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Arthropod-borne Infectious Diseases and Arthropods as Disease Agents in Human and Animal Health

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Arthropod-borne Infectious Diseases and Arthropods as Disease Agents in Human and Animal Health

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Ixodes ricinus, *Cimex lectularius*, *Ceratophyllus* sp. und *Anopheles maculipennis* (von links oben nach rechts unten). Fotos: Dr. H. Bellmann. Aus: Aspöck, H. (Hrsg.): Amöben, Bandwürmer, Zecken ... : Parasiten und parasitäre Erkrankungen des Menschen in Mitteleuropa. Linz: Oberösterreichisches Landesmuseum 2002, mit freundlicher Genehmigung.

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Inhalt

Preface

Growing globalization of trade, expanding worldwide travel activity and global changes in micro- and macroclimatic conditions increase the risk of importation to and establishment in Europe of formerly exotic arthropods capable to transmit novel vector-borne pathogens. Thus, in recent years various invasive mosquito species expanded their range in Europe, for example the Japanese bush mosquito or the Asian tiger mosquito, which have a high vector competence for various pathogens. The presence of several new arthropod-borne viruses and exotic nematodes document an increasing entry of these pathogens. Regarding arthropod pests especially the appearance of the small hive beetle in Italy causes concern.

The symposium "Arthropod-borne infectious diseases and arthropods as disease agents in human and animal health" which was jointly organized by the German Academy of Sciences Leopoldina, the *Friedrich-Loeffler-Institut* and the *Humboldt-Universität zu Berlin*, brought together internationally recognized experts in the fields of taxonomy, epidemiology, vector and pathogen research, and biology of arthropod pests in order to promote scientific exchange between the individual disciplines and to support coordinated programmes.

The auditorium agreed that a basic prerequisite for the successful control of arthropods and the pathogens they transmit is to conduct coordinated research in the areas of (infection)biology and epidemiology, including revision of arthropod taxonomy using new OMICS technologies. While research on arthropods, in particular on their taxonomy, has been declining in the past decades, the global increase in infectious diseases transmitted by arthropod vectors and the returning recognition of arthropods as significant pests sparked renewed scientific interest in different countries by a variety of experts with in-depth knowledge of their specific areas. The challenge is to bring this multitude of expertise together for a coordinated effort. This symposium was intented to provide a first platform. To maintain a high level of awareness even over longer periods it is now understood also at the highest political level that neglected tropical diseases are no longer restricted to developing countries with no significant global impact. The expansion of vector populations and the increase in vector-borne infections, exemplified by the recent catastrophic epidemic of zika virus in Brazil, transmitted by *Aedes aegypti* which had been nearly eradicated from this part of the world a few decades ago, is a tell-tale sign that action is needed. This includes raising the awareness in the medical community of new pathogens transmitted by vectors.

The involvement of citizens in research in the frame of Citizen Science Projects particularly in the field of research on arthropods and arthropod borne diseases becomes increasingly important. The public evening event at the historic animal anatomical theater in the "Langhansbau" on the campus of the *Humboldt Universität*, which focussed on the diversity of

arthropods from a molecular biological point of view, offered an opportunity to gain deeper insights into current research on the topic.

The organizers would like to thank Merial, Bayer HealthCare, and the Interdisciplinary Center of Infection Biology and Immunity (ZIBI) for supporting the symposium.

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Session I
Taxonomy goes OMICs: Molecular versus Morphological Methods in Taxonomic Research

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Approaches to Infer Local Vectorial Capacity: From Rapid Assays to Population Genomics and Transcriptomics

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Abstract

Variability in vectorial capacity among mosquitoes and other disease vectors is perversely the only clear constant. "Anophelism without malaria", the observation that malaria transmission varied widely among areas all seemingly inhabited by the same *Anopheles* species, led to the identification of cryptic species. This notion was later expanded to the recognition of cryptic populations: when vector populations with differing vectorial capacity have recognizable genetic signatures rapid assays can be used in the assessment of risk. However, hybridization/mixing or significant behavioural and/or physiological differences between recently diverged populations can make rapid assays insufficient and require more thorough multilocus genotyping. Further, new studies show that prior experiences such as larval crowding, type of food, or the microbiome can also affect vectorial capacity. Recent advances in genomic and transcriptomic analyses offer hope. I summarize the relevant literature in order to stimulate a discussion on current practices, aims, limitations and future directions.

Zusammenfassung

Die Variabilität in der Vektorkapazität ist unter Stechmücken und anderen Krankheitsvektoren unglücklicher Weise die einzige klare Konstante. „Anophelismus ohne Malaria", d. h. die Beobachtung, dass die Malariaübertragung in den Gebieten, die scheinbar von den gleichen *Anopheles*-Arten bewohnt werden, erheblich variiert, führte zur Bestimmung kryptischer Arten. Diese Vorstellung wurde später zur Anerkennung von kryptischen Populationen erweitert: Wenn die Vektorpopulationen mit verschiedenen Vektorkapazitäten erkennbare genetische Unterschiede aufweisen, können Schnell-Assays zur Abschätzung der Risiken verwendet werden. Hybridisierung/Vermischung oder deutliche Unterschiede in Verhalten und/oder Physiologie zwischen seit kurzem divergierenden Populationen können Schnell-Assays als unzureichend erscheinen lassen und erfordern sorgfältiges Multilokus-Genotyping. Neue Studien haben gezeigt, dass frühere Erfahrungen, etwa der Raupenbesatz, die Art des Futters oder das Mikrobiom, die Vektorkapazität ebenfalls beeinflussen können. Neue Fortschritte in der Genom- und Transkriptom-Analyse machen Hoffnung. Ich fasse die relevante Literatur zusammen, um die Diskussion gegenwärtiger Praktiken, Ziele, Einschränkungen und zukünftiger Entwicklungsrichtungen voranzubringen.

1. Introduction

In past centuries, yellow fever, lymphatic filariasis, dengue and malaria raged across temperate and tropical regions resulting in thousands of deaths and severe morbidity. The realization late in the 19th century of the role of mosquitoes as vectors of the agents of these diseases led to highly productive research and eventually to unprecedented worldwide coordinated efforts for their control and prevention (Gubler 1998). However, since the late 1960s subpar sanitation in the world's fast growing cities and the lack of concerted efforts to control urban mosquitoes have led to an increase in abundance and re-expansion of the invasive mosquitoes,

Aedes aegypti and *Culex quinquefasciatus* in the world's tropical cities and *Cx. pipiens* in temperate cities (Lemon et al. 2008). Additionally, the widespread movement of cargo and people has facilitated the expansion worldwide of new mosquito species, specifically *Aedes albopictus*, a species with both tropical and temperate populations, and *Ae. japonicus japonicus*, a cold-adapted species (Lounibos 2002). As a result, the likelihood of someone being bit by a mosquito inside or near his or her home is currently high across the world (Halasa et al. 2012, and references therein). Not altogether surprisingly epidemics of lymphatic filariasis, dengue, chikungunya and now zika viruses have exploded in the world's tropical cities and threaten temperate cities (Lucey and Gostin 2016).

However, although infected travellers regularly arrive in temperate cities teaming with potential mosquito vectors, so far with the exception of West Nile virus (WNV), arboviral diseases vectored by invasive mosquitoes have only had limited incursions into the temperate zone (Christofferson 2016). Clearly, a complete understanding of the factors underlying arboviral epidemiology is the key to the development and implementation of strategies for controlling these diseases in the tropics and preventing their expansion to the temperate zone. Although well-developed infrastructure such as good housing and sanitation as well as access to health services, overall the norm in the developed world, are obviously important, the presence of dense urban populations of potentially capable vectors surely paves the way for epidemics when hosts infected with disease agents arrive (Bonilauri et al. 2008, Guzman and Harris 2015). In fact West Nile virus has infected thousands, likely millions, of American citizens contributing to the untimely death of many, indicating that North American residents are being routinely bit by *Culex* mosquitoes, and has become endemic in North America in spite of extensive measures for its control (Kramer et al. 2007).

The epidemiology of mosquito-borne diseases depends on the vectorial capacity of local populations. Vectorial capacity is defined as the ability to spread disease among hosts and reflects (*1*.) the ability of local mosquito populations to become infected and transmit a specific pathogen (vector competence) and (*2*.) their ecology and behaviour such as their abundance, longevity and choice of blood-hosts (Garrett-Jones 1964).

While the realization that vectorial capacity can differ among mosquito infested areas arose early from work on *Anopheles* vectors of human malaria in Europe (Bates 1940, Jetten and Takken 1994, Sedaghat et al. 2003), this notion is still often dismissed in the epidemiology and control of diseases transmitted by invasive mosquitoes. I propose the reasons underlying this dichotomy are primarily methodological: it is hard to detect and quantify differences in vectorial capacity among populations without some independent way to tell them apart. In what follows I will discuss the evidence of intraspecific variability in vectorial capacity in invasive species, briefly summarize current DNA-based strategies for population identification, focusing on their operational use. I will also discuss some of the newer findings using genomics and transcriptomics. My objective is to stimulate the development of more precise and accurate strategies to identify and control populations of critical invasive vectors with minimal waste of insecticides and effort.

