | 4 | |
| | | | | **Sub-Total** | | | **52** | |
| Control Torr | Hamburg | Harbor, flower market | 11.7.2011 | egg batch breed | Torr | nd | 6 | |
| | Hamburg | St. Pauli, batch7 | 13.7.2011 | egg batch breed | Torr | nd | 11 | |
| | Hamburg | St. Pauli, batch3 | 20.7.2011 | egg batch breed | Torr | nd | 4 | |

Table B.1: Collection and identification details of samples used in this study. Abbreviations: nd=not detemined; Pip=*Cx. pipiens* s.s.; Torr=*Cx. torrentium.* * Identity assumed according to Rudolf et al. (2013) ; ** Identity derived from only one PCR ID.

| **Group** | **Federal State/ Country** | **Locality** | **Coll. Date** | **Coll. method/ Stage** | **Male ID** | **PCR-ID** | **No. of indiv. Bivariate analyses** | **Multivar. Analysis** |
|---|---|---|---|---|---|---|---|---|
| | Hamburg | St. Pauli, batch2 | 20.7.2011 | egg batch breed | Torr | nd | 5 | |
| | Hamburg | St. Pauli, batch4 | 22.7.2011 | egg batch breed | Torr | nd | 10 | |
| | Hamburg | St. Pauli, batch6 | 22.7.2011 | egg batch breed | Torr | nd | 5 | |
| | Hamburg | Ohlsdorf cemetary, vase | 3.8.2011 | larval breed | Torr | nd | 7 | |
| | | | | **Sub-Total** | | | **48** | |
| Test Pip | Schleswig-Holstein | Wyk/Föhr | 11.9.2012 | indoor resting | nd | Pip | 10 | |
| | Schleswig-Holstein | Warwerort | 19.8.2012 | BG+ | nd | Pip | 25 | 7 |
| | Schleswig-Holstein | Warwerort | 22.8.2012 | BG+ | nd | Pip | 38 | |
| | Schleswig-Holstein | Wulksfelde | 24.6.-5.8.2012 | BG+/-, GT | nd | Pip | 15 | 8 |
| | Hamburg | Barmbek | 1.7.2011 | larval breed | Pip+ Torr | Pip | 10 | 2 |
| | Hamburg | Cranz | 30.7.2011 | larval breed | Pip+ Torr | Pip | 2 | 2 |

Table B.1: Collection and identification details of samples used in this study. Abbreviations: nd=not detemined; Pip=*Cx. pipiens* s.s.; Torr=*Cx. torrentium.* * Identity assumed according to Rudolf et al. (2013) ; ** Identity derived from only one PCR ID.

| **Group** | **Federal State/ Country** | **Locality** | **Coll. Date** | **Coll. method/ Stage** | **Male ID** | **PCR-ID** | **No. of indiv. Bivariate analyses** | **Multivar. Analysis** |
|---|---|---|---|---|---|---|---|---|
| | Hamburg | Fuhlsbüttel | 29.7.-1.8.2012 | EVS | nd | Pip | 2 | |
| | Hamburg | Groß Borstel, "Eppendorfer Moor" | 4.8.2012 | EVS | nd | Pip | 5 | |
| | Hamburg | Hamburg | 1967/68 | nd | nd | Pip | 2 | |
| | Hamburg | Hummelsbüttel, Teetzpark | 14.7.2012 | larval breed | Pip+ Torr | Pip | 12 | 12 |
| | Hamburg | Ohlsdorf cemetary | 20.8-22.10.2012 | BG- | nd | Pip | 6 | 2 |
| | Hamburg | Ohlstedt | 5.9.-11.11.2012 | indoor resting | nd | Pip | 4 | |
| | Hamburg | Ohlstedt | 1.7.-22.8.2012 | BG+ | nd | Pip | 3 | 1 |
| | Hamburg | Poppenbüttel | 2.4.-14.8.2012 | BG+ | nd | Pip | 11 | |
| | Hamburg | St. Pauli, batch8 | 1.8.2011 | egg batch breed | Pip | Pip | | 10 |

Table B.1: Collection and identification details of samples used in this study. Abbreviations: nd=not detemined; Pip=*Cx. pipiens* s.s.; Torr=*Cx. torrentium.* * Identity assumed according to Rudolf et al. (2013) ; ** Identity derived from only one PCR ID.

| **Group** | **Federal State/ Country** | **Locality** | **Coll. Date** | **Coll. method/ Stage** | **Male ID** | **PCR-ID** | **No. of indiv. Bivariate analyses** | **Multivar. Analysis** |
|---|---|---|---|---|---|---|---|---|
| | Lower Saxony | Ashausen | 14.-22.8.2012 | BG+ | nd | Pip | 5 | |
| | Lower Saxony | Vietze-Höhbeck | 18.8.2012 | larval breed | nd | Pip | 2 | 2 |
| | Hesse | Heubach | 12.8.2011 | larval breed | Pip+ Torr | Pip | 7 | |
| | Hesse | Birkenau-Reisen | 21.12.2012 | indoor resting | nd | Pip | 2 | |
| | Baden-Württemberg | Weinheim | 18.2.2013 | indoor resting | nd | Pip | 1 | |
| | France | Cevennes | 5.8.2011 | larval breed | Pip+ Torr | Pip | | 4 |
| | | | | **Sub-Total** | | | **162** | |
| Test Torr | Schleswig-Holstein | Wulksfelde | 8.-21.7.12 | GT | nd | Torr | 8 | 8 |
| | Hamburg | Barmbek | 1.7.2011 | larval breed | Pip+ Torr | Torr | 4 | 4 |
| | Hamburg | Cranz | 30.7.2011 | larval breed | Pip+ Torr | Torr | 4 | 4 |

Table B.1: Collection and identification details of samples used in this study. Abbreviations: nd=not detemined; Pip=*Cx. pipiens* s.s.; Torr=*Cx. torrentium.* * Identity assumed according to Rudolf et al. (2013) ; ** Identity derived from only one PCR ID.

| **Group** | **Federal State/ Country** | **Locality** | **Coll. Date** | **Coll. method/ Stage** | **Male ID** | **PCR-ID** | **No. of indiv. Bivariate analyses** | **Multivar. Analysis** |
|---|---|---|---|---|---|---|---|---|
| | Hamburg | Fuhlsbüttel | 28.7.2012 | indoor resting | nd | Torr | 1 | 1 |
| | Hamburg | Ohlsdorf cemetary | 14.7.-20.8.2012 | BG+/- | nd | Torr | 17 | 8 |
| | Hamburg | Ohlstedt | 1.7.-22.8.2012 | BG+ | nd | Torr | 8 | 2 |
| | Hamburg | Ohlstedt | 20.-26.9.2011 | indoor resting | nd | Torr | 3 | |
| | Hamburg | Ohlstedt | 2.10.2012 | indoor resting | nd | Torr | 1 | |
| | Hamburg | Poppenbüttel | 10.-11.7.2012 | BG+ | nd | Torr | 3 | |
| | Hamburg | St. Pauli, batch6 | 22.7.2011 | egg batch breed | Torr | Torr | | 10 |
| | Lower Saxony | Vietze-Laasche, forest ditch | 18.8.2012 | larval breed | Pip+ Torr | Torr | 10 | 10 |

Table B.1: Collection and identification details of samples used in this study. Abbreviations: nd=not detemined; Pip=*Cx. pipiens* s.s.; Torr=*Cx. torrentium.* * Identity assumed according to Rudolf et al. (2013) ; ** Identity derived from only one PCR ID.

| **Group** | **Federal State/ Country** | **Locality** | **Coll. Date** | **Coll. method/ Stage** | **Male ID** | **PCR-ID** | **No. of indiv. Bivariate analyses** | **Multivar. Analysis** |
|---|---|---|---|---|---|---|---|---|
| | Lower Saxony | East Friesia, West-overledingen-Völlen, cow stable | 14.10.2008 | indoor resting | nd | Torr | 1 | |
| | Hesse | Heubach | 10.8.2011 | egg batch breed | Torr | Torr | 20 | |
| | Hesse | Heubach | 10.8.2011 | larval breed | Torr | Torr** | | 10 |
| | Hesse | Heubach | 12.8.2011 | larval breed | Pip+ Torr | Torr | 9 | |
| | France | Cevennes | 5.8.2011 | larval breed | Pip+ Torr | Torr | | 5 |
| | | | | **Sub-Total** | | | **89** | |
| | | | | **Total** | | | | **122** |

Table B.2: Classification accuracy of the original dataset and classification accuracy of the bootstrap samples in the validation procedure (median classification accuracy over all bootstraps with bootstrap percentile confidence intervals) for all individuals, *Cx. pipiens* s.s. and *Cx. torrentium* using the left or right wing.

| | **Left Wing** | | | **Right wing** | | |
|---|---|---|---|---|---|---|
| **Group** | N | Original [%] | Bootstrap [%] | N | Original [%] | Bootstrap [%] |
| **all** | 78 | 100 | 100 (100, 97.5) | 82 | 100 | 100 (100, 98.78) |
| **pipiens** | 35 | 100 | 100 (100, 97.5) | 40 | 100 | 100 (100, 97.56) |
| **torrentium** | 43 | 100 | 100 (100, 97.5) | 42 | 100 | 100 (100, 97.56) |

Table B.3: Classification accuracy using the mean bivariate (indices $r_{2/3}/r_3$) and univariate (vein $r_{2/3}$) parameters.

| | | | | **Correct classifications (%)** |
|---|---|---|---|---|
| **Groups** | **Species** | **N** | **Index** | $\mathbf{r_{2/3}}$ |
| **Control** | pipiens | 52 | 98 | 96 |
| | torrentium | 48 | 98 | 100 |
| **Test** | pipiens | 162 | 91.4 | 90 |
| | torrentium | 89 | 91.4 | 97.8 |

## Figures

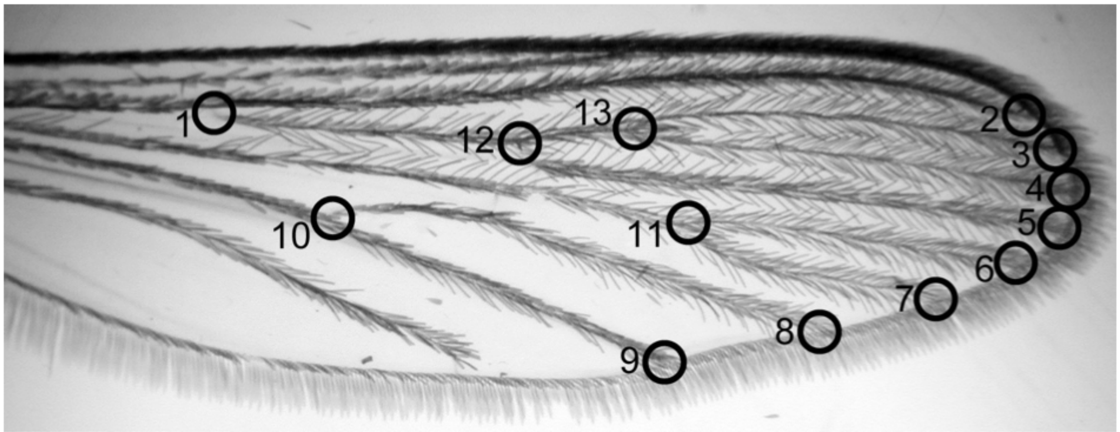

Figure B.1: *Culex* sp. female right wing. The positions of the 13 morphometric landmarks are indicated with open circles.

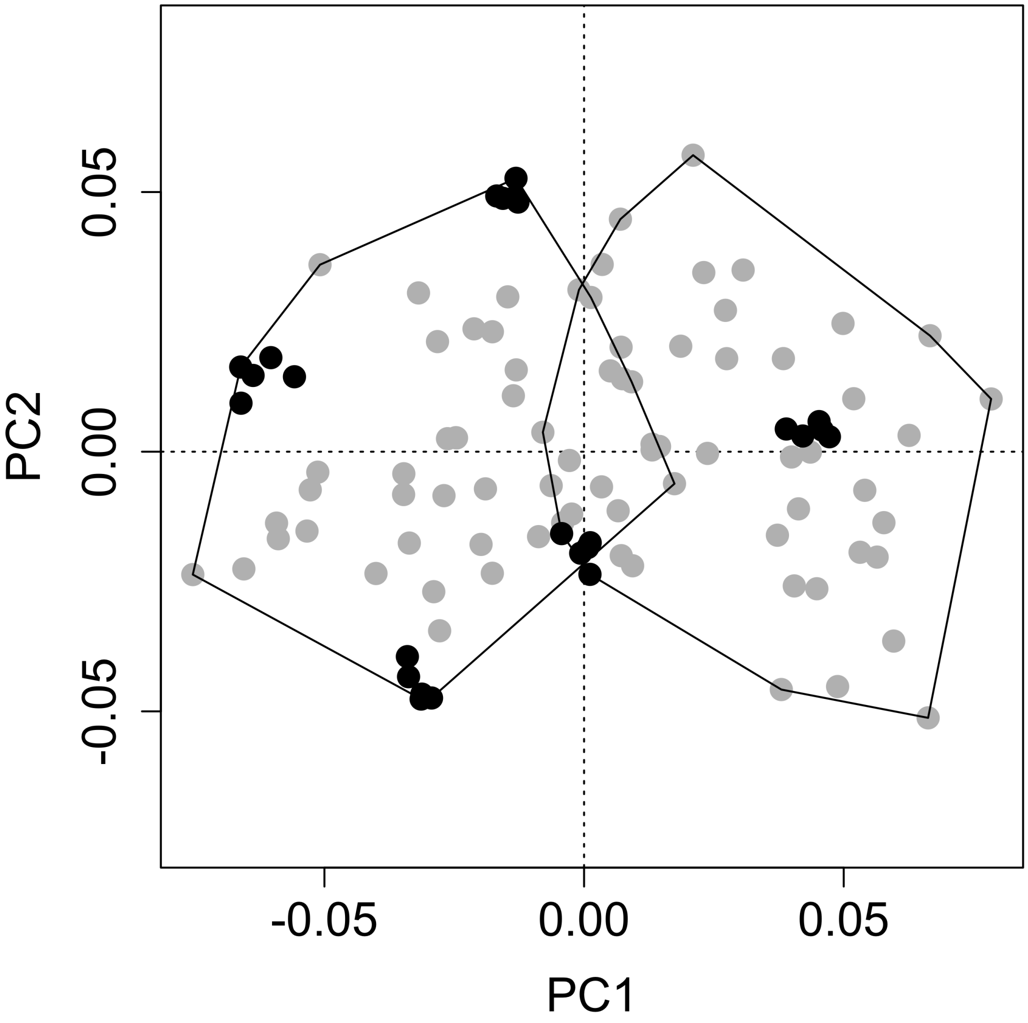

Figure B.2: Principal component analysis of the shape variance of the mosquito female right wings. Principal component 1 (PC1) accounted for 37.9% of the total shape variance of the total sample versus PC2, which accounted for 17.6%. N=82 (*Cx. pipiens*=40, *Cx. torrentium*=42). PC1 separated the two species (left: *Cx. torrentium*, right: *Cx. pipiens*). Each grey point indicates a different specimen. Precision of measurement: Black dots indicate the five mosquitoes for which we repeated the collection of raw landmark coordinates four times (original data point plus four repeated measurements).

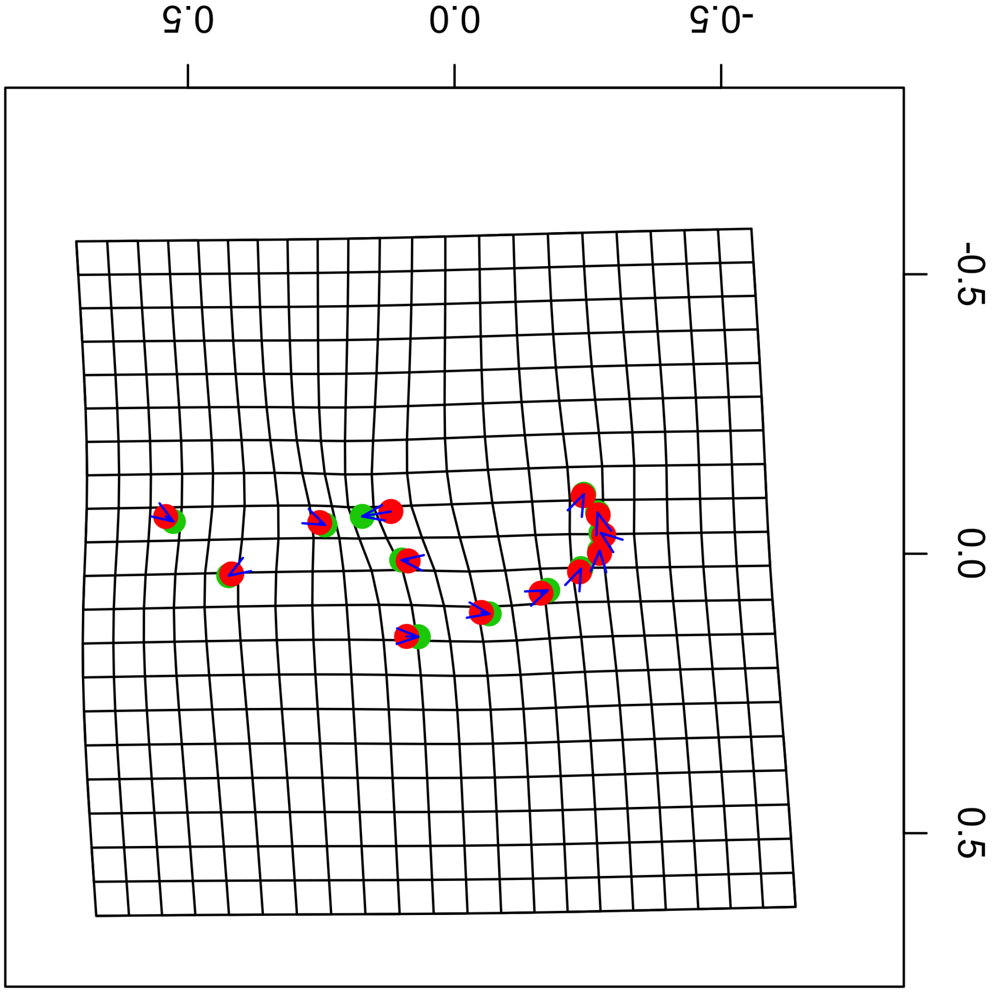

Figure B.3: The thin-plate spline grid shows the variation in shape of the female right wing (species differences; *Culex torrentium* - *Cx. pipiens*). Reference shape is represented by *Cx. torrentium* (red circles), target shape by *Cx. pipiens* (green circles), blue arrows indicate direction of change.

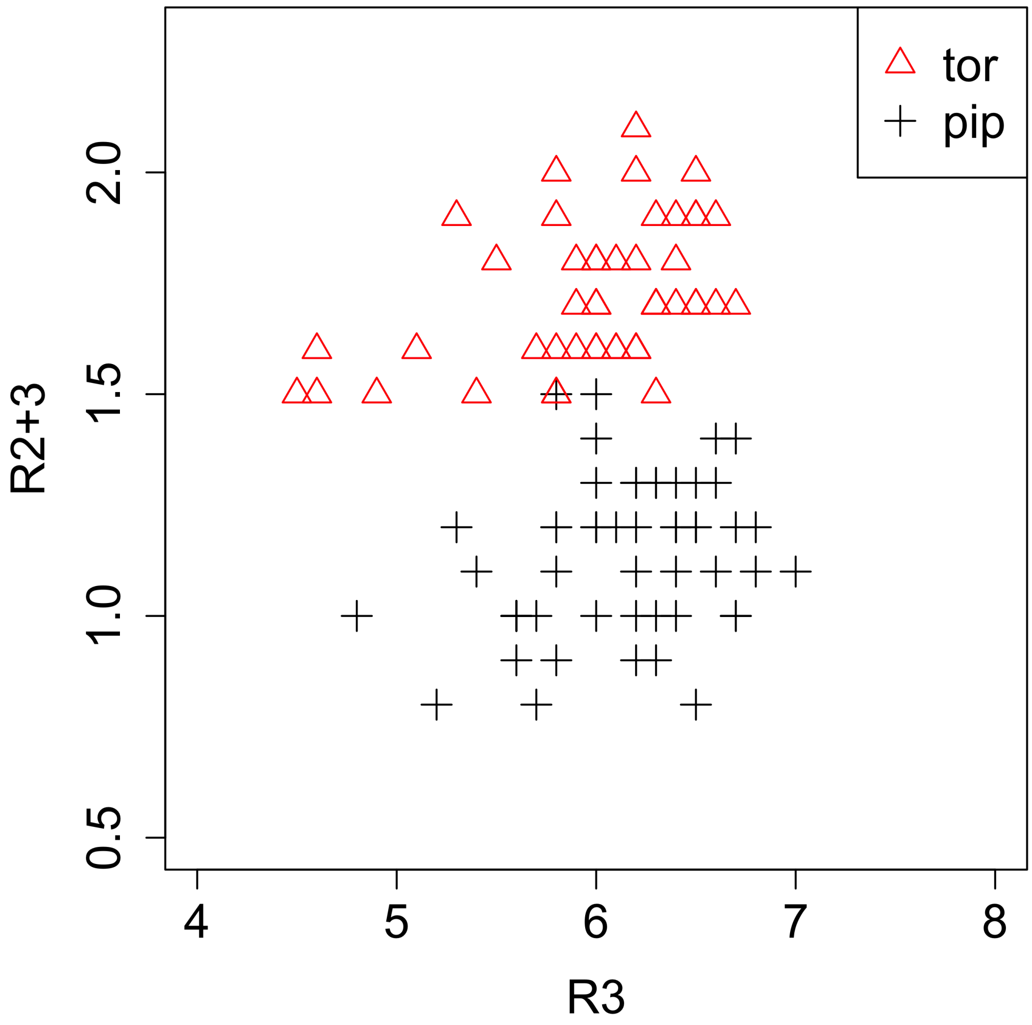

Figure B.4: Bivariate, pairwise distribution scatterplot of absolute measures of wing veins $r_3$ versus $r_{2/3}$. Measures are given in scale units. The number of dots is smaller than the actual number of specimens due to overlapping dots with the same data pairs. Labels: Pip *Cx. pipiens* s.s.; Tor *Cx. torrentium*.

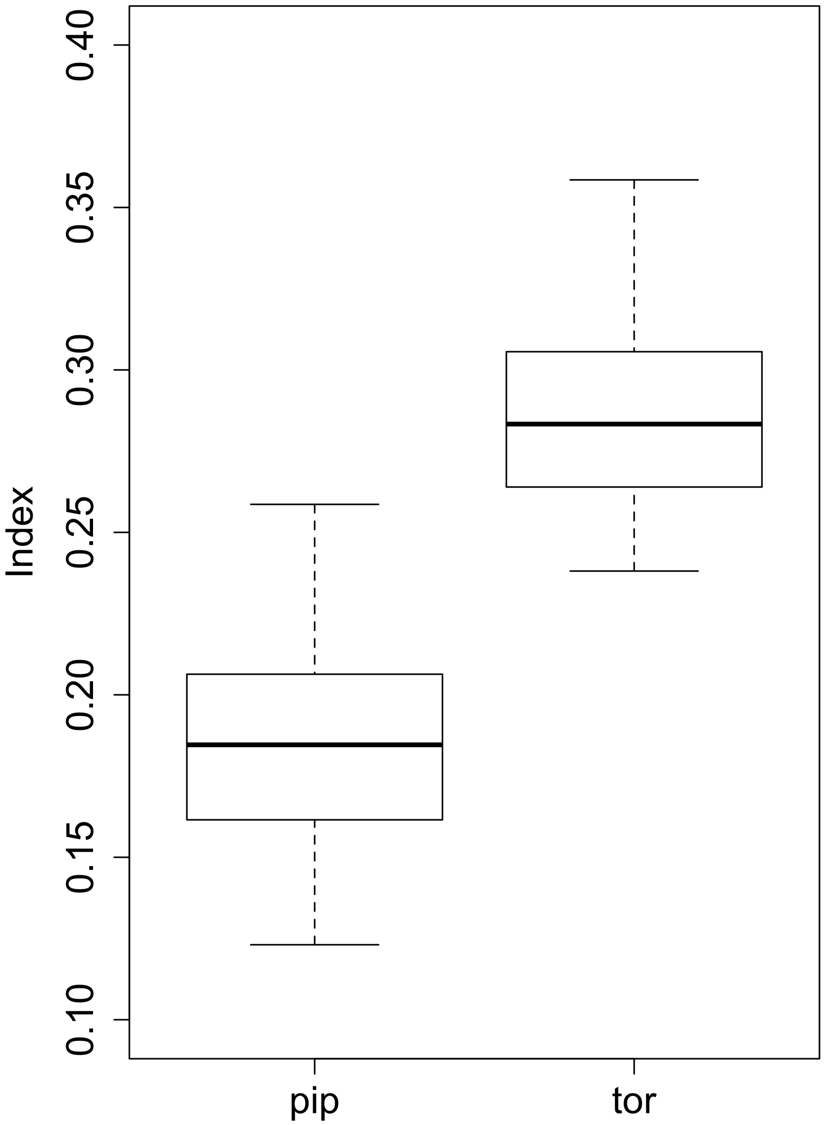

Figure B.5: Boxplot of $r_{2/3}/r_3$ wing vein indices of the control group, including the medians, quartiles, and percentiles.

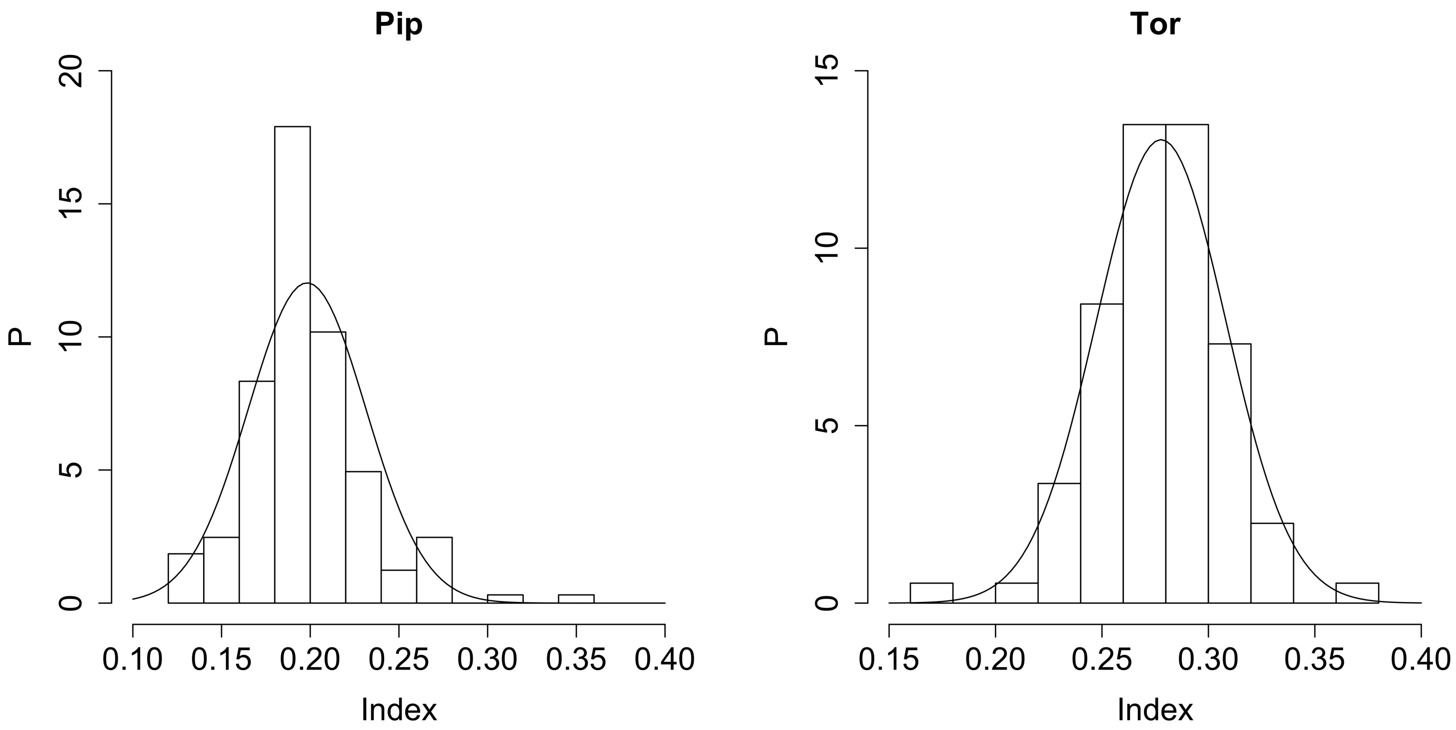

Figure B.6: Frequency histograms and graphical validation for normal distribution of the $r_{2/3}$ / $r_3$ wing vein indices of test group *Cx. pipiens* (left) and *Cx. torrentium* (right). Frequency measure is for absolute counts.

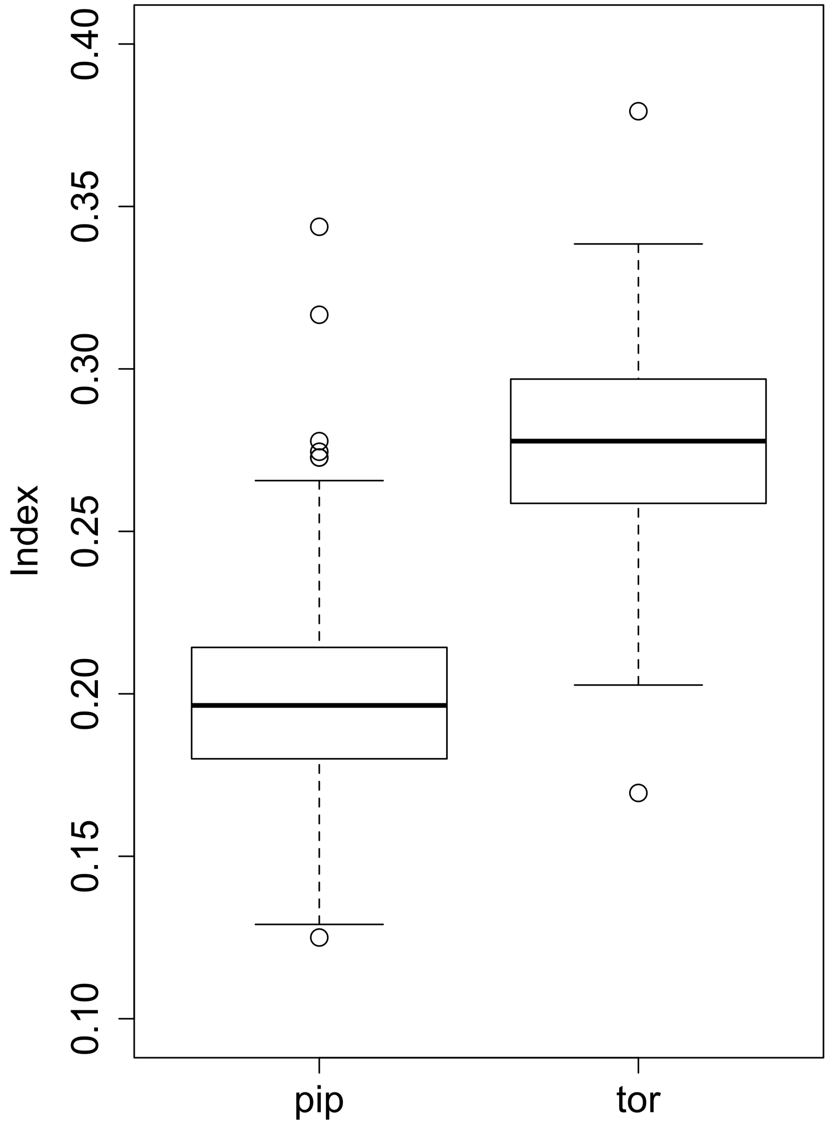

Figure B.7: Boxplot of $r_{2/3}$ / $r_3$ wing vein indices of the test group, including the medians, quartiles, percentiles, and outliers.

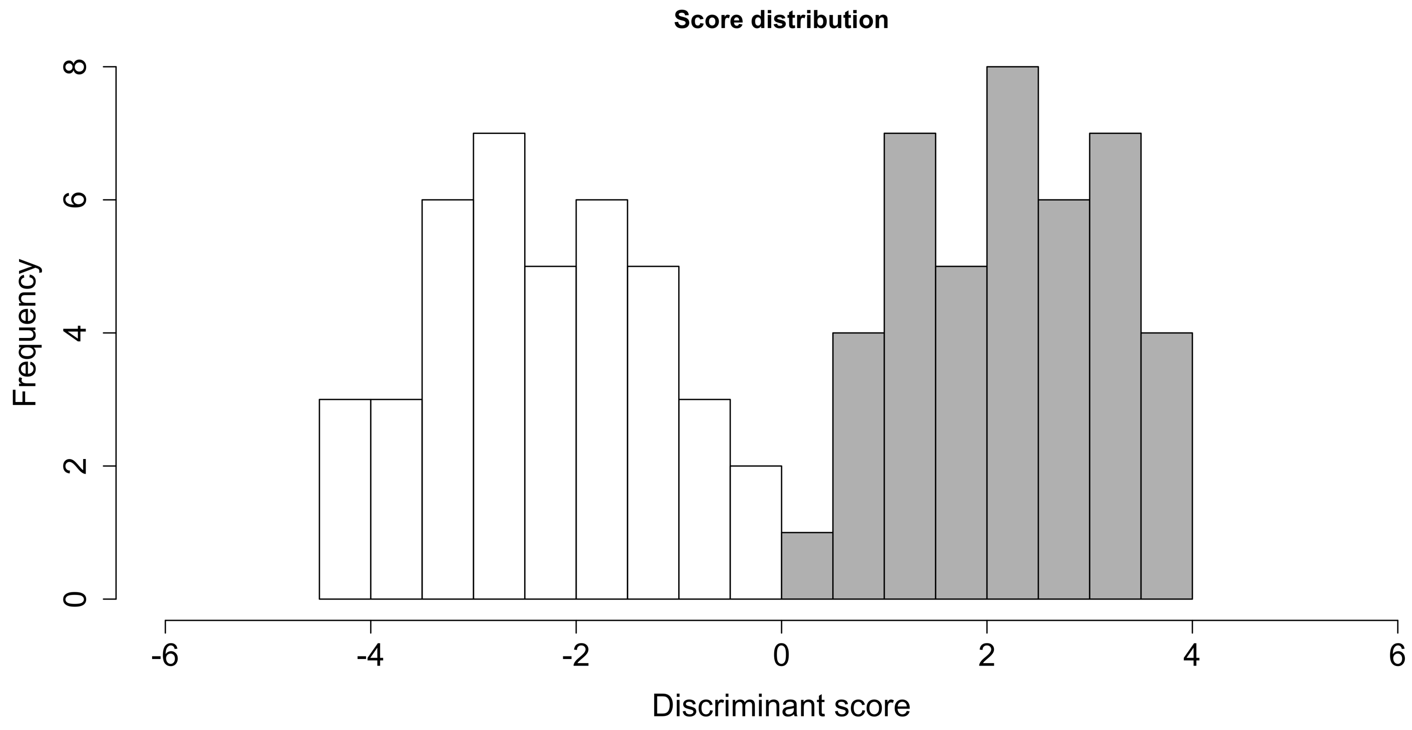

Figure B.8: BResults of the discriminant analysis of the female right wing. Negative values of the discriminant score indicate *Cx. pipiens* (white), positive values of the discriminant score indicate *Cx. torrentium* (grey). All mosquito individuals were correctly classified.

## B.2 Distribution of the various *Anopheles maculipennis* group members from Germany identified by newly developed real-time PCR assays

in preperation for *Parasites & Vectors*: **Lühken, R.**, Czajka, C., Steinke, S., Jöst, H., Schmidt-Chanasit, J., Kiel, E., Krüger, A., & Tannich, E. (in preperation). Distribution of the various *Anopheles maculipennis* group members from Germany identified by newly developed real-time PCR assays.

### Abstract

#### Background

Due to their role as vectors of malaria, the *Anopheles maculipennis* group members were intensively studied in the past, but in course of the eradication of malaria in Germany in the middle of the $20^{\text{th}}$ century, the interest in this field of research significantly declined. However, with the recent description of a new member of the species group (*An. daciae*) in 2004, research interests increased again. Nevertheless, a comprehensive analysis of the species distribution of all four members (*Anopheles atroparvus*, *Anopheles daciae*, *Anopheles maculipennis* s.s., and *Anopheles messeae*) known for Germany is still missing. Different multiplex PCR approaches were already available, which all require post-PCR analysis, i.e. agarose gel electrophoresis. Real-time PCR (RT PCR) allow differentiation in a closed tube format and thus is faster and minimize the risk of amplicon contamination. Summarized, the present work aimed to analyse the species distribution of *An. maculipennis* group members using a RT PCR assay for identification.

### Method

Between 2011 and 2013, a total of 722 individuals and 95 pools of the *An. maculipennis* group were collected from 72 different sites in Germany. These samples were analysed with a newly developed RT PCR assay for the identification of the four species using nucleotide differences in the internal transcribed spacer 2 (ITS2) gene. All gathered data were used for species distribution modelling using random forest variable selection with twelve variables: bioclimatic (4), altitude (1), and land cover (7).

### Results

Two RT PCRs were developed that allow the differentiation of single individuals of all group members. First using a multiplex RT PCR to differentiate *An. atroparvus*, *An. maculipennis* s.s., and *An. daciae*/*An. messeae*, and secondly the differentiation of *An. daciae*/*An. messeae* with a lightcycler RT PCR with subsequent melting curve analysis. However this approach was not sensitive for mixed *An. maculipennis* group pools. Therefore, one singleplex RT PCR were developed for each of the *Anopheles maculipennis* group species without differentiation between *An. daciae* and *An. messeae*. The overall prevalence of *An. daciae*/*An. messeae* was highest (92.47% of all pools), which can be further seen in detail for single individuals: *An. daciae* (6.93% of all single individuals) and *An. messeae* (69.53%). The prevalence of the other two species was relative low: *Anopheles maculipennis* s.s. (13.30% of all single individuals, 10.75% of all pools) and *An. atroparvus* (1.80%, 1.08%). Three *An. daciae*/*An. messeae* specimens revealed double peaks in the melting curves. This indicates that these specimens are hybrids between *An. daciae* and *An. messeae*. Species distribution models demonstrated that bioclimatic variables, as well as altitude and land cover are useful variables for the prediction of the species distributions in Germany. *Anopheles atroparvus* was restricted to the coastal regions and northwestern Germany. In contrast, *An. maculipennis* s.s. had a wide distribution, with a lower occurrence probability in eastern and southern Germany. Finally, the *An.*

*messeae* showed a decreasing occurrence probability from the north to the south, whereas *An. daciae* did not occur in western and southern Germany.

### Conclusion

This study developed a RT PCR assay to differentiate the *An. maculipennis* group members of Germany. Most remarkable was the detection of suspected hybrids between *An. daciae*/*An. messeae* and the lower prevalence of *An. atroparvus* compared to historic data. The decline of *An. atroparvus* might be explained by the differences in the overwintering strategy compared the other *Anopheles* species. *An. atroparvus* is expected to be more dependent on shelter sites in the immediate vicinity to cattle hosts that were lost as a result of a changed farm building design.

## Introduction

In the past, substantial research on mosquitoes in Germany has focused on the *Anopheles maculipennis* group due to their role as vectors for the transmission of malaria parasites. In multiple studies, the taxonomy and vector competence of the different group members as well as their spatial distribution and ecology were investigated (e.g. Martini et al., 1931; Weyer, 1938, 1941). However, with the eradication of malaria in the middle of the last century, this field of research has become greatly neglected in Germany (Weyer, 1956). Malaria eradication in Germany was accomplished by advances in diagnostics, treatment and vector control, e.g. intensive drainage of marshes, swamps, and fens (reviewed by Maier, 2004) as well as improvement of housing and sanitation (e.g. Bruce-Chwatt & de Zulueta, 1980).

Although time periods of malaria transmission in course of the climate change scenarios are considered to increase and likewise the risk for re-emerging of tertian malaria (Schröder & Schmidt, 2013), new epidemics of malaria in Germany are not expected (reviewed by Maier et al., 2003). However, besides malaria parasites other human pathogens have been detected in mosquitoes of the *An. maculipennis* group, such as Ťahyňa virus (Aspöck et al., 1970), West Nile virus (Filipe, 1972), Sindbis and Batai viruses (Jöst et al., 11b,c), as well as the filaria species *Dirofilaria immitis* and *D. repens* (Cancrini et al., 1997, 2006; Kronefeld et al., 14a). In these studies, members of the *An. maculipennis* group were not discriminated. Thus it remains to be established whether all of them have vector competence for the various pathogens.

According to present knowledge, the *An. maculipennis* group comprises a total of eleven species (reviewed by Danabalan et al., 2013). Four of them have been detected in Germany so far. Three members, namely, *An. atroparvus*, *An. maculipennis* s.s., and *An. messeae* have been known for decades (Weyer, 1938). With a preference for brackish water bodies, *An. atroparvus* was considered to dominate in particular in the coastal areas, but also at inland sites with elevated salinity (reviewed by Mohrig, 1969; Becker et al., 2010). In contrast, *An. messeae* is considered to be the most widespread member of the group in Germany, but explicitly absent along the coastlines and brackish water bodies. The species is considered to have preference for large water bodies. Finally, the species *An. maculipennis* s.s. is expected to be widespread in Germany colonizing a wide variety of breeding sites, but is the only group member expected to be found at altitudes more than 1,000 m above sea level (reviewed by Becker et al., 2010). In 2004, *Anopheles daciae*, a new member of the *An. maculipennis* group was described (Nicolescu et al., 2004). Due to substantial similarities in egg morphology and rDNA sequences, this species was previously misclassified as *An. messeae* (Nicolescu et al., 2004). Recent studies on the distribution of *Anopheles* species in Germany using molecular methods have indicated that *An. daciae* is present (Kronefeld et al., 2012; Weitzel et al., 2012) and widely distribution in Germany (Kronefeld et al., 14b). Interestingly, in England and Wales, *An. daciae* was demonstrated to be even more abundant than *An. messeae* (Danabalan et al., 2013).

Although the explicit role of the different species of the *An. maculipennis* group as vectors for the transmission of pathogens is largely unknown, there is reason to assume that the species indeed differ in vector competence (Bates, 1940; Kitzmiller et al., 1967; Takken et al., 02a). Previous attempts to differentiate the various members of the *An. maculipennis* group were mainly driven by the understanding of ecological differences of the species, as this information was of importance for the understanding of malaria transmission and the development of control measures (e.g. Hackett & Missiroli, 1935). The need for species differentiation of the *An. maculipennis* group members is supported by a recent example reviewed by Danabalan et al. (2013), suggesting that the patchy distribution of tertian malaria in some endemic regions is due to differences in vector competence between *An. messeae* or *An. daciae* for the transmission of *Plasmodium vivax* (Linton et al., 2005; Vladimirescu et al., 2006).