2. A Short History

Soon after *Anopheles maculipennis* mosquitoes were indicted as vectors of human *Plasmodium* in Europe, local researchers observed the consistent absence of malaria cases in areas

where the species was seemingly abundant. This observation later dubbed "Anophelism without malaria" provided the impetus for careful analyses of local populations and the discovery of several phylogenetically closely related species (sibling species) with significant differences in vectorial capacity to human malaria (Jetten and Takken 1994). In fact, ecological and/or behavioural characters such as temperature or salinity tolerances, ability to mate in confined spaces or associations with specific blood hosts, which incrementally restrict gene flow among populations and ultimately lead to speciation, are often also critical drivers of vectorial capacity (Brown et al. 2014, Severson and Behura 2012, Takken and Knols 1999). It should be noted, that while some species are identifiable morphologically, the incremental understanding of the many *Anopheles* complexes across the World involved the use of state-of-the-art cytogenetic and, more recently, genotypic, genomic and transcriptomic approaches (Lawniczak et al. 2010). Knowing which sibling or cryptic species is present and their abundance therefore is epidemiologically relevant and informs expectations of disease risk and strategies for malaria control (Jetten and Takken 1994). Of note, these early experiences shaped research on *Anopheles* and have led to the important discovery that epidemiologically critical variation may also occur among different populations of the same species (White et al. 2011).

3. Intra-Specific Variation in Vector Competence

Recent studies on vector competence have revealed a complex genetic basis such that closely related individuals in the same population can range from permissive to refractory to specific disease agents (Beerntsen et al. 2000). Therefore, from an epidemiological standpoint "vector competence" is an average population attribute that however is also dependent on the viral titre and viral strain (Lambrechts et al. 2009). This means that demographic processes such as random population bottlenecks or selection (such as after insecticide applications) can swiftly change the vector competence of local populations. Recent work has also shown that vector competence can be altered by events acting during development, such as larval competition (Alto et al. 2008) or rearing temperature (Carrington et al. 2013), and by the composition of the microbiome (Weiss and Aksoy 2011, Jupatanakul et al. 2014). Not surprisingly, field studies revealed wide variation in competence at very fine spatial and temporal scales (Bennett et al. 2002, Kilpatrick et al. 2010). While the identification of the specific mechanisms underlying vector competence may one day allow the control of vector-borne diseases (Tabachnick 2013), perversely for now such broad intra-specific variation in vector competence negates its operational use. Instead, we fall back on the idea that some species are, on average or in our collective experience, better vectors of specific disease agents than others.

4. Intra-Specific Variation in Vectorial Capacity

The use of recognizable species as our compass, however, is undermined by the fact that highly relevant ecological and/or behavioural characteristics may also differ significantly among populations. As summarized in Table 1, four of the worldwide invasive mosquito species, namely *Aedes aegypti*, *Ae. albopictus*, *Culex pipiens* and *Cx. quinquefasciatus*, have populations differing in ecology and behaviour. For example, the Asian tiger mosquito, *Aedes albopictus* has temperate and tropical "forms" (for lack of a better term, unfortunately species

Tab. 1 Summary of documented differences in characteristics relevant to vectorial capacity among populations of worldwide invasive mosquito species. Some populations are referred as subspecies or forms, neither recognized categories by the Zoological Code of Nomenclature (*Anonymus* 1999). "Geography" is approximate, and "% H" refers to the relative (approximate) percentage of human blood meals obtained from the literature. Taxonomy follows Wilkerson et al (2015).

Species	Populations	Characteristics	Geography	%H	References[1]
Aedes aegypti (tropical)	*Ae. aegypti formosus* (Aaf)	Feral, non-human primate biter, tree-holes	Africa	<5	Lounibos 1981, Mattingly 1957, McClelland and Weitz 1963, Powell and Tabachnick 2013, Sylla et al. 2009, Trpis and Hausermann 1978
	Ae. aegypti formosus	Peridomestic[2]	Africa	–	
	Ae. aegypti aegypti (Aaa)	Peridomestic, human biter	Worldwide	~90	
	Ae. aegypti aegypti	Domestic[3]	Africa, Worldwide?	>99	
	Aaa × Aaf	Peridomestic, human biter	W Africa	~50	
Aedes albopictus	temperate	Diapausing eggs, natural containers, feral/peridomestic	N Asia, Worldwide	30–75	Faraji et al. 2014, Ponlawat and Harrington 2005, Urbanski et al. 2012
	tropical	Non-diapausing, natural containers, feral/peridomestic	S Asia, Worldwide tropical	90–100	
Culex pipiens (temperate)	*Cx. p. pipiens* f. pipiens	Diapausing, feral, bird biter, eurigamous[4]	Africa, Europe	<5	Goddard et al. 2002, Kassim 2011, Molaei et al. 2006, Osorio et al. 2014, Sawabe et al. 2010, Takken and Verhulst 2013, Turell et al. 2014, Vaidyanathan and Scott 2007
	Cx. p. pipiens f. molestus	Non-diapausing Domestic	Worldwide, underground in temperate zone	50–100	
	Cx. p. pallens	Diapausing, peridomestic, bird biter, stenogamous[4]	N Asia	~40	
	Cx. p. pipiens f. pipiens × f. molestus	Diapausing, peridomestic, bird/mammal biter, stenogamous	USA, S Europe, N Africa	5–50	
Cx. pipiens × *Cx. quinquefasciatus*	Variable degree of hybridization	Variable, stenogamous	USA, Madagascar, Argentina, Japan	–	Kothera et al. 2009, Micieli et al. 2013, Savage et al. 2007, 2008, Strickman and Fonseca 2012, Urbanelli et al. 1997
Culex quinquefasciatus (tropical, non-diapausing)	Old World	bird biter	Asia, Africa, Australia, Hawaii	10–50	Takken and Verhulst 2013, Agrawal 2015, Chaves et al. 2009, Garcia-Rejon et al. 2010, Gomes et al. 2003, Janssen et al. 2015, Mattingly 1962a, b, Mboera and Takken 2003, Richards et al. 2010, Zinser et al. 2004
	New World	bird biter	Americas, Hawaii	5–50	
	Domestic[3]	human-biter	Worldwide	>90	

[1] "References" includes the most relevant and recent references for the taxonomy, characteristics and geography. Reviews were included when available.
[2] "Peridomestic" refers to mosquitoes that commonly lay eggs in artificial water containers (small or large) associated with or within suburban or urban areas.
[3] "Domestic" mosquitoes live inside houses and bite people exclusively; they lay eggs in water containers indoors or in sewers and underground locations. They are also stenogamous (see below).
[4] "Stenogamous" and "Eurigamous" refers to individuals willing and un-willing to mate in confined spaces (indoors, for example), respectively. Eurigamous populations commonly swarm around cues prior to mating.

definitions have not caught up with taxonomic usefulness [Wilkerson et al. 2015]). Temperate forms start laying diapausing eggs (that survive the winter and hatch in the spring) as daylength decreases below threshold in early fall, while tropical forms are incapable of doing so (Urbanski et al. 2010). The two forms have differing patterns of genetic diversity consistent with the hypothesis that the temperate form may have become associated with early farming communities in temperate East Asia and Indochina approximately 9,000 years ago (Porretta et al. 2012). If that scenario is true, then association with humans (i.e. domestication) may have facilitated the evolution of diapause, a complex physiological process in that species (Lounibos et al. 2003, Reynolds et al. 2012). Due to its recent worldwide expansion, extensive population genetic and phenotypic analysis in this species are still lagging and likely contribute to some of the controversy regarding the importance of this species as a disease vector.

Domestication seems to have also been the primary driver of differentiation in *Aedes aegypti*. Feral forms of *Ae. aegypti*, namely *Ae. aegypti formosus* (the accepted terminology), are restricted to Africa, the native range. Recent studies using first a panel of high-resolution microsatellite loci and then a dense array of thousands of Single Nuclear Polymorphic (SNP) loci indicate that current worldwide human-associated populations of *Ae. aegypti*, namely *Ae. aegypti aegypti*, originated from a single "out-of-Africa" event of a domesticated form (Brown et al. 2014, 2011), although newer domestication events appear to be occurring in African cities (Brown et al. 2014). In fact this phenomenon, where native species adapt to and invade human modified habitats becoming "native invasive" (Simberloff et al. 2011) may play a much larger role in urban vector-borne disease transmission than is currently accepted (Johnson et al. 2015).

Another worldwide invasive, *Culex pipiens* is native to the cooler mountaintops of Africa and to Europe where it is a widespread canopy dwelling bird-biting mosquito (Cornel et al. 2003, Medlock et al. 2012). Using high-resolution microsatellite loci (Fonseca et al. 2004) demonstrated that populations of *Cx. pipiens pipiens* form molestus, which occur worldwide and are predominantly mammalian biters, have a unique genetic signature indicating a single origin (Bahnck and Fonseca 2006, Fonseca et al. 2004). In contrast to *Ae. albopictus*, domestication of *Cx. pipiens* appears to have involved loss of ability to diapause possibly because it occurred in association with the old civilizations of North Africa or the Arabian Peninsula (Fonseca et al. unpublished). Of note, loss of diapause did not prevent expansion of *Cx. p. pipiens* form molestus to the temperate zone: since this mosquito is capable of developing in water with very high organic content, populations has spread north by exploiting the vast warm underground sewers of today's modern large cities (Byrne and Nichols 1999). Moreover, hybrids of domestic and feral forms capable of diapause but also capable of traveling in association with humans arrived in the New World and are widespread in North America (Fonseca et al. 2004, Strickman and Fonseca 2012, Fonseca et al. unpublished). Why such hybrids are not also widespread in Europe is unclear and may be due to the competitive advantage of native *Cx. pipiens*. Increasingly, however, the presence of hybrids of *Cx. pipiens* form pipiens and molestus is being documented in large European cities (Rudolf et al. 2013). From an epidemiological standpoint, hybridization (or mixing) between domestic and feral forms has the effect of creating populations that can become infected with zoonotic disease agents, such as West Nile virus (WNV), because they are primarily bird biters but bridge these diseases to humans (Farajollahi et al. 2011, Fonseca et al. 2004). This hypothesis has been supported by follow up studies (Fritz et al. 2015, Kilpatrick et al. 2007, Osorio et al. 2012) and underscores the epidemiological relevance of examining the genetic ancestry of local populations of invasive species.