Amplification of specific mosquito DNA fragments by PCR has been proven to constitute a reliable method for the differentiation of members of the *An. maculipennis* group (Talbalaghi & Shaikevich, 2011; Danabalan et al., 2013; Kronefeld et al., 14b). The PCR protocols reported so far, however, require further processing of the amplicon, e.g. by agarose gel electrophoresis, which is time-consuming and prone to false-positive results due to possible cross-contamination. Closed-tube, real-time PCR (RT-PCR) methods can circumvent these problems. These methods allow specific detection of the amplicon by binding to one or two fluorescence-labeled probes during PCR. Thus, further downstream analysis is not required, which reduces the time needed to obtain results. Accordingly, RT-PCR is in particular helpful for high though-put screening when large numbers of samples have to be processed as it is the case during nation-wide mosquito monitoring or surveillance programs. Here we report on a series of newly developed RT-PCR assays that allow differentiation of the various members of the *An. maculipennis* group present in Germany. These assays were applied on mosquitoes recently collected at different sites throughout the country to study the distribution and ecology of the four *An. maculipennis* group members in Germany.

## Material and Methods

### Sampling

Between May 2011 and May 2013, mosquitoes were collected at 72 sampling sites distributed over Germany using primarily $CO_2$ suction traps or gravid traps, but collection methods also included the sampling of immature stages from different breeding sites. All individuals were transported to the laboratory, frozen, and morphologically identified to the group level (Becker et al., 2010), i.e. *An. maculipennis* group. Subsequently, individual collections from the same trap and date were pooled to up to 25 specimens per pool. All samples were placed in sterile vials, and subsequently maintained froozen until being processed.

### DNA extraction and multiplex RT PCR assay

Each mosquito pool was triturated in 500 mL of cell culture medium (high glucose Dulbecco's modified Eagle's medium (Sigma-Aldrich) with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2.5 mg/mL amphotericin B) using two stainless steel beads (5 mm; Qiagen) in a TissueLyser (Qiagen) for 2 min at 50 oscillations per second. The suspensions were clarified by centrifugation (5,000x g for 1 min), and the supernatant was used for DNA extraction with AquaGenomicTM (protocol for *Drosophila* samples, MultiTarget Pharmaceuticals) or QIAamp viral RNA mini kit according to the manufacturer's instructions. Reference samples of the four species were obtained by application of a species-specific multiplex PCR for selected specimens (Kronefeld et al., 14b).

The extracted DNA of single individuals was analysed by a newly designed multiplex RT PCR for the internal transcribed spacer 2 (ITS2) gene using the forward primer *An. maculipennis* group F (5'-CTGCAGGACACATGAACACC-3'), the reverse primer *An. maculipennis* group R (5'-CAAGTTGAAACCTGGGGTTG-3'), and the three probes *An. atroparvus* P (5'-Rox-TCATAACCCAAGCCAAACG-BHQ2-3'), *An. maculipennis* P (5'- FAM-ATTTGACCCAGGTCAAACTACG-BHQ1–3'), and *An. daciae/An. messeae* P (5'-JOE- GACCCATTCAAGTCAAACTACGT-BHQ1-3'). Multiplex RT PCR was performed in a 20 µL reaction volume. The PCR mixture contained 10 µl HotStarTaq Mastermix (Quiagen), 2 µl MgCl2 (25 mM), 0.45 µl forward primer (2 pmol/µl), 0.45 µl reverse primer (13.3 pmol/µl), 0.45 µl of each probe (2.2 pmol/µl), 0.8 µl BSA (1 mg/ml, ThermoFischer), 2 µl template DNA, and 2.95 µl H20. Amplification was carried out with the following thermoprofile: 15 min at 95°C, 50 cycles of 15 s at 95°C, 30 s at 67°C-52°C, i.e. annealing beginning at 67°C and ending at 56.5°C, and 30 s at 72°C, and 30 s at 40°C. The sequence alignments of the loci used for primer and probe design are shown in Figure S1.

A lightcycler RT PCR were developed to differentiate all positive *An. daciae/An. messeae* samples. This PCR targeted a nucleotide difference in the internal transcribed spacer 2 (ITS2) gene (position 436), which was proposed to reliably differentiate both species (2013). This RT PCR included the primer pair *An. daciae/An. messeae* F (5'-TGCAGGACACATGAACACC-3') and *An. daciae/An. messeae* R (5'-ATGCTTAAATTTAGGGGGTAGTC-3'), the *An. daciae/An. messeae* sensor probe labelled with fluorescein at the 3'-end (5'-ACCAGCTCCTAGCCG-Fluorescein-3'), and the *An. daciae/messeae* anchor labelled with the Light-Cycler 640 at the 5'-end and blocked with phosphate at the 3'-end (5'-Light-Cycler 640-GGAGCTCATGGGCCTCAAATAATGTG-Phosphate-3'). The PCR was performed in a 10 µL reaction volume. The PCR mixture contained 1 µl Puffer BD (ohne MgCl2, 10x; Pharmacia), 1 µl Solution S (Solis), 0.8 µl MgCl2 (25 mM), 0.2 µl forward primer (10 pmol/µl), 1 µl reverse primer (10 pmol/µl), 0.4 µl of each anchor and sensor (5 pmol/µl), 0.2 µl dTNP (10 mM each base; Roth), 0,2 µl Taq-Polymerase (5 U/µl; FirePol), 2 µl template DNA, and 2.8 µl H20. Amplification was carried out with the following thermoprofile: 3 min at 94°C, 45 cycles of 30 s at 94°C, 60 s at 52°C, and 1 min at 72°C, and 10 min at 72°C. The melting curve analysis was performed with a LightCycler 480$^{®}$ system (Roche Diagnostics, Germany) by raising the temperature to 95°C for 2 min, lowering the temperature to 40°C for 2 min, and increasing the temperature to 85°C with continuous fluorescent acquisition followed by a cool down to 40°C. *Anopheles messeae* showed a peak around 48°C and *An. daciae* around 57°C. The sequence alignments of the loci used for primer and probe design are shown in Figure S2.

Finally, the extracted DNA of pooled individuals was analysed with three different singleplex RT PCRs: 1) *An. atroparvus*: forward primer 5.8S-UN (5'-TGTGAACTGCAGGACACATG-3'; published by (Proft et al 1999)), reverse primer AAT (5'- CGTTTGGCTTGGGTTATGA-3'; published by (Proft et al 1999)), and the newly designed probe Mac. Univ. P (5'-FAM-AACGCATATTGCGCATCG-BHQ1-3'). 2), *An. maculipennis* s.s.: forward primer 5.8S-UN (5'-TGTGAACTGCAGGACACATG-3'; published by (Proft et al 1999)), reverse primer AMA (5'-TATTTGAGGCCCATGGGCTA-3'; published by (Proft et al 1999)), and the newly designed probe Maculipennis P (5'-FAM-ATTTGACCCAGGTCAAACTACG-BHQ1–3'), and 3) *An. daciae*/*An. messeae*: forward primer 5.8S-UN (5'-TGTGAACTGCAGGACACATG-3'; published by (Proft et al 1999)), reverse primer AMS (5'-GACGCCTCACGATGACCTT-3'; published by (Proft et al 1999)), and the newly designed probe Mac. Univ. P (5'-FAM-AACGCATATTGCGCATCG-BHQ1-3'). These three singleplex RT PCRs were performed in a 20 µL reaction volume. The PCR mixture contained 10 µl HotStarTaq Mastermix (Quiagen), 2 µl MgCl2 (25 mM), 0.45 µl forward primer (0.2 pmol/µl), 0.45 µl reverse primer (0.3 pmol/µl), 0.45 µl probe (0.05 pmol/µl), 2 µl template DNA, and 4.65 µl H20. Amplification was carried out with the following thermoprofile: 15 min at 95°C, 50 cycles of 30 s at 95°C, 30 s at 69°C-62°C, i.e. annealing beginning at 69°C and ending at 62°C, and 30 s at 72°C, and 30 s at 40°C. The sequence alignments of the loci used for primer and probe design are shown in Figure S3.

The specificity of the singleplex RT PCRs were evaluated by execution of the PCR reaction mixes with four different trials: 1) unmixed DNA template of the species A (100 ng), for which the RT PCR was developed, 2) unmixed, but extensively diluted DNA template of the species A (4 ng), 3) 1:25 mixed samples with 4 ng DNA of species A and 48 ng DNA of each species B and C, and 4) unmixed DNA template (100 ng) of the species B or C .

### Data analysis

*Anopheles maculipennis* group presence-absence data produced in this study together with the data published by two other recent German studies (Weitzel et al., 2012; Kronefeld et al., 14b) were used to model the distribution of the four species. A total of twelve environmental variables bioclimatic (4), altitude (1), and land cover (7) (Table B.5) were extracted. For each sampling site, we calculated the proportion of each land cover variable in a 2,000 m buffer and the mean value of the bioclimatic variables and altitude variable for the same buffer size.

These variables were used in a modelling approach using random forest variable selection, which was found to be robust also if the number of response data is small relative to the number of predictors (Strobl et al., 2007). This variable selection method was applied as described in detail by Bradter et al. (2013). In random forest, several classification or regression trees are built from random subsets of the data set (Breiman, 2001; Liaw & Wiener, 2002). It uses a selection procedure based on the unscaled permutation importance (Genuer et al., 2010). Each predictor is permutated in turn and the prediction error, i.e. OOB error, before and after permutations is used as a measure of variable importance (Liaw & Wiener, 2002; Strobl et al., 2008). A training set is created by sampling 2/3 of the data set (with replacement) for each classification tree, which is then used to predict the remaining 1/3 of the data. The proportion of false classified classes over trees is the OOB error (Breiman, 2001; Liaw & Wiener, 2002).

There were five steps to identify the number of predictors suitable for the model interpretation (Genuer et al., 2010): (1) all predictors were ranked by the unscaled permutation importance (average value over 50 repetitions); (2) a regression tree was fitted to the curve of the plot of standard deviations of the importance measures ordered by their mean importance. Variables with mean importance less than the smallest predicted value of the regression tree model are discarded; (3) the OOB errors for the models (average over 50 repetitions) were computed by starting with the most important variables and adding the other predictors in order of their ranking; (4) the model with the smallest OOB error, augmented with the standard deviation of the 50 repetitions, was selected, and finally (5) the nested model with OOB error smaller than this with fewer predictors was selected. Parameters which have to be specified in the random forest were used as proposed by Genuer et al. (2010): number of trees built in the forest ntree=2000, the number of predictors available at each node split mtry=p/2 with p as number of predictors, and for the calculation of the OOB error default values were used.

For each species and the selected variables with random forest, we computed a logistic regression. As condcuted by Bradter et al. (2013), we applied Moran eigenvector filtering for all models selected with random forest variable selection to account for spatial autocorrelation (Dray et al., 2006; Griffith & Peres-Neto, 2006). Spatial eigenvectors were added until RSA was no longer significant at the 0.05 level.

Area under the receiver operating characteristic curve (AUC) was used to compare prediction performance of the models (Fielding & Bell, 1997). AUC thresholds were interpreted as proposed by Hosmer & Lemeshow (2000): 0.7–0.8 is considered an „acceptable“ prediction; 0.8–0.9 is „excellent“ and >0.9 is „outstanding“. Although this index is also criticized to be unreliable (Lobo et al 2008), we predominantly referred to AUC, because it is the most commonly used performance indicator for species distribution models. However, as recommended by Lobo et al. (2008), we present further accuracy indices: root mean square error (RMSE), overall correct classification rate (CCR), sensitivity (SENS), specificity (SPEC), positive predictive value (PPV), negative predictive value (NPV), true skill statistic (TSS), Cohen's kappa (KAPPA) (for accuracy indice formulas see Liu et al., 09b). For threshold dependent indices (e.g. CCR or KAPPA) and prevalence prediction, requiring binary results, presence and absence were differentiated using a threshold value set to achieve the observed prevalence in data set (Freeman & Moisen, 2008).

Bootstrapping was used to evaluate the models with a corrected AUC value (Kattwinkel et al., 2009; Gray et al., 2010). We generated 1000 bootstrap data sets (with replacement) for each dataset. Models were refitted with each bootstrap sample. The mean difference of the AUC values of the bootstrap samples compared to the AUC of original dataset was calculated and subtracted from the original AUC to determine the corrected $\mathrm{AUC}_{\mathrm{cor}}$.

Data visualization and statistical analyses were conducted with QuantumGIS (QGIS Development Team, 2009) and R (R Core Team, 2014) using functions from the packages randomForest (Liaw & Wiener, 2002), rpart (Therneau et al., 2014), Hmisc (Harrell, 2014), and spdep (Bivand, 2014).

## Results

A series of real-time PCR assays were developed to differentiate the various members of the *An. maculipennis* group in Germany. All assays target the internal transcribed spacer 2 (ITS2) gene, which has been previously used to reliably differentiate the four members (Talbalaghi & Shaikevich, 2011; Danabalan et al., 2013; Kronefeld et al., 14b). Two of the assays have been designed for the analysis of single individuals of *An. maculipennis* s.l. The first assay is a multiplex RT-PCR based on two primer sequences that are conserved between the four species amplifying a DNA fragment of about 200 base pairs and 3 different tacman probes, specific for *An. atroparvus*, *An maculipennis* s.s. and *An. messeae*/*An. daciae*, respectively. For further differentiation between *An. messeae* and *An. daciae* an assay was developed targeting one of the five nucleotides within ITS2 gene that deviate between the two species. This assay amplifying a 429 bp fragment is based on melting curve analysis using fluorescence resonance energy transfer (FRET) probes. As both assays use primer sequences that are conserved between the different members of the *An. maculipennis* group, they are not applicable to pools of mosquitoes. Accordingly, three singleplex RT PCRs were developed using primers specific for *An. atroparvus*, *An. maculipennis* s.s., and

*An. daciae*/*An. messeae*, respectively, which allow specific amplification of the different species even from mixed samples. Assay optimization as well as sensitivity and specificity testing of the various assays were performed using DNA from the four species that had been identified by classic PCR as previously described and subsequent DNA sequencing. Performances of the assays were specific for the different species. However, there was minor signal reduction, when the PCRs were run with mixed DNA samples, but no cross-reactivity was observed with primers specific for *An. atroparvus* or *An. maculipennis* s.s. (Figure B.9). On the other hand, the singleplex RT PCR for *An. daciae*/*An. messeae*, revealed some cross-reactivity with samples containing DNA from *An. atroparvus* or *An. maculipennis* s.s. only. Nevertheless, these samples had considerable higher Ct values, which were far beyond of values from samples with a 1:25 dilution. Therefore, the result could be easily attributed to the respective species even in pooled samples containing up to 25 mosquitoes.

The newly developed PCR assays were subjected to mosquito DNA isolated from a total of 722 single *An. maculipennis* group individuals as well as from 95 pools containing 2 to 25 individuals (mean=8.0). From the single specimen, 13 (1.8% of all single individuals) were positive for *An. atroparvus*, 96 (13.30%) positive for *An. maculipennis* s.s., and 50 individuals were identified as *An. daciae* (6.93%). The vast majority of 502 individuals were classified as *An. messeae* (69.53%). 58 individuals (8.03%) remained undifferentiated as *An. daciae*/*An. messeae*, because the respective PCR revealed no signal. These samples were all characterized by high Ct values in the multiplex RT PCR suggesting poor DNA quality, which did not allow amplification of the larger 429 bp fragment. Analyses of the 95 pools indicated, one pool (1.08% of all pools) positive for *An. atroparvus*, 10 pools (10.75%) positive for *An. maculipennis* s.s., and 86 pools (92.47%) positive for *An. daciae*/*An. messeae.* Interestingly, three single *An. daciae*/*An. messeae* specimens showed peak characteristics for both species in the melting curve analysis compatible for hybrid formation (Figure B.6).

The performance of the final models for all four species was of acceptable quality (Table B.6). Accuracy indices generally demonstrated, that the species predictions were better than chance predictions for all four species. However, as several of the species showed a rather low prevalence, the specificity (i.e. correct absence) was mostly higher. Analysis of the deviance between the model with and without spatial eigenvectors was not significant at the 0.05 level in each case suggesting that spatial autocorrelation has little or no influence on the patterns of *An. maculipennis* group species in Germany.

The probability of occurrence of *An. atroparvus* increased with decreasing maximum temperature of warmest month (variable BIO5) and with increasing minimum temperature of coldest month (variable BIO6) (Table B.7). The probability of occurrence was highest in the coastal regions and north-western Germany (Figure B.11). *An. maculipennis* s.s. showed a positive response to increasing annual mean temperature (variable BIO1) and a negative response to increasing maximum temperature of the warmest month (variable BIO5) (Table B.7). Furthermore, the models indicate that the probability of species occurrence significantly decreased with increasing proportion of the land cover wetlands. The species was well spread all over Germany and only showed a lower probability of occurrence in eastern and southern Germany (Figure B.12). Finally, the genetically very close sibling species showed a contracting response to the maximum temperature of the warmest month (variable BIO5) and altitude (Table B.7): *An. messeae* had a negative response to the maximum temperature of the warmest month (variable BIO5) and altitude (Table B.7), while *An. daciae* showed the opposite response. Additionally, the probability of occurrence for *An. messeae* was negatively correlated with the proportion of the variable agricultural and *An. daciae* was negatively correlated with the proportion of pasture. Finally, *An. daciae* showed a positive response for the annual mean temperature (variable BIO1) and a negative response for the minimum temperature of the coldest month (variable BIO12) and annual precipitation (variable BIO12). The predicted distribution of *An. daciae* and *An. messeae* showed a strong overlap (Figure B.14 and Figure B.13). However, *An. messeae* showed a higher probability of occurrence in northern Germany, while *An. daciae* was absent from north-western Germany and southern Germany.

## Discussion

Classical PCR assays for the differentiation of members of the *An. maculipennis* group in Germany requiring downstream analysis of the amplicons e.g. gel electrophoresis were already available (Talbalaghi & Shaikevich, 2011; Danabalan et al., 2013; Kronefeld et al., 14b). However, RT PCR is considerable faster and minimizes the risk of laboratory contamination (Fotedar et al., 2007; Parija, 2011), which is especially a problem when large sample collections are analysed as it is the case in large-scaled pathogen monitoring programs. The newly developed assay reported here allow differentiation of all four *An. maculipennis* group members with two RT PCRs. Furthermore, the assays are suitable for the detection of *An. atroparvus*, *An. maculipennis* s.s., and *An. daciae*/*An. messeae* in mixed *An. maculipennis* group pools, which are commonly used in surveillance programs for mosquito-borne pathogens to reduce the number of samples to be analysed. However, we were not able to develop a single multiplex RT PCR assay able to differentiate all four members within a single PCR reaction. Further genome sequencing is probably needed to identify DNA regions, which are more appropriate to develop such a PCR assay.

The most interesting change in the species distribution of the *An. maculipennis* group, is a significant decline in the distribution of *An. atroparvus*. At the beginning of the 20th century, the halophilic species was a common species in coastal areas and inland salt sites (Weyer, 1938). However, as already demonstrated by other studies from Germany (Kronefeld et al., 14b; Krüger et al., 2014) and The Netherlands (Takken et al., 02a,b), this species is hardly observed anymore. It might be argued that this due to the sampling methods used. Previous knowledge on the distribution of the *An. maculipennis* group in Germany were restricted to egg sampling (Weyer, 1938), as this is the only stage which can be used for morphological differentiation of the species (Becker et al., 2010). In contrast, the study presented here merges data from different sampling methods (different suction-traps, gravid traps, and immature sampling). All trapping methods can be considered to have a different trapping efficacy for different mosquito species (Silver, 2008). The majority of mosquitoes in this study was collected with different suction traps, which are known to have quite different efficacy for the different mosquito species (e.g. Lühken et al., 2014), but the trapping efficacy was not yet compared for the different members of the *An. maculipennis* group. However, a decline in distribution of *An. atroparvus* was also detected in different areas, while suitable breeding sites were still available and studied (Takken et al., 02a,b; Kronefeld et al., 14b; Krüger et al., 2014). Furthermore, the species distribution model build for *An. atroparvus* indicated a much wider potential distribution. The model results showed a distribution pattern, which was already observed by Weyer (1938) with a high prevalence in the coastal regions with a fast decline in the Baltic Sea region, i.e. north-eastern Germany, and a slower decline of the species occurrence in the North Sea region, i.e. north-western Germany. Van Seventer (1970) considered surface water pollution to have a significant impact on decreasing *An. atroparvus* populations in The Netherlands. Takken et al. (02a) and Takken et al. (02b) summarized that the decrease might be the result of the loss of suitable resting sites for hibernation as a result of changes in farm building designs, which provide only few dark and sheltered resting sites. This connection is considered to be the result of behavioural differences of the *An. maculipennis* members: *An. atroparvus* has a different overwintering behaviour compared to *An. daciae/An. messeae* and *An. maculipennis* s.s. (Weyer, 1938, 1941). While the latter two show dormancy without activity and blood feeding, *An. atroparvus* stays active and regularly feeds blood. Therefore, *An. atroparvus* might be expected to be more dependent on shelter sites in the immediate vicinity to cattle hosts. In contrast, Weyer (1941) did not observe any differences between the fitness of blood-fed versus unfed overwintering *An. atroparvus* or *An. daciae/An. messeae*. Furthermore, both species were reported to be present at the same overwintering sites. Takken et al. (02b) argued that *An. daciae/An. messeae* is able to hibernate in alternative, natural overwintering sites (e.g. dense shrubs, hollow trees, or animal burrows), thus it is less dependent on shelters close to cattle hosts. Moreover, our species distribution models demonstrated an increasing prevalence of *An. atroparvus* with increasing minimum temperature of coldest month, which indicate sensitivity for low temperatures during winter period and the demand for appropriate shelter sites.

The species distribution models revealed a wide distribution of *An. maculipennis* s.s. and the probability of occurrence was only slightly restricted in eastern and southern Germany. It is generally assumed that the species can cope with high alterations (Mohrig, 1969; Becker et al., 2010), but we did not find a significant impact of the variable on the distribution of the species.

As already highlighted by Kronefeld et al. (14b), there is lack of knowledge on specific differences between *An. daciae* and *An. messeae*. In addition, it remains to be established whether *An. daciae* indeed represents a separate species. Interestingly, our RT PCR results indicate the occurence of hybrids harbouring ITS2 sequences of both *An. daciae* and *An. messeae*. Whether those hybrids are fertile and able to reproduce requires further investigations.

Finally, there is also lack of knowledge on the ecological differences between *An. daciae* and *An. messeae* (e.g. breeding sites, phenology, or resting sites). The species distribution models indicated that both species generally had a large overlap of distribution in Germany. However, *An. messeae* showed a negative response to the maximum temperature of the warmest month and the variable altitude, resulting in a gradual decrease of the probability of occurrence from north to south. In contrast, *An. daciae* showed a positive response to both variables and therefore did not show such a strong north-south gradient in the probability of occurrence. Due to the negative relationship to minimum temperature and annual precipitation, the species revealed a lower probability of occurrence in north-western and southern Germany.

### Conclusion

This study presents RT PCR assays, which allow differentiation of single individuals of the four members of the *Anopheles maculipennis* group present in Germany, namely *An. atroparvus*, *An. daciae*, *An. maculipennis* s.s., and *An. messeae*. Furthermore, the assays enable identification of the species without differentiation *An. daciae/An. messeae* in pools of mosquitoes up to 25 individuals. Analysis of the species prevalence demonstrated, that *An. messeae* is by far the most common species of the group in

Germany, far behind followed by *An. maculipennis* s.s., and the relative rare species *An. daciae* and *An. atroparvus*. Most remarkable was the detection of suspected hybrids between *An. daciae*/*An. messeae* and the low number of positive sites for *An. atroparvus*. Compared to historic data, *An. atroparvus* was only detected at few sites, which might be explainable with differences in the overwintering strategy compared the other group members. Moreover, species distribution models for Germany were developed, which indicate that bioclimatic variables, as well as altitude and land cover are important variables explaining the different species distributions.

## Competing interests

The authors declare that they have no competing interests.

## Author's contributions Status (18.12.2014)

Designed the study: AK, EK, JSS, ET. Performed the mosquito collection: HJ, RL, SS. Conducted the PCRs: CC, RL. Conducted statistical analysis: RL. Wrote the paper: RL. Contributed to the manuscript drafting: ET.

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## Tables

Table B.5: Twelve Predictors used in the random forest variable selection for the four members of the *Anopheles maculipennis* group known for Germany

| **Variable** | **Resolution** | **Variable ID** | **Variable description** | | **Usage of the variables in the model building** | **Usage of the variables in the prediction** | **Source** |
|---|---|---|---|---|---|---|---|
| Corine Land Cover 2006 raster data | 100 m | | Aggregated land cover variable | CLC_ CODE | Proportion of each aggregated land cover variable in a 2000 m buffer around each sampling point | Aggregated proportion for each aggregated land cover variable in a 2000 m raster | http://www.eea.europa.eu/ |
| | | 1 | Unvegetated | 111-142, 331-335 | | | |
| | | 2 | Agricultural | 211-223 | | | |
| | | 3 | Pastures | 231 | | | |
| | | 4 | Forest | 311-313 | | | |
| | | 5 | Herbaceous vegetation | 321-324 | | | |
| | | 6 | Wetlands | 411-423 | | | |
| | | 7 | Water bodies | 511-523 | | | |
| Altitude | 30 arc-seconds (~1 km) | 8 | Altitude | | Mean of a 2000 m buffer around each sampling point | Pixel value | http://www.worldclim.org/bioclim |

Table B.5: Twelve Predictors used in the random forest variable selection for the four members of the *Anopheles maculipennis* group known for Germany

| **Variable** | **Resolution** | **Variable ID** | **Variable description** | | **Usage of the variables in the model building** | **Usage of the variables in the prediction** | **Source** |
|---|---|---|---|---|---|---|---|
| BIO1 | 30 arc-seconds (~1 km) | 9 | Annual Mean Temperature | | Mean of a 2000 m buffer around each sampling point | Pixel value | http://www.worldclim.org/bioclim |
| BIO5 | 30 arc-seconds (~1 km) | 10 | Max temperature of warmest month | | Mean of a 2000 m buffer around each sampling point | Pixel value | hhttp://www.worldclim.org/bioclim |
| BIO6 | 30 arc-seconds (~1 km) | 11 | Min temperature of coldest month | | Mean of a 2000 m buffer around each sampling point | Pixel value | http://www.worldclim.org/bioclim |

Table B.5: Twelve Predictors used in the random forest variable selection for the four members of the *Anopheles maculipennis* group known for Germany

| **Variable** | **Resolution** | **Variable ID** | **Variable description** | | **Usage of the variables in the model building** | **Usage of the variables in the prediction** | **Source** |
|---|---|---|---|---|---|---|---|
| BIO12 | 30 arc-seconds (˜1 km) | 12 | Annual Precipitation | | Mean of a 2000 m buffer around each sampling point | Pixel value | http://www.worldclim.org/bioclim |

Table B.6: Accuracy indices of the final models for the four members of the *Anopheles maculipennis* group known for Germany

| **Accuracy indices** | ***An. atroparvus*** | ***An. maculipennis* s.s.** | ***An. daciae*** | ***An. messeae*** |
|---|---|---|---|---|
| **$AUC_{cor}$** | 0.79 | 0.69 | 0.86 | 0.72 |
| **RMSE** | 0.26 | 0.44 | 0.39 | 0.43 |
| **KAPPA** | 0.32 | 0.25 | 0.56 | 0.40 |
| **TSS** | 0.32 | 0.25 | 0.56 | 0.40 |
| **CCR** | 0.90 | 0.68 | 0.80 | 0.71 |
| **SPEC** | 0.94 | 0.76 | 0.86 | 0.65 |
| **SENS** | 0.38 | 0.48 | 0.70 | 0.75 |
| **PPV** | 0.88 | 0.75 | 0.88 | 0.84 |
| **NPV** | 0.97 | 0.84 | 0.78 | 0.59 |

Table B.7: Parameters of the final models determined by random forest variable selection using twelve predictors (Table 1) for the four members of the *Anopheles maculipennis* group known for Germany

| **Model variables/ Species** | ***An. atroparvus*** | ***An. maculipennis* s.s.** | ***An. daciae*** | ***An. messeae*** |
|---|---|---|---|---|
| **(Intercept)** | 0.805585 | 1.061042 | -0.001528 | 1.2275557 |
| **BIO1** | | 0.001994 | 0.00008954 | |
| **BIO5** | -0.002813 | -0.003980 | 0.006794 | -0.0019438 |
| **BIO6** | 0.003224 | | -0.002688 | |
| **BIO12** | | | -0.001968 | |
| **Altitude** | | | 0.0005074 | -0.0008685 |
| **Agricultural** | | | | -0.3317367 |
| **Pastures** | | | -0.3314 | |
| **Wetlands** | | -1.301920 | | |

## Figures

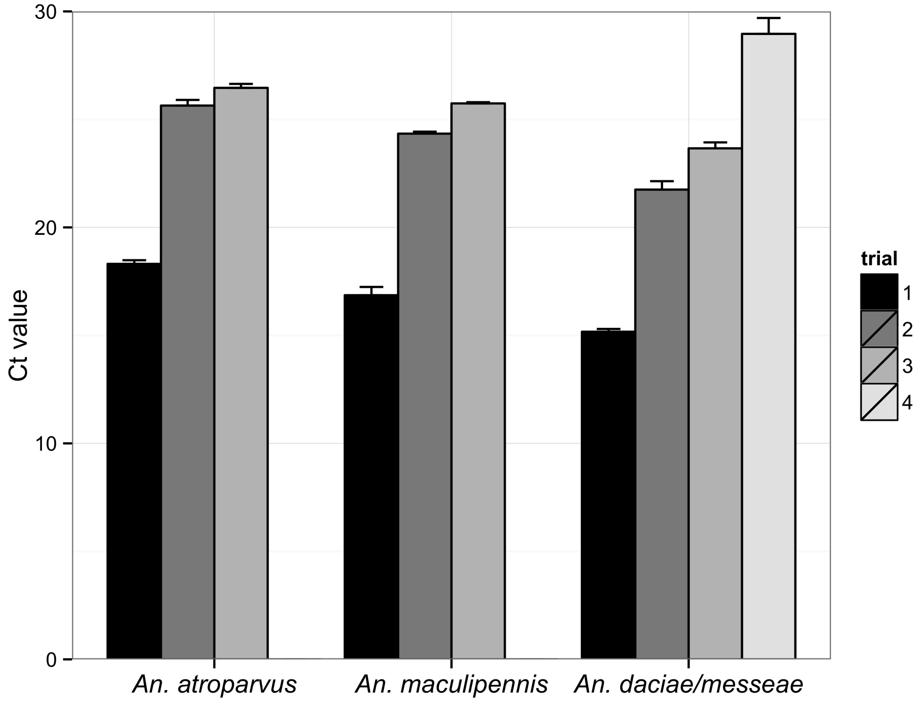


Figure B.9: The specificity of the single-plex RT PCRs were evaluated by execution of the PCR reaction mixes within four different trials: 1) unmixed DNA template of the species A (100 ng), for which the PCR was developed, 2) unmixed, but extensively diluted DNA template of the species A (4 ng), 3) 1:25 mixed samples with 4 ng DNA of species A and 48 ng DNA of each species B and C, and 4) unmixed DNA template (100 ng) of the species B or C. Values presented are the mean and standard deviation of two independent experiments. No bar indicate no detection.

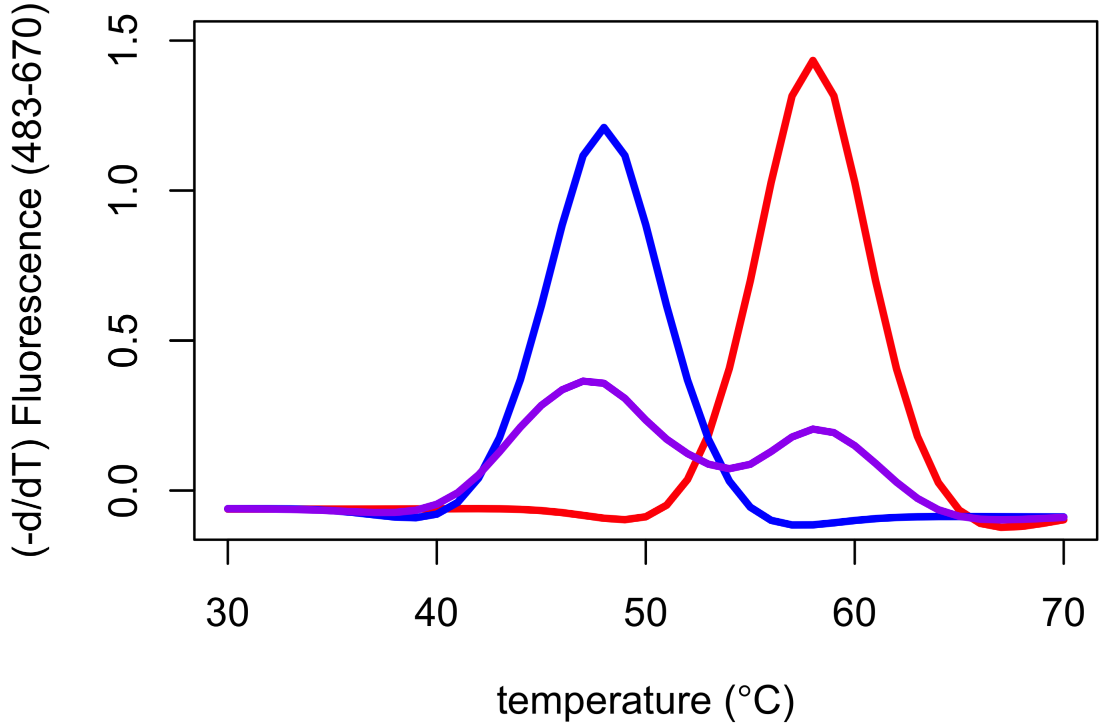


Figure B.10: Negative derivative melting curves of the real-time PCR for the differentiation of *Anopheles daciae*/*Anopheles messeae* (blue=*An. messeae*, red=*An. daciae*, purple=hybrid of *An. daciae* and *An. messeae*).

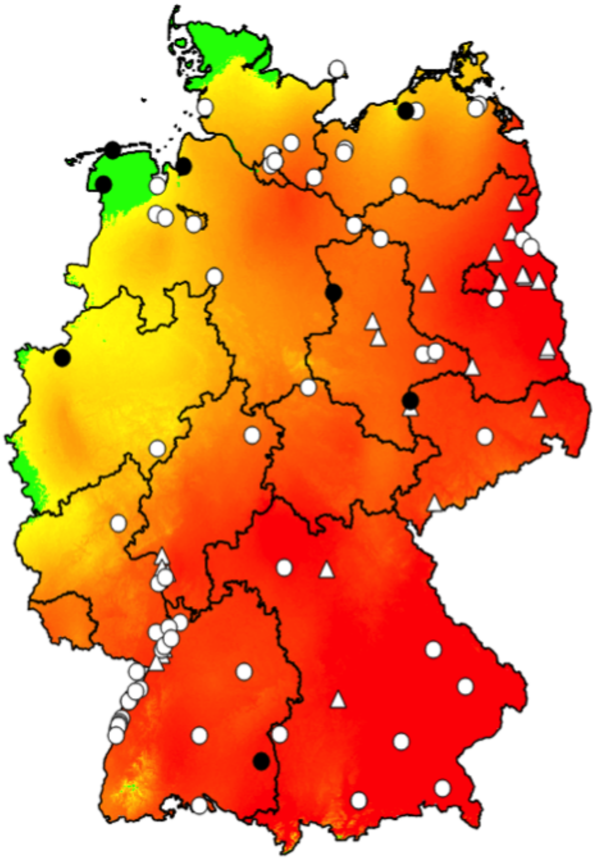


Figure B.11: Distribution of *Anopheles atroparvus* in Germany. Distribution data: dots=data gathered in this study, triangles=data from other studies (Weitzel et al., 2012; Kronefeld et al., 14b); black=presence, white=absence. Prediction of species distribution: probability of occurrence increase from red over orange to yellow, green indicate presence according the selected threshold.

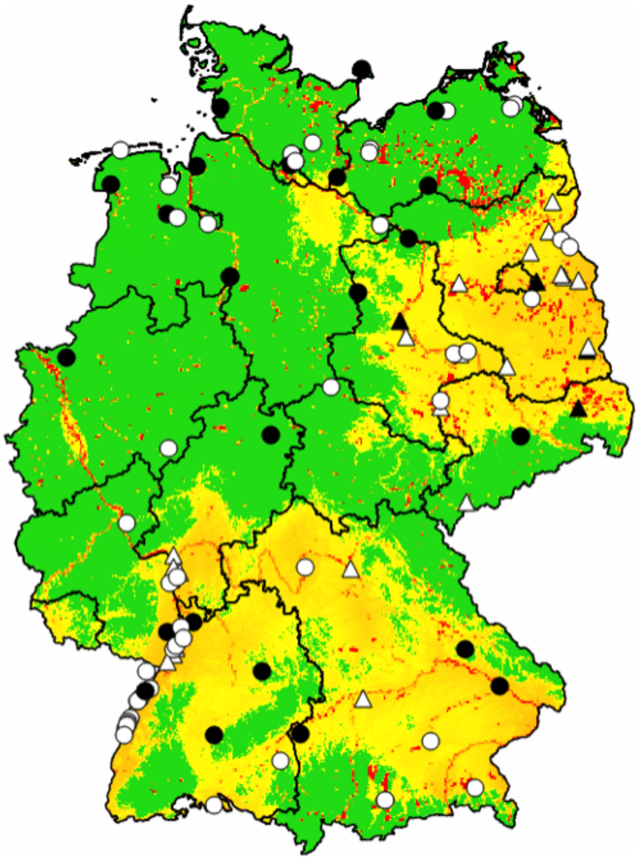


Figure B.12: Distribution of *Anopheles maculipennis* s.s. in Germany. Distribution data: dots=data gathered in this study, triangles=data from other studies (Weitzel et al., 2012; Kronefeld et al., 14b); black=presence, white=absence. Prediction of species distribution: probability of occurrence increase from red over orange to yellow, green indicate presence according the selected threshold.

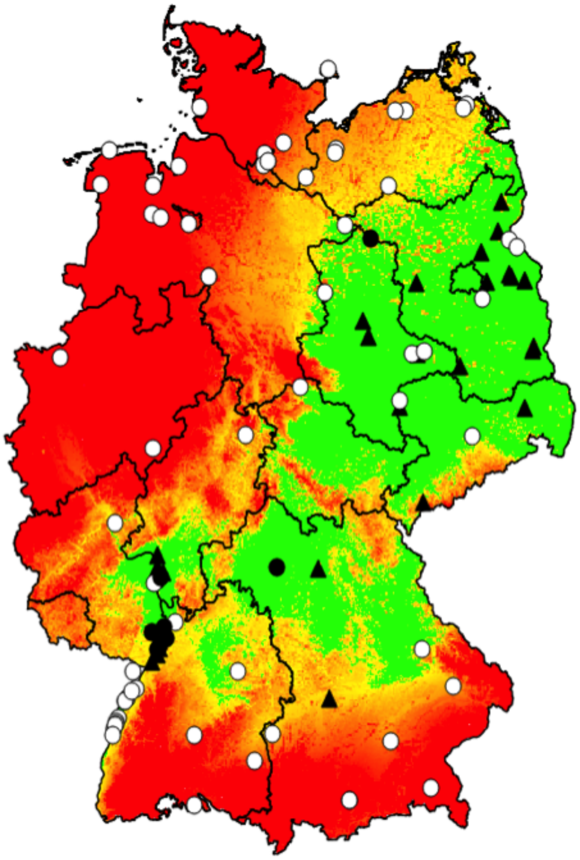


Figure B.13: Distribution of *Anopheles daciae* in Germany. Distribution data: dots=data gathered in this study, triangles=data from other studies (Weitzel et al., 2012; Kronefeld et al., 14b); black=presence, white=absence. Prediction of species distribution: probability of occurrence increase from red over orange to yellow, green indicate presence according the selected threshold.

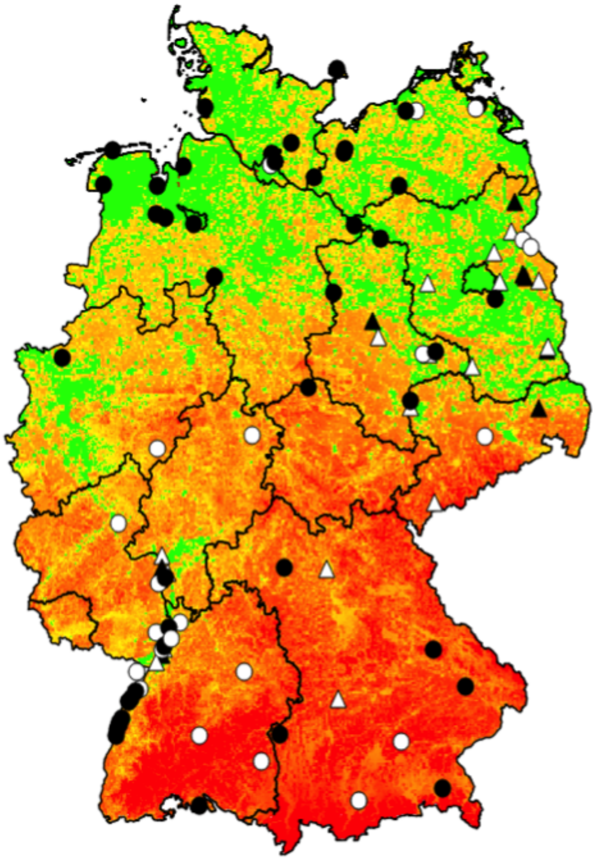
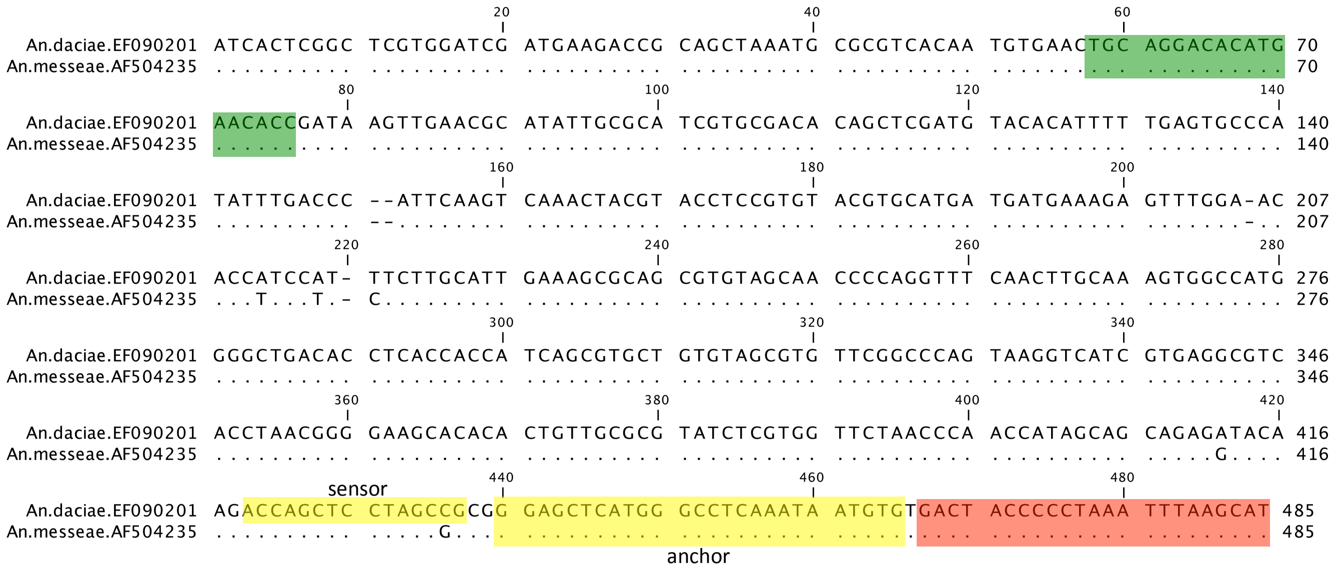


Figure B.14: Distribution of *Anopheles messeae* in Germany. Distribution data: dots=data gathered in this study, triangles=data from other studies (Weitzel et al., 2012; Kronefeld et al., 14b); black=presence, white=absence. Prediction of species distribution: probability of occurrence increase from red over orange to yellow, green indicate presence according the selected threshold.

## Supplementary material

```
                                            20                    40                    60
                                             |                     |                     |
       An.daciae.EF090201 ATCACTCGGC TCGTGGATCG ATGAAGACCG CAGCTAAATG CGCGTCACAA TGTGAACTGC AGGACACATG 70
      An.messeae.AF504235 .......... .......... .......... .......... .......... .......... .......... 70
    An.atroparvus.AY634534 .......... .......... .......... .......... .......... .......... .......... 70
 An.maculipennis.AF469852 .......... .......... .......... .......... .......... .......... .......... 70
                                 80                   100                   120                   140
                                  |                     |                     |                     |
       An.daciae.EF090201 AACACCGATA AGTTGAACGC ATATTGCGCA TCGTGCGACA CAGCTCGATG TACACATTTT TGAGTGCCCA 140
      An.messeae.AF504235 .......... .......... .......... .......... .......... .......... .......... 140
   An.atroparvus.AY634534 .......... .......... .......... .......... .......... .......... .......... 140
 An.maculipennis.AF469852 .......... .......... .......... .......... .......... .......... ........T. 140
                                           160                   180                   200
                                             |                     |                     |
       An.daciae.EF090201 TATTTGACCC --ATTCAAGT CAAACTACGT ACCTCCGTGT ACGTGCATGA TGATGAAAGA GTTTGGA-AC 207
      An.messeae.AF504235 .......... --........ .......... .......... .......... .......... .......-.. 207
   An.atroparvus.AY634534 .......T.A TA.CC....C .....GG... .....ACC.. .....G.-.T .........G ..C....T.. 209
 An.maculipennis.AF469852 .......... --.----G.. .......... .......G.. .......... .......... .......-.. 203
                                220                   240                   260                   280
                                  |                     |                     |                     |
       An.daciae.EF090201 ACCATCCAT- TTCTTGCATT GAAAGCGCAG CGTGTAGCAA CCCCAGGTTT CAACTTGCAA AGTGGCCATG 276
      An.messeae.AF504235 ...T...T.- C......... .......... .......... .......... .......... .......... 276
   An.atroparvus.AY634534 G......T.T C........C ...GT..T.. .......... .......... .......... .......... 279
 An.maculipennis.AF469852 .......T.- C......... ....A..... .......... .......... .......... .......... 272
                                           300                   320                   340
                                             |                     |                     |
       An.daciae.EF090201 GGGCTGACAC CTCACCACCA TCAGCGTGCT GTGTAGCGTG TTCGGCCCAG TAAGGTCATC GTGAGGCGTC 346
      An.messeae.AF504235 .......... .......... .......... .......... .......... .......... .......... 346
   An.atroparvus.AY634534 .......... .......... .......... .......... .......... .TC....... .........T 349
 An.maculipennis.AF469852 .......... .......... .......... .......... .......... .TC....... .........T 342
                                360                   380                   400                   420
                                  |                     |                     |                     |
       An.daciae.EF090201 ACCTAACGGG GAAGCACACA CTGTTGCGCG TATCTCGTGG TTCTAACCCA ACCATAGCAG CAGAGATACA 416
      An.messeae.AF504235 .......... .......... .......... .......... .......... .......... .....G.... 416
   An.atroparvus.AY634534 .........A .......CAG ...C....T. ......A... ..AC-C.... .......... .......... 418
 An.maculipennis.AF469852 .......... ..G....... .......... ......A... ..---..... .......... .......... 409
                                           440                   460                   480
                                             |                     |                     |
       An.daciae.EF090201 AGACCAGCTC CTAGCCGCGG GAGCTCATGG GCCTCAAATA ATGTGTGACT ACCCCCTAAA TTTAAGCAT 485
      An.messeae.AF504235 .......... .....G.... .......... .......... .......... .......... ......... 485
   An.atroparvus.AY634534 .......... .....A.... .......... .T........ .....A.... .......... ......... 487
 An.maculipennis.AF469852 .C...G.... ....T----- -...C..... .......... .......... .......... ......... 472
```

**Fig. S1:** Sequence alignment the internal transcribed spacer 2 (ITS-2) gene for the multiplex real-time PCR for the identification of *Anopheles atroparvus*, *An. maculipennis*, and *An. daciae*/*messeae* from single *Anopheles maculipennis* group individuals: green = forward primer, red = reverse primer, yellow = probes



```
                                             20                   40                   60
An.daciae.EF090201   ATCACTCGGC TCGTGGATCG ATGAAGACCG CAGCTAAATG CGCGTCACAA TGTGAACTGC AGGACACATG 70
An.messeae.AF504235  .......... .......... .......... .......... .......... .......... .......... 70
                              80                  100                  120                  140
An.daciae.EF090201   AACACCGATA AGTTGAACGC ATATTGCGCA TCGTGCGACA CAGCTCGATG TACACATTTT TGAGTGCCCA 140
An.messeae.AF504235  .......... .......... .......... .......... .......... .......... .......... 140
                                        160                  180                  200
An.daciae.EF090201   TATTTGACCC --ATTCAAGT CAAACTACGT ACCTCCGTGT ACGTGCATGA TGATGAAAGA GTTTGGA-AC 207
An.messeae.AF504235  .......... --........ .......... .......... .......... .......... .......-.. 207
                              220                  240                  260                  280
An.daciae.EF090201   ACCATCCAT- TTCTTGCATT GAAAGCGCAG CGTGTAGCAA CCCCAGGTTT CAACTTGCAA AGTGGCCATG 276
An.messeae.AF504235  ...T...T.- C......... .......... .......... .......... .......... .......... 276
                                        300                  320                  340
An.daciae.EF090201   GGGCTGACAC CTCACCACCA TCAGCGTGCT GTGTAGCGTG TTCGGCCCAG TAAGGTCATC GTGAGGCGTC 346
An.messeae.AF504235  .......... .......... .......... .......... .......... .......... .......... 346
                              360                  380                  400                  420
An.daciae.EF090201   ACCTAACGGG GAAGCACACA CTGTTGCGCG TATCTCGTGG TTCTAACCCA ACCATAGCAG CAGAGATACA 416
An.messeae.AF504235  .......... .......... .......... .......... .......... .......... .....G.... 416
                                        440                  460                  480
An.daciae.EF090201   AGACCAGCTC CTAGCCGCGG GAGCTCATGG GCCTCAAATA ATGTGTGACT ACCCCCTAAA TTTAAGCAT 485
An.messeae.AF504235  .......... .....G.... .......... .......... .......... .......... ......... 485
```

**Fig. S2:** Sequence alignment the internal transcribed spacer 2 (ITS-2) gene for the multiplex real-time PCR for the differentiation of *Anopheles daciae* and *An. messeae* from single *An. daciae/messeae* individuals: green = forward primer, red = reverse primer, yellow = anchor and sensor

**Fig. S3:** Sequence alignment the internal transcribed spacer 2 (ITS-2) gene for the singleplex real-time PCR for the identification of *Anopheles atroparvus, An. maculipennis*, and *An. daciae/messeae* from mixed *Anopheles maculipennis* group pools: green = forward primer, red = reverse primer, yellow = probes

# Appendix C

# Species distribution

## C.1 Physico-chemical characteristics of *Culex pipiens* s.l. and *Culex torrentium* breeding sites in Germany

submitted to the Journal of Medical Entomology: **Lühken, R.**, Steinke, S., Leggewie, M., Tannich, E., Krüger, A., Becker, S., & Ellen Kiel (submitted). Physico-chemical characteristics of *Culex pipiens* s.l. and *Culex torrentium* breeding sites in Germany.

### Abstract

*Culex pipiens* s.l. (L.) and *Culex torrentium* Martini, 1925 are two widely distributed mosquito species in Europe. Due to difficulties in morphological discrimination, the current knowledge on differences in the breeding ecology is fragmentary. Therefore, this study evaluated the relation between the presence-absence of both species at

various types of breeding habitats in response to physico-chemical parameters, using a recently developed molecular assay that allows reliable species specific typing of larvae and pupae. The results revealed that the two species often occur in sympatry at the studied breeding sites and there were no substantial differences concerning presence-absence of the two species with regard to the various environmental parameters investigated.

## Introduction

*Culex pipiens* s.l. (L.) is a common species in Europe, Asia, Africa, Australia, and North and South America (Harbach, 2012), and proved to be widespread in Germany (Mohrig, 1969). *Culex torrentium* Martini, 1925, in contrast, appeared to be a rare species for many years (Mattingly, 1951; Mohrig, 1969). However, later studies indicated that the species is probably widespread in Europe (Service, 1968; Jupp, 1979; Gillies & Gubbins, 1982; Snow & Ramsdale, 1999; Hutchinson et al., 2007; Hesson et al., 2011, 2014) including Germany (Küpper et al., 2006; Weitzel et al., 2011; Rudolf et al., 2013; Werblow et al., 2014). Nevertheless, it remains to be clarified, whether the distribution of *Cx. torrentium* has really changed during recent years or whether this impression is due discrimination problems in the past (Rudolf et al., 2013; Börstler et al., 2014; Krüger et al., 2014). Weitzel et al. (2011) highlighted that the interest in the species differentiation did not start until differing vector capacities of the two species were discovered. Since as the conventional characters for females are inaccurate (e.g. pre-alar scales easy fall off Service, 1968; Jupp, 1979) and other female features had been largely ignored (e.g. wing radial veins Börstler et al., 2014), only the males of both species were differentiated by hypopygial characters (Becker et al., 2010).

Problems in the morphological differentiation also apply for the immature stages of *Cx. pipiens* s.l. and *Cx. torrentium*. Although different characters for the identification have been applied, e.g. siphonal index or branching of setae (Harbach et al., 1985; Vinogradova, 2000), these did not withstand verification (Jupp, 1979; Dahl, 1988). Becker et al. (2010) proposed differences in the number of larval abdominal setae as unique characters separating both species. However, as discussed by Weitzel et al. (2014), well preserved specimens are required in order to use this character, which additionally might be variable in *Cx. pipiens* s.l.. Therefore, previous studies on the breeding ecology of both species were depended on the rearing of immature stages to adults (Service, 1968; Jupp, 1979; Gillies & Gubbins, 1982), which is time-consuming and difficult to apply when large collections are investigated. However, these difficulties were recently overcome by the introduction of new molecular DNA-typing protocols, which allow reliable differentiation between *Cx. pipiens* s.l. and *Cx. torrentium* at all developmental stages including immature larvae (Hesson et al., 2010; Rudolf et al., 2013).

Both, *Cx. pipiens* s.l. and *Cx. torrentium*, are considered to be predominantly ornithophilic (Service, 71a) and thus considered to be potential enzootic vectors for arboviruses in bird populations (Lundström, 1999). However, there are differences in the vector competence. *Culex torrentium* is an efficient vector for Sindbis virus in experimental laboratory challenge experiments (Lundström et al., 99a,b), and probably the main enzootic vector for Sindbis in Sweden (Hesson et al., 2011). *Culex pipiens* s.l., on the other hand, seems to have only moderate vector competence for Sindbis virus (Lundström et al., 99a,b). Therefore, a more detailed knowledge on the breeding ecology of the two species is of importance to understand their distribution and and associated pathogens.

So far, only a few studies have focused on the evaluation of breeding site parameters to understand the distribution of both species, which however resulted in contradictary conclusions. Different authors highlighted that *Cx. torrentium* can be classified as a “clean water species” (Scherpner, 1960; Mohrig, 1969; Vinogradova, 2003), preferring water bodies with low content of nitrogen compounds, and, deduced from low values of potassium permanganate consumption, a low amount of oxidable organic substances (Scherpner, 1960). However, this was generally concluded to be incorrect (Hesson et al., 2014), as it was also observed that the species colonizes polluted water bodies with high organic content (Scherpner, 1960; Gillies & Gubbins, 1982; Ishii & Sohn, 1987; Struppe, 1989; Raymond, 1995). Furthermore, *Cx. torrentium* was assumed to be a cold adapted species (Scherpner, 1960; Struppe, 1989), and should be more widespread at high altitudes (Mattingly, 1951; Sicart, 1954; Service, 1968; Mohrig, 1969; Struppe, 1989). Contradicting this assumption, the species was also sampled from water bodies with higher temperatures (Küpper et al., 2006) and it has been collected in many warm Mediterranean countries (reviewed by Hesson et al., 2014). According to a recently published study in Sweden, the proportion of *Cx. torrentium* has increased at elevated altitudes and the sites where this species was collected had reduced minimum monthly temperatures Hesson et al. (2011). Therefore, it was concluded that temperature probably is a prominent determinant for the distribution of both species, but the exact causal connection remains to be determined.

As already highlighted by Hesson et al. (2011), the information on the breeding ecology of *Cx. pipiens* s.l. and *Cx. torrentium* is, at best, fragmentary, and a comprehensive study on the breeding ecology of both species is still missing. Furthermore, due to problems concerning the morphological identification, results from previous studies have to be interpreted with caution. The study presented here aimed to investigate the correlation between physico-chemical parameters, water bodies and the distribution of immature stages of *Cx. pipiens* s.l. and *Cx. torrentium.*

## Material and Methods

In 2012, between August 22 and August 31 samples were taken from 90 water bodies on 20 cattle farms well spread all over Germany. Prior to mosquito collections, several parameters were measured in the field in order to characterize the various breeding sites. This list comprises the following parameters: type of water body (e.g. tyre, pond, etc.), character of habitat (near-natural, artificial), size, water depth, shade (low, medium, high), hydrology (permanent, temporary), dynamic (stagnant, non stagnant), watercolor (clear, brown, green, red, yellow, green-yellow, yellow-brown), bank vegetation (yes, no), and proportion of cover of different aquatic vegetation (terrestrial plants, helophytes, hyrdophytes, Lemna spp., filamentous algae). Furthermore, pH (WTW, Weilheim, Germany; pH 3210), conductivity and salinity (WTW, Weilheim, Germany; Cond 3110) as well as temperature and oxygen content (WTW, Weilheim, Germany; CellOx 325) were measured in the field. Water hardness was also determined directly on-site (Aquamerck®, Merck, Germany; carbonate hardness test). Additionally, water samples (0.6 L) were frozen at approximately -18 °C, transported to the laboratory and used to identify selected parameters in the laboratory including ammonium, nitrate, nitrite, phosphate, total nitrogen, total phosphorus, total iron, total chloride, as well as total organic carbon.

From small water bodies (e.g. buckets, tires) the entire water content was drawn with a little bucket and rinsed through a sieve (500 µm) while from larger water bodies (e.g. ditches or ponds), immature mosquitoes were collected with a dip net 25 cm x 25 cm x 70 cm (500 µm). Mosquitoes were morphologically identified using the key published by Becker et al. (2010). Depending on the number of individuals collected, up to 25 larvae or pupae *Cx. pipiens* s.l./*Cx. torrentium* were selected from each colonized water body and identified on the species level using a recently published molecular DNA typing assay (Rudolf et al., 2013).

The relation between the presence-absence of both species and the collected environmental factors was analysed according to the method described by Mereta et al. (2013), which use the classification trees to model the presence-absence of Anopheles larvae based on environmental factors. The calculation of classification trees in R (R Core Team, 2014) used the J48-function with default parameter settings from the weka package (Hornik et al., 2009; Witten, 2011). Three-fold cross-validation was applied for model validation, i.e. the dataset was randomly split in three subsets, and each subset in turn was used for validation and the other two subsets for training. Model performance was evaluated by the mean percentage of correctly classified instances (CCI), i.e. percentage of true positive and true negative prediction, and Cohen's kappa statistic $\varkappa$, i.e. proportion of correctly predicted cases with correction for chance effects. CCI values higher or equal to 70% and $\varkappa$ higher or equal to 0.4 were considered reliable (Gabriels et al., 2007).

## Results

*Cx. pipiens* s.l. larvae were present in 64 water bodies (71% of all water bodies) and *Cx. torrentium* was sampled from 47 water bodies (52% of all water bodies). Both species occurred together in 38 water bodies (42% of all water bodies). Neither *Cx. pipiens* s.l. nor *Cx. torrentium* preferred specific water bodies and both colonized the same types of habitats (e.g. ponds or tyres) (Table C.1). In addition, water bodies tested positive for the two species comprised a wide range of different habitats concerning hydrology or shade (Table C.2). Furthermore, the classification tree models had a bad predictive performance for both species, with a CCI of 66.6% and $\varkappa$ of 0.07 for *Cx. pipiens* s.l. and a CCI of 50.0% and $\varkappa$ of 0.03 for *Cx. torrentium*, indicating no evidence for a statistically significant relation between the presence-absence of the two species and specific environmental parameters as measured in this study.

## Discussion

*Cx. pipiens* s.l. and *Cx. torrentium* probably differ in their vector competence for the transmission of pathogens (Lundström et al., 99a,b; Hesson et al., 2011). Therefore, the identification of differences regarding their breeding ecology might help to understand the distribution of both species and associated pathogens. However, Medlock et al. (2005) summarized that there are no bionomic differences between the species, e.g. both regularly colonize the same breeding habitats (Scherpner, 1960; Jupp, 1979; Gillies & Gubbins, 1982; Sulaiman & Service, 1983; Ishii & Sohn, 1987; Raymond, 1995; Vinogradova et al., 2007; Weitzel et al., 2009; Werblow et al., 2014). At least on the level of breeding sites and based on the environmental parameters studied, the data presented here confirm this assumption.

We did not find differences regarding the breeding sites of *Cx. pipiens* s.l. and *Cx. torrentium*, which were both present in most of the water bodies, irrespective of the span measured for most of the parameters. Furthermore, the results did not explain differences in the prevalence of the two species.

Mohrig (1969) described *Cx. torrentium* to be a "wild mosquito", which is not as synanthrope as *Cx. pipiens* s.l.. In contrast, several studies identified a wide variety of breeding sites for both species including artificial habitats (Scherpner, 1960; Gillies & Gubbins, 1982; Hesson et al., 2011). Weitzel et al. (2014) and Hesson et al. (2014) found *Cx. torrentium* to be even more frequent in artificial compared to natural breeding sites, which is supported by this study.

As previously summarized by Hesson et al. (2014), *Cx. torrentium* should not be classified as a "clean water species" like it was named in earlier studies (Scherpner, 1960; Mohrig, 1969; Vinogradova, 2003). This is in agreement with the results presented here indicating that many breeding sites where *Cx. torrentium* was sampled contained high concentrations of ammonium, nitrate, nitrite, phosphate, and organic carbon. Therefore, our data could not corroborate any clean water preference of *Cx. torrentium* similar to other studies indicating the occurrence of this species in organic rich water bodies (Scherpner, 1960; Gillies & Gubbins, 1982; Ishii & Sohn, 1987; Struppe, 1989; Raymond, 1995). Also in line with these results, both species proved to be able to tolerate relative high values of conductivity, salinity, and chloride.

Aiming to explain the distribution of *Cx. torrentium*, another hypothesis stated that this species should be cold adapted (Scherpner, 1960; Struppe, 1989). Again, our study supports the criticism by Hesson et al. (2011), who rejected this hypothesis. In the breeding sites of *Cx. torrentium* we measured water temperatures exceeding 32 °C, which is in line with Küpper et al. (2006) who demonstrated this species tolerates temperatures up to 30 °C.

As final remarks regarding the presence-absence of both species and the recorded parameters, Onyeka (1980) highlighted that vegetated ponds are favored by *Cx. pipiens* s.l.. We regularly found this species, together with *Cx. torrentium*, even in artificial breeding sites without any vegetation. Furthermore, as Scherpner (1960) already discovered, the chemical parameter “total content of iron” did not explain the presence-absence pattern of both species.

In Germany, both species commonly occur in sympatry (Rudolf et al., 2013; Werblow et al., 2014). However, it was also recognized that both do not show exactly the same distribution and frequency (Rudolf et al., 2013; Hesson et al., 2014; Werblow et al., 2014). Following the theory that sympatric coexistence of two species requires at least one kind of resource partitioning (e.g. Macarthur & Levins, 1967), the ecological difference between both species remains unknown. There are hints suggesting niche separation in the adult stage, which might be worth to be studied in more depth in future. Hesson et al. (2014) described a higher prevalence of *Cx. torrentium* in northern Europe and a wider distribution of *Cx. pipiens* s.l. in southern Europe. In central Europe, the distribution maps of these species display a strong overlap. The authors explained the observed pattern by a different oviposition strategy: in Sweden *Cx. torrentium* lay eggs between May and middle July, while *Cx. pipiens* s.l. can produce eggs throughout the year. This might suggest some kind of a partial temporal niche separation. Furthermore, Service (1994) demonstrated that both species have different swarming sites, which might suggest a prezygotic isolation Sawadogo et al. (2013). Moreover, while the hibernation sites of *Cx. pipiens* s.l. had been identified in several studies (Service, 1968; Jupp, 1979; Gillies & Gubbins, 1982; Sulaiman & Service, 1983; Börstler et al., 2014), the main overwintering sites of *Cx. torrentium* are still unknown. Börstler et al. (2014) summarized that *Cx. torrentium* may avoid cellars or other peridomestic overwintering shelters.

Lastly, Silvertown (2004) highlighted the general problem of identifying the appropriate niche axes to study a priori. Accordingly, it might be possible that additional factors, which were not included into this study such as dietary niche separation, might be responsible for a partitioning within the breeding sites along some other axis. Dahl et al. (1993) demonstrated selective particle uptake by larvae of different mosquito larvae, and Gilbreath et al. (2013) found resource partitioning between the larvae of two regularly sympatric mosquito species.

Nevertheless, it can be concluded that both species probably have a strong overlap in their realized ecological niche, but there might be a divergence in respect to other breeding site parameters, not studied in detail so far. Therefore, future research on the differences in the breeding ecology of both species should have a focus on the evaluation of relative species abundances and experimental studies to test competition theory (e.g. see Silvertown, 2004).

## Acknowledgments

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## Tables

Table C.1: Presence of *Culex pipiens* s.l. and *Culex torrentium* regarding binomial, categorical, and ordinal variables (numbers in brackets indicate the total number (first column) respectively the percentage of water bodies for each category of each variable).

| | **Presence of *Culex pipiens* s.l.** | **Presence of *Culex torrentium*** | **Presence of both species** |
|---|---|---|---|
| **Type** | | | |
| Barrel lid (1) | 1 (100.0%) | 0 (0.0%) | 0 (0.0%) |
| Bin (2) | 2 (100.0%) | 1 (50.0%) | 1 (50.0%) |
| Bucket (6) | 5 (83.3%) | 4 (66.7%) | 3 (50.0%) |
| Cistern (1) | 1 (100.0%) | 0 (0.0%) | 0 (0.0%) |
| Ditch (5) | 2 (40.0%) | 1 (20.0%) | 0 (0.0%) |
| Drinking trough (2) | 2 (100.0%) | 1 (50.0%) | 1 (50.0%) |
| Foil (1) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| Lake (1) | 1 (100.0%) | 1 (100.0%) | 1 (100.0%) |
| Pond (9) | 6 (66.7%) | 6 (66.7%) | 6 (66.7%) |
| Puddle (5) | 5 (100.0%) | 3 (60.0%) | 3 (50.0%) |
| Rain barrel (5) | 4 (80.0%) | 3 (60.0%) | 3 (60.0%) |
| Rain collection basin (2) | 1 (50.0%) | 1 (50.0%) | 1 (50.0%) |
| Stone pot (1) | 1 (100.0%) | 1 (100.0%) | 1 (100.0%) |
| Toy (1) | 1 (100.0%) | 0 (0.0%) | 1 (100.0%) |
| Tyre (47) | 32 (68.1%) | 27 (57.1%) | 20 (42.6%) |
| Well (1) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| **Type of habitat** | | | |
| Artificial (70) | 50 (71.4%) | 38 (54.3%) | 30 (42.9%) |
| Near natural (20) | 14 (70%) | 9 (45.0%) | 8 (40.0%) |

Table C.1: Presence of *Culex pipiens* s.l. and *Culex torrentium* regarding binomial, categorical, and ordinal variables (numbers in brackets indicate the total number (first column) respectively the percentage of water bodies for each category of each variable).

| | **Presence of *Culex pipiens* s.l.** | **Presence of *Culex torrentium*** | **Presence of both species** |
|---|---|---|---|
| **Hydrology** | | | |
| Indifferent (2) | 2 (100.0%) | 2 (100.0%) | 2 (100.0%) |
| Permanent (15) | 8 (53.3%) | 5 (33.3%) | 4 (26.7%) |
| Temporary (73) | 54 (74.0%) | 40 (754.8%) | 32 (43.8%) |
| **Dynamic** | | | |
| Not stagnant (5) | 2 (40.0%) | 1 (20.0%) | 0 (0.0%) |
| Stagnant (85) | 62 (72.9%) | 46 (54.1%) | 38 (44.7%) |
| **Shade** | | | |
| High (15) | 11 (73.3%) | 11 (73.3%) | 9 (60.0%) |
| Medium (24) | 11 (45.8%) | 11 (45.8%) | 7 (29.2%) |
| Low (38) | 33 (86.8%) | 17 (44.7%) | 15 (39.5%) |
| No (12) | 9 (75.0%) | 8 (66.7%) | 7 (58.3%) |
| **Water colour** | | | |
| Clear (17) | 9 (52.9%) | 7 (41.2%) | 5 (29.4%) |
| Brown (3) | 3 (100.0%) | 1 (33.3%) | 1 (33.3%) |
| Green (7) | 6 (85.7%) | 5 (71.4%) | 5 (71.4%) |
| Red (4) | 3 (75.0%) | 3 (75.0%) | 2 (50.0%) |
| Yellow (9) | 6 (66.7%) | 6 (66.7%) | 5 (55.6%) |
| Greenyellow (10) | 6 (60.0%) | 4 (40.0%) | 3 (30.0%) |
| Yellowbrown (40) | 31 (77.5%) | 21 (52.5%) | 17 (42.5%) |
| **Bank vegetation** | | | |
| Yes (18) | 11 (61.1%) | 7 (38.9%) | 6 (33.3%) |

Table C.1: Presence of *Culex pipiens* s.l. and *Culex torrentium* regarding binomial, categorical, and ordinal variables (numbers in brackets indicate the total number (first column) respectively the percentage of water bodies for each category of each variable).

| | **Presence of *Culex pipiens* s.l.** | **Presence of *Culex torrentium*** | **Presence of both species** |
|---|---|---|---|
| No (72) | 53 (73.6%) | 40 (55.6%) | 32 (44.4%) |

Table C.2: Mean, minimum, and maximum physico-chemical parameters of the water bodies investigated as well as for the water bodies colonized by *Culex pipiens* s.l. and *Culex torrentium* respectively.

| | **All water bodies** | | **Presence of *Culex pipiens* s.l.** | | **Presence of *Culex torrentium*** | |
|---|---|---|---|---|---|---|
| | **Mean (SD)** | **Min-Max** | **Mean (SD)** | **Min-Max** | **Mean (SD)** | **Min-Max** |
| **Habitat characteristics** | | | | | | |
| Size [m$^2$] | 40.2 (149.9) | 0.0-900.0 | 10.1 (27.2) | 0.0-160.0 | 10.9 (29.7) | 0.0-160.0 |
| Water depth [m] | 0.3 (0.4) | 0.0-1.5 | 0.3 (0.4) | 0.0-1.5 | 0.3 (0.4) | 0.0-1.5 |
| **Water physical/chemical variables** | | | | | | |
| Temperature [°C] | 20.2 (3.8) | 13.1-32.6 | 19.6 (4.1) | 13.1-32.6 | 19.3 (3.8) | 13.1-32.6 |
| PH | 7.7 (0.8) | 6.0-10.0 | 7.6 (0.8) | 6.2-9.9 | 7.6 (0.8) | 6.2-9.9 |
| Conductivity [µS/cm$^{-1}$] | 476.1 (560.1) | 13.5-3110 | 506.4 (629.4) | 14.3-3110.0 | 436.8 (535.4) | 14.3-3110.0 |
| Salinity [PSU] | 0.2 (0.3) | 0.0-1.6 | 0.2 (0.3) | 0.0-1.6 | 0.2 (0.3) | 0.0-1.6 |
| Dissolved oxygen [mg/L$^{-1}$] | 6.7 (5.2) | 0.0-35.0 | 6.8 (5.5) | 0.0-35.0 | 6.2 (3.9) | 0.2-15.2 |
| Hardness | 5.8 (6.6) | 0.0-35.0 | 6.2 (7.0) | 0.0-35.0 | 5.9 (7.3) | 0.0-35.0 |

Table C.2: Mean, minimum, and maximum physico-chemical parameters of the water bodies investigated as well as for the water bodies colonized by *Culex pipiens* s.l. and *Culex torrentium* respectively.

| | **All water bodies** | | **Presence of *Culex pipiens* s.l.** | | **Presence of *Culex torrentium*** | |
|---|---|---|---|---|---|---|
| | **Mean (SD)** | **Min-Max** | **Mean (SD)** | **Min-Max** | **Mean (SD)** | **Min-Max** |
| Ammonium (NH$^{4+}$) [mg/L$^{-1}$] | 5.1 (12.3) | 0.0-99.9 | 6.0 (14.1) | 0.1-99.9 | 3.5 (5.7) | 0.0-29.9 |
| Nitrate (NO$^{3-}$) [mg/L$^{-1}$] | 20.4 (35.3) | 0.0-245.2 | 16 (21.3) | 0.8-160.7 | 19.8 (33.1) | 0.0-172.1 |
| Nitrite (NO$^{2-}$) [mg/L$^{-1}$] | 0.2 (0.5) | 0.0-4.0 | 0.2 (0.6) | 0-4.0 | 0.3 (0.6) | 0.0-4.0 |
| Phosphate (PO$_4^{3-}$) [mg/L$^{-1}$] | 8.3 (16.7) | 0.0-90.5 | 9.3 (18.1) | 0.1-90.5 | 6.3 (14.4) | 0.0-90.5 |
| Total nitrogen [mg/L$^{-1}$] | 13.3 (16.5) | 0.6-101.0 | 13.5 (16.9) | 1.5-101.0 | 10.7 (10.0) | 1.0-43.2 |
| Total phosphorus [mg/L$^{-1}$] | 3.5 (6.0) | 0.0-29.9 | 4.0 (6.4) | 0.0-29.9 | 2.8 (5.0) | 0.1-29.9 |
| Total iron (Fe$^{2+}$) [mg/L$^{-1}$] | 3.1 (4.3) | 0.0-17.7 | 3.3 (4.4) | 0.0-17.7 | 2.2 (3.6) | 0-16.1 |
| Total chloride (Cl$^{-}$) [mg/L$^{-1}$] | 88.8 (157.1) | 0.5-933.6 | 104.2 (181.8) | 0.5-933.6 | 76.3 (169.0) | 0.6-933.6 |
| Total organic carbon [mg/L$^{-1}$] | 249.4 (428.5) | 0.0-3200.0 | 287.1 (475.0) | 6.8-3200.0 | 232.4 (491.1) | 7.5-3200.0 |
| | | | | | | |
| | | | | | | |

Table C.2: Mean, minimum, and maximum physico-chemical parameters of the water bodies investigated as well as for the water bodies colonized by *Culex pipiens* s.l. and *Culex torrentium* respectively.

| | **All water bodies** | | **Presence of *Culex pipiens* s.l.** | | **Presence of *Culex torrentium*** | |
|---|---|---|---|---|---|---|
| | **Mean (SD)** | **Min-Max** | **Mean (SD)** | **Min-Max** | **Mean (SD)** | **Min-Max** |
| **Aquatic vegetation** | | | | | | |
| Terrestrial plants [%] | 0.3 (1.8) | 0-10 | 0.2 (1.3) | 0-10 | 0.0 (0.0) | 0-0 |
| Helophytes [%] | 2.2 (13.5) | 0-90 | 0.3 (1.8) | 0-10 | 2.1 (13.2) | 0-90 |
| Hydrophytes [%] | 3.2 (12.4) | 0-70 | 3.5 (12.6) | 0-70 | 2.4 (10.9) | 0-70 |
| Lemna spp. [%] | 5.1 (20.0) | 0-95 | 5.0 (20.4) | 0-95 | 3.9 (18.9) | 0-95 |
| Filamentous algae [%] | 3.7 (15.8) | 0-95 | 2.7 (12.6) | 0-80 | 2.6 (12.9) | 0-80 |

## C.2 Topsoil conditions correlate with the emergence rates of ***Culicoides chiopterus*** and ***Culicoides dewulfi*** (Diptera: Ceratopogonidae) from cowpats

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### Abstract

*Culicoides chiopterus* (Meigen), 1830 and *Culicoides dewulfi* Goetghebuer, 1936 (Diptera: Ceratopogonidae) are considered to develop exclusively in dung, but do not necessarily show an equal distribution and abundance on livestock farms in Northern Europe. Recent modelling studies identified soil parameters to explain these differences. The present study addressed the question whether topsoil conditions underneath cowpats correlate with the number of emerging *C. chiopterus* and *C. dewulfi.* We recorded the emergence of biting midges from 24 cowpats over a period of four weeks and analysed samples from the topsoil. In agreement with species distribution models based on remote data, our results detected the correlation of soil moisture, organic matter, and soil texture with the number of emerging *C. chiopterus* and *C. dewulfi.* With increasing soil moisture the number of emerging adults increased for both species and the amount of organic matter was positively correlated with the number of emerging *C. chiopterus.* In contrast, soil textures showed conflicting results, i.e., a positive and negative relationship with the same variables. According to our results, soil underneath dung can explain the number of emerging *Culicoides*

species. The knowledge of these effects might improve the interpretation of large-scaled distribution models for dung-breeding biting midges.

## Introduction

*Culicoides chiopterus* (Meigen), 1830 and *Culicoides dewulfi* Goetghebuer, 1936 are considered to be vectors of veterinary important pathogens (Mellor et al., 2000; Meiswinkel et al., 2007; Dijkstra et al., 2008), among which the bluetongue virus (BTV) got the highest attention in Central and Northern Europe. BTV can cause non-contagious disease of ruminants, resulting in huge economic losses worldwide, e.g., the BTV epidemic 2006-2010 caused losses of more than 254 million euro in Germany (Conraths et al., 2012). The more recent epidemic of the Schmallenberg Virus (SBV) also highlighted the importance of biting midges as vectors of pathogens (Elbers et al., 2013). Here again, *C. chiopterus* and *C. dewulfi* are suspected to be important vectors (De Regge et al., 2012; Rasmussen et al., 2012).

*Culicoides chiopterus* and *C. dewulfi* are expected to breed exclusively in dung (Kettle & Lawson, 1952). Therefore, both species were assumed to be commonly present on livestock farms, as it was also proposed for the dung-breeding *Culicoides brevitarsis* (Cannon & Reye, 1966). As summarized by Scolamacchia et al. (2013), light traps have been found to underestimate *C. chiopterus* (Carpenter et al., 2008; Viennet et al., 2011), but it is generally considered that the trapping results are proportional to the real midge population at the sampling sites. Nevertheless, several studies demonstrated that the distribution and abundance of both species differ (Nielsen et al., 2010; Scolamacchia et al., 2013). According to the statistical analyses of light trap data, soil parameters of the surrounding environment affected the abundance of these species in opposite ways: the abundance of *C. dewulfi* showed a negative correlation with moisture-retentive soils and a positive correlation with organic content, whereas *C. chiopterus* behaved quite the opposite way (Scolamacchia et al., 2013).

The objective of this study was to compare the colonization densities of *Culicoides* biting midges in cowpats with a set of edaphic parameters characterizing the topsoil underneath. A better knowledge of this aspect of *Culicoides* breeding ecology will help to understand the distribution of *Culicoides* species, e.g. interpretation of modelling results using soil information.

## Methods

On 4 April 2011, we randomly selected twelve cowpats from one pasture on each of two livestock farms close to the city of Oldenburg (Lower Saxony, Germany), which are situated in a distance of 8 km to each other. One of the farms was characterized by relative wet conditions, i.e. marsh land, and the other farm is located on comparatively dry geest land soils. These farms represent typical dairy farms situated in rural regions in Northern Germany. The pastures from which we collected the cowpats were located at a distance of approximately 250 m from the stable. The pastures had not been used for grazing during the winter, i.e., from October until we took the cowpat samples. Therefore, it is reasonable to assume that the cowpats were approximately 5 to 6 months old.

The sampling design aimed at larvae overwintering within the cowpats. Thus, the cowpats were probably only colonized by one developmental stage, which is an advantage for a comparison of cowpat colonisation. Emergence traps (see figure in Lühken et al., 2014) were set up on all of the cowpats we selected for our experiment, which were situated in different situation of soil wetness. The traps of a pyramidal form (area: 0.8 $m^2$, height: 32 cm) covered the entire area of every cowpat. When exposed in the field, the bottom rim was thoroughly covered with soil in order to prevent the midges to invade from outside. Traps were emptied two times in intervals of 14 days. The samples were sorted in the laboratory, and biting midges were determined to group level (Obsoletus group, Pulicaris group or other Ceratopogonidae). Males and females of the Obsoletus group were determined to the species level based on morphological characters (Campbell & Pelham-Clinton, 1960), whereas female *Culicoides obsoletus* and *Culicoides scoticus* were considered indistinguishable (e.g. Mathieu et al., 2012).

On 2 May 2011, which was the last day of the experiment, we took three soil samples (depth=5 cm, area=2 cm) from underneath each cowpat. In order to record soil wetness, these substrates were transported to the laboratory in plastic bags. Fresh weight was measured immediately (Sartorius CP324S, precision: 0.001 g) and the dry weight was assessed after the substrate had been dried at 105 °C for two days and cooled down to room temperature in the desiccator. Afterwards, the samples were exposed to 400 °C for four hours and cooled down in the desiccator again in order to measure the ash weight. The difference between fresh and dry weight expresses the water content of the substrate, i.e. soil moisture, while the difference between dry weight and ash weight gives the amount of organic matter. Finally, we analysed the grain size distribution of the samples. Each sample was flushed through a sieve cascade (1000 µm, 500 µm, 200 µm, 63 µm, and 20 µm) with tap water. The finest fraction was collected ($<$ 20 µm) in a collection pan. The sieve residues from each mesh size were dried at room temperature and weighted. This resulted in a percentage distribution of six grain size classes: grain size 6 ($\geq$ 1000 µm, very coarse sand), grain size 5 ($\geq$ 500 µm, $<$ 1000 µm, coarse sand), grain size 4 ($\geq$ 200 µm, $<$ 500 µm, medium sand), grain size 3 ($\geq$ 63 µm, $<$ 200 µm, fine sand), grain size 2 ($\geq$ 20 µm, $<$ 63 µm, silt), and grain size 1 ($<$20 µm, clay). All results of the three soil samples from underneath the same cowpat were averaged.

Statistical analyses of the biting midge abundance was conducted according to the publication of Goodenough et al. (2012). The authors described a new method of variable selection called “Regression with Empirical Variable Selection (REVS)”, which were demonstrated to have a better performance than full, stepwise or all-subsets models. For both species (*C. chiopterus* and *C. dewulfi*), all-subset regressions were conducted. The function uses branch-and-bound techniques to calculate all combinations of variables for each level, while level means the number of variables per model at any one time. The best model with the highest $R^2$ at each level was selected. From this subset of models, the amount of empirical support was quantified for each variable by the number of models the variable was included in. Finally, models were built according to the empirical support for each variable. The first model only includes the variable with the most empirical support, the second model includes this variable and the variable with the next most empirical support and so on. These models were than compared using the delta AIC (Akaike information criterion) values. Models with a delta AIC $> 10$ were interpreted as not supported, while models with delta AIC $<$ 2 were interpreted as strongly supported. Additionally, the commonly used stepwise regression was applied using the step-function in R. The best REVS model and the best stepwise model according to their AIC values, where compared with adjusted $R^2$, AIC, delta AIC and p-values. See Goodenough et al. (2012) for further details on the modelling strategy and the R script. Leave-one-out cross-validation of the predictive accuracy of each model was applied to estimate the mean square prediction error using the R library boot. To quantify the severity of multi-collinearity, variance inflation factors (VIF) were calculated for each predictor. VIF $> 10$ were interpreted as severe collinearity. Spatial autocorrelation was accessed via variogram plots for the residuals of the linear regression models (Zuur et al., 2009). The data structure of the environmental variables was evaluated with Spearman's rank correlation.

## Results

We collected a total of 2,792 *Culicoides* biting midges belonging exclusively to the Obsoletus group. Six cowpats (25%) were not colonised by biting midges. The emergence per cowpat varied considerably a high variability (mean = 116.3, max = 804, min = 0). The majority (2,397 individuals, 85.9%) were determined as *C. chiopterus* and a further 366 individuals (13.1%) as *C. dewulfi*. 23 male individuals (0.8%) were identified as *C. scoticus*. Three female individuals (0.1%) were identified as either *C. obsoletus* or *C. scoticus*, as the morphological differentiation of these two species is difficult. Two further females and one male (0.1%) were not determined to species level because important identification characters were destroyed.

Although the stepwise and REVS model had a similar delta AIC, adjusted $R^2$ and p-value for *C. chiopterus*, the stepwise model also included several collinear factors (VIF $> 10$) (Tab. C.3). The REVS model for *C. dewulfi* had a better performance compared to the stepwise model. For *C. chiopterus*, 56% of the variation in the number of emerged adults could be explained and 40% of the variation for *C. dewulfi*. The variogram plots for the residuals of the linear regression models did not indicate spatial autocorrelation for any of the models.

Generally, stepwise and REVS models included similar sets of variables (Tab. C.3). Both species showed a positive response to increasing soil moisture, which was included in all REVS and stepwise models. *C. chiopterus* showed a negative correlation with organic matter in the REVS and stepwise model, in contrast *C. dewulfi* was not significantly affected by this variable. In REVS and stepwise models *C. chiopterus* emergence was positively correlated with grain size 6 ($\geq$ 1000 µm) and negatively correlated with grain size 4 ($\geq$ 200 µm, $<$ 500 µm). The REVS model for the species showed a positive correlation with grain size 2 ($\geq$ 20 µm, $<$ 63 µm) and the stepwise model a negative correlation with grain size 3 ($\geq$ 63 µm, $<$ 200 µm) and grain size 5 ($\geq$ 500 µm, $<$ 1000 µm). Furthermore, there was a significant site effect for *C. chiopterus*. *Culicoides dewulfi* emergence numbers were positively correlated with grain size 1 ($<$ 20 µm) in the REVS model and negative with grain size 3 ($\geq$ 63 µm, $<$ 200 µm) and grain size 4 ($\geq$ 200 µm, $<$ 500 µm) in the stepwise model.

According to the explorative data analysis, the small and large grain size variables (grain size 1, 2, 5, 6) only showed a small range of values. Several of the grain size variables were strongly correlated with each other (Tab. C.4). Furthermore, a significant positive correlation was observed between soil moisture and amount of organic matter.