Not surprisingly, considering its long association with humans, the evolutionary history of *Culex quinquefasciatus*, a tropical non-diapausing species that occurs worldwide, is also quite complex. *Cx. quinquefasciatus* is the principal urban and suburban vector of *Wuchereria bancrofti*, the agent of periodic human lymphatic filariasis, but also of bird malaria and West Nile virus. Patterns of genetic differentiation among 28 populations of this species across the tropical zone appear to indicate this mosquito arrived in the New World much earlier than European settlers and likely from Australasia (Fonseca et al. 2006). This ancestry would explain why *Cx. quinquefasciatus* in both Australasia and North America are predominantly bird biters and important zoonotic vectors (Fonseca et al. 2000). However, this species has populations with known endophilic and anthropophilic behaviour that earned it the name of southern "house" mosquito. *Cx. quinquefasciatus* are in fact principal vectors of periodic lymphatic filariasis in east Africa, south Asia, Brazil and the Caribbean (Raccurt et al. 1988) and recently their abundance and anthropophilic behaviour has led to the hypothesis that they may be significant vectors of zika virus in Brazil (Ayres 2016). While those specific populations appear to have unique genetic signatures indicative of a complex history (Fonseca et al. 2006) clearly further research is needed into their behaviour, ecology and vector competence.

5. How to Tell Epidemiologically Relevant Populations Apart

The advent of high-resolution genetic analysis (Brown et al. 2014, 2011, Fonseca et al. 2004, 2006, 2009, 2010), as well as of rapid assays that allow cost-effective genotyping of thousands of specimens (Bahnck and Fonseca 2006, Smith and Fonseca 2004) has allowed the recognition of native and derived forms of invasive species, which often have epidemiologically distinct traits (listed in Tab. 1). However, these studies also revealed local differentiation and multiple introductions of different populations of the same or closely related species often followed by mixing (or hybridization). Of note, hybridization of species forcefully made to overlap by human intervention does not constitute evidence they are not distinct species since having been allopatric most of their evolutionary lives they have not developed hybridization barriers (Mooney and Cleland 2001).

Epidemiologically, however, these demographic events create highly adaptive populations exhibiting a wide array of behaviours and ecologies (detailed references associated with Tab. 1). Much like their vector competence (Tabachnick 2013), the behaviour and ecology of populations of invasive species appear to be multigenic and may change quickly (Egizi et al. 2015). One of the few constants is that successful invasive species have become associated with human settlements, moved with people around the world and, importantly, recognize humans as a source of blood. In fact recent work has shown that *Ae. aegypti aegypti*, the domestic form, has a odorant receptor specifically tuned to human odor (Mcbride et al. 2014). Likewise, comparisons of the transcriptomes of *Cx. p. pipiens* form pipiens and form molestus uncovered rapid evolution of loci involved in olfaction and blood feeding as well as immunity, the latter possibly a consequence of a life in sewers where form molestus thrives (Price and Fonseca 2015).

Next steps are to identify the genetic basis of variation in ecological and behavioural relevant traits, as exemplified by work on odor receptors and diapause (Denlinger and Armbruster 2014, Mcbride et al. 2014), in order to be able to recognize populations with the "right" combination of traits. Proximally, we must recognize that populations differ over time

and space such that "yesterday's epidemiology may not predict today's". For example, highly endophilic and anthropophilic populations of *Ae. aegypti*, once widespread were significantly curbed by insecticide intensive campaigns of the 1940s and 1950s. Indoor applications of insecticides, a premier strategy for control of yellow fever vectors have been shown to select for exophilic behaviour in *Ae. aegypti* (Pates and Curtis 2005) and could be part of the reason why truly domestic (indoor) populations of this species are now harder to find (Brown et al. 2014). Although reconstructing the history of long ago events conclusively is always difficult, real time examinations of more recent expansions, such as that of *Ae. j. japonicus*, support the hypothesis that most invasive populations are the outcome of multiple introductions of the same species, followed quickly by local differentiation and human-mediated mixing (Egizi and Fonseca 2015, Egizi et al. 2016, Fonseca et al. 2010, Kaufman and Fonseca 2014).

In summary, by the very nature of their expansion worldwide, invasive species have become domesticated and therefore are overall likely to reach high local abundance around humans while also recognizing them as sources of blood, a dangerous combination. However, rapid evolution associated with demographic bottlenecks or insecticide applications and subsequent mixing among differentiated populations have created a lattice of phenotypes that make broad extrapolations across geography and across time ineffective. While most high-resolution population level approaches currently available only allow the reconstruction of demographic history they may be combined with loci (genes) phenotypically relevant. Such a tool will finally allow the assessment of the vectorial capacity of mosquito populations and track changes over time and space in disease transmission risk, particularly after specific interventions.

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Rolle der Wissenschaft im Globalen Wandel

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Gesellschaftliche Probleme verlangen heute sehr häufig eine Widerspiegelung im Bereich der Wissenschaften. Als Nationale Akademie der Wissenschaften ist die Leopoldina in zunehmendem Maße gefordert, auch Beratung bei Fragen zu liefern, die über Länder und Kontinentgrenzen hinausgreifen: Klimawandel, der Einsatz erneuerbarer Energien, Fragen der Gesundheitsversorgung, die Einrichtung einer effektiveren Landwirtschaft zur Bekämpfung von Hunger in Krisengebieten und die sich wandelnde Altersstruktur von Bevölkerungen in vielen Staaten sind nur einige Beispiele für entsprechende Gebiete mit dringendem Forschungsbedarf. Sie bilden Herausforderungen für die Gesellschaften, die nur in internationaler, oft globaler Zusammenarbeit zu bewältigen sein werden. Daher wählte die Leopoldina 2012 das Thema „Rolle der Wissenschaft im Globalen Wandel“ für ihre Jahresversammlung. Der Band umfasst Beiträge zu den Themenkomplexen „Die Erde im Globalen Wandel“, „Herausforderungen des Globalen Wandels“ und „Lösungswege von Problemen des Globalen Wandels“ sowie zu den gesellschaftlichen und politischen Implikationen der mit dem globalen Wandel verbundenen Prozesse.

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Pathways to Becoming Pests and Vectors: Lessons from the Simuliidae

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Abstract

The family Simuliidae includes at least 45 economically important pest species, more than 25 vectors of disease agents of humans, and probably hundreds of vectors of disease agents of domestic animals and wildlife. An understanding of how simuliid vectors arise and why only certain species of simuliids become pests can provide the basis for predicting and preventing pest and vector problems. The historical process of becoming a prominent vector involved the evolution of anautogeny and repetitive blood feeding, augmented by dispersal from the cool, mountainous, northern ancestral habitat of simuliids into lowland and tropical areas where multiple generations could be achieved and an increased range of potential host species became available. The process of attaining pest status occurs through two primary pathways, both of which generate large populations of simuliids typically associated with pest problems. One pathway involves habitat generalization – development in the majority of streams and rivers in an area – and the other involves habitat specialization centered around development in large rivers. The latter pathway is typical of the world's major pests of humans and domesticated animals, and includes the livestock-killing species.

Zusammenfassung

Die Familie der Simuliidae umfasst mindestens 45 ökonomisch wichtige Schädlingsarten, mehr als 25 Vektoren von Krankheitserregern für Menschen und wohl Hunderte Vektoren von Krankheitserregern für Haustiere und Wildtiere. Eine Erkenntnis darüber, wie Simuliid-Vektoren entstehen und warum nur gewisse Arten von Mitgliedern der Simuliidae-Familie Schädlinge werden, kann die Grundlage für die Prognose und das Verhindern von Schädlings- und Überträgerproblemen bilden. Der Geschichtsprozess der Entwicklung eines maßgebenden Vektors ging einher mit der Entwicklung der Nicht-Autogenität und dem repetitiven Blutsaugen, verstärkt durch die Ausbreitung von dem kalten, gebirgigen, nördlichen angestammten Lebensraum der Mitglieder der Simuliidae-Familie in das Flachland und in tropische Gebiete, wo mehrere Generationen entwickelt werden konnten und eine größere Vielfalt möglicher Wirtsarten vorhanden war. Der Prozess des Erreichens eines Schädlingsstatus tritt durch zwei grundlegende Wege ein, wobei beide große Bestände von Mitgliedern der Simuliidae-Familie schaffen, die typischerweise mit Schädlingsproblemen in Verbindung gebracht werden. Ein Weg betrifft die Verallgemeinerung des Lebensraums – Entwicklung in dem Großteil der Ströme und Flüsse in einem Gebiet – und der andere die Lebensraumspezialisierung um die Entwicklung in großen Flüssen. Der letztere Weg ist typisch für die weltbedeutendsten Schädlinge für Menschen und Haustiere und schließt die Arten, die Nutztiere töten, ein.

1. Introduction

Blood-feeding arthropods share certain attributes, notably their persistent attacks on animals, including humans, and their potential to transmit disease agents. These attributes carry negative societal value – threats to public health and animal welfare – and bring blood-feeding arthropods into the orbit of scientific investigation. A global community of medical-veterinary biologists forms around these arthropods, with common goals of eliminating disease threats and suppressing pests below economic thresholds.

Each group of blood-feeding arthropods brings its own unique set of biological attributes, economic and societal consequences, and research challenges. Black flies (Simuliidae), for instance, are among the few insects that have routinely killed animals by direct attacks. Death typically results from exsanguination (withdrawing so much blood that it becomes too thick to transport oxygen efficiently) or, more commonly, from toxic shock – simuliotoxicosis – from injected salivary components (Wilhelm et al. 1982, Adler and McCreadie 2009, Schnellbacher et al. 2012). Unlike mosquitoes, which are well known for their ability to translocate among continents, no definitive cases of invasive black flies are known, despite a historically demonstrated ability to colonize even the most remote oceanic islands (Adler et al. 2005, Craig et al. 2001). New threats from black flies, instead, are likely to result from natural environmental events, such as El Niño and local flooding (Ballard 1994, Adler et al. 2004); human encroachment into undisturbed natural areas (Basáñez et al. 2000); and anthropogenic habitat disturbances such as construction of dams (Fredeen 1985).