## Discussion

This study compared the colonization densities of *Culicoides* biting midges in cowpats with a set of edaphic parameters characterizing the topsoil underneath. The results confirm the correlation of topsoil conditions with the number of emerging biting midges from cowpats. Soil moisture and organic matter as well as soil texture were significant variables in the models for the number of emerging *C. chiopterus* and *C. dewulfi*, whereby these variables explained a significant amount of the emergence variation. The relative large numbers of not colonized cowpats match previous studies on dung-breeding *Culicoides* (e.g. Zimmer et al., 2014).

The emergence of both species was positively correlated with increasing soil moisture. Furthermore, the model indicated a significant site effect for *C. chiopterus* with higher emergence rates at the farm characterized by relative wet conditions, i.e. marsh land. Similar results were obtained for closely related species in other studies: in the United Kingdom, the genus *Culicoides* and the species *C. obsoletus* was able to colonize a wide range of soil moisture (10.3–63.0%), but statistical analysis indicated a positive association of the probability of occurrence and soil moisture (Harrup et al., 2013). The modelling study by Peters et al. (2013) identified soil moisture as an important environmental variable explaining the distribution of *C. imicola*. Additionally, Scolamacchia et al. (2013) highlighted the availability of moisture as an important factor, for the distribution of *C. chiopterus*, *C. pulicaris* s.s., and *C. obsoletus*/*C. scoticus*. These results match the general considerations regarding the breeding sites of *Culicoides* species of the Obsoletus group, which are considered to be moist but not waterlogged (Nevill et al., 2007).

Scolamacchia et al. (2013) reported on a negative correlation between the organic matter of surrounding soils and the abundance of *C. chiopterus* in light traps, but a positive relationship for *C. dewulfi*. Our study supported the modelling results only for *C. chiopterus*, but there was no significant effect for *C. dewulfi*. In general, the amount of organic matter has a positive correlation with the water holding capacity of the soil (reviewed by Saxton & Rawls, 2006). Therefore it remains ambiguous why in our study the number of emerging *C. chiopterus* and *C. dewulfi* lack a positive response for the amount of organic matter. Moreover, further studies need to analyse why opposing responses occur in these species regarding the effect of organic matter (Scolamacchia et al., 2013), even though the number of both species positively correlated with increasing soil moisture.

An increasing proportion of smaller grain sizes correlated with a higher number of emerging *C. dewulfi*, while an increasing proportion of larger grain sizes had a negative effect in the stepwise model. Since the water holding capacity increases with increasing proportion of the small grain size fraction (reviewed by Saxton & Rawls, 2006), our results can be interpreted as an increasing number of emerging *C. dewulfi* with increasing water holding capacity, thus matching a general preference of *Culicoides* for moist conditions. However, our results contradict the modelling of Scolamacchia et al. (2013), whereby *C. dewulfi* prefers less moisture-retentive soils. Furthermore, our models showed conflicting correlations between *C. chiopterus* and the soil texture variables. The abundance of the species seems to be positively affected by an increasing proportion of substrate with larger grain size and at the same time a positive correlation with the proportion of substrate with smaller grain sizes. According to Scolamacchia et al. (2013), *C. chiopterus* positively correlated with moisture-retentive soils, i.e. soils with a higher proportion of smaller grain sizes. We assume that the conflict in our results might be caused by the usage of several correlated variables, which make it difficult to identify the driving variables in our models. Furthermore, the range of values was small in some of the significant grain size parameters, and, therefore might be unimportant by themselves, but might be substitute variables for not involved data.

Although we did not collect environmental parameters from the actual breeding sites of *C. chiopterus* and *C. dewulfi*, i.e. cowpats or the interplay between the environmental conditions in the topsoil and the conditions in the cowpat, our study indicated a significant correlation of topsoil conditions and the number of emerging *C. chiopterus* and *C. dewulfi* from cowpats. This study therefore gave support for recent modelling studies, which demonstrated that the soil moisture (Peters et al., 2013) and soil texture (Scolamacchia et al., 2013) are important factors explaining the distribution of *Culicoides* species.

## Acknowledgment

We would like to give special thanks to Esther Timmermann for technical assistance during data collection. Furthermore, sincerest thanks are given to the farmer families who supported our study.

# Tables

Table C.3: Final Regression with Empirical Variable Selection models (REVS) and stepwise models for the number of emerging *Culicoides chiopterus* and *C. dewulfi* from cowpats. estimate: estimated regression parameter, SE: standard error, t: t-value, p: p-value, VIF: variance inflation factor, AIC: Akaike information criterion, $R^2$: coefficient of determination, MSE: mean square prediction error.

| **Species/ model/ parameter** | **estimate** | **SE** | **t** | **p** | **VIF** | **AIC** | **delta AIC** | **Adj $R^2$** | **p** | **MSE** |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Culicoides chiopterus*** | | | | | | | | | | |
| **Best REVS model** | | | | | | 234.591 | 0.000 | 0.561 | 0.002 | 1885.762 |
| Intercept | -192.800 | 203.622 | -0.947 | 0.357 | | | | | | |
| site | 379.144 | 91.968 | 4.123 | 0.001 | 2.785 | | | | | |
| soil moisture | 11.275 | 2.815 | 4.005 | 0.001 | 1.305 | | | | | |
| grain size 2 ($\geq$ 20 µm, $<$ 63 µm) | 36.355 | 11.134 | 3.265 | 0.005 | 2.177 | | | | | |
| organic matter | -15.892 | 5.688 | -2.794 | 0.012 | 2.807 | | | | | |
| grain size 6 ($\geq$ 1000 µm) | 84.237 | 53.156 | 1.585 | 0.131 | 1.647 | | | | | |
| grain size 4 ($\geq$ 200 µm, $<$ 500 µm) | -11.197 | 4.830 | -2.318 | 0.033 | 1.958 | | | | | |
| | | | | | | | | | | |
| **Best stepwise model** | | | | | | 234.926 | 0.335 | 0.565 | 0.003 | 2482.687 |

Table C.3: Final Regression with Empirical Variable Selection models (REVS) and stepwise models for the number of emerging *Culicoides chiopterus* and *C. dewulfi* from cowpats. estimate: estimated regression parameter, SE: standard error, t: t-value, p: p-value, VIF: variance inflation factor, AIC: Akaike information criterion, $R^2$: coefficient of determination, MSE: mean square prediction error.

| **Species/ model/ parameter** | **estimate** | **SE** | **t** | **p** | **VIF** | **AIC** | **delta AIC** | **Adj $R^2$** | **p** | **MSE** |
|---|---|---|---|---|---|---|---|---|---|---|
| Intercept | 3479.522 | 927.671 | 3.751 | 0.002 | | | | | | |
| site | 458.657 | 112.836 | 4.065 | 0.001 | 4.128 | | | | | |
| soil moisture | 10.641 | 2.860 | 3.721 | 0.002 | 1.306 | | | | | |
| organic matter | -11.566 | 6.686 | -1.730 | 0.103 | 33.038 | | | | | |
| grain size 6 ($\geq$ 1000 µm) | 93.473 | 57.957 | 1.613 | 0.126 | 1.466 | | | | | |
| grain size 5 ($\geq$ 500 µm, $<$ 1000 µm) | -49.868 | 11.781 | -4.233 | 0.001 | 44.038 | | | | | |
| grain size 4 ($\geq$ 200 µm, $<$ 500 µm) | -37.213 | 10.949 | -3.399 | 0.004 | 30.615 | | | | | |
| grain size 3 ($\geq$ 63 µm, $<$ 200 µm) | -82.103 | 39.897 | -2.058 | 0.056 | 4.323 | | | | | |
| ***Culicoides dewulfi*** | | | | | | | | | | |
| **Best REVS model** | | | | | | 165.259 | 0.000 | 0.407 | 0.002 | 1268.449 |
| Intercept | -45.120 | 16.206 | -2.784 | 0.011 | | | | | | |
| soil moisture | 2.157 | 0.643 | 3.357 | 0.003 | 1.051 | | | | | |

Table C.3: Final Regression with Empirical Variable Selection models (REVS) and stepwise models for the number of emerging *Culicoides chiopterus* and *C. dewulfi* from cowpats. estimate: estimated regression parameter, SE: standard error, t: t-value, p: p-value, VIF: variance inflation factor, AIC: Akaike information criterion, $R^2$: coefficient of determination, MSE: mean square prediction error.

| **Species/ model/ parameter** | **estimate** | **SE** | **t** | **p** | **VIF** | **AIC** | **delta AIC** | **Adj $R^2$** | **p** | **MSE** |
|---|---|---|---|---|---|---|---|---|---|---|
| grain size 1 (< 20 µm) | 79.814 | 24.742 | 3.226 | 0.004 | 1.051 | | | | | |
| | | | | | | | | | | |
| **Best stepwise model** | | | | | | 167.345 | 2.086 | 0.375 | 0.006 | 1316.142 |
| Intercept | 338.213 | 139.435 | 2.426 | 0.025 | | | | | | |
| soil moisutre | 2.028 | 0.659 | 3.079 | 0.006 | 1.048 | | | | | |
| grain size 4 ($\geq$ 200 µm, < 500 µm) | -4.441 | 1.419 | -3.130 | 0.005 | 2.443 | | | | | |
| grain size 3 ($\geq$ 63 µm, < 200 µm) | -4.000 | 1.734 | -2.307 | 0.032 | 2.386 | | | | | |

Table C.4: Spearman's rho rank correlation coefficients and range of the sampled topsoil parameters. Significant correlations ($p<0.05$) are indicated in bold

| | **range (mean, (max, min))** | **soil moisture** | **organic matter** | **grain size 6 (≥ 1000 µm)** | **grain size 5 (≥ 500 µm, < 1000 µm)** | **grain size 4 (≥ 200 µm, < 500 µm)** | **grain size 3 (≥ 63 µm, < 200 µm)** | **grain size 2 (≥ 20 µm, < 63 µm)** |
|---|---|---|---|---|---|---|---|---|
| **soil moisture** | 20.3% (4.9%, 41.9%) | **1.00** | | | | | | |
| **organic matter** | 14.4% (8.0%, 25.9%) | **0.42** | | | | | | |
| **grain size 6 (≥ 1000 µm)** | 1.0% (0.0%, 3.2%) | -0.06 | -0.12 | | | | | |
| **grain size 5 (≥ 500 µm, < 1000 µm)** | 2.1% (0.5%, 5.4%) | -0.04 | 0.17 | **0.56** | | | | |
| **grain size 4 (≥ 200 µm, < 500 µm)** | 23.8% (14.5%, 46.7%) | 0.12 | 0.2 | 0.31 | **0.58** | | | |
| **grain size 3 (≥ 63 µm, < 200 µm)** | 64.6% (47.7%, 74.0%) | 0.03 | -0.35 | 0.14 | -0.16 | **-0.53** | | |
| **grain size 2 (≥ 20 µm, < 63 µm)** | 7.6% (2.0%, 18.2%) | -0.11 | 0.11 | **-0.55** | **-0.59** | **-0.76** | -0.06 | |
| **grain size 1 (< 20 µm)** | 0.2% (0.0%, 0.9%) | -0.24 | 0.08 | **-0.59** | **-0.52** | **-0.7** | 0.12 | **0.83** |

## C.3 *Culicoides* biting midge density in relation to the position and substrate temperature in a cattle dung heap

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### Abstract

Dung heaps offer warm breeding sites, which might be suitable for a continuing development or even emergence at low air temperatures in winter. Therefore, this study collected substrate samples from the outer surface and core of a cattle dung heap at the beginning of the winter period. We aimed to analyze the density of immature *Culicoides* in relation to substrate position and temperature. We took samples from the outer layer and core of the dung heap at different heights. Floatation was used to extract *Culicoides* larvae from the dung heap samples. In order to rear larvae individually, we separated them in glass tubes. A total of 229 *Culicoides* larvae were extracted from the dung heap samples. Highest densities (99.1% of all larvae) were recorded for the outer layers of the dung heap but hardly any in the core (0.9 % of all individuals). While the density of larvae was negatively correlated with increasing substrate temperatures, *Culicoides* larvae were found in a temperature range between 7.9 and 38.0 °C (mean 16.6 °C). Extracted larvae were reared to adults. All male individuals were identified as *Culicoides* obsoletus (Meigen), 1818 and all female individuals as *C. obsoletus*/*Culicoides scoticus*. It can be concluded that dung heaps offer temperature conditions, which allow the survival and probably also the development to adults for immature *Culicoides* also under harsh climate conditions in winter.

### Short communication

The Bluetongue virus emerged in Europe since the late 1990s, transmitted by several species of biting midges (Diptera: Ceratopogonidae) of the genus *Culicoides* (Mehlhorn et al., 2009; Wilson & Mellor, 2009). Moreover, biting midges were also involved in the transmission of the spread of the Schmallenberg virus since 2011 (De Regge et al., 2012; Rasmussen et al., 2012). Both viral diseases resulted in huge economic costs worldwide (Bath, 1989; Velthuis et al., 2010; Conraths et al., 2012).

At least in Northern Europe, biting midges need an overwintering strategy to resist harsh climate conditions with low winter temperatures. Although not studied in detail, *Culicoides* species in temperate climate zones are expected to hibernate in a late larval stage (Szadziewsi et al., 1997). Moreover, vector surveillance studies in Northern Europe indicated very low but regular activity of adult *Culicoides* also in winter (Losson et al., 2007; Bauer et al., 2009; Clausen et al., 2009; Hoffmann et al., 2009; Kiel et al., 2009; Mehlhorn et al., 2009). These findings raised a discussion whether or not these individuals represent an overwintering adult population or newly emerged imagines (Losson et al., 2007). The hibernation strategy of biting midges is probably an important factor influencing the overwintering of the Bluetongue virus or Schmallenberg virus (Koenraadt et al., 2014), which is still an unsolved riddle.

Hörbrand & Geier (2009) hypothesized a positive correlation between the number of *Culicoides* in light-trap samples and the distance of the trap to dung heaps. A study carried out on different farm sites in the UK highlighted that cattle dung heaps are colonized by large numbers of overwintering immature biting midges, which were predominantly colonized by overwintering *Culicoides obsoletus* (Harrup et al., 2013). Dung heaps offer warm breeding sites (Husted, 1994), which might be suitable for a continuing development or even emergence at low air temperatures in winter. Thus, for example, Harrup et al. (2014) hypothesized that the first *Culicoides* emerging in the year originate from dung heaps in which immature stages develop faster.

Depending on the amount of decaying organic material, it seems reasonable that the temperature differs within a dung heap. However, a study focusing on the link between the colonization densities of immature *Culicoides*, the position in a dung heap, and the temperature pattern within a dung heap was not conducted previously. Such knowledge might bring us closer to an understanding of the overwintering ecology of biting midges. We thus evaluated the density of immature *Culicoides* in response to the substrate temperature within a dung heap in Northern Europe at the beginning of the winter period.

This study was performed at the end of October 2008 on a cattle farm in the marshland region of Northwest Germany. The week before, frosty weather had just started and air temperatures at night were below 0 °C. Livestock on this farm comprises about 250 cattle, and the dung heap was located 3–10 m from two cattle stables. The dung had been piled during the last 6 months to a height of approximately 3.30 m and a circumference of 42 m (measured at 1.70 m height). Its outer shape was a broad-based flat pyramid.

At first, we took samples from the outer layer of the dung heap, i.e., surface of the dung heap, at different heights: upper layer ≈2.00–2.75 m, medium layer ≈1.00–1.75 m, and lower layer 0–0.75 m (Fig. C.1). From each layer, we took six samples. Additionally, four samples were taken from the top of the dung heap (top ≈3.00–3.30 m). Afterwards, we took samples from the core of the dung heap. To do so, we stepwise trenched the core of the dung heap in three vertical layers (V1–V3; Fig. C.1). Each layer was sampled at eight random positions each. Before substrate sampling, we recorded the substrate temperature at every position with a penetration substrate thermometer (PCE–EN 882 Environment Meter, PCE Group, Meschede, Germany). All samples were taken with a stainless steal grab sampling device. This grab covered a surface area of 15 cm×11 cm and sampled a total volume of 456 ml down to a maximal depth of 7.8 cm. Each sample was stored in 250-ml freezer bag (Ziploc-bags, Toppits, Minden, Germany) and transported to the laboratory. Wet weight of each sample was measured with a laboratory scale (Sartorius, LE4202S).

*Culicoides* larvae were extracted from these samples via a floating method (for methodological details see Steinke et al., 2014). In order to rear larvae individually, we separated them in 48-ml glass tubes with 8 cm in height and 3 cm in width, which were covered with a cotton gauze. As a reservoir for moisture, the bottom of each tube was grouted with gypsum (approximately 0.5–1.0 cm in height). The gypsum was mixed with some charcoal to prevent massive growth of bacteria and fungi.

A total of 229 *Culicoides* larvae were transferred individually on a teaspoon of dung (5–10 g), which had been carefully floated and sieved previously to exclude invertebrates from the rearing device. For both rearing experiments, the temperature was adjusted to a mean of 21.5 ± 2.0 °C. All samples were regularly wetted with tap water and controlled for adult *Culicoides* every 2–4 days for the following 4 months. Emerged adults were preserved in 70% ethanol.

Colonization densities (number of *Culicoides* larvae per kilogram substrate) were calculated for each sample. *Culicoides* were identified according to morphological characters (Campbell & Pelham-Clinton, 1960). Data analyses were done with the program R (R Core Team, 2014) with the package ggplot2 (Wickham, 2009) for the graph. A total of 229 *Culicoides* larvae were extracted from the dung heap samples. Of all *Culicoides* larvae (227 individuals), 99.1% were found in samples from the outer layer (Fig. C.2). Only 2 larvae (0.9% of all individuals) were extracted from the core material. Most larvae in the outer layer were sampled from the lower layer (204 individuals, 89.1% of all individuals; Fig. C.2), i.e., the bottom layer of the dung heap. Much lower numbers were present in samples from the medium layer (23 larvae, 10.0% of all individuals), and no larvae were found in the upper layer and the top layer. The number of *Culicoides* larvae per kilogram differed significantly between the horizontal layers (Kruskal-Wallis test, $P<0.05$).

Biting midges were present in samples with temperatures ranging from 7.9 to 38.0 °C (mean 16.6 °C) (Fig. C.2). There was a significant negative correlation between the number of individuals per kilogram substrate and the substrate temperature (spearman rank correlation, $P<0.01$, $\text{rho}=-0.39$). From the 229 larvae, 49 individuals (21.4%) completed development to adults in the glass tubes. Except for 2 males with damaged hypopygia, all males (28 individuals, 57.1% of all adults) were identified as *C. obsoletus*, while all females (21 individuals, 42.9% of the adults) were identified as

*C. obsoletus*/*Culicoides scoticus*. Biting midges are generally expected to colonize the top 2–8 cm of breeding substrates (Mullens & Rodriguez, 1992; Blackwell & King, 1997). We also recorded highest densities of immature *Culicoides* in the outer substrate layer. Furthermore, larval densities were highest close to the ground, which might indicate a preference for oviposition near to the ground. This conclusion seems to fit to results published by Service (71b), who recorded decreasing numbers of several *Culicoides* species with increasing trap height. However, our result might also be biased by the composition of the dung heap material. Fresh material was generally added on top, but *Culicoides* seem to prefer older, more composted manure (González et al., 2013).

Development of different *Culicoides* species was shown to be temperature dependent e.g. Veronesi et al. (2009). We found *Culicoides* larvae in a temperature range of 7.9 to 38.0 °C. In our study, development to adults in the laboratory was possible at a temperature around 20 °C. Therefore, it might be concluded that *Culicoides* emergence should also be possible in the field during winter at least at some positions of a dung heap, which offers adequate temperatures for the development of immature biting midges. This might explain the low, but regular, trapping success during winter surveillance (Losson et al., 2007; Bauer et al., 2009; Clausen et al., 2009; Hoffmann et al., 2009; Kiel et al., 2009; Mehlhorn et al., 2009). Adults emerging from dung heaps during winter have the opportunity to stay in a comparatively warm and moist microclimate of a dung heap. If a dung heap is located close to the stable, females should be able to fly to the stable, stay inside after blood meal, and fly back to the dung heap for oviposition without being strongly affected by harsh winter temperatures, e.g., Clausen et al. (2009) caught adult *Culicoides* inside stables during the winter period. But even if a complete life cycle is not possible, due to the different temperatures in the dung heap, a desynchronized emergence of small numbers of *Culicoides* adults is imaginable also during the entire winter period. Furthermore, our results supports the hypotheses by Harrup et al. (2014) that the first *Culicoides* emerging in the year probably originate from warm breeding sites like dung heaps.

Future research regarding the ecology of biting midges should focus on the life-history parameters of *Culicoides* species and should determine temperature thresholds and time of development under different temperature regimes, which was already done for *Culicoides imicola* (Veronesi et al., 2009). This information can be used in mechanistic models to predict *Culicoides* phenology, e.g., probability of winter emergence from dung heaps. Furthermore, it is just as important that future breeding site studies do not omit the winter period, which is not included in most of the studies.

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## Figures

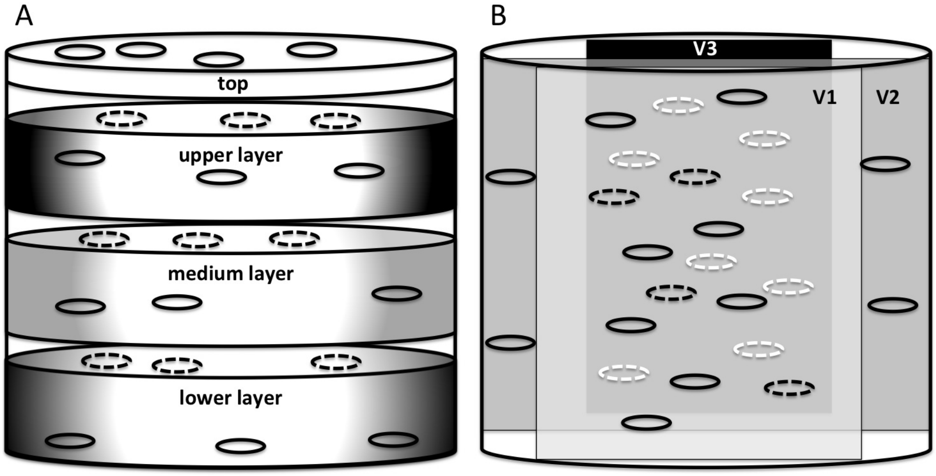

Figure C.1: **A** Schematic diagram of peripheral sampling of dung heap at 26 spots on horizontal layers (top=≈3.00–3.30 m, upper layer=≈2.00–2.75 m, medium layer=≈1.00–1.75 m, lower layer=0–0.75 m). **B** Schematic diagram of core sampling of dung heap at 22 spots, which were randomly chosen on three vertical core layers (V1–V3).

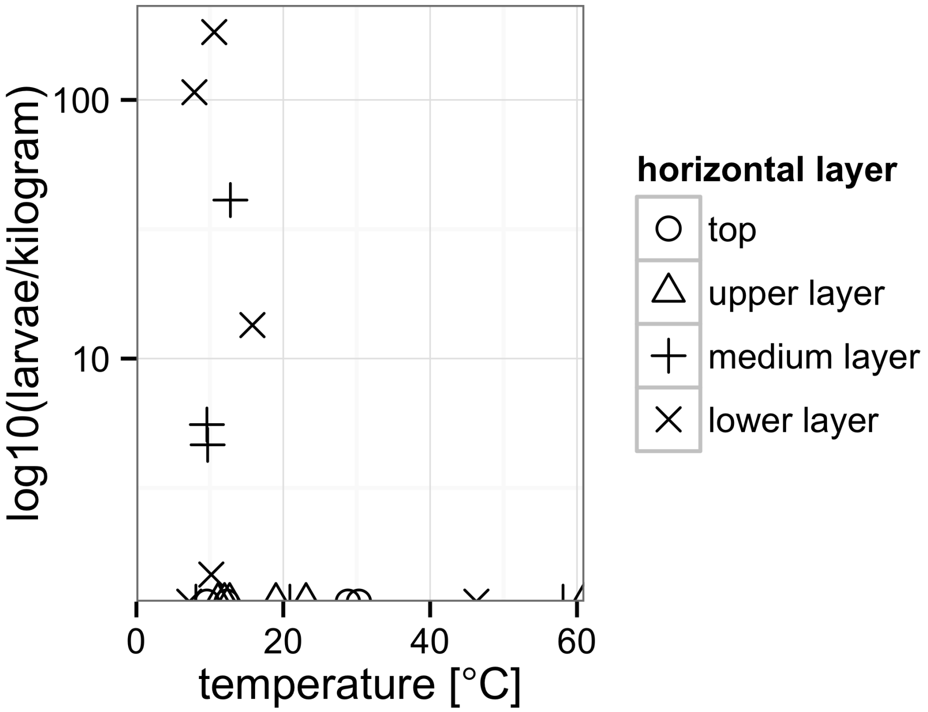

Figure C.2: Immature *Culicoides* densities per kilogram recorded in the outer layer of a dung heap in response to sampling spot temperatures. Further differentiated according to the four horizontal layers: top (N=4), upper layer (N=6), medium layer (N=6), and lower layer (N=6).

## C.4 A tool for simulating the spread of invasive mosquitoes

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### Abstract

There are many concerns about the spread and establishment of exotic mosquito species in Europe, some of which are highly competent to transmit pathogens. The fast spread of some invasive mosquito species, e.g. the Asian bush mosquito, might be explained by a combination of natural dispersal and human transport. Therefore, the aim of this study was the development of a software tool that is capable to model the local spatial-temporal spread of invasive mosquitoes through natural dispersal and their transportation through human transport via cars. The evaluation of the tool shows that it is capable to simulate the spread but also reveals the need for more biological data about invasive mosquito species.

### Introduction

Mosquitoes can cause significant nuisance and are important vectors of several pathogens (Hubálek, 2008). Therefore, this group of insects was and is subject of research all over the world, but especially in areas with health concerns due to mosquito-borne diseases, e.g. Africa (Godfray, 2013). Nevertheless, in view of the worldwide range expansion of several mosquito species, some of which are highly competent to transmit pathogens, the interest in this research topic is also increasing in other geographical areas (Benedict et al., 2007). Especially climate change and globalisation are considered to facilitate the spread and establishment of these species (Benedict et al., 2007; Ying Zhang et al., 2008).

There are many concerns about the introduction and establishment of exotic mosquito species in Europe, some of which are highly competent to transmit pathogens (Medlock et al., 2012). The worldwide most important invasive mosquito species is the Asian tiger mosquito (*Aedes albopictus*). The species is known to be a highly competent vector of several pathogens and is considered to be the vector, which caused the autochthonous transmission of Chikungunya virus (Grandadam et al., 2011; Rezza et al., 2007) and Dengue virus (Gjenero-Margan et al., 2011; La Ruche et al., 2010; Schmidt-Chanasit et al., 2010) in Southern Europe. Surveillance studies at potential introduction sites in Germany identified motorway service stations as important gateways (Becker et al., 2013; Kampen et al., 2012; Pluskota et al., 2008; Werner et al., 2012). Individuals of the Asian tiger mosquito are considered to enter cars or trucks in Southern Europe, where the species is established since several years and transported by transit traffic.

The fast spread of another invasive mosquito species in Germany, the Asian bush mosquito (*Ochlerotatus japonicus*), highlighted the necessity of dispersal analyses and the demand for modelling tools to predict the spread of invasive mosquito species. The species was firstly detected in the year 2008 in Southern Germany at the border to Switzerland. During the following years, the species was found to spread fast in Southern Germany and additional populations were detected in Northern Germany (reviewed by Kampen & Werner, 2014). The fast spread of the Asian bush mosquito might be explained by a combination of natural dispersal and human transport (Werner & Kampen, 2013). The distribution and habitat preference of invasive mosquitoes were analysed in several studies in Europe. At least for the Asian tiger mosquito there are several habitat models on the basis of climate data and landscape parameters, which give information on the actual and potential distribution in

Europe (Fischer et al., 2014). However, there are only few studies on the spatial-temporal spread of invasive mosquito species via traffic (Tatem et al., 2006). This applies in particular to the small scaled, regional spread through natural dispersal and human transport (e.g. cars). Information on the potential spread of invasive species especially can help to develop an adequate surveillance program and control strategies.

Therefore, the aim of this study was the development of a software tool that is capable to model the local spatial-temporal spread of invasive mosquitoes through natural dispersal and their transportation through human transport via cars.

## Mosquito Tool

A software tool for predicting a possible exposure of mosquitoes has to take the passive transport by vehicles as well as the active dispersal of mosquitoes into account. These two processes take place on different scales in time and space. Such multi-scale modelling is often used to model physical phenomena (Weinan, 2011). Hoekstra et al. (2010) studied the modelling of complex automata by coupling cellular automata with different temporal and spatial scales. A framework for modelling ecological systems as multi-scale models has already been proposed (Speckels et al., 2003; Sonnenschein & Vogel, 2001). The theoretical background of this framework models complex spatial processes on different scales by so-called hierarchical asymmetric cellular automata, which allow the coupling of cellular automata (layers) with different spatial and time scales as well as restricted interactions between layers. The *Mosquito Simulation Tool* (*MoSiTo*) presented here follows this approach. It consists of two automata: the *MosquitoCA* on a spatial fine-scale layer and the Tool *VASim*, which models the dissemination of mosquitos by traffic on a coarse spatial level.

Both components of *MoSiTo* have been prototypically implemented in Python as a Plugin for the geographic information system QGIS (version 1.8 and 2.x, resp.) and can also be used independently from each other.

### Traffic network Tool VASim

Tourist traffic from regions with established mosquito populations are considered as a potential reason for mosquito dispersal. The traffic network tool *VASim* (Mengelkamp, 2014) models this transport.

The transport can be viewed as a generalized (asymmetric) cellular automaton (Sonnenschein & Vogel, 2001) where the cells represent starting points, stops (at resting places, motorway stations), and destinations of vehicles. The neighbourhood relation is determined by a traffic route from the starting point to the destination with stops in between.

The movement of cars on a traffic route and the release of mosquitos at stops is modelled by the cells' state: The cell at the starting point of the route is initialized with a *vehicle object*. Each *vehicle object* (*VO*) represents a set of cars, which traverse the same route. It is characterized by parameters to describe its behaviour:

- The minimal distance between *stops* ($d_{min}$) and its *mileage* determine where the vehicle object will stop and possibly release mosquitoes.
- The distribution of the number of mosquitoes inside the *VO* at the start of the simulation and the probability for *mosquitoes leaving* the vehicle at every single *stop* determine how many mosquitos will be initially in the *VO* and leave it at a stop.

Beside the information about the *VO*, the cell stores the distance to the prior cell (attribute *length*) and the number of mosquitoes, which have been released (attribute *mosquitoes*). The behaviour is modelled as transfer step and update step:

- The transfer step moves the *VO* information to the next cell:

  $\mathrm{cell[i+1].VO = cell[i].VO}$

- The update step describes the release of mosquitos inside a cell:

  cell[i].VO.mileage+ = cell[i].length

  If cell[i].VO.mileage > random$_\mathrm{k}$m(cell[i].VO.dmin) :

  mosquitos_out = random_Mosquitos(cell[i].VO.inside)

  $cell[i].mosquitos+ =$mosquitos_$out = random$

  $cell[i].VO.inside- =$mosquitos_out = random

  $cell[i].VO.mileage = 0$

The *random-functions* allow random deviations from the minimal distance between stops of a vehicle and from average number of mosquitos released.

Hence, a traffic route defines a one-dimensional cellular automaton. As it is possible to assign an arbitrary number of routes to one simulation scenario, a more complex neighbourhood relation is possible.

For initialization of *VASim* at least one traffic route between arbitrary starting points and destinations and a list of possible *stops* along the route (rest areas, service stations) must be specified as xml-documents. *VASim* extracts the cell attributes and topology from these inputs. The documents can be generated by the web services OpenRouteService (http://openrouteservice.org) and the OSM tool Nominatim (http://nominatim.openstreetmap.org), which are based on OSM XML OpenStreetMap data. The vehicle objects at the starting position of each route are initialized according to user-specified probability distributions for the initial number of mosquitos inside and the release of mosquitoes.

So far *VASim* does not model time explicitly. In one simulations step the *VO* is transferred from one cell to the following - the real time, which corresponds to this transition, depend on the distance between the *stops*, i.e. *length* of the cells.

### MosquitoCA

The *MosquitoCA* (Klich et al., 2013) models the autonomous dispersal and the possibility of establishing a population as a fine-scale 2-dimensional cellular automaton with a regular grid of cells. The dynamic is based on the static data about the area using the Corine landcover information (http://www.eea.europa.eu/) and integrates climatic information about the regional temperature.

Each cell is characterized by its habitat quality factor (hab∈[0,1]), the number of its adult mosquitoes (*adults*) and mosquito larvae (*larvae*), which vary during the simulation. The maximum mosquito or larvae capacity of cells depends on the habitat quality. Hence the optimal capacities of adult mosquitos ($A_{max}$) or larvae ($L_{max}$) are reduced by the habitat quality factor. The size of the cells depends on the flying range of the mosquito species in the given time step.

The dynamics of the number mosquitoes per cell depends on the mortality rate ($m$), reproduction rate ($r$), the length of a gonotrophic cycle ($u$), i.e. the time span of alternate feeding and laying of eggs, and the larvae's development rate $d$. $u$ and $d$ depend on climatic factors (temperature) and are defined as in Linard et al. (2009). The variable $P$ describes the length of a time step in the simulation and corresponds to one day.

The default dynamic of the cellular automaton is based on rules for reproduction, mortality, and dispersion based on the MALCAM model published by Linard et al. (2009) with some minor improvements concerning the different habitat qualities. The dynamic of adults and larvae inside each cell is determined by

$$\mathrm{larvae(t)}{+}{=}\mathrm{P}\times(\mathrm{adults(t\text{-}1)}\tfrac{r}{u}\text{-}\mathrm{d}\times\mathrm{larvae(t\text{-}1)})\times\mathrm{hab}\times(1\text{-}\tfrac{larvae(t-1)}{(L_max*hab})$$

$$\mathrm{adults(t)+ = P \times (d\,larvae(t-1) - m\,adults(t-1)) \times hab \times (1 - \tfrac{adults(t-1)}{A_max*hab})}$$

Alternatively to this predefined dynamics inside each cell, user specified rules can be specified and used in *MosquitoCA*.

For each cell the number of leaving mosquitos is computed as in (Linard et al., 2009) by

$leaving(t) = adults(t) \times P/u.$

We simplified the MALCAM model for the entering of cells: the flow of mosquitos from cell[i] to cell[j] is computed by cell[i].leaving(t) weighted by the normalized habitat quality factor of cell[j]:

$cell[j].entering(t) = cell[i].leaving(t)$

The simulation of *MosquitoCA* results in a map, which shows the distribution and abundance of mosquitoes in the cellular automaton.

### Coupling

*MosquitoCA* and *VASim* are only loosely coupled: In a first step, *VASim* computes the *initial occurrences* of *mosquitos* at the *stops* along the routes for a given set of routes and *vehicle objects*. These stops comply with cells of the *MosquitoCA*. This allows to check, whether the suspected traffic flows are able to transport mosquitos that far. Starting from these *stops* as initial places, *MosquitoCA* computes the *dispersal* of mosquitoes.

In the next version of *MoSiTo*, we plan to integrate a refined time concept: as the survival rate of transported mosquitos and the behaviour of the mosquitos depend on the time of the day, in future version *VASim* cells will transfer mosquitos numbers tagged with a time stamp information.

### Evaluation scenarios

For ensuring the correct implementation of the underlying models, the *MoSiTo* layers *MosquitoCA* and *VASim* have been successfully tested separately with virtual scenarios. Due to the limited availability of data about the detailed behaviour of neozoa and their spread, we based our real world evaluation on existing publications.

### Scenario 1: Spread of Mosquitos

The fast spread of the invasive Asian bush mosquito (*Ochlerotatus japonicus*) in Germany highlights the necessity of dispersal analyses and the demand for modelling tools to predict the spread of invasive mosquito species. The species was firstly detected in the year 2008 in Southern Germany at the border to Switzerland. During the following years, the species was found to spread fast in Southern Germany (reviewed by Kampen & Werner, 2014). This spread is probably due to natural dispersal and, therefore, is a good case study to evaluate the *MosquitoCA* tool.

Huber et al. (2012) presented a map where the Asian bush mosquito has been found in 2011. Their study only included punctually data. Therefore, raster grids cells in a resolution of a topographic map (1:25.000, 18 km×10 km) were defined as colonized, if at least one positive point lay in the respective grid cell. As this scale is much to coarse for a detailed simulation, we initialized the automaton with 135×55 cells of size approx. 0.01×0.01 square degree (1.1 km×1.1 km) and focused to the infested area in the south of Baden-Württemberg, using a daily rates of 0.1 (mortality $m$) and 1.0 (reproduction rate $r$), and a constant temperature of 20°C. The habitat qualities of the cells were derived from the Corine landcover data 2006 on a 250 m×250 m grid. Based on expert knowledge the landcover types were classified as "not suitable", "medium suitable" and "very suitable" and each cell was assigned a habitat quality of 0.01, 0.5 or 10, resp. As the cell size of *MosquitoCA* and the Corine grid differed, the landcover in the centre of the cell determined its habitat quality.

In order to start *MosquitoCA* with the map of 2011, about 20 cells in the infested area were initialized with 1000 mosquitoes and 5000 larvae and (pre-)simulated for 25 time steps (days). Figure C.3 shows the occurrence of mosquitoes and after the pre-simulation phase with 2011 distribution data (Huber et al., 2012). The infected areas from 2011 show a medium to high occurrence of mosquitoes. Below the simulated occurrence after about 120 time steps can be seen with a map section of the 2014 distribution.

Our modelling results showed a much faster spread of *Ochlerotatus japonicus* compared to the observed spread in the real-world. This might be explained by different reasons: 1) the input data do not have a very good quality and are only based on a very broad meshed mosquito survey. Colonized areas with very low *Ochlerotatus japonicus* might be not detected. Therefore, the currently published distribution maps might not reflect the actual distribution of the species. 2) Furthermore, our model use several assumptions and simplifications, e.g. each cell have the same, relatively high temperature (20°C) and there are no temperature variations in space and time, which significantly accelerate the spread in our model. Therefore, the incorporation of upper and lower development temperature thresholds might give a more realistic picture.

### Scenario 2: Transport and Spread

Further *Ochlerotatus japonicus* foci were detected in Northern Germany (reviewed by Kampen & Werner, 2014), which appeared to be too far away to be explained by natural dispersal, but might be explained by human transport via car traffic (reviewed by Werner & Kampen, 2013). Therefore, this scenario 2 was used to evaluate the *VASim* tool in the first place. Beyond that, subsequent simulation of the spread with the tool *MosquitoCA*, starting from service stations might explain findings of the main traffic routes.

In scenario 2, the object of investigation was the possibility of an introduction of mosquitoes from southern Germany by traffic which is passing the motorway junction Weilheim (nearby Heilbronn and the most northern point of *Ochlerotatus japonicus* observation in the federal state Baden-Württemberg (Huber et al., 2012)) and going north in direction to motorway junction Hilden (nearby Düsseldorf). North bound motorway routes in this area were generated by OpenRouteService and service stations along the routes were determined by the web service Nominatim. The “fastest route” via the motorways A67 and A3 crosses the north eastern region of further *Ochlerotatus japonicus* foci (Kampen et al., 2012).

*VASim* was initialized with three routes having each 1000 *vehicle objects*. Each *VO* was meant to represent a set of cars with an average of 60 *mosquitoes* per *VO*, an initial, uniform distributed *initial mileage* of 50 up to 250 km, and a *minimum distance between stops* of 200 km. Figure C.5 shows the number of *VOs* ("Cars") and the final number of exposed mosquitos per station. The stations have been sorted by their (Euclidean) distance from the start at Weilheim. Figure C.5 shows that the number of released mosquitos correlates linearly to the number of cars. Despite the uniform distribution of the initial mileage, waves of resting cars become obvious.

The *Ochlerotatus japonicus* observations from (Kampen et al., 2012) were located by manually georeferencing the sketch (Figure C.4). The resulting map was validated using Open Street Map data locations of cemeteries.

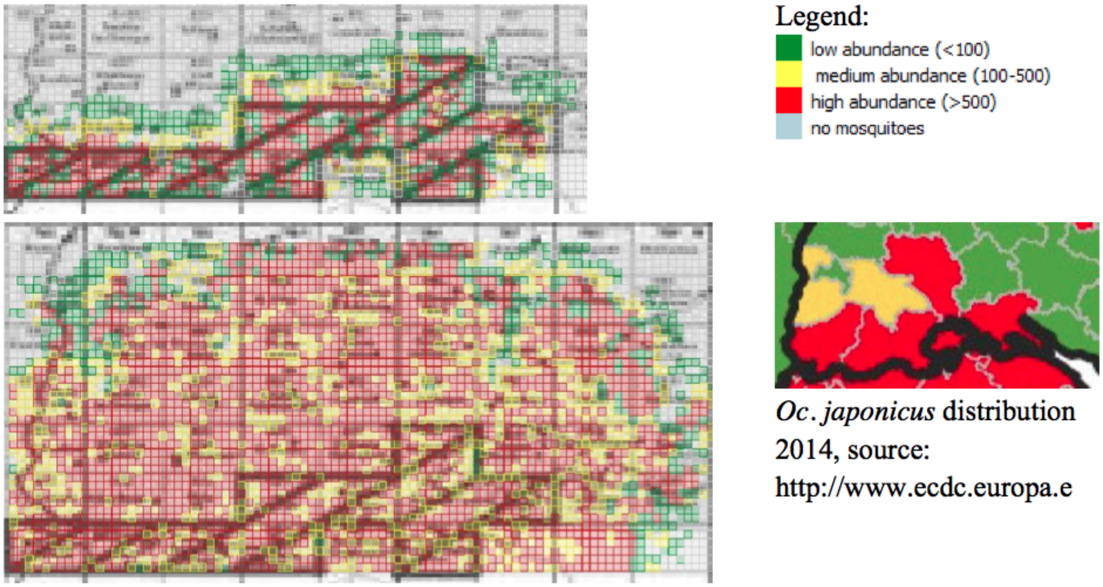
Figure C.6 shows the modelling results of scenario 2 produced with the *MosquitoCA* tool. For the service station Siegburg West at km 299, an exposure of 1460 Mosquitos was computed by *VASim* and simulated by *MosquitoCA* in order to prove, if the nearby findings at locations in the north, east and south-west can be attributed to exposures at Siegburg West. Each of these positive sites has a distance of about 5 km. With the parameters of the scenario, Corine landcover data with a resolution of 100m×100m and nearly equally sized cells (0.0015×0.0015 square degrees), we found, that only the observations in the eastern location can be explained. Figure C.6 shows the simulation after 100 and 200 simulation steps.

As stated above, the tools of *MoSiTo* are only loosely coupled and communicate asynchronously via data files. As a next step, it is planned to allow a tighter and timed coupling between the two layers, which will also allow the integration of seasonal changes in traffic flows as well as in the *mosquitoes' survival* conditions into the model.

## Conclusion

The research on invasive mosquito species predominantly focus on static information about the recent and future distribution based on environmental data and lacks studies and tools to predict and understand the spatial-temporal spread especially on the regional and local scale. The tool *MoSiTo* with its layers *VASim* and *MosquitoCA* is the first approach, combining the natural dispersal and the transportation through human transport via road traffic. From the vector ecologist's perspective, the tools can help to understand the historic dispersal of exotic mosquito species. However, the ultimate objective of this project is the establishment of a tool, which allows predicting the future spread and distribution of the exotic mosquito species. This tool offers extensive application possibilities, e.g. an exact geographic definition for the design of mosquito surveillance or control programs. The next steps in this ongoing project are the evaluation of the system by real world scenarios as well as the enhancement of the layers by a refined concept of time.

## Figures



*Oc. japonicus* distribution 2014, source: http://www.ecdc.europa.e

Figure C.3: Simulated mosquito occurrences after pre-simulation (left, upper) and after 120 timesteps (left, lower). Positive *Ochlerotatus japonicus* raster grids in 2011 (Huber et al., 2012) in a resolution of a topographic map (1:25.000, 18km x 10km) are edged dark gray (left, upper). Small sketch in the right show the observation from *Oc. japonicus* in this region from 2014.

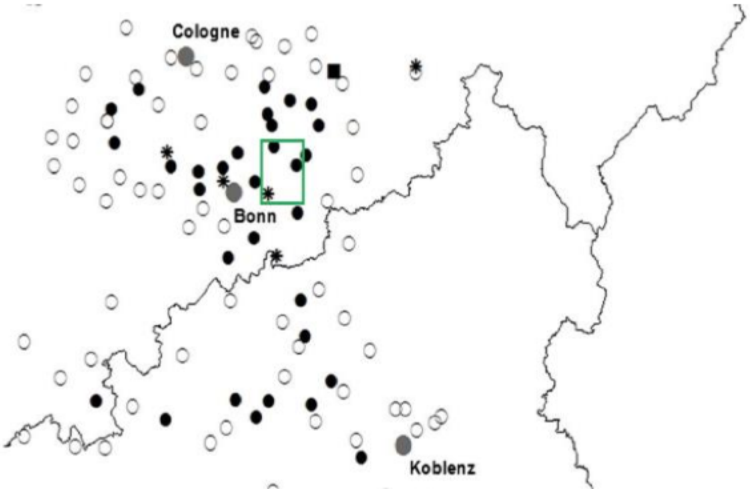


Figure C.4: Mosquito findings in (Kampen et al., 2012), dark points: presence of *Ochlerotatus japonicus*. Green frame: location of scenario 2.

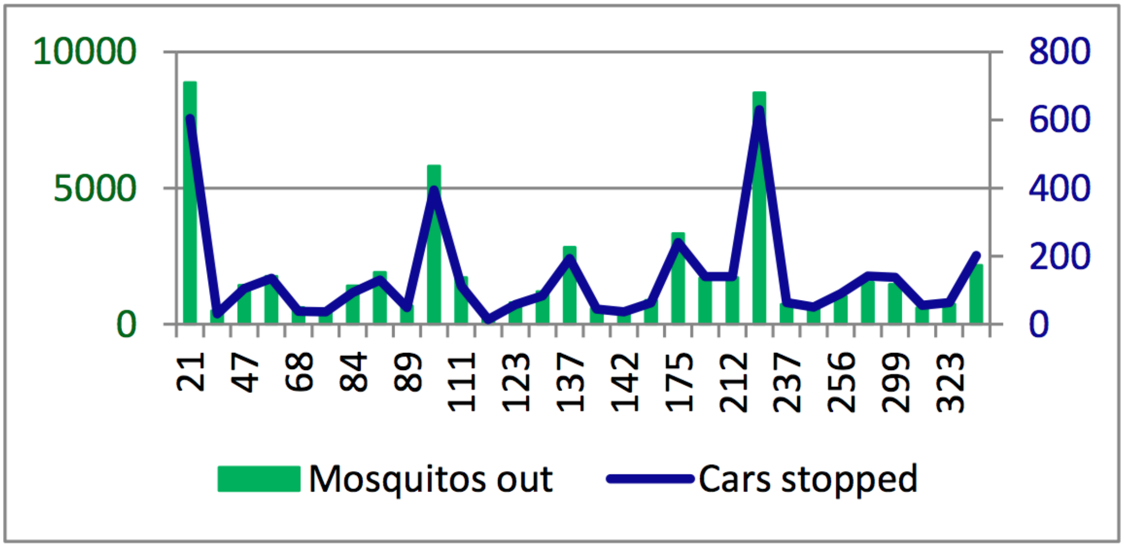


Figure C.5: Distribution of stopping cars (right axis) and released mosquitoes (left axis) per stations. The X-axis shows the distance of the stations to the starting point at Weilheim.

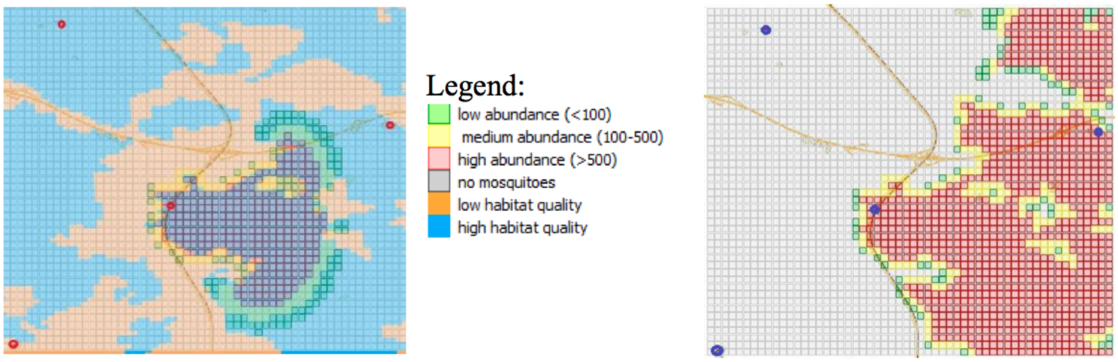


Figure C.6: Simulation starting at station Siegburg West after 100 (left) and 200 simulation steps (right). In the left picture, a map of habitat quality is shown in the background. Due to land use barriers, the dispersal is only directed eastwards.

## C.5 Comparison of single- and multi-scale models for the prediction of *Culicoides* biting midge distribution in Germany

in preperation: **Lühken, R.**, Kiel, E., Gethmann, J.M., Staubach, C., Conraths, F.J., Kranz, P., & Steffenhagen, P. (in preperation). Comparison of single- and multi-scale models for the prediction of *Culicoides* biting midge distribution in Germany.

### Abstract

This study analysed *Culicoides* presence-absence data from 46 sampling sites in Germany, where monitoring had been carried out from April 2007 until May 2008. *Culicoides* presence/absence data were analysed in relation to data from the Automatic Topographic Cartographic Information System (ATKIS®), in order to study whether the prevalence of biting midges is correlated to land cover data around the trapping sites. We differentiated eight scales, i.e. buffer zones with radii of 0.5, 1, 2, 3, 4, 5, 7.5, and 10 km around each site and chose several land cover variables. We built eight single-scale models based on averaged generalized linear models and two multi-scale models (averaged generalized linear models and generalized linear models with random forest variable selection) for each species. Model performance indicators suggest that ATKIS® land cover data can be successfully used to build species distribution models for *Culicoides* biting midges. There were no significant differences between performances indicators of models build with land cover data from different buffer zones around the trapping sites. The same applies for multi-scale models, which did in the majority of cases not show significant differences if compared to single-scale models. However, the overall performance of multi-scale was higher. Furthermore, multi-scale models mostly fulfilled the best performance for the different species using the index area under the receiver operating characteristic curve. However, as

also presented in this study, the relevance of the different variables could significantly differ between different scales including the number of species affected and the positive or negative direction. This is an even more severe problem if multi-scale models are concerned in which one model could have the same variable on different scales but different directions, i.e. negative and positive direction of the same variable on different scales. Thus, multi-scale modelling is a promising approach to model the distribution of *Culicoides* species, accounting much more for the ecology of biting midges, which use different resources (breeding sites, hosts, etc.) on different scales, but do not resolve general interpretational problems due to missing information on the ecology of biting midges.

## Introduction

Bluetongue disease (BTD) is a reportable non-contagious viral infection of ruminants, which occurred in Germany for the first time in late summer 2006 (Conraths et al., 2012). Several species of the genus *Culicoides* (Diptera: Ceratopogonidae) are considered to be potential vectors of the bluetongue virus (BTV) (Meiswinkel et al., 2007; Dijkstra et al., 2008), while the concrete vector competence of the different species is still unresolved. The European BTD epidemic was caused by the bluetongue virus of serotype 8 (Conraths et al., 2012), which was introduced into Central Europe on a still unknown way (Mintiens et al., 2008). In 2006 and 2007, a massive spread of BTD was observed in Germany and, at the end of 2007, nearly all federal states were affected. Until spring 2008, more than 17,000 cattle, sheep, and goat died from this disease, resulting in total costs of approximately 250 million euros. Hence, Germany decided to start a compulsory vaccination program in 2008. The quite recent epidemic of the Schmallenberg virus in Europe again highlights the importance of *Culicoides* species as vectors of pathogens (Beer et al., 2013), as these are also here considered to be the main vectors (De Regge et al., 2012; Rasmussen et al., 2012).

Following the outbreaks of BTD, the European Commission directed a surveillance program, which included a monthly vector monitoring on farms in the restriction zones (Mehlhorn et al., 2009). From April 2007 to May 2008, vector surveillance with a common sampling protocol was carried out in Germany. Thus, for the first time, a comparable data set on the distribution of *Culicoides* species in different regions of Germany was achieved (Werner, 2010). Although this project predominantly focused on the detection of BTV in *Culicoides*, the collected data can also be used for species distribution models, i.e. evaluate the factors correlated with the prevalence of the different species.

Although there is huge lack of knowledge about the causal connection between environmental variables and the distribution of biting midges, several studies modelled biting midge distribution and phenology using different sets of environmental data (Purse et al., 2004; Calvete et al., 2008; Purse et al., 2011; Rigot et al., 2012; Kluiters et al., 2013). These modelling approaches use environmental data from various scales, e.g. all environmental data at one scale (e.g. 1 km, Kluiters et al., 2013) or at different scales (e.g. between 1 and 8 km, Calvete et al., 2008; Purse et al., 2011). However, as previously shown (Hamer & Hill, 2000), the selection of the spatial scales affects the outcome of the modelling, e.g. decrease the variance explained or bias regression coefficients, which might result in wrong conclusions and interpretations (reviewed by Bradter et al., 2013). Therefore, a selection of the appropriate scale is important to allow accurate species distribution modelling.

Furthermore, as generally described by Bradter et al. (2013), species distribution can also be affected by land cover variables from multiple scales, e.g. if breeding sites, resting sites, and hosts of *Culicoides* biting midges are distributed over several scales. Land cover changes with distance to the farm buildings, where sampling of biting midges commonly takes place (e.g. Kiel et al., 2009). The environment is generally modified most intensively around the main buildings in order to optimize the farm management. The percentage of other, natural land cover variables (e.g. forest), increase with increasing scales around the trapping sites. Depending on the breeding and resting sites, host preferences or the species-specific flight range of *Culicoides*, this should result into different scale-specific variables, which are useful for the prediction of biting midge distribution. Therefore, an impact of multiple spatial scales relative to the trapping sites might be expected. A land cover variable can either be a predictor on several scales, e.g. grassland as breeding site on a local scale, hosts are scattered on the grassland on a medium scale, and resting sites are on the edge of the grassland were the vegetation might be higher on the largest scale. Moreover, different variables can be predictors on different scales, e.g., a species breeds in the forest in large distance, the hosts are present in direct vicinity of the trapping sites, and the resting sites are in medium distance.

In this study, we investigated the performance of single- and multi-scale models to predict the distribution of *Culicoides* species on farms in western Germany with land cover variables on different scales. Biting midge data were obtained from light-trap sampling and landscape variables from the Automatic Topographic Cartographic Information System (ATKIS®). As little is known about the ecology and flight range of *Culicoides* biting midges, an a priori selection of the appropriate scale for the modelling of species distribution was not possible. Therefore, we extracted the same landscape variables on eight different spatial scales (radii of 0.5, 1, 2, 3, 4, 5, 7.5, 10 km), which were used separately for single-scale models or all together for multi-scale modelling approaches for the prediction of species distribution. The objectives of this study were: (1) the evaluation of the spatial scales, which gave the best predictions for the species distribution of different biting midge species, (2) the evaluation if multi-scale models increase predictive ability, and (3) the determination of the most important landscapes variables for the prediction of *Culicoides* species distribution on the different scales.

## Materials and methods

### *Culicoides* and landscape data

In this study, we analysed a dataset from 46 trapping sites, covering a gradient from northwest to southwest Germany. At every site, adult *Culicoides* were sampled for 14 months (April 2007 until May 2008). Sampling was conducted during the first seven consecutive days of each month, using the BG-sentinel trap with black light following a standardized sampling protocol (Mehlhorn et al., 2009). All traps were placed in the immediate vicinity of the predominant residences of cattle. The prior objective of this monitoring was to document the distribution and spread of BTV, but did not aim on the distribution and abundance of the biting midge vectors. Therefore, most *Culicoides* samples were sorted to group level only. Species identification was restricted to aliquots and based on morphological characters (Werner, 2010). These aliquots were restricted to a maximum of 10% of the total *Culicoides* sample. During the monitoring, the total number of trapped *Culicoides* ranged from zero to several thousands of individuals (Mehlhorn et al., 2009). Therefore, the number of *Culicoides* determined to species level differed strongly between the study months and trapping sites. Thus, only aggregated presence-absence data over a timespan of 14 month were analysed in this study. Furthermore, species with a prevalence of less than 10% or more than 90% were excluded.

In order to analyse the land cover of each trapping site, we referred to a selection of land cover attributes provided by ATKIS®, which were assumed to be important for *Culicoides* biting midges. The ATKIS® primary data sources are aerial orthophotos (ALK = automated real estate map) and the German topographic raster base map (DTK5). ATKIS® provides linear and polygon vector data with a resolution of 1:5,000 +/- 2.5 m positional accuracy. We analysed eight circular zones (radii of 0.5, 1, 2, 3, 4, 5, 7.5, and 10 km) around each trapping site. The same 14 landscape attributes were measured for all scales (Tab. C.5). This data collection was carried out in ArcGIS9.2 (ESRI Redlands, CA, USA).

### Statistical analyses - Selection of scales for the variables included in the multi-scale models

Selection of variables for the multi-scale models was applied as proposed by Bradter et al. (2013). This preceding reduction of variables had the aim to prevent that several variables on neighbouring scales are selected for the final model, e.g. because variables of neighbouring scales are often highly correlated with each other (Fig. C.7). Another advantage of such exclusion of variables is a significant reduction of computation time.

We used univariate binomial logistic regression models for presence or absence data of each *Culicoides* species for each variable and all eight scales. Due to the small sample size (n=46), we used the corrected form of the Akaike information criterion (AICc), which indicates the best compromise between model complexity and likelihood for each model. The predictors of the different variables on the eight different scales were selected, if the (1) AICc was at least two lower than the AICc of the null model (Intercept only), (2) the AICc was less than the next smaller or larger scale, and (3) the AICc was less than the AICc on the second smaller or larger scale (not applicable for the smallest and largest scale). With this method, we selected all local minima of the AICc, which had at least a difference of two compared to the null model for each predictor and each scale.

### Statistical analyses - Single- and multi-scale models built with model-averaging

According to methods for species distribution modelling applied in other studies (e.g. Kattwinkel et al., 2009; Gray et al., 2010), single-scale and multi-scale generalized linear models were built in four steps: (1) highly correlated variables (Spearman's rho $\geq 0.7$) were excluded. For each highly correlated pair, the variable with the largest mean correlation with other variables were dropped. (2) Univariate binomial logistic regression models were calculated for all variables on all scales for each species. Variables with $p \geq 0.15$ were excluded from further analysis as they were not regarded as statistically significant. (3) Multivariate binomial logistic regression models were built with every combination of the remaining variables from the previous two steps. We considered all possible models and did not use a stepwise model selection strategy, which are often criticised, e.g. because the results of this methods depend on the order in which variables enter the model (Burnham & Anderson, 2002; Whittingham et al., 2006). The large number of variables in different buffer zones per species results in a very large number of possible models. Such a brute force method might therefore not be the optimal approach (Burnham & Anderson, 2002). However, we had not enough information about the ecology of biting midges for an a priori exclusion of variables or restriction to a subset of possible models. (4) If several models were obtained for one species, model averaging was conducted (Burnham & Anderson, 2002). Model averaging approaches are considered to overcome problems such as overfitting or variable selection, which are found in modelling approaches aiming for a single best model (Burnham & Anderson, 2002). The Akaike weight, using AICc for calculation, can be interpreted as a measure of the strength of evidence for each model. We selected a 95% confidence set of models by sequentially summing the Akaike weights until 0.95 was reached. According to Burnham & Anderson (2002), this set of models can be interpreted as having a 95% confidence that the best approximating model is included. The final averaged models were built by multiplication of model coefficients with corresponding Akaike weight $\omega$ (for an example see Strauss & Biedermann, 2006). Weighted coefficients were summed for each variable including all models per species on each buffer zone (single-scale models) or all buffer zones (multi-scale models).

### Statistical analyses -Multi-scale models with random forest variable selection

Although the modelling approach with model averaging of multiple generalized linear models are considered to be relative robust against overfitting (Burnham & Anderson, 2002), the large number of potential land cover variables included in the multiple models might cause such problems. Additionally, the exclusion of highly correlated variables can lead to the inclusion of variables on not meaningful, spatial scales. Therefore, for multi-scale models only, we used an second modelling approach using random forest for the variable selection, which was found to be robust even if the number of response data is small in comparison to the number of predictors (Strobl et al., 2007). This variable selection method was applied as described in detail by Bradter et al. (2013). In random forest, several classification or regression trees are built from random subsets of the data set (Breiman, 2001; Liaw & Wiener, 2002). The procedure uses a selection based on the unscaled permutation importance (Genuer et al., 2010). Each predictor is permutated in turn and the prediction error, i.e. OOB ("out of bag") error, before and after permutations is used as a measure of variable importance (Liaw & Wiener, 2002; Strobl et al., 2008). A training set is created by sampling 2/3 of the data set (with replacement) for each classification tree, which is then used to predict the remaining 1/3 of the data. The proportion of false classified classes over trees is the OOB error (Breiman, 2001; Liaw & Wiener, 2002).

There were five steps to identify the number of predictors suitable for the model interpretation (Genuer et al., 2010): (1) all predictors were ranked by the unscaled permutation importance (average value over 50 repetitions); (2) a regression tree was fitted to the curve of the plot of standard deviations of the importance measures ordered by their mean importance. Variables with a mean importance of less than the smallest predicted value of the regression tree model are discarded; (3) the OOB errors for the models (average over 50 repetitions) were computed by starting with the most important variables and adding the other predictors in order of their ranking, (4) the model with the smallest OOB error, augmented with the standard deviation of the 50 repetitions, was selected, and finally (5) the nested model with OOB error smaller than this with fewer predictors was selected. Parameters which have to be specified in the random forest were used as proposed by Genuer et al. (2010): number of trees built in the forest ntree=2000, the number of predictors available at each node split mtry=p/2 with p as number of predictors, and for the calculation of the OOB error default values were used.

### Spatial autocorrelation

For all models built with model averaging, Moran eigenvector filtering was applied for the full model without highly correlated and non-significant variables (Powney et al., 2010). If significant, these eigenvectors were added to the model and included in the model averaging procedure. Furthermore, as recommended by Bradter et al.

(2013), we applied Moran eigenvector filtering for all multi-scale models selected with random forest variable selection (Dray et al., 2006; Griffith & Peres-Neto, 2006). Spatial eigenvectors were added until RSA was no longer significant at the 0.05 level.

### Performance assessment

Nagelkerke's R squared ($R^2{}_N$) was used as a measure of model calibration (Hosmer & Lemeshow, 2000). Area under the receiver operating characteristic curve (AUC) was used to compare prediction performance (Fielding & Bell, 1997). AUC thresholds were interpreted as proposed by Hosmer & Lemeshow (2000): 0.7–0.8 is considered an „acceptable" prediction; 0.8–0.9 is „excellent" and >0.9 is „outstanding". Although this index is criticized as unreliable by some authors (Lobo et al., 2008), we predominantly referred to AUC, because it is the most commonly used performance indicator for species distribution models. However, as recommended by Lobo et al. (2008), we present further accuracy indices: root mean square error (RMSE), overall correct classification rate (CCR), sensitivity (SENS), specificity (SPEC), positive predictive value (PPV), negative predictive value (NPV), true skill statistic (TSS), Cohen's kappa (KAPPA) (for accuracy indice formulas see Liu et al., 09b). For threshold dependent indices (e.g. CCR or KAPPA) and prevalence prediction, requiring binary results, presence and absence were differentiated using a threshold value set to achieve the observed prevalence in data set (Freeman & Moisen, 2008).

We used bootstrapping 95% percentile confidence intervals to evaluate the statistical differences between the model performances on different scales (Liu et al., 09a; Pearman et al., 2008). We generated 1000 bootstrap data sets (with replacement) for each species on each scale (single- and multi-scale models). Models were refitted with the bootstrap data set. 95% confidence intervals (upper and lower 2.5% quartiles of the distribution) were calculated for each accuracy index. Non-overlapping confidence intervals were interpreted as significant differences between the scales. A threshold of 0.7 for the lower 2.5% quantiles of the AUC, i.e. $AUC_{2.5}$, was used to select acceptable models.

### Software

Data visualization and statistical analyses were conducted with R (R Core Team, 2014) using functions from the packages ggplot2 (Wickham, 2009), plyr (Wickham, 2011), qgraph (Epskamp et al., 2012), randomForest (Liaw & Wiener, 2002), and spdep (Bivand, 2014).

## Results

Eighteen species of the 26 species had a prevalence higher than 10% and lower 90%, and thus were used in this modelling study. From these, 57 models for thirteen species fulfilled our performance criteria, i.e. at least one single- or multi-scale model with $\mathrm{AUC}_{2.5} \geq 0.7$. Only seven of these models for the species *C. albicans* (0.5 km, 2 km, and multi-scale models built with model-averaging), *C. lupicaris* (3 km), *C. newsteadi* (0.5 km), and *C. riethi* (1 km and 2 km) provided a better model fit with spatial eigenvectors, indicating that spatial autocorrelation has little or no influence on the presence-absence at the other scales. $\mathrm{R}^2{}_\mathrm{N}$ ranged from 0.2 to 0.5, which can be considered to be good for logistic regression models Hosmer & Lemeshow (2000); Kattwinkel et al. (2009). Moreover, according to the other accuracy indices, the performance of these models was satisfactory and indicated a better prediction than just by chance (Fig. C.8). Most of the *Culicoides* species studied here had a relative high prevalence resulting in a higher specificity and positive predictive value compared to sensitivity and negative predictive value.

The accuracy indices mostly did not show statistically significant differences, i.e. they had overlapping confidence intervals for the different species and scales (Fig. C.9). Nevertheless, the mean accuracy indices overall were generally slightly higher for multi-scale models compared to single-scale models (Fig. C.8, Fig. C.9)

Summarizing multi- and single-scale models, nearly all species were influenced by "agricultural/urban" and "forest" variables, while around 50% of the species were also influenced by “water” variables. However, looking into more detail, the percentage of species showing correlations with the different land cover variables can strongly differ between the different models and scales (Fig. C.10), while the weights of the different variables in the models built with model averaging did not show strong differences between the different variables (Fig. C.11).

For most of these species (9 out of 13), multi-scale models showed best performance, i.e. highest $\mathrm{AUC}_{2.5}$ value per species (listed in supplementary table C.6). According to the mean AUC, seven of these models were characterized by an excellent, another six ones by an outstanding performance. These models were exemplarily applied for three different artificial landscape configuration to evaluate the impact of the different land cover variables on the distribution of *Culicoides* species: 1) increasing “grassland” and decreasing “forest” variables (Fig. C.12), 2) increasing “arable land” and decreasing “forest” variables (Fig. C.12) and 3) increasing “water” variables (Fig. 6). Some of the *Culicoides* species responded with a wide range under these scenarios: e.g. *C. grisescens* in the scenario increasing “grassland”, decreasing “forest” variables (Fig. C.12) or *C. dewulfi* in the scenario with increasing “water” variables (Fig. C.12). In contrast, *C. lupicaris* did not occur under the three applied scenarios (Fig. C.12). In the best model, the species had a negative association with the proportion of “fallow land” on the 3 km scale not studied in the three scenarios (Tab. C.6). However, the other species showed a distinct response under at least one of the scenarios, e.g. *C. chiopterus*, *C. scoticus*, and *C. stigma* were more restricted to the left of the gradient for the “forest” variables (low “grassland”/low “arable land”, and high “forest” variables), while *C. clastrieri* were more restricted to the right end of the gradient (Fig. C.12).

Under the same landscape scenarios, the presence-absence predictions changed in dependence of the applied single-scale models on the different scales (Fig. C.13). In addition, the landscape context could be important. For example, the distribution of *C. chiopterus* is affected by “grassland” (Tab. C.6), but the prevalence predictions differ depending on the scale this variable change (Fig. C.13).

## Discussion

Species distribution modelling is the most important method to predict species distribution in general, including *Culicoides* biting midges. Since the availability of digital datasets of land cover, temperature, or potential hosts is continuously increasing, several studies used these data also to predict the prevalence of biting midge species, e.g. using the Normalized Difference Vegetation Index (Calvete et al., 2008; Kluiters et al., 2013; Purse et al., 2004) or land cover data (Kirkeby et al., 2009; Purse et al., 2011). These data are available or used on different scales raising the question, which spatial scale or scales should be chosen to reach best predictions for different biting midge species. At the same time, there are huge knowledge gaps on the ecology of *Culicoides* species, which would allow choosing the appropriate scale of the predictors, e.g. missing information on the flight range or resting sites. Therefore, an a priori selection of appropriate scaling of the variables used for *Culicoides* distribution modelling is not possible. According to the review by Bradter et al. (2013), the wrong scale of a predictor can result in different problems. It may, e.g., decrease the variance or can bias the regression coefficients. This, in turn, might result in wrong conclusions and interpretations of the final model.

Active dispersal of *Culicoides* is generally expected to be limited. Concerning *Culicoides* nuisance, Kettle (1984) identified a zone of about 500 m around the farmyard to be most important. A substantial reduction of the number of adult *C. molestus* and *C. subimmaculatus* was achieved by measures targeting breeding sites within this radius. Furthermore, *Culicoides* abundance was found to decrease with increasing distance to potential hosts or breeding sites(Kettle, 1984; Lühken & Kiel, 2012; Rigot et al., 2012; Kirkeby et al., 2013). Moreover, it has been proposed that the direct surroundings of farms provide a huge number of potential breeding sites (Foxi & Delrio, 2010; González et al., 2013; Zimmer et al., 2008, 2014).

In this study, all traps were placed in immediate vicinity to the predominant residences of the cattle directly on the farms, thus, it might be expected that *Culicoides* species are captured in short distance from their breeding sites and land cover information on the smaller scales around the light-traps should have the highest predictive performance. However, in the majority of cases, the model performance did significantly not differ between the models based on variables from different buffer zones. This matches the study by Kirkeby et al. (2013), where the covariate "distance to the breeding site" also did not explain differences in *Culicoides* trapping. One explanation for this result might be that the dispersal of *Culicoides* is much higher than generally expected. Indeed, the small number of data available from mark-release-recapture studies, indicated dispersal distances between two and six kilometres (reviewed by Kirkeby et al., 2013). Another explanation for the lack of higher performance of models on smaller scales could be the underlying data for *Culicoides*. They represent aggregated presence-absence data from a sampling conducted over several months. Therefore, the probability might have been high to trap rare *Culicoides* species.

A comprehensive interpretation of our modelling results is hampered by different circumstances: According to our data, the same variables (e.g. "forest" variables) had a significant correlation with each other on different scales or with other variables. At the same time, highly correlated variables should not be included in the same statistical regression models, e.g., because then small changes in the model or data can result in strong changes of the coefficient estimates (reviewed by Dormann et al., 2013). Therefore, as conducted in this study, it might be recommended to conduct a threshold-based pre-selection to exclude highly correlated variables. However, a preliminary exclusion of variables can result in problems regarding the interpretation of final models and omitted variables have to be considered in the conclusions to be drawn (Dormann et al., 2013). Furthermore, as presented in this study, several species were influenced by different land cover variables on different scales or the same variables have a different algebraic sign (positive or negative) on different scales, e.g. a negative correlation with forest in the model on the local scale and a positive correlation with forest in the model on a larger scale. This causes problems for the interpretation, which even increases in multi-scale models where one final model can include the same variable on different scales with different algebraic signs, e.g. a negative and positive correlation with the forest variable on different scales in the same model.

Nevertheless, at this point, we try to draft out a short ecological interpretation of the predicted prevalence of *Culicoides* species under the chosen scenarios, as we interpret the data from the present point of knowledge. Immature biting midges are generally expected to occupy semi-aquatic larval breeding sites (Kettle, 1984). Kluiters et al. (2013), for example, mentioned a correlation of the abundance of the Obsoletus group and number of water sources. However, in our analysis, the probability of the occurrence of Obsoletus group species was not necessarily associated with high values of the "water" parameters. While *C. scoticus* did not occur under the chosen scenario with increasing values of the "water" variables, *C. dewulfi* and *C. chiopterus* presence were predicted for a wide span regarding these parameters.

In contrast, *C. newsteadi* appeared to be more restricted to low values of the "water" variables in the species distribution modelling, while Kettle & Lawson (1952) published that *C. newsteadi* prefer pools with decaying vegetation. Achieving a similar result, Foxi & Delrio (2010) found this species to be correlated with grassed pools. In our study, an increasing probability of occurrence of *C. newsteadi* and the variable "grassland" was evident. This result corresponds with the study published by Foxi & Delrio (2010), where the species occurred in grassed pools.

*Culicoides chiopterus* and *C. dewulfi* are expected to breed exclusively in cattle dung (Kettle & Lawson, 1952). Therefore, both species should be commonly present on livestock farms, as it was also proposed for the dung-breeding *C. brevitarsis* (Cannon & Reye, 1966). Contradicting this assumption, *C. chiopterus* associated with high proportions of "forest" in our models, while *C. dewulfi* was correlated with high to medium proportions of the same variable.

The breeding sites of *C. scoticus* are not known very well. However, previous studies recorded *C. scoticus* breeding in fungus, mud ruts, silage residues or marshy areas (reviewed by Harrup et al., 2013). Additionally, Harrup et al. (2013) mentioned a close association of the species with *C. obsoletus* and identified several natural or near natural breeding sites like broadleaved leaf litter and vegetation, marginal vegetation around open water, or organically enriched substrates. According to our modelling results, the presence of *C. scoticus*, just as *C. chiopterus* was restricted to high proportions of “forest”.

Ecological information on *C. albicans*, *C. clastrieri*, *C. fagineus*, *C. grisescens*, *C. lupicaris*, *C. riethi*, *C. stigma*, and *C. vexans* is scarce. These species are mostly caught in relative small numbers and therefore are considered to be not involved in virus transmission. Nevertheless, information on these species would probably enable us to better understand the general species distribution, ecology, and the diversity of taxa in the genus *Culicoides*. Breeding sites of *C. lupicaris* seem to be diverse, e.g. old and composted manure, grass with fallen leaves, mud or freshwater lagoons (Kettle & Lawson, 1952; Ramilo et al., 2012; González et al., 2013). The species was not present in the three modelling scenarios we evaluated, but the models indicated a negative association with the proportion “fallow land”. In accordance with results reported by Harrup et al. (2013) in the UK, the distribution of *C. albicans* was characterized by a positive statistical response to increasing “grassland”. *Culicoides stigma* is considered to have a preference for wet, muddy breeding sites (Kettle & Lawson, 1952; Zimmer et al., 2014), but resembles *C. chiopterus* and *C. scoticus* in our study, with predicted presence under a high land cover of “forest” variables. In contrast, the probability of occurrence for *C. clastieri* was highest where the proportion of the “forest” parameters was low. Regarding the response to hydrological situations, the single species habitat models revealed a restriction of *C. clastieri*, *C. griesicens*, and *C. vexans* to the highest proportions of the “water” parameters, e.g. González et al. (2013) also found *C. vexans* breeding in pond microhabitats. Finally, *C. fagineus*, *C. riethi*, and *C. nubeculosous* had the highest probability of occurrence at high proportions of “arable land”.

Our analysis was restricted to *Culicoides* presence-absence data from 46 sampling sites, as part of a wide-meshed monthly monitoring over 14 months in Germany not primarily focused on entomological data, but virus detection in biting midges. However, additional data on species abundance or data covering longer time periods with shorter sampling intervals do not exist at present. Nevertheless, the available data give a first impression on land cover variables explaining the distribution of the German *Culicoides* fauna. Moreover, the German land cover data ATKIS® were successfully used to develop species distribution models for thirteen *Culicoides* species, including *C. chiopterus*, *C. dewulfi*, and *C. scoticus* as potential vectors of the BTV and Schmallenberg virus (Meiswinkel et al., 2007; Dijkstra et al., 2008; De Regge et al., 2012; Rasmussen et al., 2012).

Furthermore, our study showed that multi-scale modelling is a promising approach to model the distribution of *Culicoides* species. Although multi-scale models did often not show significant differences compared to single-scale models, the overall performance of these models was higher. Furthermore, multi-scale models principally fulfilled the best performance for the different species using the AUC values. A multi-scale approach offers the opportunity to include a diverse set of variables from different scales. This is especially important for hematophagous insects, e.g. when breeding sites, resting sites, or host density have to be taken into account for modelling, which are generally distributed across several scales.

Finally, this study again demonstrated a general problem in the interpretation of *Culicoides* species distribution modelling. Although several studies increased our knowledge on the breeding sites different *Culicoides* species are able to colonize (Foxi & Delrio, 2010; González et al., 2013; Harrup et al., 2013; Zimmer et al., 2014), the causal connections with environmental parameters remain mostly unknown. Therefore, besides the evaluation of different modelling techniques and the implementation of further environmental parameters, there is an urgent need for experimental studies on these relationships.

## Acknowledgement

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(University of Bochum), Dr. Martin Geier and Dr. T. Hörbrand (Biogents) permitting us to analyse *Culicoides* data they sampled during the German BTD-monitoring, which was funded by the German Federal Ministry of Food and Agriculture (BMEL). Finally, many thanks to Ute Bradter providing the R code for the multi-scale variable selection and the random forest variable selection as published in Bradter et al. (2013).

# Table

Table C.5: ATKIS® land cover variables used for species distribution modelling.

| **forested areas / woodland** | **abbrevation** |
|---|---|
| deciduous forest/coniferous forest (undifferentiated) | deco |
| deciduous forest | deci |
| coniferous forest | coni |
| other forest (unspecified) | othf |
| forest (sum of all forest) | fore |
| other vegetation (unspecified) | othe |
| | |
| **agricultural & urban** | |
| arable land | acre |
| grassland | gras |
| garden | gard |
| fallow land | fall |
| settlement | sett |
| | |
| **water bodies** | |
| ditch length | ditc |
| stream length | stre |
| water | wate |

## Figures

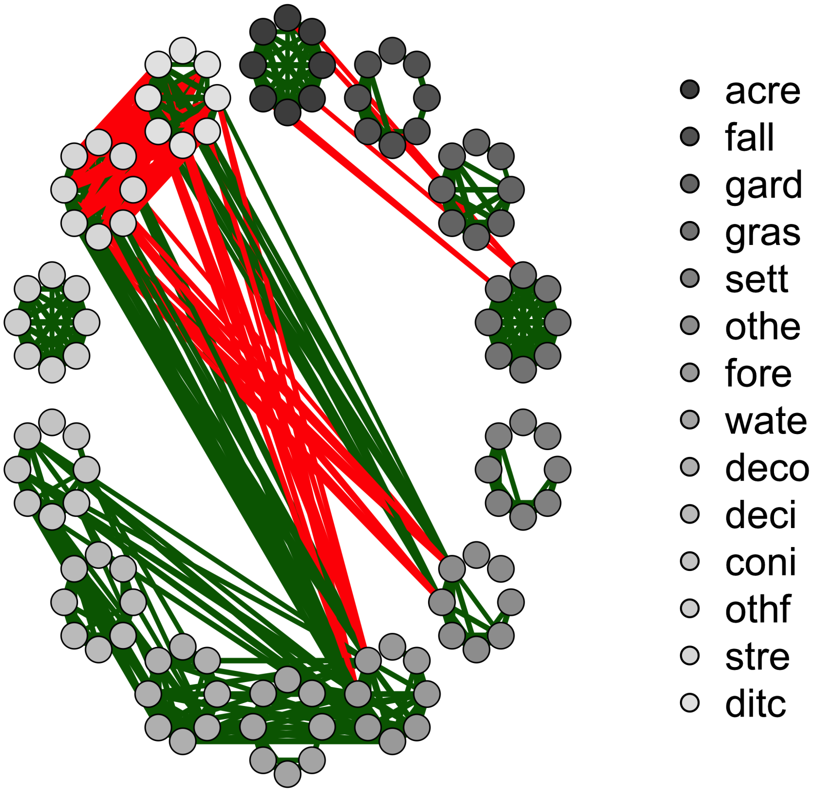


Figure C.7: Correlation network of all 114 predictors. The eight different scales of the 14 variables are grouped each. All correlations with a Spearman rho ≥ 0.7 are indicated by a connection (red = negative correlation, green = positive correlation). See Table C.5 for the abbreviation of the coefficients.

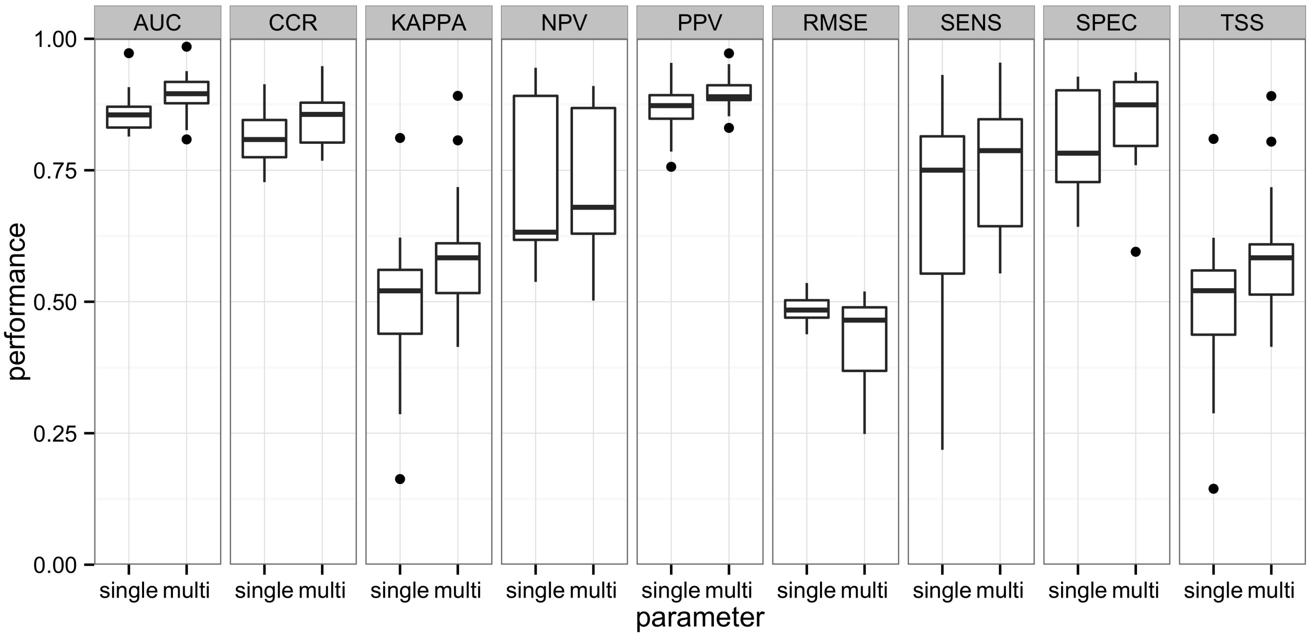


Figure C.8: Performance of the species models with AUC $\geq 0.7$. For each performance criterion [area under the receiver operating characteristic curve (AUC), overall correct classification rate (CCR), Cohen's kappa (KAPPA), negative predictive value (NPV), positive predictive value (PPV), root mean square error (RMSE), sensitivity (SENS), specificity (SPEC), true skill statistic (TSS)], the range and distribution of values for all models is shown. For each criterion, the left boxplot represents the single-scale model (single) and the right boxplot the multi-scale (multi) model.

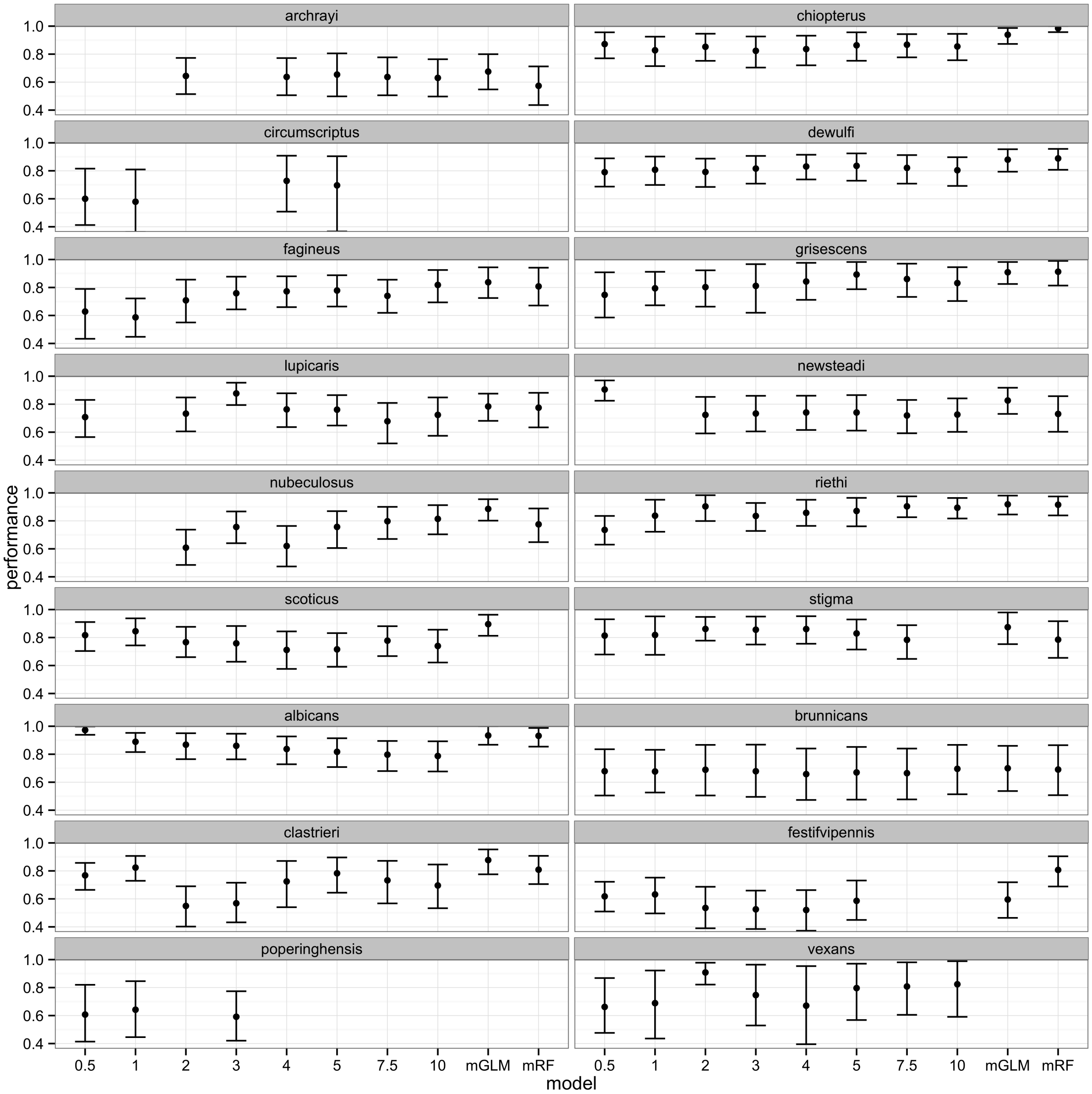


Figure C.9: AUC values with 95% bootstrapped confidence intervals (upper and lower 2.5% quantiles of the distribution) for all *Culicoides* species with a prevalence between 10% and 90% and the different models. Single-scale models on eight different scales and multi-scale models (multi-scale model built with model-averaging, mGLM, and random forest variable selection, mRF) are shown.