Understanding the process of becoming a pest or a vector, particularly the evolutionary process, which has an underlying genetic basis, could enhance opportunities for proactive pest management and vector control. Sufficient commonality exists between pests and vectors to discuss both in the same conceptual framework. And a single species, such as *S. meridionale* and some members of the *S. amazonicum* group, can be both a pest and a vector (Adler et al. 2004, Shelley et al. 2010).

Vector status is an inherent biological property of an organism, fine-tuned by selection pressures levied by the behavioural, physiological, and structural features and defenses of the host and parasite. Pest status is not an inherent biological property. It is the result of human interests conflicting with organismal traits, and it typically manifests as direct attacks on humans, domesticated animals, and wildlife (Crosskey 1990, Adler et al. 2004, Werner and Adler 2005, Adler and McCreadie 2009), including endangered species (Adler et al. 2007, King and Adler 2012). The term 'pest', as used here, follows the definition applied by Adler et al. (2016): "a species that in some part of its range has caused economic losses or has been a target for management".

The objective here is to review and synthesize the pathways and processes involved in becoming pests and vectors, the associated life-history traits that promote these processes, and the role of taxonomy and systematics in directing an understanding of pest and vector biology and the strategies for control.

2. Pests and Vectors – Background

2.1 Taxonomic and Ecological Scope of Pests and Vectors

A global total of 2,177 valid species of Simuliidae are formally recognized (Adler and Crosskey 2015a), representing about 0.2 % of all insects and 1.4 % of all Diptera (Adler and Foottit 2009). About 98 % of the 2,177 formally described species of black flies feed on vertebrate blood (Adler 2009), setting the scene for pest and vector problems. Roughly 45 species fit the definition of a pest, about 1.5 % are demonstrated vectors of the causal agents of human diseases, and about 1 % are vectors of organisms that cause diseases in livestock and poultry (Adler and McCreadie 2009, Adler et al. 2010). The majority (ca. 94 %) of pests

and vectors (Adler and McCreadie 2009) are concentrated in the largest and most derived genus, *Simulium*, with 80 % of the world's simuliid species (Adler and Crosskey 2015a). Efforts to suppress and control pests and vectors are complicated by the structural homogeneity of many species of simuliids. Nearly all vectors, for example, are complexes of several to nearly 50 isomorphic species (Conn et al. 1989, Charalambous et al. 1996, Post et al. 2007, Adler et al. 2010, Shelley 2010). The enormous pool of simuliid-borne pathogens and parasites in wildlife (Hellgren et al. 2008, Murdock et al. 2015, Lotta et al. 2016) suggests the potential for additional disease problems in domesticated animals and perhaps in humans. The unexpected explosion of the previously obscure mosquito-borne zika virus from geographically restricted wild primates and arboreal mosquitoes to widespread mosquitoes and humans on a multicontinental scale provides a telling example of possibilities that could occur in the Simuliidae (Fauci and Morens 2016).

In contradistinction to the economic and public health burdens, the beneficial value of simuliids as key ecological organisms – "ecosystem engineers" – in lotic and terrestrial environments can be enormous (Malmqvist et al. 2001, 2004a). So, too, do larvae have value as biological indicators of water quality (Carle et al 2015). Suppression programmes, therefore, should be designed to maximize not only the level of control, but also the ecological value of the species targeted for control.

An important caveat for programmes battling simuliid pest problems and vector-borne diseases is that success can bring its own set of new problems. The remarkable effectiveness of the biological control agent *Bacillus thuringiensis israelensis* (*Bti*) against simuliids was associated with a significant decrease in research on natural enemies, portending concern over a shift to alternative strategies if resistance to *Bti* should develop (Adler et al. 2004). The economic and public health success of West Africa's colossal Onchocerciasis Control Programme (OCP), as of 2001, had freed 25 million hectares of once-abandoned land for resettlement and agriculture (Richards et al. 2001), bringing "massive landscape changes" with associated ecological consequences (Resh et al. 2004).

2.2 Vector Problems

The only two documented simuliid-borne human diseases are filarial-associated mansonellosis and onchocerciasis (river blindness). Mansonellosis is a mildly pathogenic disease presumably endemic to the New World (Shelley and Coscarón 2001). Human onchocerciasis, however, is Afrotropical in origin, having been introduced to Latin America with the slave trade (Procunier and Hirai 1986, Zimmerman et al. 1994, Morales-Hojas et al. 2007, Crainey et al. 2016). Onchocerciasis remains a major public health threat in large areas of Africa south of the Sahara Desert (*WHO* 2105) where its control is often hampered or interrupted by war, civil strife, and infrastructural inadequacies or breakdowns, as in the 2014–2015 Ebola epidemic. Human onchocerciasis has been nearly eradicated from the New World, largely through a long-term ivermectin programme. It remains in only two of the previous 13 New World foci – the Yanomami region of the Amazonian highlands of Brazil and Venezuela (*WHO* 2015). Although onchocerciasis periodically enters North America, it is unlikely to become established in the Nearctic Region (Adler et al. 2010, Encarnacion et al. 1994) or, for that matter, in the Palearctic Region. These regions lack appropriate vectors, suitable climate, or the factors necessary to achieve a critical density of parasites for endemic transmission.

Simuliid-borne diseases of domesticated animals are more numerous and widely distributed than are the human diseases. They include avian trypanosomiasis, bovine onchocerciasis, canine onchocerciasis, leucocytozoonosis, vesicular stomatitis, and perhaps tularemia (Adler 2009). Some of these diseases are abundant and widespread (Valkiūnas 2004), whereas others are sporadic (e.g. Hassan et al. 2015).

2.3 Vector Prerequisites

The two fundamental biological requirements for a simuliid vector include anautogeny (i.e., egg development dependent on blood feeding) and acquisition of more than one blood meal (i.e., one meal to acquire the parasites and one to deliver them). These characteristics are found throughout the family Simuliidae, predisposing a wide range of species to becoming vectors.

Vector efficiency has been augmented by key evolutionary events. Over evolutionary time, for instance, black flies began dispersing from their ancestral habitat – cool, mountainous streams in northern areas (Currie and Adler 2008) – into lowlands and eventually into tropical areas. The probability of transmission increased with adaptation to tropical environments where more generations per year (multivoltinism) became possible (Adler et al. 2010) and a broad range of host species became available. Accordingly, all vectors of the parasites that cause human diseases are multivoltine, completing up to 15–20 generations per year (Crosskey 1990).

The choice of blood host will factor into the vector potential of each species of black fly. A reasonably strong correlation exists between claw structure and host category – mammal *versus* bird. An unadorned talon typically reflects mammalophily, whereas a thumblike lobe at the base of the talon signifies ornithophily (Malmqvist et al. 2004b). Exceptions, however, exist (Lotta et al. 2016). Specific host use by black flies is insufficiently explored and, in most cases, the full range of host species used by a simuliid species is unknown. The effects of blood chemistry of each vertebrate host species on simuliid fitness also are unknown beyond a few studies that indicate significant differences among host species (Mokry 1980a, b). Studies of simuliid saliva suggest general adaptations to the use of avian *versus* mammalian blood (Cupp and Cupp 1997). Introduction or removal of alternate hosts, domesticated (e.g., cattle) or wild, to an area can alter the risk of infection for other hosts in the area (Krüger et al. 2005).

2.4 Pest Problems

Pest problems caused by simuliids typically derive from biting and blood feeding, persistent swarming, crawling on the skin, and flying into facial orifices (Crosskey 1990, Adler et al. 2004, Adler and McCreadie 2009). Less common pest problems include, for example, dense mating swarms obstructing bicycle paths (e.g., *Simulium vittatum* complex) and mass emergences fouling and obscuring vehicle windshields (e.g., *Cnephia pecuarum*) (Adler et al. 2004). In most cases, pest status is associated with abundance. Exceptions include situations in which even a single black fly can be unacceptable – accidentally embedding in paper products during milling operations or flying obstinately about the face of a person intent on playing a game of golf (Gray et al. 1996, Adler et al. 2004).

3. Factors Driving Pest Status and Vector Problems

3.1 Generalists and Specialists

Pest and vector problems are related to population size of the individual species. This relationship suggests that an understanding of the generators of abundance – organismal attributes that allow populations of a species to proliferate – might provide insights into the process of becoming a pest or exacerbating a vector problem. Premiere among these organismal attributes is the choice of breeding habitat. The 40-plus simuliid pests of the world typically can be classified as habitat generalists or habitat specialists, based on the size of the watercourses in which the immature stages develop.

Species that exploit the majority of watercourses in an area are likely to achieve large populations in that area. These are the habitat generalists – species that occupy a range of streams and rivers, from a meter to roughly a hundred meters in width. They are the species that can be found in any watercourse during routine sampling. Examples of habitat generalists that are pest species include *Prosimulium mixtum*, *Simulium equinum*, *Simulium erythrocephalum*, *Simulium ornatum*, *Simulium venustum*, and *Simulium vittatum*. Habitat generalists might have a broader physiological tolerance of environmental conditions, such as oxygen concentration, pH, and particulate load in the watercourses, although this relationship has not been tested. Tolerance of a broad range of aquatic conditions carries with it the ability to inhabit polluted watercourses that exclude most macroinvertebrates and fish (competitors and predators) but allow a few species of simuliids, including those predisposed to becoming pests, to achieve high densities (Adler et al. 2004).

To declare a species a habitat generalist first requires sorting out potential cryptic species that would give the illusion of a single species with a broad habitat range. The original concept of *Simulium tuberosum* was that of a widely distributed, ecologically versatile Holarctic species occurring in the majority of streams and rivers in any given area. Subsequent cytogenetic analyses revealed that so-called *S. tuberosum* is actually a complex of at least 10 species in the Nearctic Region alone (Landau 1962, Mason 1984, Adler et al. 2004), each breeding in its own size range of streams and rivers, and exhibiting different levels of prevalence by ecoregion – each species a habitat specialist (Adler and McCreadie 1997). *Simulium venustum* was viewed originally as a wide-ranging species, both ecologically and geographically. Cytogenetic analyses revealed that it, too, consists of cryptic species – at least 10 in North America – each with a well-defined ecology (Rothfels et al. 1978, McCreadie and Colbo 1993, Adler et al. 2004). *Simulium venustum* sensu stricto, nonetheless, remains ecologically adaptable to a wide range of watercourses (Adler et al. 2004). Thus, as currently recognized, *S. venustum* s. s. is a bona fide generalist. *Simulium pertinax*, a Neotropical pest that colonizes the majority of streams in an area, is a single species, based on chromosomal analyses (Campos et al. 2001). As a caveat, however, species defined on morphological and chromosomal criteria still might hold additional cryptic species awaiting discovery when subjected to molecular analyses (Low et al. 2016).

Habitat specialists occupy a narrower range of breeding sites. They include, for example, headwater species and big-river species. Spring-fed, headwater streams are typically small and shallow, with low productivity and reduced oxygen levels. The specialists of spring-fed habitats typically do not reach population densities that cause pest problems. As an extreme example, species that are specialized to the extent that they also are rare (McCreadie and Adler 2008) do not cause pest problems.

The specialists most capable of achieving high population densities are the big-river species. Among the premiere examples are the Australasian *Austrosimulium pestilens*, members of the Neotropical *S. amazonicum* and *S. oyapockense* complexes, the Palearctic *S. maculatum*, the Nearctic *Cnephia pecuarum*, and most notably, the members of the Holarctic *S. jenningsi-malyschevi-reptans* clade, which includes about 25 % of the most significant pest species in the world, such as five of the nine livestock killers (*S. colombaschense*, *S. kurense*, *S. luggeri*, *S. reptans*, and *S. vampirum*) and the world's worst nuisance pest (*S. jenningsi*) (Adler et al. 2016).

Thus, pest status can be reached by two principal pathways, first described and outlined by Adler et al. (2016): (*1*) colonization of a wide variety of streams and rivers (habitat generalization) and (*2*) colonization of large rivers more than 100 m wide (habitat specialization). Both colonization strategies promote the production of population levels sufficient to cause an economic or public health problem as pests, vectors, or both.

3.2 Case Study

Here I summarize an example of pest status acquired *via* colonization of large rivers (specialization), using a representative case study of *Simulium colombaschense*, originally developed and presented by Adler et al. (2016). *Simulium colombaschense* was the most destructive simuliid pest in history, killing as many as 22,000 livestock per year, inspiring the first biological study of the Simuliidae (in 1795), and generating its own mythology in the Iron Gate region of the Danube River between Romania and Serbia.

Simulium colombaschense is a member of the *S. jenningsi-malyschevi-reptans* clade (Adler and Huang 2011, Senatore et al. 2014, Adler et al. 2016). Phylogenetic analysis suggests that an ability to specialize in the largest rivers on Earth evolved in the ancestor of the *S. jenningsi-malyschevi-reptans* line (Adler et al. 2016).

The distribution of *S. colombaschense* is greater than the distribution of the pest problem, which is restricted to the area along the Danube River and Italy's Adige River. Populations of *S. colombaschense* in southern Italy, Greece, and Slovakia, for example, are not pests, despite being near livestock areas (Adler et al. 2016). A working hypothesis is that hidden biodiversity exists within *S. colombaschense*, associated with biological traits that result in differential pest status. *Simulium colombaschense*, thus, invites a taxonomic analysis.

A strong case has been made for chromosomal rearrangements as drivers of speciation in the Simuliidae (Rothfels 1989). Chromosomal differences, therefore, might reasonably be expected among populations that have undergone differential selection. The polytene chromosomes have been used for decades to evaluate simuliids for possible hidden diversity, such as cytotypes and cryptic species (Rothfels 1979, 1989, Adler and Crosskey 2015b).

Chromosomal analyses of *S. colombaschense* across its range revealed five cytologically distinct forms, at least two of which are reproductively isolated cryptic species (Adler et al. 2016). The pests of historical infamy, Cytoforms 'A' and 'B', inhabit the largest rivers, that is, the Danube and the Adige. The other three cytoforms ('C', 'D', and 'E') are not pests; they inhabit smaller rivers. 'D' is known from only one river in Greece and is reproductively isolated from 'C'.

The distributions of the five cytoforms reveal an intriguing trend. The cytogenetic profiles (e.g., polymorphism frequencies) of the cytoforms remain consistent within rivers but not across rivers (Adler et al. 2016). Cytoform 'A' is chromosomally cohesive for at least

1,000 km along the Danube River, but is distinct from Cytoform 'E' in another river 200 km distant, although no topographic barriers to gene exchange exist. Nor do physical barriers exist between 'A' and 'B'. Similarly, Cytoforms 'C' and 'D', in separate rivers less than 50 km apart with no physical barriers to gene exchange, are reproductively isolated. A pattern emerges: Populations within rivers, regardless of distance up or down river, are chromosomally cohesive, whereas populations between rivers, even if separated by shorter distances, are chromosomally distinct.

A similar pattern might be expected in other big-river species. *Simulium jenningsi* and *S. maculatum*, for instance, present compelling examples – abundant pests that breed in numerous large rivers across their ranges – that would be amenable to further testing of the pattern. Big-river vectors to which the pattern might apply include *S. amazonicum*, *S. guianense*, and *S. oyapockense*, all Neotropical vectors of *Onchocerca volvulus*, the causal agent of human onchocerciasis.

An irony is associated with the pattern of unique chromosomal cohesiveness of populations in large rivers. Big-river species can produce enormous numbers of females capable of dispersing to initiate subsequent generations (Amrine 1982). Although no apparent geographical barriers exist to dispersal and gene exchange – no large mountain ranges, for instance – the cytoforms of *S. colombaschense* within rivers, nonetheless, maintain their integrity while differing significantly from populations in adjacent rivers. Why? The pattern requires explanation.

The return of females to natal watercourses to oviposit would concentrate and increase populations in particular habitats. But from the perspective of an individual female fly, why return to the natal waterway? Adler et al. (2016) suggested that larger rivers are scarce and this scarcity makes this particular habitat more difficult for dispersing females to find. Selection, therefore, should favour females that return to their natal river. River fidelity builds and maintains river-specific chromosomal profiles. Habitat generalists, on the contrary, would be expected to show less site fidelity and not have site-specific chromosomal profiles. Of the generalist pests investigated, no evidence for site fidelity has been discovered (Rothfels 1981, Hunter and Jain 2000). Thus, the degree of habitat specialization might be expected generally to reflect the degree of site fidelity.

Adaptations demonstrate the relation of habitat specialists to their particular environment. Big-river species, for example, have various structural characters that would adapt the larvae and pupae for life in strong currents, such as stout labral fans, short antennae, strong silk for adhesion to substrates, many microhooks on the prolegs to engage the silk, a gradually expanded and streamlined larval body, a short pupal gill, and a shoe- or boot-shaped pupal cocoon to provide protection from abrasive suspended particles (Adler et al. 2016).

4. Conclusions

Understanding the factors that drive pest and vector problems can aid the identification of genes responsible for the enabling traits (i.e., adaptations). Thus, genes might be identified that code for traits associated with big-river specialization, such as strong silk, or associated with habitat generalization, such as tolerance of a broad range of oxygen levels. The option of genetically disrupting expression of these characters could provide a proactive approach to pest management and vector control.

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Towards High-Throughput Identification of Arthropod Vectors by Mass Spectrometry

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Abstract

Accurate and high-throughput identification of vector arthropods is of paramount importance in the surveillance and control programmes that are becoming more common due to changes in the geographic range and extent of many arthropod-borne diseases, including in Europe. Protein profiling by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) technically fulfils these requirements for identification, and various research groups have recently established reference databases for some vector taxa. However, their approaches vary greatly in terms of sample processing, equipment used, data acquisition and analysis. Furthermore, these databases are typically stored on local drives ("in-house databases") and are thus not accessible to the public. In a consortium of an academic institution and a private company, we have established the largest database of vector arthropods, and for the first time we showed that spectra obtained on mass spectrometers from different companies can be analysed using this database. Thus, a centralized high-quality database (created by expert taxonomists and experienced users of mass spectrometers), which is easily amenable to customer-oriented identification services, is feasible and should be made available in a cost-efficient manner in the near future *via* an online platform.