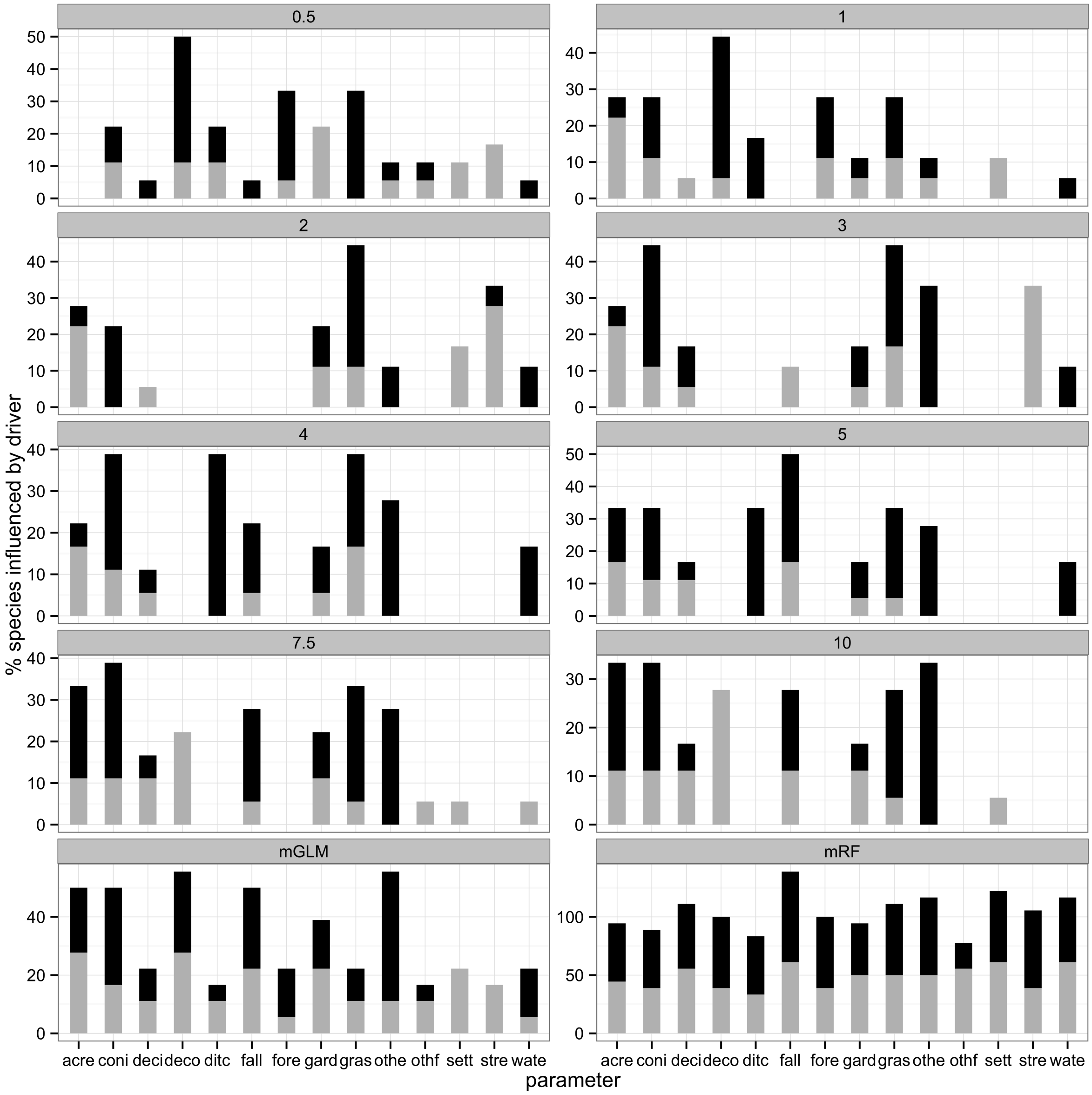


Figure C.10: Percentage of species influenced by each variable in the different models (gray = positive coefficient, black = negative coefficient). Single-scale models on the eight different scales and multi-scale models (multi-scale model built with model-averaging = mGLM and random forest variable selection = mRF) are shown. For the abbreviations of the coefficients, please refer to Table C.5.

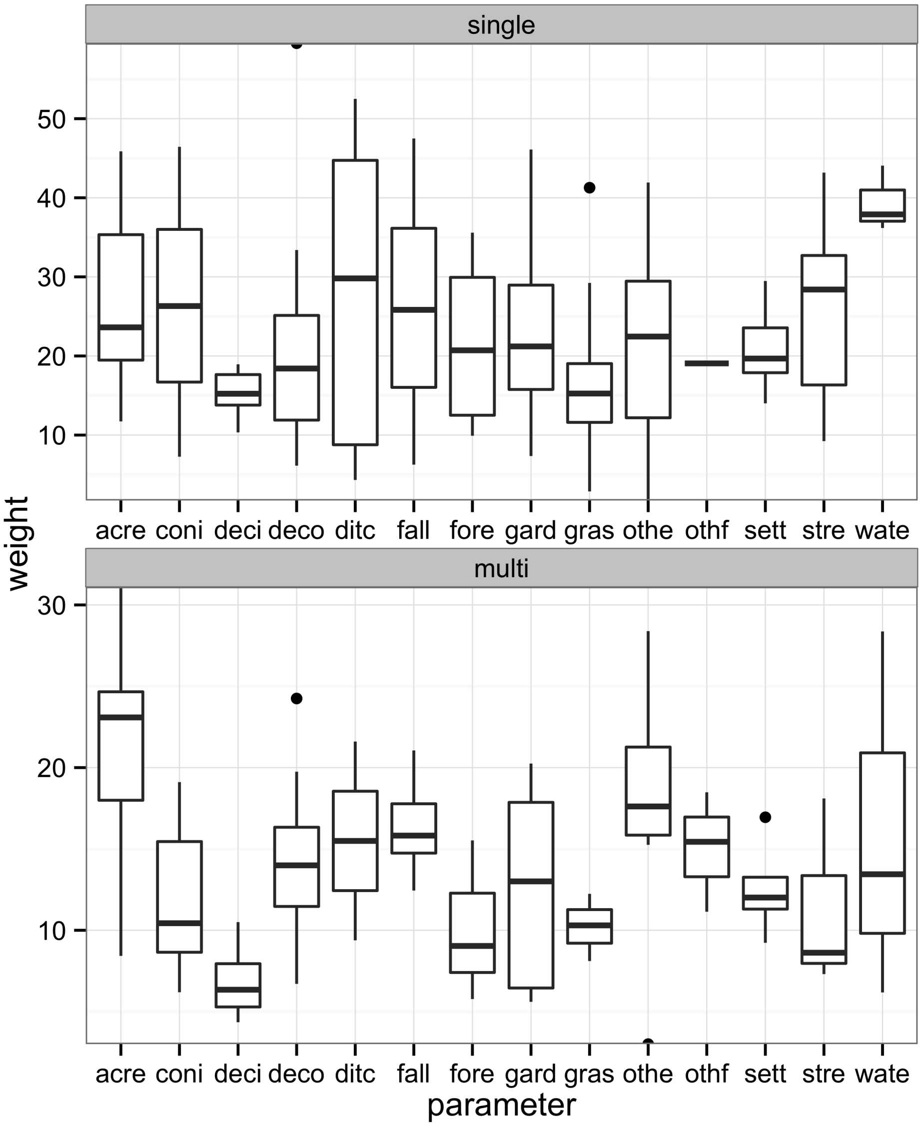
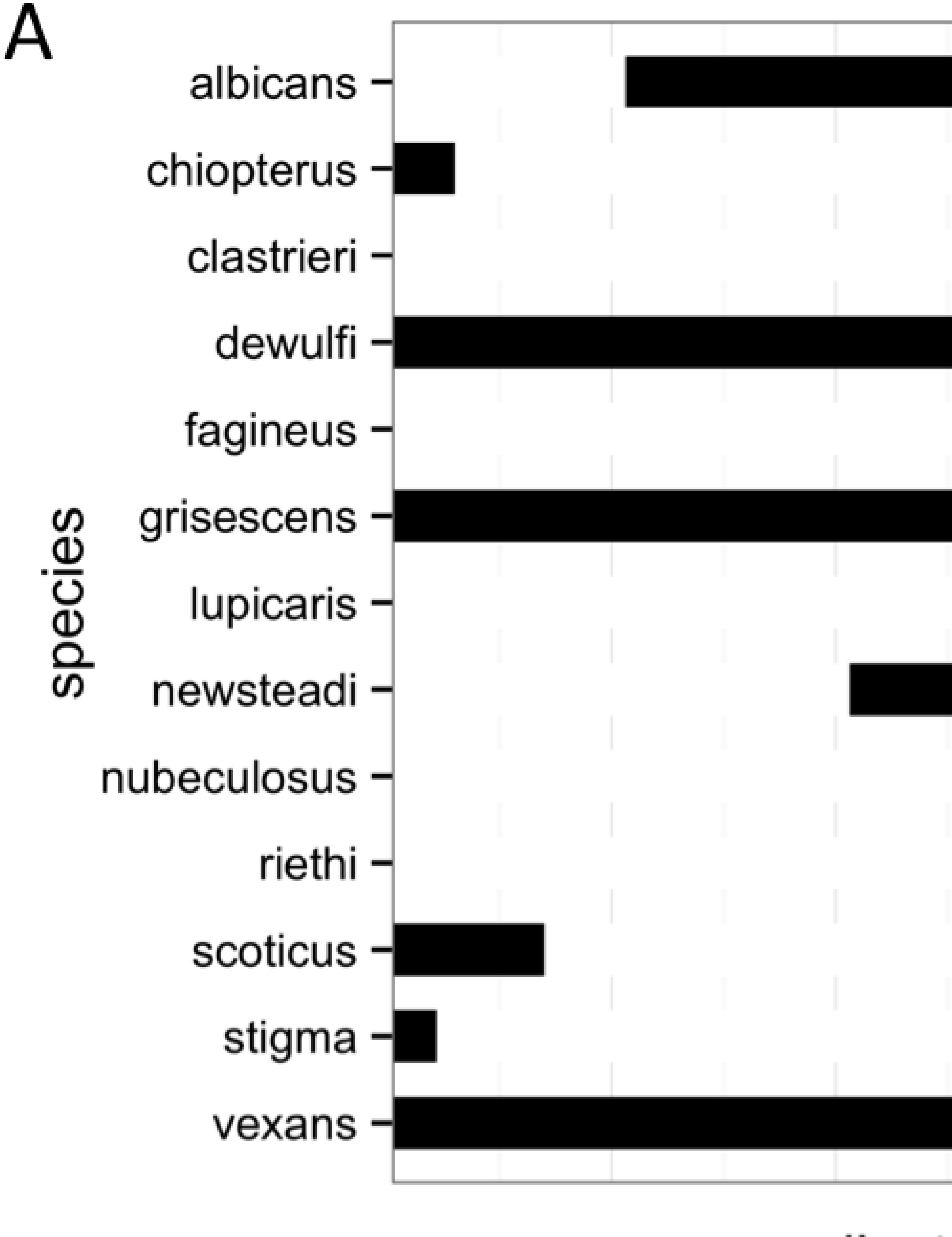
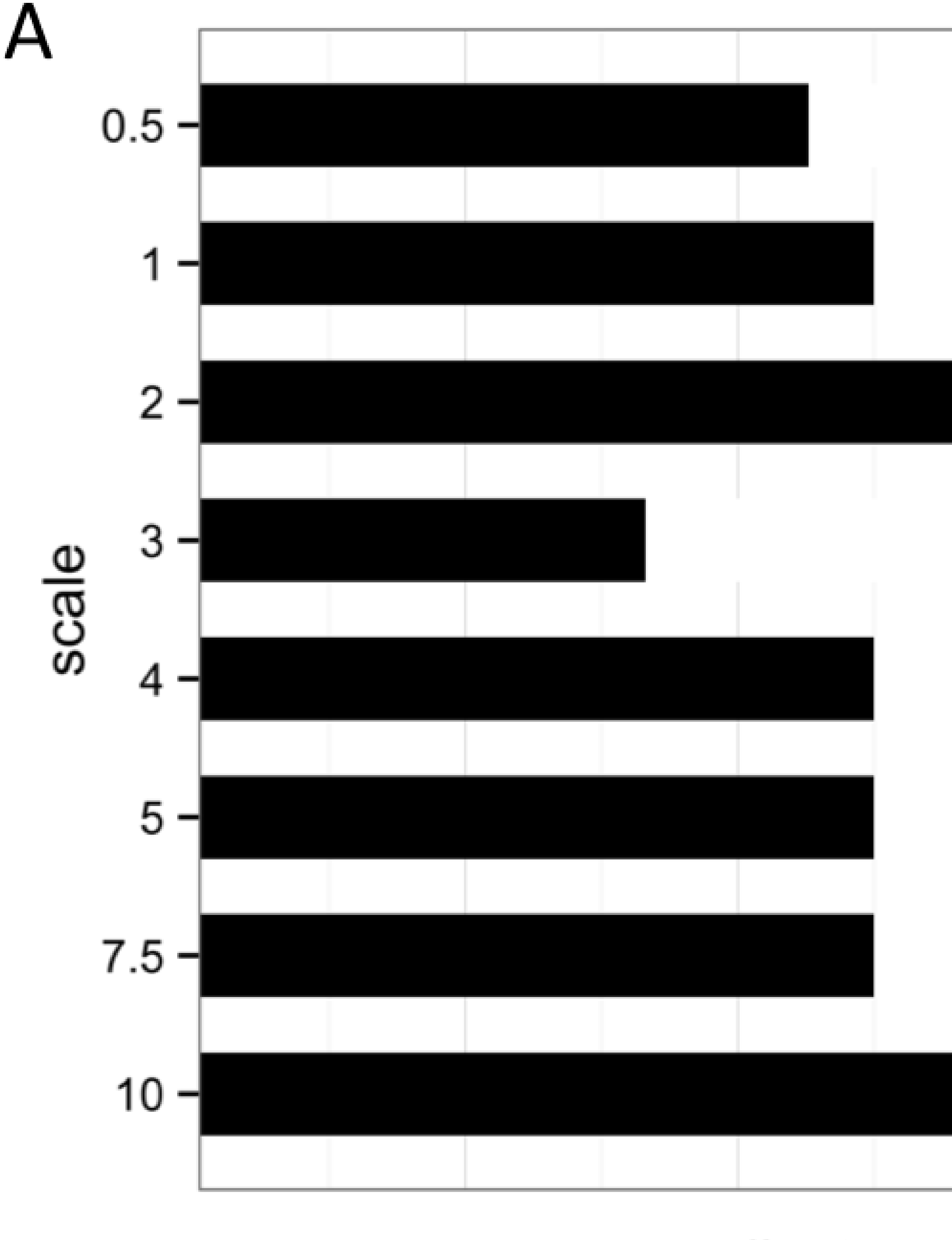


Figure C.11: Range and distribution of factor weights for single- and multi-scale models built with model-averaging separately shown for multi-scale and single-scale models. For the abbreviations of the coefficients, please refer to Table C.5.


A
species
albicans
chiopterus
clastrieri
dewulfi
fagineus
grisescens
lupicaris
newsteadi
nubeculosus
riethi
scoticus
stigma
vexans


A
scale
0.5
1
2
3
4
5
7.5
10

## Supplementary material

Table C.6: Regression coefficients estimates for variables retained in the best model for each vector species considered (multi-scale model built with model-averaging = mGLM and random forest variable selection = mRF). For the abbreviation of the coefficients, please refer to Table C.5.

| **Species** | **Model** | **Coefficient** | **Value** |
|---|---|---|---|
| albicans | single-scale 0.5 km | (Intercept) | 0.34171803 |
| albicans | single-scale 0.5 km | gard 0.5 km | -0.07082257 |
| albicans | single-scale 0.5 km | gras 0.5 km | 0.010794714 |
| albicans | single-scale 0.5 km | deco 0.5 km | 0.012398087 |
| albicans | single-scale 0.5 km | coni 0.5 km | -0.006940595 |
| albicans | single-scale 0.5 km | stre 0.5 km | -0.001750673 |
| albicans | single-scale 0.5 km | ditc 0.5 km | -9.25E-06 |
| albicans | single-scale 0.5 km | spatial eigenvector | 0.9211279 |
| chiopterus | mRF | (Intercept) | 1.386264173 |
| chiopterus | mRF | ditc 10 km | -0.003857233 |
| chiopterus | mRF | stre 7.5 km | -0.013571942 |
| chiopterus | mRF | gras 10 km | -0.014304278 |
| chiopterus | mRF | acre 5 km | -0.000546263 |
| chiopterus | mRF | othe 10 km | 0.099545085 |
| chiopterus | mRF | deci 10 km | 0.003213419 |
| chiopterus | mRF | sett 0.5 km | -0.009461247 |
| chiopterus | mRF | gras 2 km | 0.006740964 |
| chiopterus | mRF | fall 10 km | -0.002982939 |
| chiopterus | mRF | sett 10 km | 0.021452661 |
| chiopterus | mRF | gras 0.5 km | 0.001628489 |
| chiopterus | mRF | fore 0.5 | 0.010363357 |

Table C.6: Regression coefficients estimates for variables retained in the best model for each vector species considered (multi-scale model built with model-averaging = mGLM and random forest variable selection = mRF). For the abbreviation of the coefficients, please refer to Table C.5.

| **Species** | **Model** | **Coefficient** | **Value** |
|---|---|---|---|
| chiopterus | mRF | deco 1 km | 0.010869548 |
| clastrieri | mGLM | (Intercept) | 0.228413841 |
| clastrieri | mGLM | fall 5 km | -0.017652888 |
| clastrieri | mGLM | gard 3 km | 0.017541841 |
| clastrieri | mGLM | othe 0.5 km | 0.049981518 |
| clastrieri | mGLM | othf 0.5 km | 0.006394879 |
| clastrieri | mGLM | wate 4 km | 0.012417449 |
| clastrieri | mGLM | deco 0.5 km | -0.00566676 |
| dewulfi | mRF | (Intercept) | 0.827457863 |
| dewulfi | mRF | acre 1 km | -0.007308604 |
| dewulfi | mRF | gard 3 km | 0.026980507 |
| dewulfi | mRF | coni 10 km | 0.029787434 |
| dewulfi | mRF | deci 7.5 km | -0.018338995 |
| fagineus | mGLM | (Intercept) | 0.068323411 |
| fagineus | mGLM | acre 10 km | 0.002862186 |
| fagineus | mGLM | deco 10 km | -0.00226265 |
| fagineus | mGLM | fall 0.5 km | 0.010475048 |
| fagineus | mGLM | othe 10 km | 0.080312522 |
| fagineus | mGLM | coni 5 km | -0.003025449 |
| grisescens | mRF | (Intercept) | -0.219210995 |
| grisescens | mRF | wate 5 km | 0.038394196 |
| grisescens | mRF | acre 5 km | 0.006148215 |

Table C.6: Regression coefficients estimates for variables retained in the best model for each vector species considered (multi-scale model built with model-averaging = mGLM and random forest variable selection = mRF). For the abbreviation of the coefficients, please refer to Table C.5.

| **Species** | **Model** | **Coefficient** | **Value** |
|---|---|---|---|
| grisescens | mRF | acre 10 km | -0.000207316 |
| grisescens | mRF | fall 5 km | 0.059130116 |
| lupicaris | single-scale 3 km | (Intercept) | 0.333139699 |
| lupicaris | single-scale 3 km | fall 3 km | -0.067209061 |
| lupicaris | single-scale 3 km | gras 3 km | 0.000753518 |
| lupicaris | single-scale 3 km | spatial eigenvector | 1.4803754 |
| newsteadi | single-scale 0.5 km | (Intercept) | 0.630726532 |
| newsteadi | single-scale 0.5 km | gard 0.5 km | -0.057528424 |
| newsteadi | single-scale 0.5 km | gras 0.5 km | 0.00335398 |
| newsteadi | single-scale 0.5 km | othe 0.5 km | -0.057765189 |
| newsteadi | single-scale 0.5 km | othf 0.5 km | -0.010234301 |
| newsteadi | single-scale 0.5 km | stre 0.5 km | -0.000580121 |
| nubeculosus | mGLM | (Intercept) | 0.478169348 |
| nubeculosus | mGLM | coni 3 km | 0.006248505 |
| nubeculosus | mGLM | deci 3 km | 0.003363109 |
| nubeculosus | mGLM | deco 10 km | -0.009467254 |
| nubeculosus | mGLM | fall 5 km | 0.067384382 |
| nubeculosus | mGLM | gras 3 km | -0.002068702 |
| nubeculosus | mGLM | othf 7.5 km | -0.005057276 |
| nubeculosus | mGLM | sett 7.5 km | -0.010238493 |
| nubeculosus | mGLM | acre 10 km | 0.00296438 |
| riethi | mGLM | (Intercept) | -0.023575559 |

Table C.6: Regression coefficients estimates for variables retained in the best model for each vector species considered (multi-scale model built with model-averaging = mGLM and random forest variable selection = mRF). For the abbreviation of the coefficients, please refer to Table C.5.

| **Species** | **Model** | **Coefficient** | **Value** |
|---|---|---|---|
| riethi | mGLM | acre 10 km | 0.005720458 |
| riethi | mGLM | deco 0.5 km | -0.000666635 |
| riethi | mGLM | deco 10 km | -0.003220398 |
| riethi | mGLM | fall 10 km | -0.009744633 |
| riethi | mGLM | gard 7.5 km | 0.058173092 |
| riethi | mGLM | othe 2 km | 0.069933914 |
| riethi | mGLM | coni 10 km | 8.63E-06 |
| scoticus | mGLM | (Intercept) | 0.67644907 |
| scoticus | mGLM | deco 0.5 km | 0.00617887 |
| scoticus | mGLM | fall 3 km | -0.047027645 |
| scoticus | mGLM | gard 1 km | -0.171415777 |
| scoticus | mGLM | gard 10 km | 0.004116653 |
| scoticus | mGLM | othe 1 km | -0.110006323 |
| scoticus | mGLM | sett 1 km | -0.003477437 |
| scoticus | mGLM | wate 7.5 km | -0.004319163 |
| scoticus | mGLM | fore 0.5 km | 0.000472524 |
| scoticus | mGLM | coni 3 km | 0.002970027 |
| stigma | mGLM | (Intercept) | 0.242125467 |
| stigma | mGLM | acre 0.5 km | -0.002395436 |
| stigma | mGLM | coni 4 km | 0.001940643 |
| stigma | mGLM | ditc 0.5 km | 0.001477518 |
| stigma | mGLM | fall 5 km | 0.013790944 |

Table C.6: Regression coefficients estimates for variables retained in the best model for each vector species considered (multi-scale model built with model-averaging = mGLM and random forest variable selection = mRF). For the abbreviation of the coefficients, please refer to Table C.5.

| **Species** | **Model** | **Coefficient** | **Value** |
|---|---|---|---|
| stigma | mGLM | sett 2 km | -0.003324499 |
| stigma | mGLM | coni 1 km | 0.00374789 |
| vexans | single-scale 2 km | (Intercept) | 0.058930728 |
| vexans | single-scale 2 km | sett | -0.002124151 |
| vexans | single-scale 2 km | wate | 0.019467968 |
| vexans | single-scale 2 km | coni | 0.017852597 |

# Appendix D

# Breeding ecology of *Culicoides*

## D.1 Impact of mechanical disturbance on the emergence of *Culicoides* from cowpats

published: **Lühken, R.**, Kiel, E., & Steinke, S. (2014). Impact of mechanical disturbance on the emergence of *Culicoides* from cowpats. *Parasitology Research*, *113*, 1283-1287.

### Abstract

The outbreaks of Bluetongue virus and Schmallenberg virus between 2006 and 2012 highlighted the need for control methods for north-western Palaearctic biting midge species. There is especially a demand for alternative control methods without application of insecticides. Therefore, the objective of this paper was to evaluate the impact of mechanical disturbance as a control method for *Culicoides* biting midges in cowpats. We used a trash rake to destroy the compact structure of cowpats and spread the dung over the ground. We expected the disturbance to cause a faster alteration of physical characteristics and drying of the dung, resulting in lower emergence from disturbed cowpats. Emerging biting midges were collected with emergence traps. The number of emerged *Culicoides* was compared between treated and control cowpats. A total of 12,979 biting midges emerged, all belonging to the Obsoletus group. The majority of the 6,758 male individuals were identified as *Culicoides chiopterus* (Meigen, 1830) (91.4%), followed by *Culicoides dewulfi* (Goetghebuer, 1936) (6.5%), and a small number of *Culicoides scoticus* (Downes & Kettle, 1952) (1.2%). Our results showed no significant differences between the emergence of *Culicoides* (males and females) from disturbed and control cowpats. The lack of differences is discussed in light of the time period chosen for the experiment and the climatic conditions (especially low temperatures) during the study period. The climatic conditions during the study did not favour desiccation effects and therefore did not cause a severe alteration of the dung and the biotic parameters. In conclusion, immature *Culicoides* showed a high tolerance against mechanical disturbance.

## Introduction

*Culicoides* biting midges are vectors of several pathogens. Currently, two viruses are of major importance in Northern Europe: the Bluetongue virus (BTV) and the 2011 newly identified Schmallenberg virus (SBV) (Hoffmann et al., 2009; Conraths et al., 2013). For several decades, the species *Culicoides imicola* was considered to be the main vector of *Culicoides*-borne diseases in Europe, but the spread of BTV serotype 8 in 2006/2008 across Northern Europe demonstrated the vector competence of indigenous north-western Palaearctic species. Because *C. imicola*, the major vector for BTV in Mediterranean regions, is absent in Northern Europe (Mellor et al., 2000), prior studies about control methods for biting midges in Northern Europe were restricted to nuisance species (e.g. *Culicoides impunctatus*) (Carpenter et al., 2008), which are currently not expected to play a central role in the transmission of BTV and SBV. On the other hand, the rapid succession of BTV and SBV epidemics from 2006 to 2012 highlighted the need for methods to control north-western Palaearctic biting midge species in order to reduce the probability for the transmission of arboviruses. BTV and SBV cause large direct and indirect economic losses (e.g. milk drop, export restrictions): worldwide, the economic damage caused by BTV has been estimated to 3 billion US$ a year (Bath, 1989). Because of the significant widespread outbreaks of BTV since this estimation, the actual figure is probably much higher.

Control methods used to reduce both attack rates and transmission of arboviruses by *Culicoides* are predominantly restricted to insecticides, which are used to treat vector resting sites, host animals or screens. Other methods focus on the prevention of vector-host contact (repellents, housing of animals, attractants to lure and kill adult biting midges) (Kettle, 1962; Mellor & Wittmann, 2002; Carpenter et al., 2008). Recent studies also demonstrated promising results with entomopathogenic fungi or a citronella (natural oil)-based repellent as alternative control agents for adult *Culicoides* (MartAnez-de la Puente et al., 2009; Ansari et al., 2011, 2010). As reviewed by Conraths et al. (2012), especially pyrethroids have been successfully used against *Culicoides* spp. and may result in a protection of livestock for several weeks. Many studies positively evaluated pour-on formulations with different pyrethroids (e.g. deltamethrin, permethrin or fenvalerate) to kill *Culicoides* on cattle and sheep (Mehlhorn et al., 08a,b; Schmahl et al., 2008, 09a,b). However, most control methods have been proven unsuccessful for the sustainable control of *Culicoides*, e.g. although pour-on formulations were effective even under field conditions (Schmahl et al., 09c), their application over the whole year is quite disadvantageous (Mehlhorn et al., 2010). In addition, Bauer et al. (2009) used a combination of insecticide-impregnated ear tags, deltamethrin pour-on and insecticide-treated mosquito fences, but they neither found a reduction of *Culicoides* density nor did the methods keep the midges from attacking the livestock. Up to now, there is no proof for a significant reduction of virus transmission by using pour-on formulations (Carpenter et al., 2008). According to the authors, the effects of pour-on formulations are too small to significantly reduce the local midge population. Field studies for other control methods (e.g. entomopathogenic fungi) or a basic knowledge on the ecology of indigenous *Culicoides* species for an effective treatment (e.g. resting habits for an efficient execution of insecticide spraying (Satta et al., 2004) are still missing.

Potential methods to control *Culicoides* larvae and pupae are insecticides and pathogens, but also habitat modifications or the destruction of breeding sites might be reasonable (Carpenter et al., 2008). The number of studies on breeding sites of potential *Culicoides* vectors is increasing (e.g. González et al., 2013; Harrup et al., 2013). Nevertheless, a comprehensive understanding on the breeding ecology is still missing, thus hampering the effectiveness of a targeted, species-specific application of insecticides. Alternative methods like pathogens as biocontrol agents for immature biting midges were identified (e.g. Unkles et al., 2004; Stephen & Kurtböke, 2011), but field applications were not conducted yet. Therefore, alternative large-scale vector control methods targeting the breeding sites are predominantly restricted to intensified drainage schemes (Carpenter et al., 2008), an agricultural method which can cause severe ecological problems (e.g. reduced species diversity) (Lu et al., 2009). For that reason, control methods that consider ecological requirements are needed. To our knowledge, up to now, no one has evaluated the effects of manual breeding site disturbance to reduce or even prevent the emergence of biting midges. In Northern Australia, Bishop et al. (2005) found decreasing emergence of biting midges (*Culicoides brevitarsis*) from cowpats, which were naturally disturbed by dung beetles. This result was attributed to the alteration of physical characteristics (e.g. thickness of crust) and faster drying of the dung. Inspired by these studies, we investigated the impact of mechanical disturbance on the emergence of *Culicoides* from cowpats as breeding sites of *Culicoides chiopterus* and *Culicoides dewulfi* (Kettle & Lawson, 1952; Dijkstra et al., 2008), both species being considered to be potential vectors of BTV and SBV in Northern Europe (Hoffmann et al., 2009; Rasmussen et al., 2012). This method should simulate the regular activities of farmers, who use harrowing in order to smooth the ground of grassland (e.g. flattening molehills). Pastures generally account for a large area around farms and are intensively frequented by livestock at least during the summer period. Therefore, we assume the disturbance of cowpats as breeding sites for biting midges might be a useful measurement to reduce the vector population and, in this context, also the transmission of arboviruses.

## Methods

On April 4, 2011, we randomly selected 46 cowpats on three farms (variable FARM) in Northern Germany. These sites represented typical dairy farms situated in rural regions. The cowpats were taken from a pasture that had not been used for grazing during the previous months, so that they were approximately 5–6 months old. Twenty-three randomly chosen cowpats were mechanically treated with a trash rake, and another 23 cowpats served as a control (variable DISTURBANCE). The disturbance treatment was carried out in a manner that destroyed the compact structure of the cowpat and spread the dung over the ground. At the same date, emergence traps were set up on all disturbed and control cowpats (Fig. D.1). The traps (0.8 m2) covered the entire area of every cowpat. Traps were emptied two times in intervals of 14 days. The samples were sorted in the laboratory, and biting midges were determined to group level (Obsoletus group, Pulicaris group or other Ceratopogonidae). Males of the Obsoletus group were determined to species level based on morphological characteristics of their hypopygia (Campbell & Pelham-Clinton, 1960).

Data analyses were done with the program R (R Core Team, 2014), using the package "ggplot2" (Wickham, 2009) for the graphs. Because of overdispersion, data of all emerged individuals of the Obsoletus group, females of the Obsoletus group, males of the Obsoletus group and *C. chiopterus* males was analysed separately with negative binomial generalised linear models through the function glm.nb from the package "MASS" (Venables & Ripley, 2002). The variables DISTURBED, FARM, and the interaction DISTURBED:FARM were implemented in the model.

According to recommendations by Zuur et al. (2009), hypothesis testing for significance of the variables and their interaction was conducted using the likelihood ratio test (function lrtest) from the package "lmtest" (Zeileis & Hothorn, 2002), dropping each term in turn and comparing the full model with a nested model. The non-significant variables with the lowest impact on the deviance of the model were excluded. The model was then refitted with the remaining terms until all terms were significant.

## Results

Beside a large amount of other insects (55,998 individuals), 12,979 biting midges were collected. All biting midges belonged to the Obsoletus group. *Culicoides* emerged from all cowpats with only one exception (97.8% of all 46 cowpats). Among the 6,758 (52.1%) males, due to damaged hypopygia, 67 individuals (1.0%) could not be identified. The majority (6,174 individuals, 91.4%) were determined as *C. chiopterus*. Only few male *C. dewulfi* (438 individuals, 6.5%) and *Culicoides scoticus* (79 individuals, 1.2%) were present. Males of all three species emerged from disturbed and control cowpats. There was a strong variance in the numbers of emerged individuals from the different cowpats (Fig. D.2). We found no significant effect of the DISTURBANCE, FARM or the interaction of DISTURBANCE and FARM on the emergence (likelihood ratio tests, $P>0.05$), neither on the total number of emerged individuals belonging to the Obsoletus group, the females or the males of the Obsoletus group, nor on the emergence of *C. chiopterus* males.

## Discussion

One option to reduce populations of biting midges is to eliminate or destroy their breeding sites (Carpenter et al., 2008). Firstly, on the local scale, a reduction of potential breeding sites can be achieved through a spatial explicit identification and their removal by an adjustment of the farm management. This includes, e.g., to avoid overflowing cattle troughs, dripping taps or reducing the amount of manure and dung piles directly on the farm. Secondly, on a larger scale, a reduction of biting midge breeding sites is generally undertaken by an intensification of soil drainage (Linley & Davies, 1971). Lowering water levels might be suitable to disturb the development of moisture associated *Culicoides* biting midges. In particular, members of the Pulicaris group are expected to be closely associated with high soil wetness and waterbodies (Kettle & Lawson, 1952; González et al., 2013), but this does not necessarily match breeding site preferences of the species belonging to the Obsoletus group. While *C. chiopterus* and *C. dewulfi* are expected to breed exclusively in dung (Kettle & Lawson, 1952; Dijkstra et al., 2008), *C. scoticus* and C. obsoletus s.s. have been sampled from a variety of breeding sites (e.g. dung heaps, manure, dead leaves) (Harrup et al., 2013; González et al., 2013). Thus, an intensification of drainage might not affect the Obsoletus group species. Therefore, in this study, we evaluated an alternative method to control *Culicoides* in cowpats. In temperate regions, *Culicoides* are generally expected to overwinter as larvae (Mellor, 1990). In Germany, adult *Culicoides* biting midges were trapped in high numbers after the winter period (Hoffmann et al., 2009). Because the first generation occurs at the end of April or early May, our control measurements at the beginning of May targeted the overwintering immature stages of two members in the Obsoletus group, which probably play an important role in BTV transmission in Germany: *C. chiopterus* and *C. dewulfi* (Mehlhorn et al., 2009). Disturbance affects the physical characteristics of dung (e.g. faster drying) and was shown to negatively affect the development of biting midges (Bishop et al., 2005). We assumed the numbers of emerging adults to be significantly reduced when cowpats had been mechanically disturbed. Surprisingly, our results did not confirm this hypothesis, i.e. no significant reduction of emerging *Culicoides* biting midges by disturbance was recorded. For the interpretation of this result, some framework conditions of our study have to be considered. First of all, the time period we chose for the experiment could be important. *Culicoides* larvae overwinter in the third or fourth larval stage (Mellor, 1990), which might be relatively tolerant against the kind of mechanical disturbance we applied. Additionally, larvae and pupae breeding in cowpats are generally exposed to harsh environmental parameters during the winter period (e.g. sunlight, temperature, snow or rain) for a comparatively long time. Hence, compared to the natural disturbance during the winter, the mechanical disturbance we applied might have been only of low impact.

Another factor we have to consider when referring to our results is temperature. Differences between our results and the results found by Bishop et al. (2005) for Northern Australia might be due to differences in the climatic conditions. The surface temperatures were much lower during our experiment (–2.9 to 9.3 °C; Germany's National Meteorological Service, www.dwd.de). Therefore, there might not have been enough desiccation to cause a faster alteration of the biotic and abiotic parameters for the immature biting midges.

Although we found no significant differences between the treated and control cowpats, this study may represent a first step towards the development of alternative methods of *Culicoides* control, which are still missing (Mehlhorn et al., 2012). Our results demonstrated huge tolerance of immature biting midges against mechanical disturbance as a single factor. However, further field studies on the impact of mechanical disturbance on *Culicoides* in Europe should be carried out either during dry summer months, in order to analyse the effects of increased desiccation, or should focus on the combination of mechanical disturbance and frost earlier or at the onset of winter. Additionally, laboratory studies under controlled conditions would be advisable to explicitly identify the driving factors, which affect the survival of immature biting midges in their breeding sites. Our study highlighted the general lack of knowledge about the breeding ecology of biting midges. Only few studies focused on the ecological niche of *Culicoides* species, i.e. the variety of factors determining the distribution and development of immature *Culicoides*, in order to identify bottleneck situations suitable for vector control.

## Acknowledgments

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## Figures

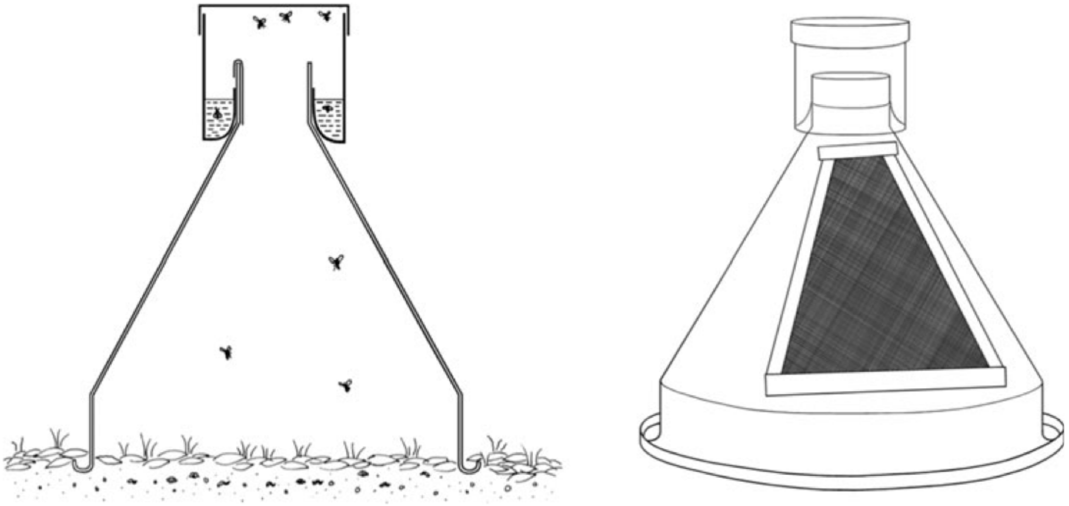

Figure D.1: Schematic drawing of the emergence traps [area=0.8 m$^2$, height= 32 cm]. Emerging arthropods are attracted by the light falling through the transparent beaker. They were trapped in a conservation liquid (four parts ethanol (96%), three parts distilled water, two parts glycerine, one part acetic acid and a small drop of washing-up liquid). Each eclector has two aeration windows covered with gauze (mesh size = 125 µm), which should ensure gas exchange and prevent the temperatures in the eclectors to become too high. When exposed in the field, the bottom rim was thoroughly covered with soil/substrate in order to prevent the midges to invade from outside (drawings: M. Stöckmann)

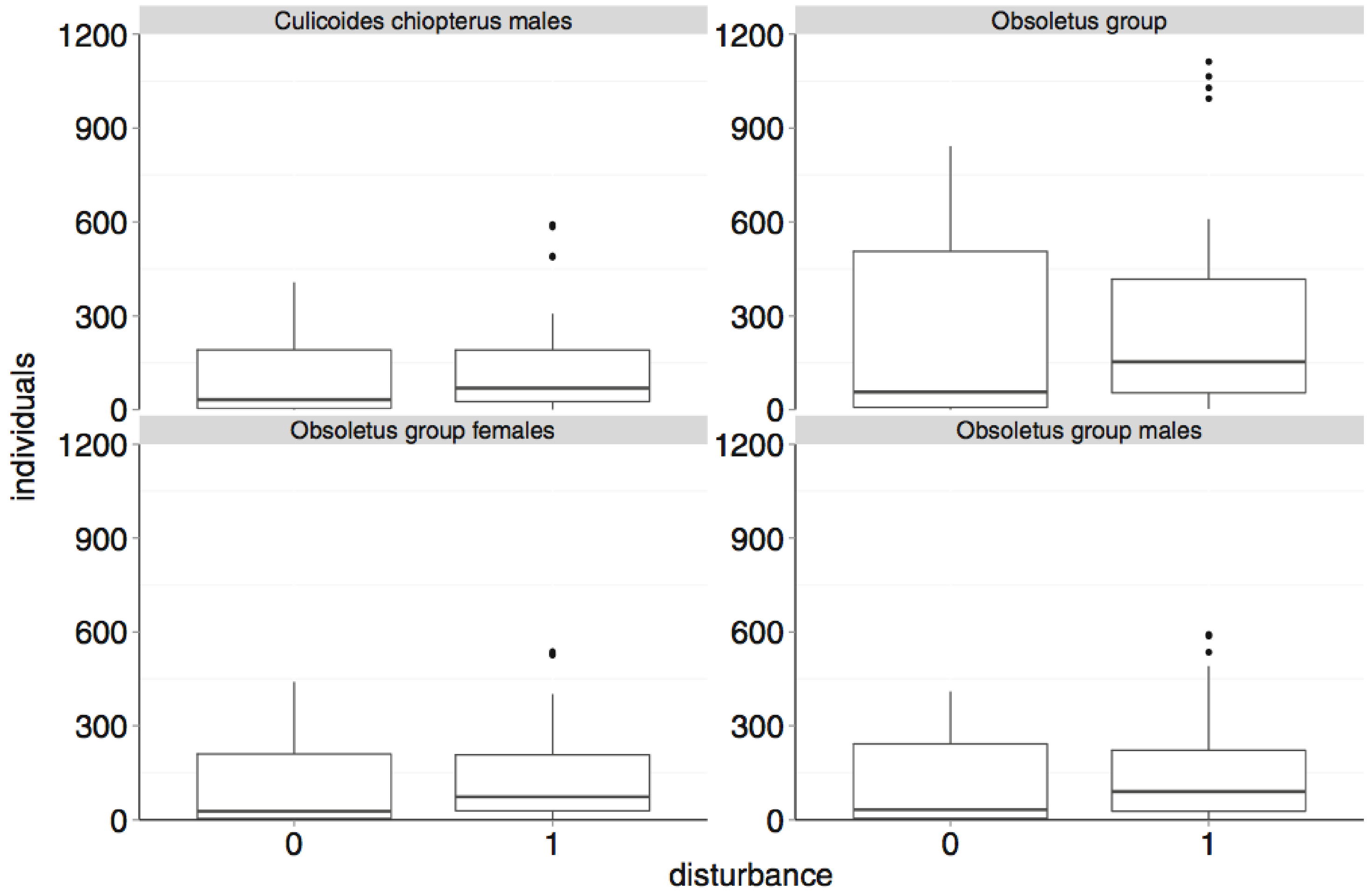

Figure D.2: Number of emerged biting midges from disturbed (disturbance=1, n=23) and control (disturbance=0, n=23) cowpats between April 4, 2011 and May 2, 2011

## D.2 Impact of flooding on the immature stages of dung-breeding *Culicoides* in Northern Europe

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### Abstract

In Northern Europe, dung-breeding *Culicoides* (e.g., *Culicoides chiopterus* (Meigen 1830) and *Culicoides dewulfi* (Goetghebuer 1936)) are considered to be important vectors of the Bluetongue virus and Schmallenberg virus. The interpretation of their distribution is difficult due to the lack of knowledge about their ecology. Previously, soil moisture and especially flooding were identified as important factors that influence the development of several biting-midge species. Therefore, this experimental study addressed the question whether flooding has a negative impact on the development of immature stages of Obsoletus group species. Ten cowpats were collected, and each was divided into four quarters and kept at different moisture regimes in a greenhouse: (1) "dry" (no water added), (2) "control" (regularly moistened), (3) "alternately flooded" and (4) "permanently flooded", to compare *Culicoides* emergence. Flooding had a significant negative impact on the emergence of *Culicoides*. No individuals emerged from the "permanently flooded" treatment and only two individuals were sampled from the "alternately flooded" treatment. In contrast, the total emergence from the non-flooded samples in the "dry" (96 individuals, 38.6% of all *Culicoides*) and "control" (151 individuals, 60.6% of all biting midges) treatments was considerably higher. Biting midges were predominantly identified as *C. dewulfi* (161 individuals, 64.7% of all *Culicoides*) and *C. chiopterus* (63 individuals, 25.3% of all *Culicoides*). There were no significant differences in emergence between the "dry" and "control" treatments. Our results highlight the importance of soil moisture on the distribution of *C. chiopterus* and *C. dewulfi*. Regarding physiological and behavioural

adaptations of other *Culicoides* species, we argue that pupae of *C. chiopterus* and *C. dewulfi* are in danger of drowning when breeding sites are flooded as they cannot float. On the contrary, our results indicate that desiccation might not be harmful to these species.