Zusammenfassung

Präzise und Hochdurchsatz-Identifizierung von Arthropodenvektoren sind von größter Bedeutung in Überwachungs- und in Kontrollprogrammen, die auch in Europa aufgrund der Änderung der geographischen Verbreitung und des Ausmaßes vieler durch Arthropoden übertragener Krankheitserreger immer häufiger werden. Protein-Profilierung durch Matrix-unterstützte Laser-Desorption/Ionisation-Flugzeitanalyse-Massenspektrometrie (MALDI-TOF MS) erfüllt technisch diese Anforderungen für die Identifizierung, und Referenzdatenbanken wurden kürzlich für einige Vektortaxa von verschiedenen Forschungsgruppen etabliert. Jedoch sind deren Ansätze in Bezug auf Probenverarbeitung, Ausrüstung, Datenerfassung und Analyse sehr unterschiedlich. Darüber hinaus sind diese Datenbanken in der Regel auf lokalen Laufwerken („*In-house*-Datenbanken") gespeichert und daher für die Öffentlichkeit nicht zugänglich. In einem Konsortium aus einer akademischen Einrichtung und einem Privatunternehmen haben wir die größte Datenbank für Arthropodenvektoren etabliert, und wir konnten zum ersten Mal zeigen, dass Spektren, welche mit Massenspektrometern verschiedener Firmen erstellt wurden, mit dieser Datenbank analysiert werden können. Somit wird eine zentrale, hochwertige Datenbank (erstellt von Taxonomie-Experten und erfahrenen Anwendern von Massenspektrometern) realisierbar, welche für eine kundenorientierte Identifizierung auf Dienstleistungsbasis leicht zugänglich ist und welche in naher Zukunft über eine Online-Plattform in kostengünstiger Weise verfügbar sein sollte.

1. Introduction

The geographic occurrence and extent of arthropod-borne diseases of medical and veterinary significance are changing. Drivers for this are, on the part of the pathogens, (*i*) the emergence of novel pathogens (e.g. *Plasmodium knowlesi*, Collins 2012, and Schmallenberg virus, Wernike et al. 2014); (*ii*) the long-distance spread of pathogens in the context of increasing globali-

zation (e.g. through the rapid movement of infected individuals) to new areas where they can be transmitted in an unpredictable way by indigenous vectors (e.g. bluetongue virus introduced into Europe in 2006 and transmitted by biting midges, Wilson and Mellor 2009; West Nile virus to the United States in 2009 and transmitted by mosquitoes, Hofmeister 2011); and (*iii*) the re-emergence of known pathogens (e.g. chikungunya, dengue, West Nile virus) through genetic adaptations to new vector species (Ciota and Kramer 2013, Coffey et al. 2013). On the vector side, changes in the risk of exposure to pathogens are caused by the spread of invasive vector species (Schaffner et al. 2009, Capelli et al. 2011, Medlock et al. 2012), e.g. the Asian tiger mosquito *Aedes albopictus* (syn. *Stegomyia albopicta*), which was the incriminated vector in recent outbreaks of chikungunya and dengue fever in southern Europe (Schaffner and Mathis 2014). Further, the distribution ranges of indigenous arthropod vectors are expanding in Europe, including those of ticks (Jaenson et al. 2012, Dergousoff et al. 2013), phlebotomine sand flies (Maroli et al. 2013, Poeppl et al. 2013) and biting midges (Nolan et al. 2008), driven by environmental and socioeconomic changes. In the coming decades, distributional shifts of arthropod-borne diseases will be further fuelled by climate change.

These examples highlight the growing importance of surveilling and controlling arthropod vector populations across Europe (Medlock et al. 2012, Alkan et al. 2013, Schaffner et al. 2013). As only few of the numerous arthropod species are important disease vectors, accurate species identification is fundamental to these aims. Morphological identification is still considered the "gold standard" method, yet species identification by morphology is often very time consuming (e.g. in case body parts need to be mounted on a microscope slide) and requires a considerable degree of proficiency and experience. Morphological identification can be difficult when using hierarchical dichotomous keys with specimens that are damaged and lack features, such as scales, that are addressed in the key and which are important in distinguishing important from less important species (e.g. *Ae. albopictus*, *Ae. cretinus*, Patsoula et al. 2006). Alternatively, interactive keys have been developed, e.g. for *Culicoides* biting midges (Mathieu et al. 2012), that are structured as multi-entry keys – although in the cited study users with advanced expertise correctly identified only 75 % of the specimens. Morphology may not reliably distinguish between closely related species in many cases, and morphological features between specimens of the same taxon may also vary depending on the environmental background (Prudhomme et al. 2012). Finally, comprehensive morphological keys may not be available for certain taxonomic groups (Ready 2013), and this is also the case for early life stages (e.g. eggs of aedine mosquitoes, Schaffner et al. 2014; larvae of biting midges, Steinmann et al. 2013).

One alternative to morphological identification is nucleic acid tests. A number of polymerase chain reaction (PCR) assays have been developed for various vector arthropods, in conventional or real-time formats and allowing for the discrimination of single (singleplex assays) or few (multiplex) genetically defined species (Bahnck and Fonseca 2006, Shone et al. 2006, Bass et al. 2008, Cetre-Sossah et al. 2008, Pages et al. 2009, Wenk et al. 2012, Minter et al. 2013, Engdahl et al. 2014, Kronefeld et al. 2014, van de Vossenberg et al. 2015). The sensitivity of PCRs is high, although identification of specimens in large pools does not seem to be straightforward (Wenk et al. 2012). Specificity is determined by the primers included in the reactions ("you identify what you are looking for"). These approaches are generally considered time-consuming and expensive, particularly if precise identification requires a follow-up by sequencing of amplicons. Loop-mediated isothermal amplification (LAMP) seems superior to PCR in some points (low cost of instrumentation, less sensitive to

inhibitory components allowing use of "quick and dirty" DNA isolations; direct visualization of DNA replication, Tomlinson 2013), but so far it has gained only very limited attention in vector entomology (Bonizzoni et al. 2009, Schenkel et al., in preparation). A caveat with these DNA amplification assays is that intra-species genetic variability at the target loci (e.g. mitochondrial COI gene, rDNA internal transcribed spacers; e.g. Dvorak et al. 2006, Wenk et al. 2012, Versteirt et al. 2015) might lead to false negative results.

High-throughput DNA sequencing (next generation sequencing, NGS) is increasingly being applied in environmental and clinical studies, allowing the identification of all organisms in a sample for which reference sequence data are available ("you identify what is included in the database"). Metabarcoding relies on PCR performed with taxa-specific primers, targeting the respective barcode region (e.g. 16S rRNA gene in bacteria, a chloroplast gene in plants, rDNA ITS in fungi, the mitochondrial COI gene in animals; Ji et al. 2013, De Barba et al. 2014, Op De Beeck et al. 2014, Fantini et al. 2015). As with "conventional PCRs", primer choice can bias the results (Pawluczyk et al. 2015). Metagenomics is an unbiased sequencing approach, i.e. the massive shotgun ("random") sequencing of genomic DNA of a sample. Current limitations to this approach include costs, the need for DNA of high quantity and quality, and some bioinformatics challenges (Wang et al. 2015). Some comparative studies on these two NGS approaches are available (e.g. Srivathsan et al. 2015).

2. Protein Profiling by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) for Arthropod Characterization

MALDI-TOF MS has revolutionized the routine identification of microorganisms such as bacteria and fungi (Patel 2015), and more than 3,000 diagnostic microbiology labs are employing this technology in Europe. The technique is very simple, requiring minimal sample preparation: sample homogenates are mixed with a matrix on steel plates; macromolecules are ionized and desorbed upon pulsed laser irradiations, then separated according to mass in a vacuum environment. MALDI-TOF MS generates a mass spectrum that can then be compared to reference spectra of species that are stored in a reference database ("you identify what is included in the database"). Reference spectra (biomarker mass patterns) are calculated with the spectra of several well defined individuals (Schaffner et al. 2014). Such databases are available commercially for clinical microbiology, and MALDI-TOF MS has been shown to be very accurate, with high inter-laboratory reproducibility (Mellmann et al. 2009). The technique is fast, delivering results within minutes, and it is cost-effective, at < 1 Euro per sample, including labour (Buchan and Ledeboer 2014), though the acquisition costs for a mass spectrometer are still high.

In recent pioneering work, MALDI-TOF MS has been evaluated for the characterization of insects from colonies, namely *Drosophila* spp. (Campbell 2005, Feltens et al. 2010) and plant aphids (Perera et al. 2005). In an initial investigation with arthropod vectors (*Culicoides nubeculosus* biting midges from a colony), we evaluated a number of parameters, including homogenization assay, age and sex of the insects, storage conditions and body parts to be analysed (Kaufmann et al. 2011). Unsurprisingly, the presence of blood in the abdomens interfered with the MALDI-TOF pattern, reducing the intensity of the midges' biomarker masses up to four days after the blood meal. Thus, as remnants of a blood meal might not easily be detectable in females, particularly when processing large numbers, the

abdomens should be removed by default before analysis. Though this can be a tedious task with small vectors, it allows the abdomen to be used for genetic analyses, confirming species identification as well as identifying the blood host. Next, we established a MALDI-TOF MS database comprising reference spectra of the 26 most abundant species in Switzerland (Kaufmann et al. 2012a, b), and were able to confirm a very high level of agreement of MALDI-TOF MS with morphology/PCR of above 98 % when analysing 1,200 randomly selected biting midges from field collections. Thus, 1,177 specimens (97.7 %) could unequivocally be assigned by MALDI-TOF MS to one of the species included in the database, whereas 14 specimens (1.2 %) yielded novel spectra. Thirteen specimens (1.1 %) could not be identified due to low spectra quality (Kaufmann et al. 2012b). We then expanded our database by including reference spectra of adult Phlebotominae (20 species; Mathis et al. 2015), Culicidae (51 species) and Simuliidae (six species, unpublished). Contrary to DNA sequencing approaches, mass spectra are specific for the different developmental stages. We thus determined mass spectra for larval stages of *Culicoides* biting midges and of Culicidae (Steinmann et al. 2013). Similar to the blood in blood-fed adults, the gut content of larvae strongly impairs the protein profile, and thus gut-less larvae or parts without gut (head and thorax in Culicidae) are analysed. Further, specific biomarker mass sets were determined for eggs of nine container-breeding aedine mosquito species, including all the major invasive and indigenous species of Europe and North America (Schaffner et al. 2014). Adult and larval stages are processed and analysed singly, but this is impractical in the case of eggs. We were able to show that up to three species can be identified when analysing pools of 20 eggs (Schaffner et al. 2014), similar to the findings with mixed bacterial species (Mahe et al. 2014). Our database is maintained by a private company (Mabritec SA, Riehen, Switzerland) and is publicly available at market prices or at cost price for contributors. Routine surveillance for the tiger mosquito *Ae. albopictus* is performed in Switzerland, based on aedine eggs collected by ovitraps and their identification with MALDI-TOF MS (Müller et al. 2013, Flacio et al. 2015). Interestingly, two unexpected species were identified with this approach, namely *Ae. koreicus*, which has been described as an invasive species in parts of neighbouring Italy (Suter et al. 2015), and *Ae. cretinus*, "the Mediterranean representative of *Ae. albopictus*" (Edwards 1921, cited in Samanidou-Voyadjoglou et al. 2005) whose adults might have been confused with *Ae. albopictus* in earlier surveillance programmes relying on adult collections.

MALDI-TOF MS has been used by few other groups for the identification of arthropod vectors in Europe: one group in France (six tick species, Yssouf et al. 2013a; five flea species, Yssouf et al. 2014a, 20 Culicidae adult, Yssouf et al. 2013b, and six larval stage species, Dieme et al. 2014); three groups in Germany (seven tick species, Karger et al. 2012; five tsetse fly species, Hoppenheit et al. 2013; seven biting midge species, Uhlmann et al. 2014); and finally one group in the Czech Republic (six phlebotomine sand fly species, Dvorak et al. 2014). Outside Europe, to the best of our knowledge, only one group has so far made use of the technique. Fortunately, MALDI-TOF MS is also becoming available in tropical Africa (Fall et al. 2015), and a reference database comprising ten *Culicoides* spp. has been established in Senegal (Sambou et al. 2015).

Although most databases were created with arthropods mainly from laboratory colonies, several approaches have proved valid for the identification of field-collected specimens (Kaufmann et al. 2012a, b, Hoppenheit et al. 2014, Yssouf et al. 2014a, Flacio et al. 2015, Mathis et al. 2015, Sambou et al. 2015). The storage of specimens (frozen, in ethanol or dry) and storage duration have emerged in several studies as critical factors determining the qual-

ity of mass spectra, but these aspects have not yet been extensively evaluated (Kaufmann et al. 2011, Dvorak et al. 2014, Yssouf et al. 2014a, Mathis et al. 2015).

In contrast to morphology but in accordance with DNA-based methods, MALDI-TOF MS was capable of separating sister taxa and cryptic sibling species, as shown for *Culicoides* (Kaufmann et al. 2012a) and Culicidae (Yssouf et al. 2013b, Schaffner et al. 2014; and own unpublished data). However, members of the *Anopheles gambiae* complex could only be differentiated by considering not only presence/absence of mass peaks but also their intensities (Muller et al. 2013). The classification of specimens from different lab colonies was also possible with highly standardized approaches (fresh mosquito specimens, same feed, etc.) (Muller et al. 2013, Dvorak et al. 2014) but such "fine typing" seems improbable with field-collected specimens. Nevertheless, closer analysis of peaks whose peptide sequences are known (determined either after proteolytic cleavage or from genomic data) and for which allelic heterogeneity is known might in the future contribute to yield insights into the population structures of arthropod vectors, which is of particular interest, e.g. in the case of invasive species, in order to track their sources and to understand their population behaviour. Such targeted peak analyses might also identify genetic markers, e.g. for insecticide resistance.

Other applications of MALDI-TOF MS in arthropod vectors include the determination of the age, sex and mating status of mosquitoes, which are important factors for pathogen transmission dynamics (Suarez et al. 2011, Hugo et al. 2013). Very recently, mass spectrometry has been used to identify bacterial pathogens in tick vectors in small studies (Fotso Fotso et al. 2014, Yssouf et al. 2015). Whether this approach is suitable for the detection of other vector-borne pathogens remains to be seen.

While it is thus straightforward to establish MALDI-TOF MS databases for the identification of arthropod vectors, and there is sound evidence for the diagnostic value of the technique, there are currently major obstacles preventing wider use. These mainly include the fact that, with the exception of our database maintained by a private company, the databases are scattered, mainly at academic institutions ("in-house databases"), which do not offer routine identification of specimens for external persons meaning such databases are not readily accessible to the public. Further, existing databases cannot be merged as the approaches used by the various research groups vary greatly in many ways. This includes the body parts analysed (from whole arthropods to only legs or wings, e.g. Karger et al. 2012, Hoppenheit et al. 2014, Yssouf et al. 2014a) and experimental protocols (e.g. simple mechanical homogenization in formic acid, e.g. Kaufmann et al. 2012a; sonication, e.g. Karger et al. 2012; or complex protein extraction with tryptic digestion and clean-up steps, e.g. Uhlmann et al. 2014). Also, differences exist with regard to the matrices that are used and the mass ranges of the peaks that are considered (e.g. 2–15 kDa or 2–20 kDA in studies on ticks; Karger et al. 2012, Yssouf et al. 2013b).

Further, MALDI-TOF MS instruments are produced by different companies, with the two most widespread instrument series being from Bruker Daltonics (Biotyper/Utralflex, Microflex) and Biomerieux (VitekMS)/Shimadzu (Axima). Several aspects differ between the systems, including the implemented software, which hampers the exchangeability of even raw data. We have recently addressed this apparent incompatibility between the MALDI-TOF MS platforms. Raw mass spectra of phlebotomine sand flies obtained by automatic routine procedure on a Bruker instrument were blindly subjected to automatic identification using our database and the software established on a Shimadzu system, yielding highly accurate results (98.3 % for laboratory-reared specimens; 94.7 % for field-collected specimens, Mathis et al. 2015).

3. Outlook

The power of the mass spectrometric identification of organisms depends on the quality of the available database (i.e. reference quality, taxonomic coverage) and its accessibility. A number of developments need to be made in order to render the MALDI-TOF MS technology universally beneficial and available for the identification of arthropod vectors. We propose the following approach: First, harmonized protocols ("standard operating procedures", SOPs) should be implemented to provide instructions as to how arthropod vectors should be collected, preserved and processed, including parameters for the acquisition of mass spectra and data handling on instruments from different companies. Based on these SOPs, a comprehensive database that comprises the reference spectra of all major arthropod vectors could then be built. This requires the participation of expert taxonomists, particularly when dealing with closely related species, and experienced users of mass spectrometers who would ensure a high standard of quality in the generation of reference spectra. We advocate the establishment of a centralized comprehensive database that might better guarantee consistency and data quality of deposited reference spectra. As accessibility (i.e. the analysis of specimens by third parties) might be an issue at scientific institutions where the capacity for analysis and the availability and willingness of an operator could be limiting factors, a centralized database at a private company seems the most practical solution. This would ensure both customer-oriented service and greater sustainability. Ultimately, the aim should be for the database to be hosted *via* a publicly accessible online platform, so that anyone with access to a MALDI-TOF MS machine can measure specimens of arthropod vectors on his or her own equipment and obtain automated species identification in a cost-efficient manner by submitting mass spectrometry data to the centralized database. Further, the platform should provide access to all SOPs and also to a user forum offering information exchange, troubleshooting, etc.

Given the increasing importance of arthropod vectors for public health, and considering the fact that an arthropod vector mass spectrometry database will by far not have the commercial significance of databases of clinically important microorganisms, core funding by public or non-profit organizations to assure open access for non-commercial institutions and to maintain the database (with regard to e.g. continued expansion, taxonomic adaptations) might be a realistic option. This would revolutionize the efficiency and scientific rigour of surveillance and control programmes of arthropod vectors and the diseases they transmit.

We hope to further contribute to reaching the aims outlined above, along with our industry partner Mabritec SA and our colleagues at COST Action TD1303 "European Network for Neglected Vectors and Vector-Borne Infections (EurNegVec)", which represents the perfect network for scientists to collaborate on developing a unified MALDI-TOF MS platform for arthropod vectors with a high standard of quality and throughput.

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Note added in proof: For a recent review an molecular identification of arthropods, including mass spectrometry see Yssouf et al. 2016.

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The Red Flour Beetle – A New Genetic Model System for Pest and Vector Control with the Option of Large-Scale RNAi Screening

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Abstract

Genetic research in pest insects is notoriously challenging due to long generation time, difficulties in stock keeping, and limited experimental possibilities. Therefore, genetic research has focused on model systems that are easier to handle in the lab and that have an extensive genetic tool kit. Within arthropods, basic research has mainly been performed in *Drosophila melanogaster*, and the results have subsequently been transferred to arthropod pest or vector species. Here, we briefly present the reasons for the dominance of a few genetic model systems and subsequently introduce some recent technical breakthroughs that allow functional genetic work to be extended to additional arthropod species. We present the red flour beetle *Tribolium castaneum* as an emerging model system representing Coleoptera, which comprise many important pests. *T. castaneum* builds on an experimental toolkit second only to *D. melanogaster*. It comprises a well annotated genome and efficient gene knockdown by environmental/systemic RNAi. Further, transgenic methods *via* transposon-mediated insertion or CRISPR/Cas9 genome editing have allowed for the misexpression of genes and the establishment of *in vivo* imaging tools, among other things. Importantly, *T. castaneum* has been set up for genome-wide RNAi screening. The *iBeetle* screen has established resources that will facilitate future unbiased screens for novel gene functions outside the well-established *Drosophila* system. We propose that the interaction of scientists working on emerging genetic model organisms and those studying pest or vector species promises to enhance the development of transgenic tools for vector and pest control.