## Introduction

Several *Culicoides* species are vectors of pathogens [e.g., Bluetongue virus (BTV), Schmallenberg virus (SBV), or African Horse Sickness virus (AHSV)], among which BTV has received the greatest attention in Northern Europe. Bluetongue virus can cause a non-contagious disease of ruminants, resulting in huge economic losses, e.g., the BTV epidemic 2006–2010 led to costs of more than 250,000,000 € in Germany alone (Conraths et al., 2012). Indigenous biting midges of the Obsoletus and Pulicaris group are considered to be the most important vectors (Ander et al., 2012). The recent epidemic caused by SBV again emphasised the importance of biting midges as vectors of pathogens and once again, members of the Obsoletus group were suspected to be the main vectors (De Regge et al., 2012; Rasmussen et al., 2012).

Due to their veterinary importance, many studies have attempted to identify the environmental factors that drive the occurrence of *Culicoides* species (Baylis et al., 1998; Conte et al., 2003). A variety of data types (e.g., temperature, landscape, land cover) have been used to develop species distribution models, however, because of the lack of basic knowledge concerning the ecology of biting midges (e.g., breeding or resting sites), a comprehensive interpretation of the modelling results is often subject to uncertainties. Several studies have demonstrated the need for information on the species to interpret distribution models for *Culicoides*. The interpretation of models for *Culicoides imicola* led to the prediction that areas with an annual rainfall greater than 1000 mm might be unsuitable for *C. imicola* (Wittmann et al., 2001), because the pupae drown when breeding sites are flooded (Nevill, 1967). Another study revealed modelling results that appear contradictory at first sight. Here, a negative impact of cattle density and a positive impact of pasture cover on the species abundance of

*Culicoides impunctatus* were found (Purse et al., 2011), however, due to the knowledge of this species' preference for rush pasture cover (Blackwell et al., 1994, 1999), Purse et al. (2011, p. 174) concluded that the correlations "are probably related to the association of this species in the larval stage with rush pasture cover that arises from light grazing and high soil water content".

Larvae of biting midges belonging to the subgenus *Avaritia* are generally expected to have a slow head-to-tail flexion, in contrast to the serpentine swimming movements of other *Culicoides* species. Furthermore, the pupae are not able to float (Cannon & Reye, 1966). This is interpreted as an adaptation to breeding sites with a relative high viscosity (e.g., dung) and might also explain their breeding site preferences in comparison to other *Culicoides* species. Members of the Pulicaris group are tolerant of, or might even prefer, waterlogged breeding sites, because the pupae can float on the water surface (Nevill et al., 2007; EFSA, 2008). Additionally, there was no negative impact of flooding on the eggs or larvae of seven South African *Culicoides* species and only a negative impact on the pupae of *C. imicola*, which drown under waterlogged conditions (Nevill, 1967).

Controlled experiments of environmental (e.g., soil characters, type of vegetation) and management factors (e.g., manure storage) can help to understand the ecological processes that affect *Culicoides* species distribution in and around farms (Scolamacchia et al., 2013). There still is a deficit of experiments to close the broad gaps of knowledge on the ecology of biting midges. Even the breeding ecology of the exhaustively investigated species *C. imicola*, which is known as the main vector of BTV and AHSV in Southern Europe, the Mediterranean region, and Africa (Mellor et al., 2000), is not fully understood and laboratory studies are particularly lacking (Peters et al., 2013). Soil moisture is expected to be an important factor influencing the occurrence of *Culicoides* species, but the connection between soil moisture and *Culicoides* larval development has not yet received sufficient experimental attention (Mellor et al., 2000), although a deeper understanding would help to interpret different patterns of species distribution.

Due to the current state of knowledge on breeding sites of *Culicoides* species in Europe, members of the Obsoletus group are thought to avoid waterlogged habitats (González et al., 2013). It was supposed that the dung-breeding members of the Obsoletus group (*Culicoides chiopterus* and *Culicoides dewulfi*) are able to colonise the driest habitats, which are occupied by immature *Culicoides* (Kettle & Lawson, 1952). However, this was not deduced from experiments on the impact of flooding on immature stages, but from studies on their breeding habitats (Hill, 1947; Zimmer et al., 2008). Therefore, this study aimed to evaluate the impact of flooding on the development of immature *C. chiopterus* and *C. dewulfi* in cowpats.

## Materials and Methods

On 4 April 2013, we selected 10 cowpats on a farm close to the city of Oldenburg (Lower Saxony, Germany). This farm represents a typical dairy farm situated in rural regions in Northern Germany, with a total area of 195 ha and 100 ha grassland, which is used as pasture or meadow. The pasture from which we collected the cowpats is located at a distance of approximately 250 m from the cowshed and is surrounded by forest, a residential area and a small stream. The pasture had not been used for grazing during the winter, i.e., from October until we took the cowpat samples. Therefore, it is reasonable to assume that the cowpats were approximately 5–6 months old.

From the centre of each cowpat, an area of 14 cm × 14 cm was sampled together with approximately 3 cm of the adjacent soil and was transported to a greenhouse. Preliminary studies demonstrated that cowpats can differ strongly in the abundance of *Culicoides*. Therefore, the sampled cowpat areas were divided into four equally sized, quadratic samples (7 cm × 7 cm) and a different treatment was applied to each sample: (1) “dry”: no water was added to the samples, (2) “control”: each sample was moistened with a pressure spray device every three days (ca. 10 mL tap water per quarter), (3) “alternately flooded”: each sample was alternately flooded with tap water (for 24 h, water level ca. 11.5 cm) or not flooded (for 48 h), and (4) "permanently flooded": each sample was permanently flooded with tap water (water level ca. 11.5 cm). Water loss due to evaporation was replaced daily with tap water.

Samples were placed under emergence traps and were covered with a collecting jar. The collecting jars were filled with saturated salt solution to catch and preserve the emerging insects (Fig. D.3). The emergence traps of the "alternately flooded" and "permanently flooded" treatments were placed in plastic trays (60 cm × 40 cm × 40 cm, five emergence traps per tray, two trays per treatment). Four small holes (Ø 1 cm) were drilled into the base of the emergence traps and were covered with gauze (mesh size: 125 µm). These holes allowed the filling and draining of the emergence traps with tap water, but the gauze prevented the cowpat material and its coloniser to be washed out.

Collecting jars of the emergence traps were emptied daily. Sampling, refilling and draining were conducted at the same time (ca. 14–16 h) for all treatments. Oxygen and water temperature in the water were measured daily in the "permanently flooded" treatment and after refilling and before draining in the "alternately flooded" treatment (WTW Oxi 330i, Sensor CellOx 325, Germany). One emergence trap in each treatment was randomly selected to record temperature data (logger: HOBO Pendant® Temperature/Alarm Data Logger 8-K, ONSET, Bourne, MA, USA). Another data logger recorded the room temperature. The temperature was measured every 4 h. After 31 days from when the last insect emerged from the cowpats (28 May 2013), the experiment was stopped. The samples were sorted and biting midges were determined to the group level (Obsoletus group, Pulicaris group or other Ceratopogonidae). Males and females of the Obsoletus group were determined to the species level based on morphological characters (Campbell & Pelham-Clinton, 1960). Data analysis was conducted with R (R Core Team, 2014), using the package (**?**) for graphs.

## Results

We collected a total of 249 *Culicoides* biting midges belonging exclusively to the Obsoletus group. All except for one cowpat (90%) were colonised by biting midges. The emergence per cowpat showed a high variability (mean = 24.9, max = 123, min = 0). The majority of the 161 individuals (64.7%) were determined as *C. dewulfi* and a further 63 individuals (25.3%) as *C. chiopterus*. Eleven male individuals (4.4%) were identified as *C. scoticus*. Three female individuals (1.2%) were identified as either *Culicoides obsoletus* or *C. scoticus* (Fig. D.4), as the morphological differentiation of these two species is difficult. A further 11 male individuals (4.4%) were not determined to species level because their hypopygia were destroyed.

Flooding proved to have a significant impact on the development of *Culicoides* from the samples (Fig. D.4). Most *Culicoides* emerged from samples in the “dry” (96 individuals, 38.6%) and “control” (151 individuals, 60.6%) treatments, whereas two individuals (0.8%) emerged from the “alternately flooded” treatment and none from the “permanently flooded” treatment. There were no significant differences between the numbers of *Culicoides* emerging from the “dry” and the “control” treatment (U-test, $p > 0.05$; Figs. D.4 and D.5). All of the three detected *Culicoides* species emerged from the “dry” and “control” treatment, but only *C. chiopterus* emerged from the “alternately flooded” treatment. There were no significant differences in *C. chiopterus* and *C. dewulfi* emergence between the “dry” and “control” treatment (U-tests, $p > 0.05$) (Figs. D.4 and D.5). Due to the low number of *C. scoticus*, we did not apply a statistical test to compare “dry” and “control” treatments for this species.

The daily mean temperatures in the emergence traps fluctuated between 15 and 20 °C (mean = 18.5 °C, SD = ± 2.2 °C), but were similar between the four treatments and were close to the room temperature. Slightly higher daily mean temperatures occurred in the “permanently flooded” treatment (Fig. D.6). Despite high fluctuations in oxygen saturation in the “permanentlyflooded” treatments,the oxygen saturation differed between the trays, where we placed the emergence traps to achieve flooding. Oxygen saturation in one of the trays dropped significantly from about 100% to approximately 50% during the first few days, but increased during subsequent days and often exceeded 100% (Fig. D.7). In the other trays, oxygen saturation decreased much more strongly and did not exceed 80% maximum oxygen saturation until the last day of biting midge emergence (Fig. D.7).

## Discussion

One main outcome of the present study was that *C. chiopterus* and C. dewulfi were not able to survive flooding of their breeding substrate. Even alternate flooding of these substrates every 24 h almost completely prevented the emergence of *Culicoides* biting midges. From our experiment, we cannot conclude whether the strong reduction in emergence is caused by a high mortality of larvae, pupae or both. However, the inability of pupae to float is considered to be the most important reason for the breeding-site selection by *Culicoides* species of the subgenus Avaritia, habitats of which are described as moist but not waterlogged (Nevill et al., 2007). This agrees with a literature review according to which the members of the Obsoletus group (*C. chiopterus*, *C. dewulfi*, *C. obsoletus* and *C. scoticus*) in the subgenus *Avaritia* (Borkent, 2014) do not occur in waterlogged habitats (González et al., 2013).

The oxygen saturation in the "permanently flooded" treatment strongly decreased at the beginning of the experiment, which might explain the failure of development. However, it can be expected that dung-breeding *Culicoides* biting midges are able to tolerate hypoxic or hypercapnic conditions, because the oxygen content within cowpats can also be low (1–2%) and that of carbon dioxide can be high (25–30%) (Holter, 1991). One counter-argument is the vertical distribution of immature stages of *Culicoides*. Eggs are laid on top of the cowpat (Bishop et al., 96b) and larvae/pupae prefer the top layer of breeding substrates (Blackwell & King, 1997; Kettle, 1977; Mullens & Rodriguez, 1992; Zimmer et al., 2008), where the oxygen concentration is higher (Holter, 1991). Additionally, while the cowpats were ageing, the oxygen concentration quickly increased, whereas that of carbon dioxide decreased (Holter, 1991). Thus, dung-breeding *Culicoides* do not have to deal with very low levels of oxygen or very high levels of carbon dioxide during immature development.

*Culicoides brevitarsis* showed a vertical movement of larvae and pupae towards areas with higher moisture in cattle dung over time (Bishop et al., 96b). Furthermore, experimental studies identified a negative correlation between the water content of dung and the abundance of *C. brevitarsis*, but these results were not supported by field studies (Campbell, 1974; Bishop et al., 2005). The prevalence of this species was not limited by its moisture content (Campbell, 1974), which implies that *C. brevitarsis* is highly adapted to dry habitats. We also did not find any statistically significant differences between cowpats in the "dry" and the "control" treatment (regularly moistened). Therefore, besides the ability of short-range migration towards zones in the cowpat with higher moisture, we assume that coprohphilic *Culicoides* possess physiological adaptations against desiccation (e.g., thickness of the cuticle), which otherwise might be disadvantageous (e.g., inhibiting oxygen uptake) under flooded conditions.

Dung-breeding *Culicoides* (e.g., *C. brevitarsis*) appear to have specific physiological and behavioural adaptations for breeding habitats that are not waterlogged: the pupae are not able to float and the larvae do not show a serpentine swimming characteristic (Cannon & Reye, 1966). Similarly, the eggs and larvae of *C. imicola* can survive flooding, but pupae drown (Nevill, 1967). In contrast, the pupae of aquatic species (e.g., Pulicaris group) are probably able to swim and do not drown when submerged and are therefore able to colonise waterlogged breeding sites (EFSA, 2008). Specific physiological or behavioural adaptations of immature stages might be the key factor in explaining the differences in the breeding site selection between different *Culicoides* species (Nevill et al., 2007), e.g., the females might use volatile chemical cues to identify suitable breeding sites. Floating *Culicoides* pupae use gaseous inclusions within the cephalothorax for buoyancy (Dyce & Murray, 1966). Among the East Australasian *Culicoides* species studied by Dyce & Murray (1966), three types of pupal physiological and behavioural adaptations to different breeding sites were distinguished: pupae of Type A are able to float after flooding, but are not able to submerge again and breed on the margins of still and slow flowing waters, pupae of tree-hole breeding species of Type B can variably float or submerge, and pupae of Type C remain submerged and burrow in the substrate as adaption for breeding sites in estuarine sands, which are regularly disturbed by flooding or desiccation depending on the tide. Additionally, a Type D was described for *C. imicola*, breeding in moist but not waterlogged soils (Foxi & Delrio, 2010), whose pupae cannot float or burrow and lie on the substratum and drown if flooded (Nevill, 1967). According to the results of this study, *C. chiopterus* and *C. dewulfi* also probably belong to Type D.

In lowland areas of Northern Europe, agricultural intensification is strongly connected to large-scale drainage via ditches (e.g., 300,000 km in the Netherlands) (Verdonschot et al., 2011). These ditches serve to drain rainwater or seepage from groundwater into rivers and lakes, thus resulting in a lower risk of flooding of farmland. Due to the sensitivity of *C. chiopterus* and *C. dewulfi* to flooding, recent agricultural practices might facilitate the populations of both species. Coprophilic *Culicoides* species are suspected to be common species and should be present if cow dung is available (Cannon & Reye, 1966), thus, the species distribution is probably independent from land cover or edaphic conditions. In contrast, both species do not necessarily show an equal distribution (Nielsen et al., 2010) and edaphic variables were found to significantly affect the abundance of both species (Scolamacchia et al., 2013). Our study revealed a clear negative impact of flooding for *C. chiopterus* and *C. dewulfi*. As for *C. imicola*, it is therefore reasonable to expect that both species regularly avoid flooded breeding sites (Foxi & Delrio, 2010). Therefore, soil moisture probably is an important factor for species of the Obsoletus group and should be included in species distribution models, as it was successfully demonstrated for *C. imicola* (Peters et al., 2013).

## Acknowledgments

We would like to give special thanks to Esther Timmermann, technical assistant in our working group, who had the initial idea for this study, and for her help during this study. Thanks to Prof. Dr. Dirk C. Albach (Head of the Research Group Biodiversity and Evolution of Plants and Director of the Botanical Garden, University of Oldenburg, Germany) and Holger Ihler (Botanical Garden, University of Oldenburg, Germany) for their permission to conduct this experiment in the greenhouse of the botanical garden. Finally, sincerest thanks are given to the farmer family who supported our study.

## Figures

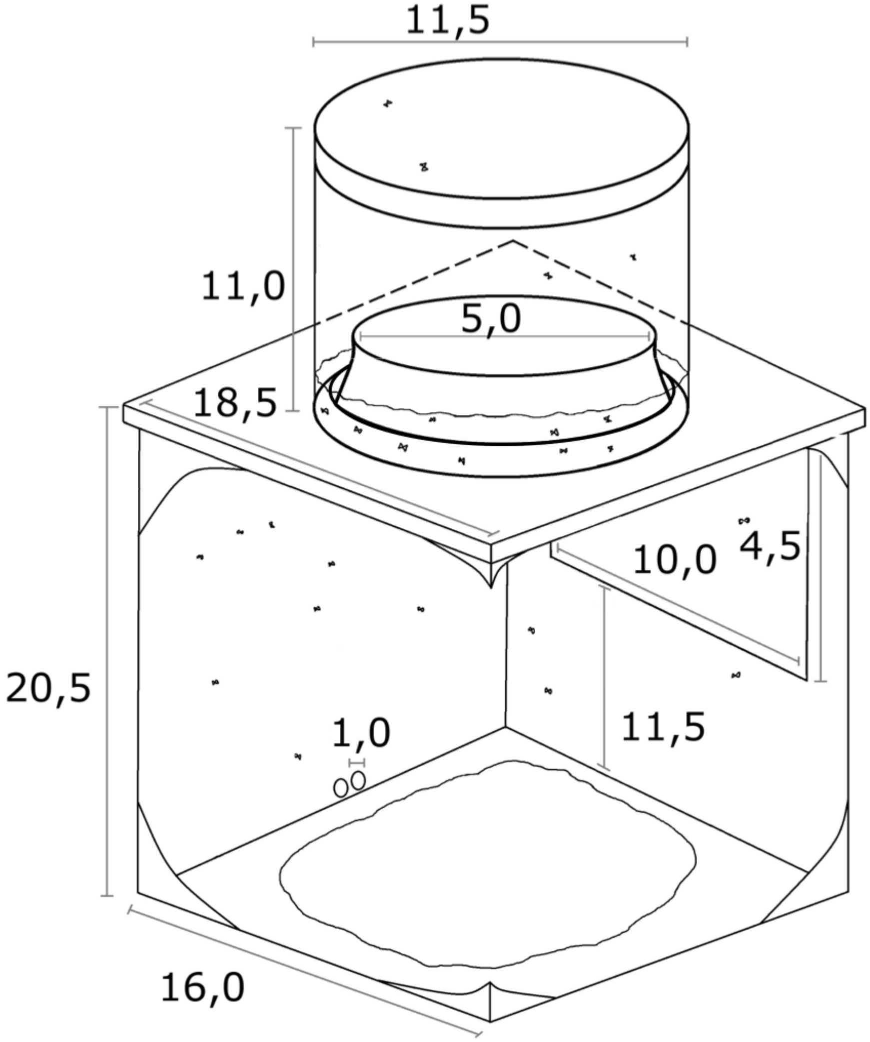

Figure D.3: Schematic drawing of an emergence trap [area = 256 cm2]. Emerging insects fly towards the light and are trapped ina saturated salt solution. Eachemergence traphas two aeration windows covered withgauze (mesh size = 125 m) to ensure gas exchange and prevent warming within the trap. Indications of size are given in cm. At the bottom of the emergence traps, four small holes (Ø 1 cm) were drilled to facilitate filling and draining and covered with gauze (mesh size = 125 m) to prevent the cowpat material and its coloniser to be washed out.

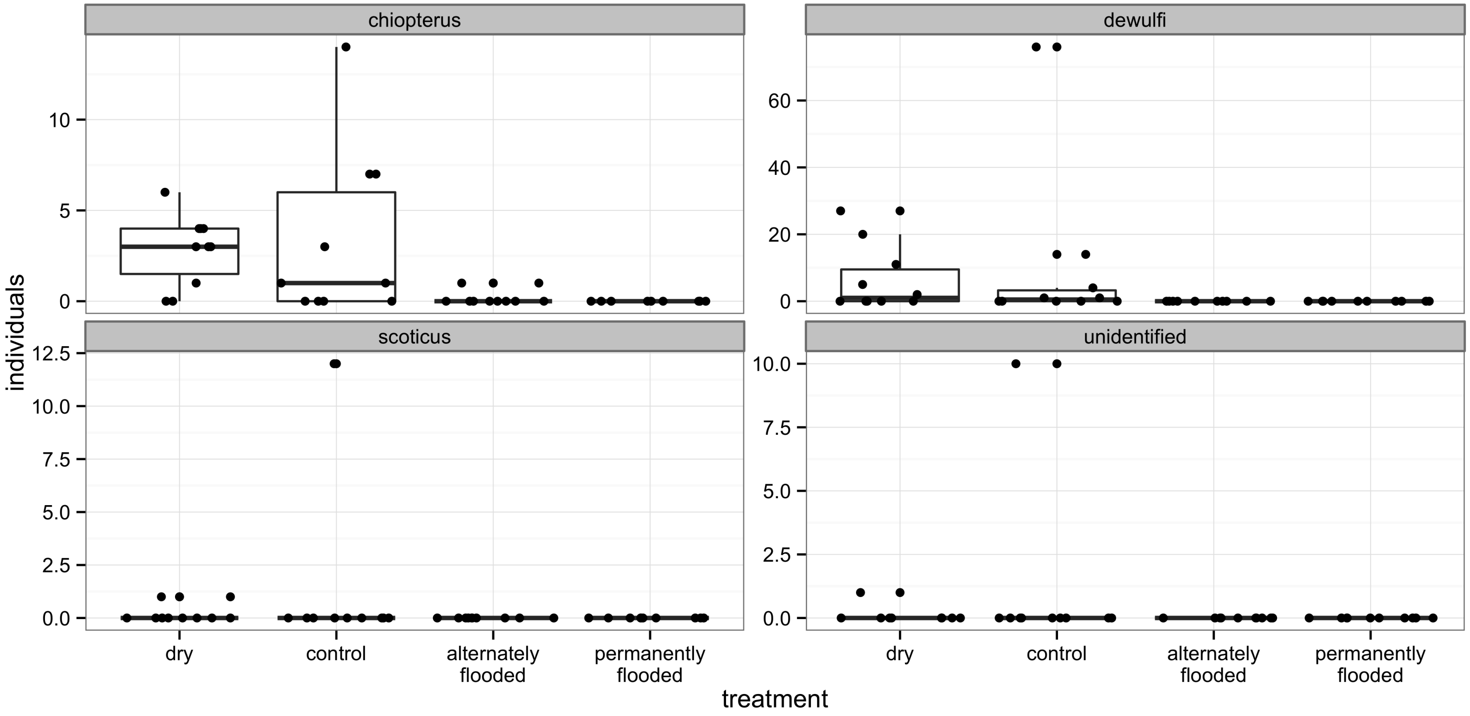

Figure D.4: (a) The number of individuals of *Culicoides* species emerging from samples in the four treatments. A random jitter was added to visualise the raw data.

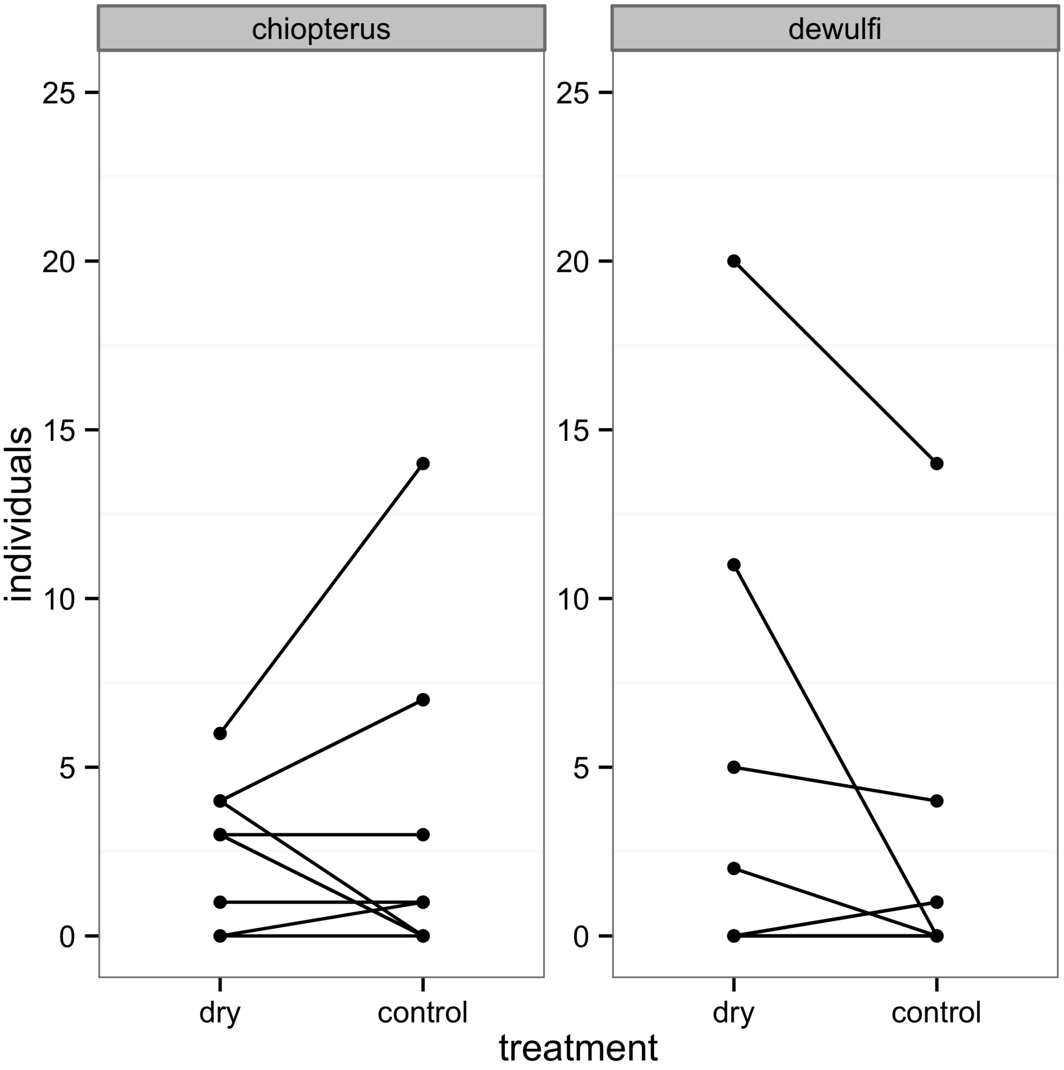

Figure D.5: The number of individuals of *Culicoides chiopterus* and *Culicoides dewulfi* emerging from samples in the "dry" and "control" treatments. The lines connect samples from the same cowpat.

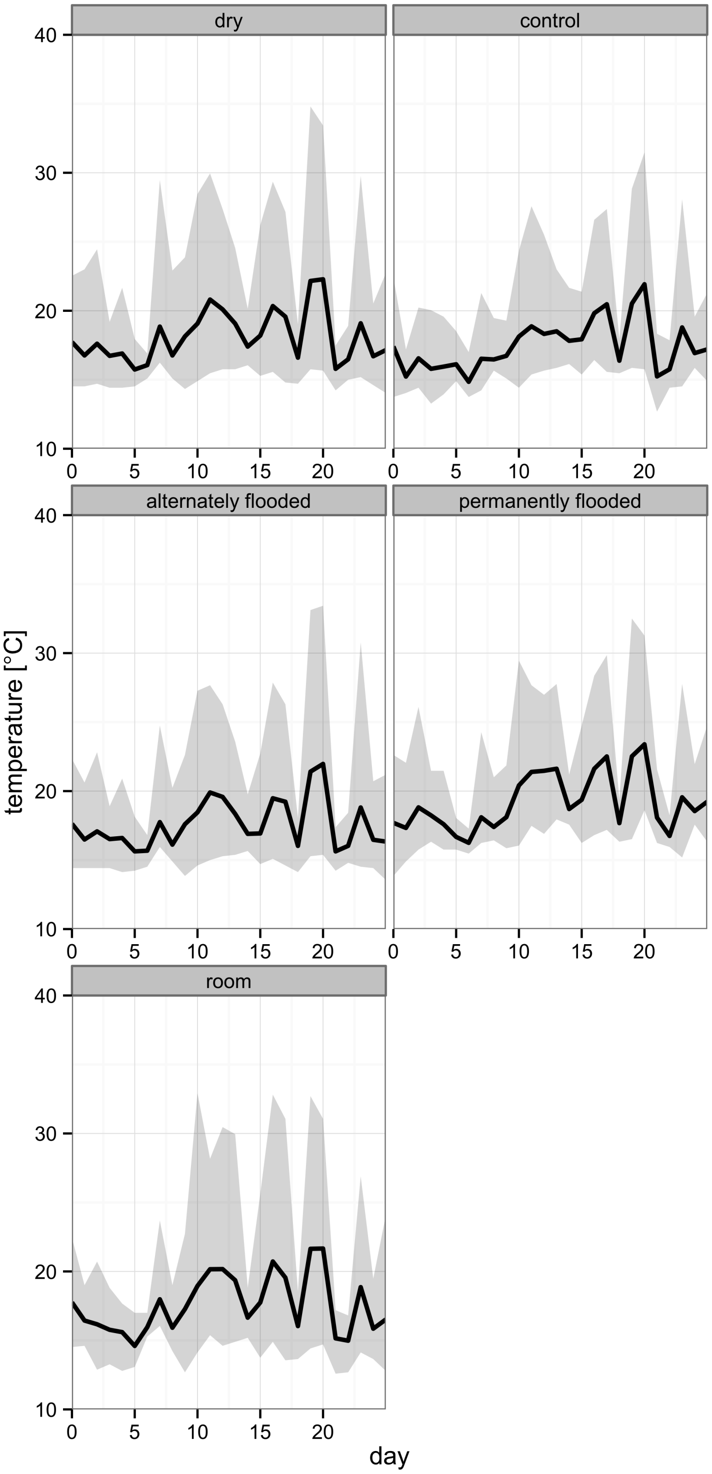

Figure D.6: Daily mean temperature, with daily maximum and minimum values measured with data loggers in the emergence traps of the four treatments, and room temperature. Data from the beginning of the experiment until the day of the last emergence of *Culicoides* biting midges are shown.

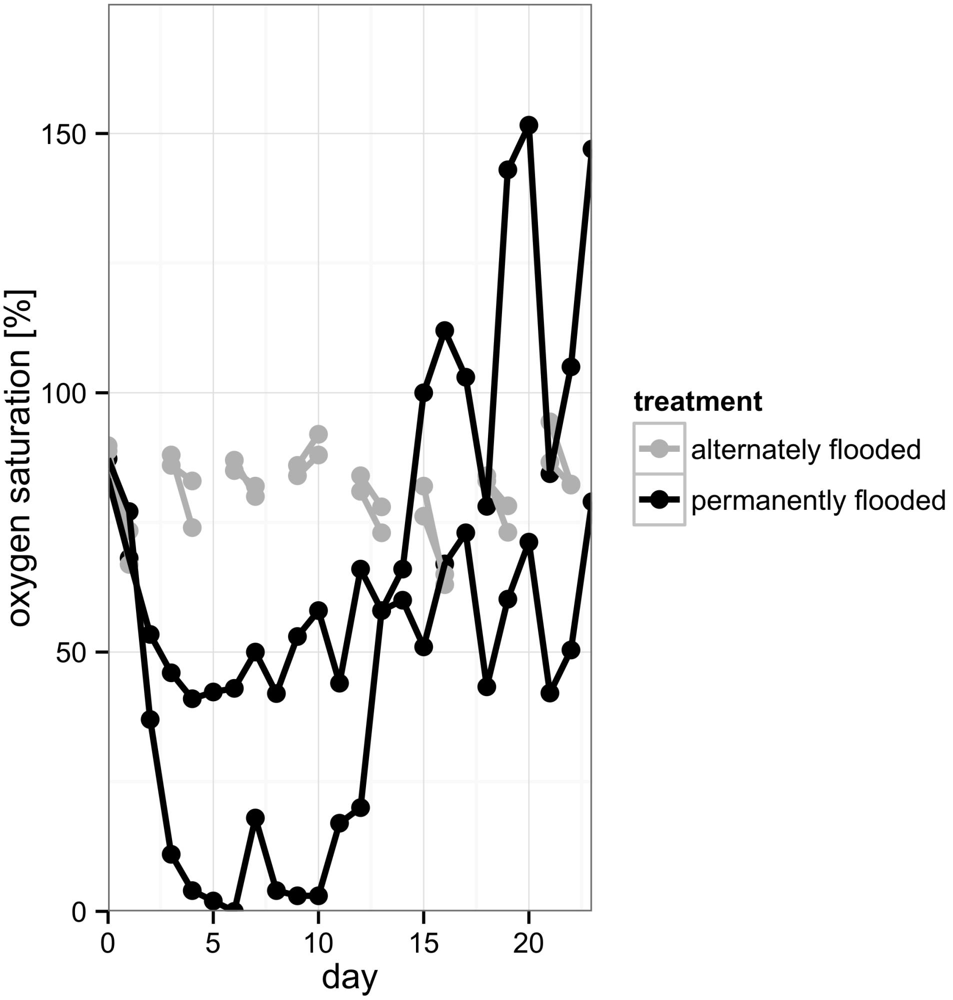

Figure D.7: Oxygen saturation in the water. Daily measurements in both plastic trays of the "permanently flooded" treatment and both plastic trays of the "alternately flooded" treatment, where oxygen saturation was measured immediately after flooding and immediately before draining (24 h period). Data from the beginning of the experiment until the day of the last emergence of *Culicoides* biting midges are shown.

## D.3 Effects of temperature and photoperiod on the development of overwintering immature *Culicoides chiopterus* and *Culicoides dewulfi*

submitted to Veterinary Parasitology: **Lühken, R.**, Steinke, S., Hoppe, N., & Kiel, E. (submitted). Effects of temperature and photoperiod on the development of overwintering immature *Culicoides chiopterus* and *Culicoides dewulfi*.

### Abstract

In areas with harsh winters, low numbers to zero adult biting midges were recorded during the winter period by a population peak in the subsequent spring. This study determined whether temperature, photoperiod, or the combination thereof affect the development of overwintering immature dung breeding *Culicoides*, resulting in this peak. Temperature had a significant impact on the development period of *Culicoides chiopterus* (Meigen), 1830 and *Culicoides dewulfi* Goetghebuer, 1936. In the trials, with constant high temperature, emergence occurred shortly after the beginning of the experiment (mean=day 9). In contrast, no individuals emerged from the other two trials, as long as the temperatures were below 10°C. In these trials, the emergence of *Culicoides* started when the temperature exceeded 20°C for some days (mean=day 33). Emergence decreased when the temperatures dropped, and vice versa when the temperatures increased again for some of the samples when temperatures increased. There was no significant difference between the two photoperiods (February or April day length; 9 h:15 h [light:dark] vs. 13 h:11 h). Our results highlight the importance of temperature on the spring emergence of *C. chiopterus* and *C. dewulfi*, both of which did not respond differently to the four temperature-photoperiod trials.

## Introduction

Several *Culicoides* species all over the world are known to be vectors of a variety of pathogens (e.g. bluetongue virus (Mellor et al., 2000), African horse sickness virus (Mellor et al., 2000) or Schmallenberg Virus (De Regge et al., 2012; Rasmussen et al., 2012). For example, the bluetongue epidemic in Europe caused losses running into several hundred million euros (Velthuis et al., 2010; Conraths et al., 2012). Therefore, several studies were conducted, aiming to determine the distribution and phenology of species relevant in terms of the disease's epidemiology (Hoffmann et al., 2009). This data can, for example, be used for the risk assessment of vector-borne pathogen transmission (Brugger & Rubel, 2013). At least in the temperate climate zone, these studies found a recurrent phenological pattern with low numbers or a total absence of *Culicoides* adults during the winter followed by a population peak in the spring. This pattern occurred in different taxa worldwide, e.g. for the Obsoletus group or Pulicaris group in Germany (Hoffmann et al., 2009), *Culicoides impunctatus* and the *Culicoides pulicaris* in Scotland (Searle et al., 2012), or *Culicoides biguttatus* in the USA (Root & Gerhardt, 1991). However, this observation might be affected by the sampling method, i.e. resulting most often from the light-trap collections, which are substantially biased for the collection of *Culicoides chiopterus* (Carpenter et al., 2008).

Although researchers paid only scant attention to the overwintering ecology of biting midges, it is generally expected that most species in areas with harsh winter conditions hibernate in the fourth larval stage (Szadziewsi et al., 1997). For these species, individuals emerging from the overwintering larval population constitute the first adult generation in the year. However, it is unclear what factor or combination of factors (e.g. temperature, photoperiod) regulate the development of the overwintering stages resulting in this peak.

The overwintering capability of vectors of the bluetongue virus or Schmallenberg virus is an important factor influencing the disease's epidemiology (Koenraadt et al., 2014). An understanding of the factors regulating the overwintering of immature biting midges might help in predicting the vector phenology and the associated viruses more precisely. It may, at least, allow for the prediction of the first population peak in spring, which was also demonstrated to affect the timing of further population peaks in the year (Lysyk, 2007; Searle et al., 2012).

Examples on crop pest species demonstrated that the knowledge of threshold temperatures/photoperiod values or degree-day models are useful in predicting the populations' peaks, as well as to time control measurements most effectively (Collier & Finch, 1985; Kim et al., 2000). The usefulness of threshold temperatures and degree-days to forecast biting midge development was, for example, indicated by studies on *Culicoides variipennis* (Vaughan & Turner, 1987) and *Culicoides impunctatus* and *Culicoides pulicaris* (Searle et al., 2012).

Temperature and photoperiods are common factors for the induction, maintenance and completion of dormancy in insects (Beck, 1962; Tauber & Tauber, 1976). Various studies indicate that temperature significantly affect the developmental period of immature biting midges. *Culicoides* emerged from the substrates in the winter period if the samples were exposed to higher temperatures in the laboratory (Foxi & Delrio, 2010; Zimmer et al., 2010; Steinke et al., 2014). Additionally, the presence of nulliparous *Culicoides* during the winter indicates low, but continuous, development to adulthood during this season of the year (e.g. Hoffmann et al., 2009). If so, *Culicoides* development should not depend on the photoperiod. In contrast, Isaev (1985) found a combined impact of warm temperature and long photoperiod on the pupation rate of *Culicoides odibilis*.

However, the driving factors for the development of overwintering *Culicoides* resulting in spring emergence are unknown. Therefore, this study aims to compare the emergence patterns of *Culicoides* from cowpats that were experimentally exposed to different temperature-photoperiod trials. It raised the question of whether different temperature-photoperiod trials, i.e. short day length (9 h:15 h [light:dark]) vs. long day length (13 h:11 h [light:dark]) and high temperature ($> 20$°C) vs. low temperature ($< 10$°C), will affect the development of overwintering immature *Culicoides* differently. Due to the preceding studies summarised above, we assumed that high temperatures, but not photoperiod, triggered the emergence of *Culicoides* from cowpats.

## Material and Methods

On 3 February 2014, we selected 15 cowpats on a farm close to the city of Oldenburg (Lower Saxony, Germany; latitude 53.1, longitude 8.1). This farm represents a typical dairy farm situated in rural regions in Northern Germany, with 195 ha and 100 ha grassland used as pasture or meadow. The pasture where the cowpats were collected is located approximately 250 m from the cowshed, surrounded by a small forest, a residential area, and a small stream. As cattle had not grazed on this pasture during the winter, i.e., from October until February, it was assumed that the sampled cowpats were four months old.

Initially, a small substrate sample (5 cm x 5 cm) was collected from the margin of the 15 cowpats, while the rest remained in the field. These rim-samples were analysed using the Berlese method (Steinke et al., 2014) to evaluate whether or not the cowpats were colonised by *Culicoides* larvae. Four days later (7 February 2014), an area of 14 cm × 14 cm was sampled from the centre of each of the nine colonised cowpats. These core-samples were taken, along with approximately 3 cm of the soil underneath for moisture-regulation, and then transported to the laboratory. Preliminary studies demonstrated that cowpats could differ strongly in the density of immature *Culicoides*. Therefore, the sampled cowpat areas were divided into four equally sized, quadratic samples (7 cm × 7 cm), which were exposed to a different temperature-photoperiod trial (Table D.1).

Samples were placed underneath emergence traps and were covered with a collecting jar (Steinke et al., 2014). The collecting jars were filled with a saturated salt solution to collect and preserve the emerging insects. The emergence traps allocated to one trial were placed in one separate lightproof wooden box (length 118.5 cm × width 51.5 cm × depth 50 cm). Each of the wooden boxes was closed with a wooden cover, and a fluorescent lamp (Osram®, Biolux T8 36W, length 121 cm) was included inside the box. The boxes were opened only to empty the collecting jars and to test the moisture of the samples.

The collecting jars of the emergence traps were emptied three days a week (Monday, Wednesday, Friday), resulting in sampling intervals of two and three days. Once a week, the samples were moistened with tap water (approximately 20 ml per sample), e.g. to prevent drought-induced pupation or emergence. Sampling and moistening was conducted at the same time around noon for all trials. Data logger (Hobo U23 Pro v2 Data Logger, Bourne, MA, USA) recorded the air temperature in each wooden box at 4 h intervals. 12 days after the last biting midge emergence was recorded (9 April 2014), the experiment was terminated. The samples were sorted and biting midges were identified to the group level (Obsoletus group, Pulicaris group, other Ceratopogonidae). Males and females of the Obsoletus group were determined to the species level based on morphological characters (Campbell & Pelham-Clinton, 1960).

Data analysis was conducted with R (R Core Team, 2014). We followed a recently proposed method to analyse the phenology of insects, which was used to identify the timing of the peak of emergence (Searle et al., 2012). The mean number of emerged individuals was calculated for each day of the sampling interval and a three-day moving average of emergence was calculated for each sample and for each species and sex. A separate generalised additive model with a poisson distribution, log link and spline smoothing was fit for each sample using the R package mgcv (Wood, 2011). Therefore, emergence data were rounded to integer values in order to provide whole the number necessary for the poisson models. The degree of smoothing was selected automatically using generalised cross-validation. The findPeaks function from the quantmod package (Ryan, 2013) was used to identify emergence peaks in the predicted values. Mean and 95% confidence intervals were calculated for the first emergence, the last emergence, the peak of emergence and the total number of emerging individuals for each sex of each species using the summarySEwithin function from the Rmisc package (Hope, 2013), with the cowpats as subjects and the trial as a within-subjects variable. Non-overlapping 95% confidence intervals were considered a sign of significant difference. We used the package ggplot2 (Wickham, 2009) for graphs and gridExtra (Auguie, 2012) for multi-panel graphs.

## Results

A total of 765 *Culicoides* biting midges emerged from the cowpats that were exposed to the four temperature photoperiod trials. All adults belonged to the Obsoletus group. The majority of the 564 individuals (73.7%) were determined to be *Culicoides chiopterus* and a further 201 individuals (26.3%) were *Culicoides dewulfi*.

Temperature proved to have a significant impact on the development period of both species. In the constant warm environment at temperatures around 22°C (trials A and B), the first specimens of *C. chiopterus* and *C. dewulfi* emerged between day 5 and day 18 (mean=day 9) of the experiment, while the last individuals emerged between day 11 and day 31 (mean=day 17) (Fig. D.8, Fig. D.10). The peak of emergence occurred between day 9 and day 20 (mean=day 13) (Fig. D.11). The number of *C. dewulfi* adults showed a second peak in trial B (Fig. D.11). However, the total emergence of *C. dewulfi* was relatively low compared to *C. chiopterus* (Fig. D.12). Therefore, this observation should be treated with caution, because small changes in the emergence of this species might result in the presence of or lack of emergence peaks and valleys.

In contrast, no *Culicoides* emerged from the samples in trials C and D, as long as the temperatures were below 9°C. Emergence started in these trials between day 30 and day 39 (mean=day 33), when temperatures rose above 24°C around day 30, and emergence finally ceased between day 37 and day 60 (mean=day 45) (Fig. D.9, Fig. D.10). The first peak of emergence occurred between day 34 and day 42 (mean=day 38) (Fig. D.11). In both trials (C and D), fewer adults emerged when the temperatures decreased to 20°C around day 40, but in a few samples, a second emergence peak for *C. chiopterus* occurred between day 47 and day 59 (mean=day 54) when the temperatures increased again.

The two different photoperiods tested in our experiment did not cause different emergence patterns (Fig. D.8, D.9, D.10, D.11, D.12). Though the first emergence, last emergence and the peak of emergence sometimes appeared to be a little bit earlier in the long day trials (A vs. B and C vs. D), these differences were not significant.

Cowpats were divided into four samples and a different photoperiod-temperature trial was applied to each sample since preliminary studies demonstrated that cowpats could differ strongly in the density of immature *Culicoides*. However, there were no significant differences in the total number of emerging individuals between the four trials for either sex of both species (Fig. D.12). Thus, the impact of different colonisation densities on the study results can be regarded as negligible.

## Discussion

At least in the temperate climate zone, there is a recurrent phenological pattern, with low numbers or the absence of *Culicoides* adults during the winter, followed by a population peak in spring (Hoffmann et al., 2009). However, the explicit factors driving the development of overwintering *Culicoides* resulting in this spring emergence are still unknown. Photoperiod and temperature induce, maintain, or terminate dormancy in many insect species (Beck, 1962; Tauber & Tauber, 1976). Indeed, this assumption, in general, is also supported for biting midges (Isaev, 1985; Searle et al., 2012). However, our study tried to identify the driving factors in a controlled experiment.

Our results gave evidence that temperature, rather than photoperiod, regulates the emergence peak in the spring for *C. chiopterus* and *C. dewulfi*. According to our results, immature specimens in the cowpats developed to adults in the laboratory at high temperatures ($>$ 20°C, independent of the photoperiod. The February or April day length in Northern Germany (9 hours daylight vs. 13 hours daylight) simulated in our experiments did not affect the timing of the first nor did it affect the peak of emergence. In contrast, with the restriction that the data were based on light-trap data, Searle et al. (2012) demonstrated a positive correlation between the day of the year with eight of daylight and the first population peak of *Culicoides pulicaris* and *Culicoides impunctatus*.

However, the connection between the development of immature biting midges under different photoperiod trials has rarely been investigated experimentally, and we compared only two different photoperiod trials for overwintering immature *Culicoides* collected from the field all at the same date. For example, the correlation with day length, demonstrated by Searle et al. (2012), and the results from our experiments can be brought into alignment. If we assume that overwintering biting midges are in diapause and if a critical photoperiod of around eight hours of daylight is supposed to be the relevant stimulus, day lengths shorter than the critical photoperiod should maintain diapause, while day lengths longer than the critical photoperiod should terminate diapause (Tauber & Tauber, 1976).

The relationship between temperature and the development of immature *Culicoides* was studied in more detail (Kitaoka, 1982; Vaughan & Turner, 1987; Allingham, 1991). In our study, we recorded the development through adulthood at high temperatures. This finding is in concordance with several emergence studies detecting adults, when substrate samples were transferred to higher temperatures in winter (Foxi & Delrio, 2010; Zimmer et al., 2010; Steinke et al., 2014).

This effect of higher temperature is also supported by various laboratory studies on the life-history parameters of immature biting midges. Veronesi et al. (2009) determined a distinct temperature range, with lower and upper temperatures between 26°C and 33°C, to limit the development of larvae of *Culicoides brevitarsis*. Temperature dependence has also been published for immature *Culicoides imicola*, with a shorter development period at higher temperatures (Allingham, 1991). Moreover, the developmental period of *Culicoides variipennis*, *Culicoides arakawae*, and *Culicoides maculatus* have been explained with different temperature thresholds (Kitaoka, 1982; Vaughan & Turner, 1987).

Our experiment supports studies that used temperature thresholds and degree-days to predict *Culicoides* development period and emergence (Vaughan & Turner, 1987; Searle et al., 2012). In trials C and D, emergence was limited to the time with higher temperatures. The emergence in both trials decreased when the temperatures dropped and emergence increased again for some of the samples when the temperatures increased. However, from our experiment, we cannot judge the lower or upper temperature threshold for dormancy, nor can we conclude whether or not larval *Culicoides* are capable of continuing development but have a shorter development period at low temperatures.

Our results might also explain the low but regular trapping of biting midges during winter light-trap surveillance (Hoffmann et al., 2009). As immature *Culicoides* seemed to depend on temperature, but not on day length, it should generally be possible to continue and complete development in the winter period as long as the temperatures are high enough. Although this seems unlikely for larvae developing in cowpats, which are exposed to harsh winter temperatures on pastures, it should be possible in habitats with more favourable temperature conditions, like indoor breeding sites or dung heaps (Husted, 1994). This also supports the hypothesis that the first *Culicoides* emerging in the year might originate from indoor breeding sites or dung heaps (Harrup et al., 2014).

Although our data indicated a clear impact of temperature but no effect of the two different photoperiods studied, further questions remain unanswered, e.g. the diapause mechanism allowing overwintering larvae of *Culicoides* to withstand harsh winter conditions. As in other taxa (Belozerov, 2008), eco-physiological information on overwintering *Culicoides* is lacking. Thus, we do not know if the immature biting midges we collected in February were in diapause (controlled endogenously) or in quiescence (controlled exogenously). Such detailed information would allow for a more accurate understanding of the *Culicoides* spring phenology. Furthermore, knowledge on the impact of the winter development of immature stages on the vector population is vague. However, this knowledge is essential in order to understand the ecology of the biting midge vectors and associated pathogens. For example, overwintering blackfly larvae (Diptera: Simuliidae) are larger adults resulting in a higher survival rate, dispersal ability and reproduction rate of adults, which in turn could result in a higher vector competence (summarised by Moor, 1982).

## Acknowledgements

We would like to give special thanks to Günter Lühken who donated the material and built the wooden boxes. Furthermore, sincerest thanks are given to the organic cattle farm Junge who again supported our studies.

# Table

Table D.1: Temperature-photoperiod trials exposed to the samples from each cowpat.

| **Trial** | **Temperature** | **Photoperiod** |
|---|---|---|
| **A** | Constant high temperature (> 20°C) | Long day photoperiod, April day, 13 h:11 h [light:dark] |
| **B** | Constant high temperature (> 20°C) | Short day photoperiod, February day, 9 h:15 h [light:dark] |
| **C** | Constant low temperature (< 10°C) at the beginning of the experiment and variable higher temperatures (> 15°C) at the end of the experiment | Long day photoperiod, April day, 13 h:11 h [light:dark] |
| **D** | Constant low temperature (< 10°C) at the beginning of the experiment and variable higher temperatures (> 15°C) at the end of the experiment | Short day photoperiod, February day, 9 h:15 h [light:dark] |

## Figures

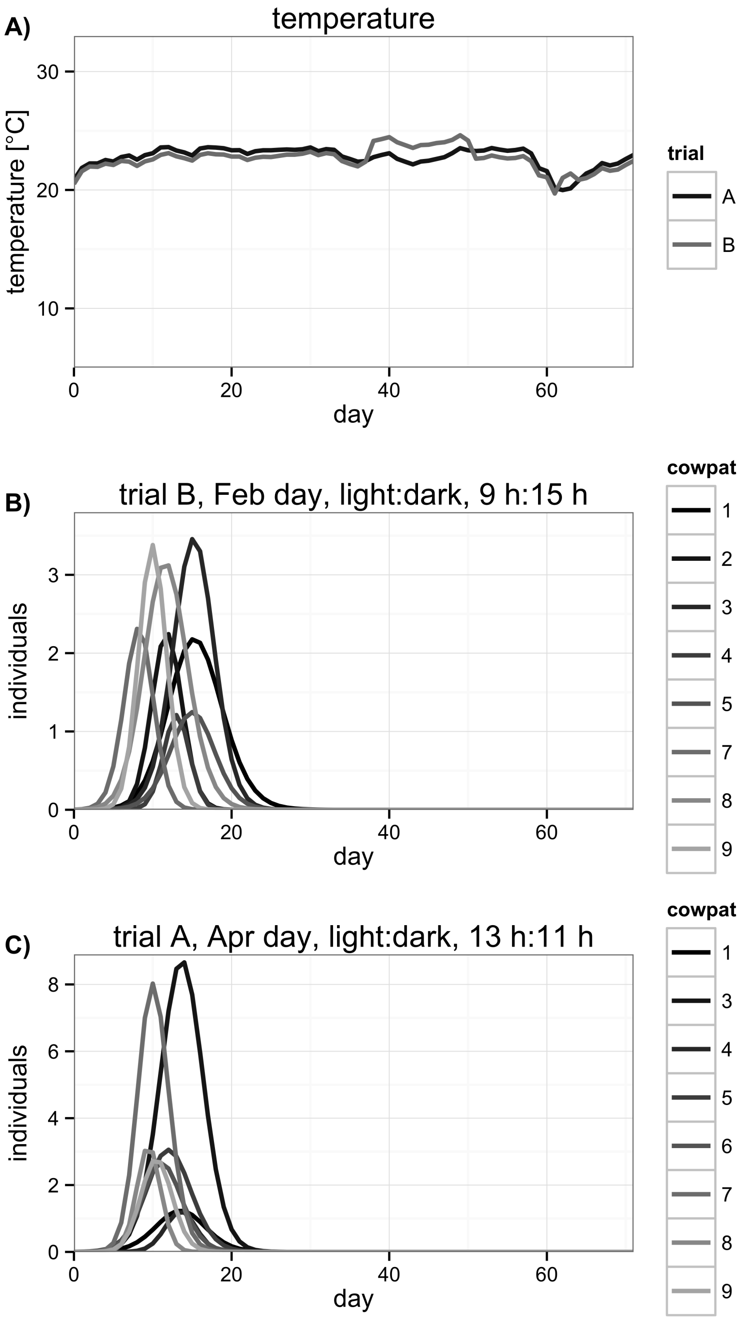

Figure D.8: **A:** Mean temperature in the wooden boxes of trials A and B, **B:** Fitted generalised additive models for the emergence of *C. chiopterus* females from the cowpats in the temperature-photoperiod trial B. **C:** Fitted generalised additive models for the emergence of *C. chiopterus* females from the cowpats in the temperature-photoperiod trial A.

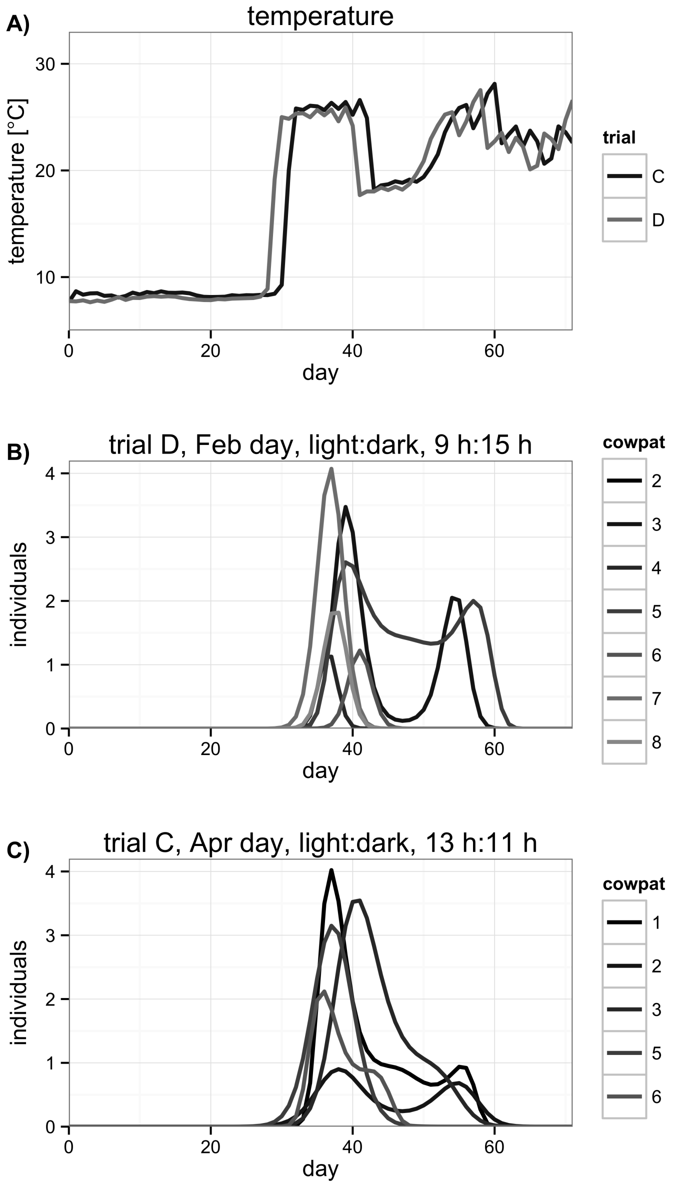

Figure D.9: **A:** Mean temperature in the wooden boxes of trials C and D, **B:** Fitted generalised additive models for the emergence of *C. chiopterus* females from the cowpats in the temperature-photoperiod trial D. **C:** Fitted generalised additive models for the emergence of *C. chiopterus* females from the cowpats in the temperature-photoperiod trial C.

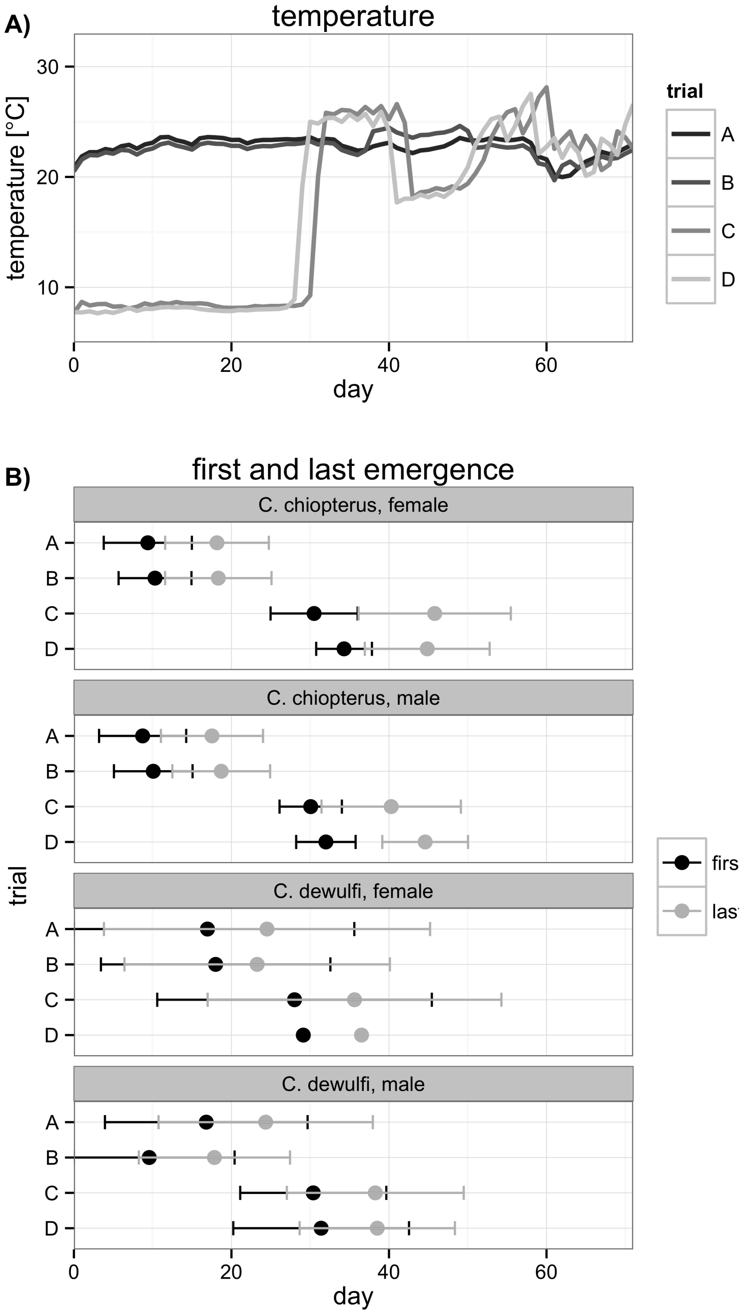

Figure D.10: **A:** Mean temperature in the wooden boxes of trials A, B, C and D, **B:** Mean and 95% confidence intervals of the first and last emergence of biting midge individuals from the cowpats in the four different temperature-photoperiod trials.

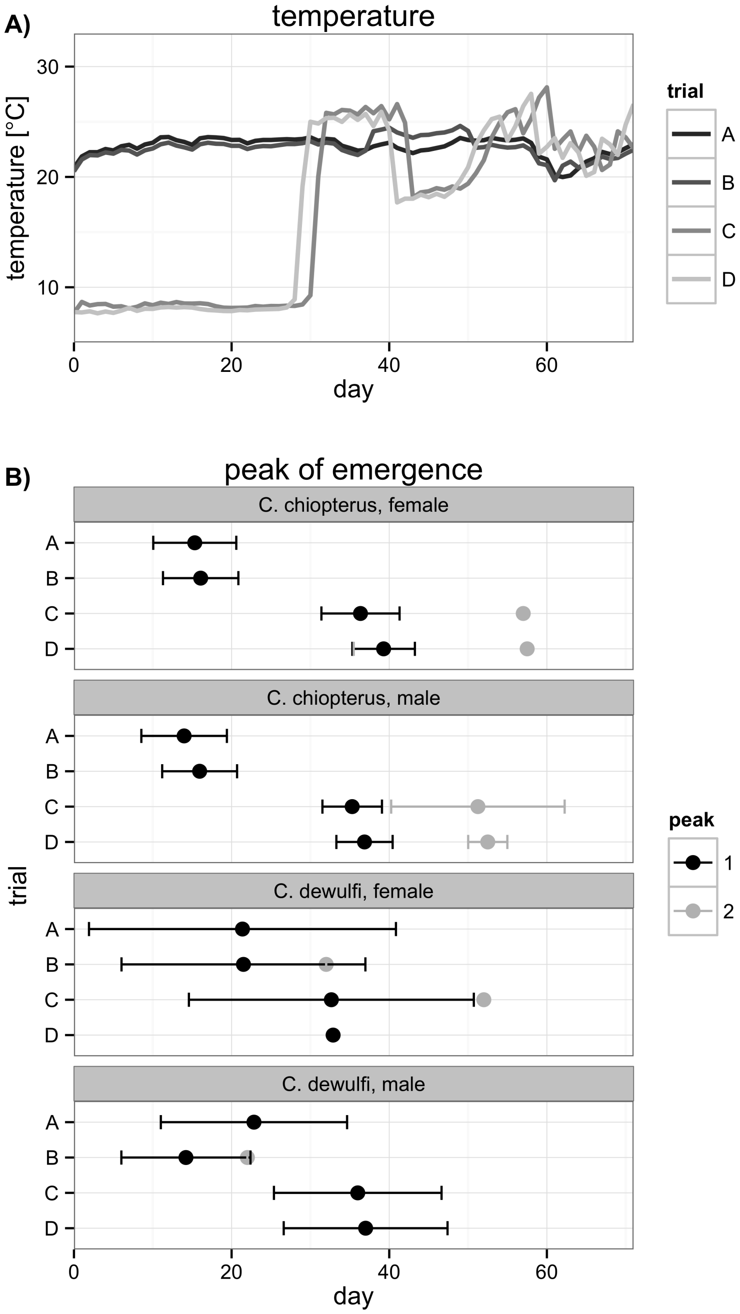

Figure D.11: **A:** Mean temperature in the wooden boxes of trials A, B, C and D, **B:** Mean and 95% confidence intervals of the first and second peak of emergence of biting midge individuals from the cowpats in the four different temperature-photoperiod trials.

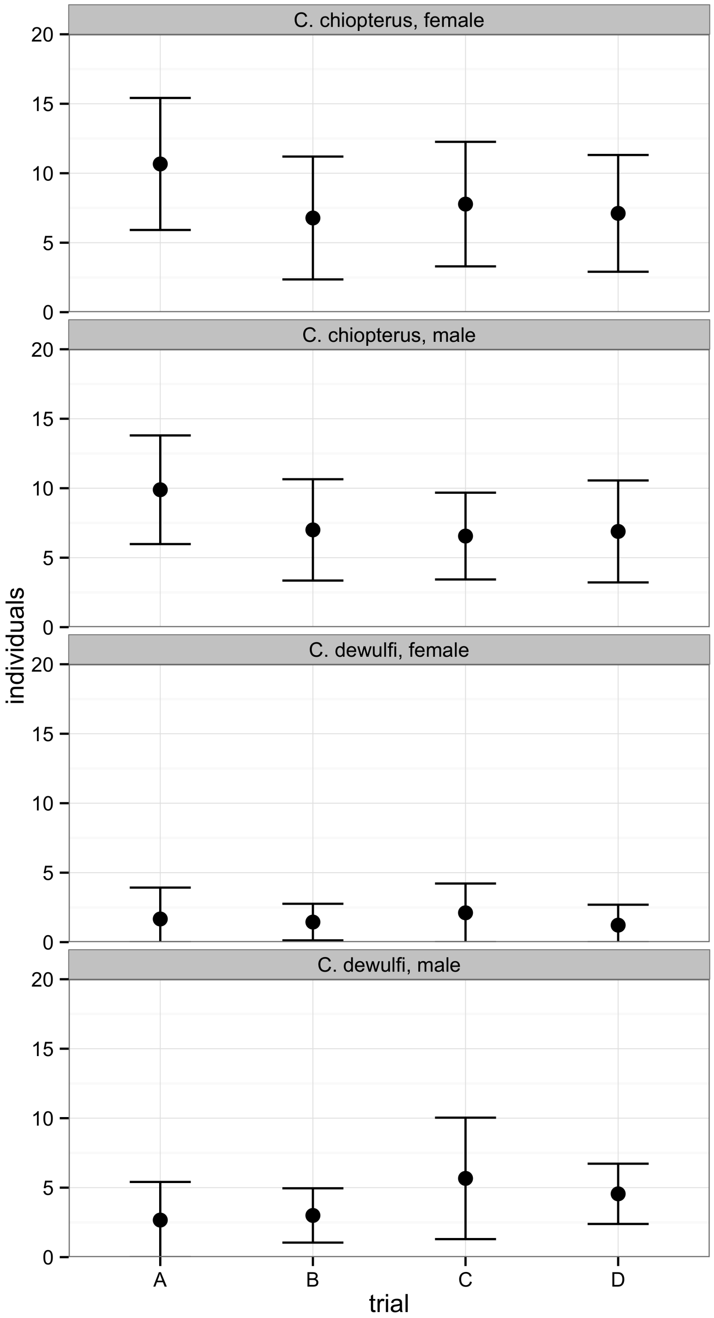

Figure D.12: Mean and 95% confidence intervals of biting midge total emergence from the cowpats in the four different temperature-photoperiod trials.

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Zimmer, J.-Y., Haubruge, E., Francis, F., Bortels, J., Simonon, G., Losson, B., Mignon, B., Paternostre, J., De Deken, R., De Deken, G., Deblauwe, I., Fassotte, C., Cors, R., & Defrance, T. (2008). Breeding sites of bluetongue vectors in northern Europe. *Veterinary Record*, *162*, 131–131. 42, 93, 223, 255, 258

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# Appendix E

# Formalities

## E.1 Curriculum vitae

**Personal data**

| | |
|---|---|
| Name | Renke Lühken |
| Date and place of birth | May 10th 1984, Varel |

**Academic Record**

| | |
|---|---|
| 2011-2014 | **PhD-student**, Carl von Ossietzky University, Oldenburg |
| 2010 | **Study abroad**, Massey University, Palmerston North, New Zealand |
| 2008-2011 | **Master-degree** in Landscape Ecology, Carl von Ossietzky University, Oldenburg |

| | |
|---|---|
| 2005-2008 | **Bachelor-degree** in Environmental Sciences, Carl von Ossietzky University, Oldenburg |

## Professional Appointments

| | |
|---|---|
| 2014–2015 | **Research assistant**, Research Group Aquatic Ecology and Nature Conservation, Carl von Ossietzky University, Oldenburg |
| 2014 | **Research assistant**, Department Molecular Parasitology, Bernhard Nocht Institute for Tropical Medicine, Hamburg. |
| 2011–2014 | **Research assistant**, Research Group Aquatic Ecology and Nature Conservation, Carl von Ossietzky University, Oldenburg |
| 2008–2012 | **Freelancer work**, Different planning offices, Breeding bird, resting bird, collision and activity range surveys |
| 2007–2011 | **Student assistant and research assistant**, Several research groups, Carl von Ossietzky University, Oldenburg. |
| 2005–2011 | **Breeding bird surveys**, Lower Saxony Water Management, Coastal Defence and Nature Conservation Agencey, Norden. |

## E.2 Publications

### Peer-reviewed

| Year | Publications |
|---|---|
| **2014** | [11] **Lühken, R.**, Kiel, E., & Steinke, S. (2014). *Culicoides* biting midge density in relation to the position and substrate temperature in a cattle dung heap. *Parasitology Research*, *113*, 4659–4662 |
| | [10] Steinke, S., **Lühken, R.**, & Kiel, E. (2014). Assessment of the abundance of *Culicoides chiopterus* and *Culicoides dewulfi* in bovine dung: A comparison of larvae extraction techniques and emergence traps. *Veterinary Parasitology*, *205*, 255–262. |
| | [9] **Lühken, R.**, Steinke, S., Wittmann, A., & Kiel, E. (2014). Impact of flooding on the immature stages of dung-breeding *Culicoides* in Northern Europe. V*eterinary Parasitology*, *205*, 289-294. |
| | [8] **Lühken, R.**, Pfitzner, W. P., Börstler, J., Garms, R., Huber, K., Schork, N., Steinke, S., Kiel, E., Becker, N., Tannich, E., & Krüger, A. (2014). Field evaluation of four widely used mosquito traps in Central Europe. *Parasites & Vectors*, *7*, 268. |
| | [7] Krüger, A., Börstler, J., Badusche, M., **Lühken, R.**, Garms, R., & Tannich, E. (2014). Mosquitoes (Diptera: Culicidae) of metropolitan Hamburg, Germany. *Parasitology Research*, *113*, 2907–2914. |

| **Year** | **Publications** |
|---|---|
| | [6] *Börstler, J., ***Lühken, R.**, Rudolf, M., Steinke, S., Melaun, C., Becker, S., Garms, R., & Krüger, A. (2014). The use of morphometric wing characters to discriminate female *Culex pipiens* and *Culex torrentium*. *Journal of Vector Ecology*, *39*, 204–212. *contributed equally to this paper |
| | [5] **Lühken, R.**, Kiel, E., & Steinke, S. (2014). Impact of mechanical disturbance on the emergence of *Culicoides* from cowpats. *Parasitology Research*, *113*, 1283–1287. |
| **2013** | [4] Becker, N., Geier, M., Balczun, C., Bradersen, U., Huber, K. Kiel, E., Krüger, A., **Lühken, R.**, Orendt, C., Plenge-Bönig, A., Rose, A., Schaub, G., & Tannich, E. (2013). Repeated introduction of *Aedes albopictus* into Germany, July to Oktober 2012. *Parasitology Research*, *112*, 1787–1790. |
| **2012** | [3] **Lühken, R.** & Kiel, E. (2012). Distance to the stable affects trapping of biting midges (Diptera, Ceratopogonidae). *Journal of Vector Ecology*, *37*, 453–457. |
| | [2] Kiel, E., Kastner, F., **Lühken, R.**, & Schröder, M. (2012). The invertebrate fauna of ditches in northern Germany. *Natur und Landschaft*, *87*, 347–350. |
| **2009** | [1] **Lühken, R.**, Kiel, E., Lieckweg, T., & Niedringhaus, R. (2009). Mosquito species on the Island of Baltrum in the southern North Sea (Germany) including information on the culicids from the Islands of Langeoog and Mellum (Diptera: Culicidae). *Studia Dipterologica*, *16*, 87–95. |

## Not peer-reviewed and book chapters

| **Year** | **Publications** |
|---|---|
| **2014** | [16] Vogel, U., **Lühken, R.**, & Kiel, E. (2014). A tool for simulating the spread of invasive mosquitoes. In: Gomez, J. M. et al. (Eds.), *Proceedings of the 28th International Conference on Informatics for Environmental Protection – Enviroinfo Oldenburg 2014* (pp. 279–286). |
|  | [15] **Lühken, R.**, Steinke, S., & Kiel, E. (2014). Potential impact of climate and landscape change on mosquitoes and biting midges in Germany. In: Lozán, J. L., Grassl, H., Karbe, L. & G. Jendritzky (Eds.), *Warnsignal Klima: Gefahren für Pflanzen, Tiere und Menschen.* 2. Auflage. Elektron. Veröffent. (Kap. 3.2.17), [German]. |
| **2013** | [14] Kiel, E., **Lühken, R.**, & Steinke, S. (2013). Vector ecology for animal health: Studies on vectors of pathogens in the circumstances of farms. *Technologie-Informationen*, *2*, 9, [German] |
|  | [13] Klich, D., Vogel, U., **Lühken, R.**, & Kiel, E. (2013). Development of a software- prototype to model the spread of mosquito species. In: *Modellierung und Simulation von Ökosystemen – Workshop Kölpinsee 2012* (pp. 177–198), [German]. |
| **2012** | [12] Krüger, L., Kiel, E., **Lühken, R.**, & Martens, A. (2012). Egg-laying strategies of the European fairy shrimp *Eubranchipus grubii* in temporary floodplain waters. *German Limnological Society. Extented abstracts of the annual conference 2010 (München/Weihenstephan). Hardegsen 2011*, 401–405. |

| Year | Publications |
|---|---|
| | [11] **Lühken, R.** & Kiel, E. (2012). Macroinvertebrate communities and diversity of brackish, ephemeral water bodies at the coast of Northern Germany. *Verhandlungen der Gesellschaft für Ökologie*, *42*, 60–61. |
| | [10] **Lühken, R.** & Kiel, E. (2012). Mosquito control and nature conservation: case study about the Wadden Sea National Park of Lower Saxony. *Communications of the German Society for General and Applied Entomology*, *18*, 27, [German]. |
| | [9] **Lühken, R.** & Kiel, E. (2012). Experiments on the preference for structures by larvae and pupae of the mosquito species *Ochlerotatus detritus* (Diptera: Culicidae). *Proceedings of the German Society for General and Applied Entomology*, *18*, 339–343, [German]. |
| **2011** | [8] **Lühken, R.** & Kiel, E. (2011). Mosquito colonisation in response to predatory taxa and abiotic factors. *Verhandlungen der Gesellschaft für Ökologie*, *41*, 253. |
| | [7] **Lühken, R.**, Lieckweg, T., & Kiel, E. (2011). Classification system of mosquito breeding sites for a dune and salt marsh complex. *German Limnological Society. Extented abstracts of the annual conference 2010 (Bayreuth). Hardegsen 2011*, 412– 416. |
| **2010** | [6] Lieckweg, T., **Lühken, R.**, Niedringhaus, R., & Kiel, E. (2010). Survey of potential breedings sites of mosquitoes on the East Frisian Islands. *German Limnological Society. Extented abstracts of the annual conference 2009 (Oldenburg). Hardegsen 2010*, 90–95, [German]. |

| Year | Publications |
|---|---|
| | [5] **Lühken, R.** & Kiel, E. (2010). *Ochlerotatus detritus* (Diptera: Culicidae): Adaptations to drought? *German Limnological Society. Extented abstracts of the annual conference 2009 (Oldenburg). Hardegsen 2010*, 103–107, [German]. |
| | [4] **Lühken, R.**, Kiel, E., & Lieckweg, T. (2010). Bluetongue disease & Asian Tiger Mosquito – Does the climate change bring exotics to Northern Germany? In: *Schriftenreihe des Landesmuseums Natur und Mensch*, *76*, 50–53, [German]. |
| | [3] **Lühken, R.**, Niedringhaus, R., & Kiel, E. (2010). *Ochlerotatus detritus* (Diptera: Culicidae): Adaptations to high salanities? *German Limnological Society. Extented abstracts of the annual conference 2009 (Oldenburg). Hardegsen 2010*, 108–112, [German]. |
| **2009** | [2] **Lühken, R.** & Kiel, E. (2009). Mosquitoes (Diptera: Culicidae) of the East Frisian Island of Baltrum in the Wadden Sea National Park Lower Saxony. *Proceedings of the German Society for General and Applied Entomology*, *17*, 323–326, [German]. |
| | [1] **Lühken, R.** Kiel, E., Lieckweg, T., & Niedringhaus, R. (2009). Uninvestigated pests – Mosquitoes (Diptera: Culicidae) on the East Frisian Islands. *Natur und Umweltschutz*, *8*, 64–69, [German] |

## E.3 Erklärung

gemäß § 10 Abs. 2 der Promotionsordnung der Fakultät für Mathematik und Naturwissenschaften der Carl von Ossietzky Universität Oldenburg vom 26.08.2011.

Hiermit erkläre ich, dass ich eine Promotion zum Dr. rer. nat. anstrebe. Es soll der Grad eines Doktors verliehen werden.

Zudem erkläre ich, dass die vorliegende Arbeit selbständig verfasst wurde und nur die angegebenen Hilfsmittel benutzt wurden. Einzelne Kapitel wurden bereits veröffentlicht, dies ist jeweils zum Beginn der Kapitel vermerkt.

Des Weiteren erkläre ich, dass diese Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung vorliegt oder vorgelegen hat.

Oldenburg, 19.12.2014

(Renke Lühken)

## E.4 Own contributions and contributions of other authors

| Publications | | Author's contributions |
|---|---|---|
| **Paper 0** | **Lühken, R.**, Steinke, S., Kiel, E. (2014). Potential impact of climate and landscape change on mosquitoes and biting midges in Germany. In: Lozán, J. L., Grassl, H., Karbe, L. & G. Jendritzky (Eds.), *Warnsignal Klima: Gefahren für Pflanzen, Tiere und Menschen*. 2. Auflage. Elektron. Veröffent. (Kap. 3.2.17), [German]. | Wrote the paper: **RL**; Contributed to the manuscript drafting: EK, SS |
| **Paper A.1** | **Lühken, R.**, Pfitzner, W. P., Börstler, J., Garms, R., Huber, K., Schork, N., Steinke, S., Kiel, E., Becker, N., Tannich, E., & Krüger, A. (2014). Field evaluation of four widely used mosquito traps in Central Europe. *Parasites & Vectors*, *7*, 268. | Conceived of the study: AK, **RL**, WPP, EK; Data collection: JB, RG, KH, AK, **RL**, NS, SS, NB, ET, WPP; Analysed the data: **RL**; Wrote the paper: **RL**; Contributed to the manuscript drafting: AK, ET, WPP, RG, EK, NS, SS |

| **Publications** | | **Author's contributions** |
|---|---|---|
| **Paper A.2** | **Lühken, R.**, & Kiel, E. (2012). Distance from the stable affects trapping of biting midges (Diptera, Ceratopogonidae). *Journal of Vector Ecology*, *37*, 453-457. | Conceived of the study: EK; Data collection: EK, **RL**; Analysed the data: **RL**; Wrote the paper: **RL**; Contributed to the manuscript drafting: EK |
| **Paper B.1** | Börstler*, J., **Lühken*, R.**, Rudolf, M., Steinke, S., Melaun, C., Becker, S., Garms, R., & Krüger, A. (2014). The use of morphometric wing characters to discriminate female *Culex pipiens* and *Culex torrentium*. *Journal of Vector Ecology*, *39*, 204-212. *contributed equally to this paper | Conceived of the study: SB, RG, AK; Data collection: JB, AK, **RL**, CM, MR, SS; Analysed the data: JB, AK, **RL**; Wrote the paper: AK, **RL**; Contributed to the manuscript drafting: SB, JB, RG, CM, MR, SS |
| **Paper B.2** | **Lühken, R.**, Czajka, C., Steinke, S., Jöst, H., Schmidt-Chanasit, J., Kiel, E., Krüger, A., & Tannich, E. (in preperation for *Parasites & Vectors*). Distribution of the various *Anopheles maculipennis* group members from Germany identified by newly developed real-time PCR assays. | Status (18.12.2014): Conceived of the study: AK, EK, JSS, ET. Performed the mosquito collection: HJ, **RL**, SS. Conducted the PCRs: CC, **RL**. Analysed the data: **RL**. Wrote the paper: **RL**. Contributed to the manuscript drafting: ET |

| **Publications** | | **Author's contributions** |
|---|---|---|
| **Paper C.1** | **Lühken, R.**, Steinke, S., Leggewie, M., Becker, S., Krüger, A., Tannich, E., & Kiel, E. (submitted to the *Journal of Medical Entomology*): Physico-chemical characteristics of *Culex pipiens* s.l. and *Culex torrentium* breeding sites in Germany. | Conceived of the study: EK, **RL**, SS; Data collection: ML, **RL**, SS; Analysed the data: **RL**; Wrote the paper: **RL**; Contributed to the manuscript drafting: SB, EK, AK, SS, ET |
| **Paper C.2** | **Lühken, R.**, Kiel, E., & Steinke, S., Fladung, R. (submitted to *Parasitology Research*). Impact of edaphic factors on the presence of dung breeding *Culicoides*. | Conceived of the study: EK; Data collection: RF, EK, **RL**; Analysed the data: **RL**; Wrote the paper: **RL**; Contributed to the manuscript drafting: EK, SS |
| **Paper C.3** | **Lühken, R.**, Kiel, E., & Steinke, S. (2014). *Culicoides* biting midge density in relation to the position and substrate temperature in a cattle dung heap. *Parasitology Research*, *113*, 4659-4662. | Conceived of the study: EK; Data collection: EK, **RL**; Analysed the data: **RL**; Wrote the paper: **RL**; Contributed to the manuscript drafting: EK, SS |

| **Publications** | | **Author's contributions** |
|---|---|---|
| **Paper C.4** | Vogel, U., **Lühken, R.**, Kiel, E. (2014). A tool for simulating the spread of invasive mosquitoes. In: Gomez, J. M. et al. (Eds.), *Proceedings of the 28th International Conference on Informatics for Environmental Protection – Enviroinfo Oldenburg 2014* (pp. 279–286). | Conceived of the study: EK, **RL**, UV; Data collection: **RL**, UV; Analysed the data: **RL**, UV; Wrote the paper: **RL**, UV; Contributed to the manuscript drafting: EK |
| **Paper C.5** | **Lühken, R.**, Kiel, E., Gethmann, J., Staubach, C., Conraths, F. J., & Kranz, P. (in preperation). Comparison of single- and multi-scale models for predicting the distribution of *Culicoides* biting midges. | Status (18.12.2014): Conceived of the study: EK; Data collection: PK, PS, Analysed the data: **RL**; Wrote the paper: **RL**; Contributed to the manuscript drafting: FJC, JG, EK, CS |
| **Paper D.1** | **Lühken, R.**, Kiel, E., & Steinke, S. (2014). Impact of mechanical disturbance on the emergence of *Culicoides* from cowpats. *Parasitology Research*, *113*, 1283-1287. | Conceived of the study: EK; Data collection: EK, **RL**; Analysed the data: **RL**; Wrote the paper: **RL**; Contributed to the manuscript drafting: EK, SS |

| Publications | | Author's contributions |
|---|---|---|
| **Paper D.2** | **Lühken, R.**, Steinke, S., Wittmann, A., & Kiel, E. (2014). Impact of flooding on the immature stages of dung-breeding *Culicoides* in Northern Europe. *Veterinary Parasitology*, *205*, 289-294. | Conceived of the study: EK, **RL**, SS; Data collection: AW, **RL**, SS; Analysed the data: **RL**; Wrote the paper: **RL**; Contributed to the manuscript drafting: EK, SS |
| **Paper D.3** | **Lühken, R.**, Steinke, S., Hoppe, N., & Kiel, E. (submitted to *Veterinary Parasitology*). Effects of temperature and photoperiod on the development of overwintering immature *Culicoides chiopterus* and *Culicoides dewulfi*. | Conceived of the study: **RL**, SS; Data collection: NH, **RL**, SS; Analysed the data: **RL**; Wrote the paper: **RL**; Contributed to the manuscript drafting: EK, SS |