Zusammenfassung

Genetische Untersuchungen in Schadinsekten sind meist schwierig, zum Beispiel weil deren Generationszeit lang oder die Stammhaltung schwierig ist und weil die experimentellen Möglichkeiten begrenzt sind. Daher waren genetische Untersuchungen lange auf wenige Modellorganismen beschränkt, die gut zu halten und die einem großen Arsenal an genetischen Werkzeugen zugänglich sind. Bisher wurden bei Arthropoden grundlegende Untersuchungen vor allem an *Drosophila melanogaster* gemacht und die Resultate dann auf Schädlinge oder Vektoren übertragen. Hier fassen wir kurz die Gründe zusammen, warum es so wenige genetische Modellsysteme gibt, und führen dann kurz in die jüngsten technischen Durchbrüche ein, die es nun erlauben, funktionelle Genetik auch in anderen Arthropoden zu betreiben. Wir stellen den Reismehlkäfer *Tribolium castaneum* als neues Modellsystem innerhalb des Taxons Coleoptera vor, in dem viele Schädlinge zu finden sind. Bezüglich funktionell genetischer Methoden muss sich *T. castaneum* nur der Fruchtfliege *D. melanogaster* geschlagen geben. Er hat eine gut annotierte Genomsequenz und zeigt effizienten Gen-Knockdown über systemische RNAi. Transgene Methoden über Transposon-vermittelte Integration oder CRISPR/Cas9-Genom-Editierung erlauben die Erstellung von *In-vivo-Imaging*- und Gen-Missexpressionslinien und anderen Werkzeugen. Ganz entscheidend ist die kürzlich entwickelte Möglichkeit in *T. castaneum*, genomweite RNAi-Screens durchzuführen. Im *iBeetle*-Screen wurden Ressourcen entwickelt, die erstmalig die genomweite unvoreingenommene Suche nach Genfunktion außerhalb von *D. melanogaster* erlauben. Wir glauben, dass eine Zusammenarbeit von Wissenschaftlern, die an der Entwicklung von neuen genetischen Modellsystemen arbeiten, mit Experten der Vektor- oder Schädlingsbiologie sehr fruchtbar sein kann, um diese neuen Techniken zur Bekämpfung von Schädlingen oder Vektoren nutzbar zu machen.

1. A Handful of Genetic Model Organisms Dominate Genetic Research

In the past decades, research in functional genetics has focused on a very few highly developed model systems like the fly *D. melanogaster*, the nematode *Caenorhabditis elegans*, the zebrafish *Danio rerio*, and the house mouse *Mus musculus*. There are several good reasons for this restriction to such a small number of model systems. The first is a practical one: for a model system to be productive in basic research, the amount of space and the costs for stock keeping need to be reasonable, and offspring needs to be available all year round. In addition, a short generation time is imperative to make genetic screens or transgenesis feasible. These requirements already restrict the number of potential model species to small, fast-developing taxa (Bolker 2012).

The second group of reasons could be termed "historical": the establishment of novel tools for genetic research usually takes years, is expensive and labor intensive, and comes with a high risk of failure (Jenner and Wills 2007). Hence, it would not make sense to establish a new model system and invest years in adapting techniques to that species if there were another system for which these pioneering efforts have already been made. For functional genetics research, the core techniques that need to be present are the knockdown or knockout of genes (e.g. by mutation) and transgenesis that allows for misexpression of genes and the establishment of *in vivo* imaging lines and other transgenic tools. Where genetic screens and the mapping of mutants are concerned, genetic maps are essential, and balancer chromosomes help to efficiently maintain homozygous lethal mutations.

One of the most crucial advantages of *D. melanogaster* over other arthropod models has been the possibility of performing saturating genetic screenings (Nüsslein-Volhard and Wieschaus 1980, St Johnston 2002). In this approach, random mutagenesis is performed, and the resulting mutants are screened for phenotypes relevant to the process under study. For mutant lines with interesting phenotypes, the gene is determined and its function is studied further. Importantly, this represents an unbiased approach that is capable of identifying novel and unexpected gene functions. Further, in *Drosophila* elegant genetic tools and a short generation time allow the screening to be extended to saturation, i.e. until each gene has statistically been mutated at least once. Therefore, almost all genes required for a certain process can be identified in *D. melanogaster*, allowing the comprehensive study of its genetic underpinnings (Nüsslein-Volhard and Wieschaus 1980, St Johnston 2002). While mutagenesis has been performed in other arthropods as well (Maderspacher et al. 1998, Pultz et al. 2000, Sulston and Anderson 1996, Trauner et al. 2009) it takes the speed of *D. melanogaster* development and a number of elaborate genetic tools to extend genetic screenings to saturation.

Alongside the more extensive tool kit of genetic model organisms, important resources accumulate over time and speed up research in a given model system. Such resources are, for instance, a well annotated genome, collections of antibodies, mutants and transgenic lines, etc. Importantly, all scientific knowledge gained in the past speeds up present research as it allows us to formulate hypotheses more precisely and to approach novel questions based on previously understood facts. As a consequence, the most mature model system will usually be chosen for studying novel questions.

In a self-reinforcing process, these practical and historical reasons have led to the dominance of *D. melanogaster* as a genetic model system for arthropods to the extent that most of what we know about arthropod genetics is actually fly genetics. Other arthropods were used

in genetics decades ago but these dropped off or were abandoned entirely. As a consequence, the advice to young scientists had long been: "Whatever your question in arthropod functional genetics may be – use *D. melanogaster* to study it. If this process cannot be studied in the fly, look for another question." Theoretically, however, one might prefer to select an organism in which the process under study is optimally represented.

Besides the many benefits, there are also restrictions that arise from focusing on a single model system. Most importantly, there is a lot of interesting and relevant biology that is not represented in *D. melanogaster* (Snodgrass 1935, Weber 1966). Research into the genetic underpinnings of these aspects has been largely neglected. It also remains difficult to judge to what degree the data gained in a fly might be representative for insects in general. Third, some processes are being studied in a suboptimal situation. For instance, despite many interesting insights found in *D. melanogaster* it remains somewhat ironic that studies into the genetic basis of ageing are currently being performed in one of the shortest-lived insects on earth (Jafari 2010).

2. Technical Breakthroughs Extend the Zoo of Model Organisms

2.1 Determining the Gene Complement via Next Generation Sequencing

Several technical breakthroughs have been changing this situation, opening up possibilities for establishing alternative model systems. First, next generation sequencing allows us to determine the genomic sequence and the gene complement of any organism (Wang et al. 2009). Beyond that, RNA-seq approaches have the power to identify the genes active at certain stages, or in specific tissues, or even in single cells (Shapiro et al. 2013). By comparing wildtype with experimental animals, the genes differentially expressed under certain treatments can be identified (e.g. Oberhofer et al. 2014, Vogel et al. 2014). Essentially, next generation sequencing can be applied to all species without constraints.

2.2 Knocking Down Gene Function via RNAi

Techniques have emerged that allow the knockdown of gene function in organisms that are not amenable to mutagenesis screening or large-scale mutant stock keeping. RNA interference (RNAi) has become the most important of these so-called reverse genetics techniques in insects (Fire et al. 1998, Meister and Tuschl 2004). RNAi is a highly conserved defence mechanism that is present in virtually all animals (Fire 2007). Specifically, the presence of double-stranded RNA (dsRNA) in cells leads to the destruction of messenger RNAs complementary to the dsRNA. As a consequence, protein levels are diminished, leading to a knockdown phenotype. Due to its conservation, RNAi can be expected to work in any animal model organism if the dsRNA can be delivered. One way of delivery is direct injection into cells, which unfortunately is only feasible in large cells. In some organisms, dsRNA is taken up by cells from the hemolymph (environmental RNAi) or may even spread from cell to cell (systemic RNAi) (Akiyama-Oda and Oda 2006, Bucher et al. 2002, Grishok 2005, Hunter et al. 2006, Jose and Hunter 2007, Liu and Kaufman 2004, Lynch and Desplan 2006, Tomoyasu and Denell 2004, Whangbo and Hunter 2008, Winston et al. 2002). While the molecular mechanism of environmental/systemic RNAi remains elusive in arthropods,

this process is extremely useful for application: a technically simple injection of dsRNA into the hemolymph of a certain stage leads to subsequent knockdown of gene function in all cells. In some insect species it is even possible to inject female animals and detect the RNAi phenotype in the offspring (e.g. Bucher et al. 2002, Liu and Kaufman 2004, Lynch and Desplan 2006, Tomoyasu and Denell 2004, Whangbo and Hunter 2008). Unfortunately, the efficiency of systemic/environmental RNAi in insects has turned out to be extremely different between taxa. In some arthropods it is efficient, leading to loss of function phenotypes, and in some species even oral delivery is possible (Araujo et al. 2006, Baum et al. 2007, Mao et al. 2007, Meyering-Vos and Müller 2007, Price and Gatehouse 2008, Tian et al. 2009, Turner et al. 2006, Zhou et al. 2008). However, in other taxa, systemic delivery of dsRNA does not result in a strong phenotype or in any phenotype at all (reviewed in Na Yu 2013, Scott et al. 2013). Essentially, this appears to be a taxon-specific trait but some trends are observed, e.g. that beetles are usually well amenable to systemic RNAi while many Lepidoptera are less so (Terenius et al. 2011). In summary, almost all arthropods will mount a strong RNAi response once dsRNA has entered their cells. However, delivery remains a major hurdle unless the species has a strong environmental RNAi response. Hence, a large number, although not all, arthropods are amenable to more or less efficient functional genetics *via* RNAi. So far, it is still difficult to predict whether RNAi will work efficiently or not, such that this has to be tested for each taxon.

2.3 Inserting Transgenes by Transposon Mediated Transgenesis

For a long time, *D. melanogaster* was the only arthropod model system in which it was possible to insert exogenous DNA. To that end, an endogenous transposon was used, the so-called P-element (Bellen 1999, Cooley et al. 1988, Peter et al. 2002). By placing the exogenous DNA into the transposon and providing a source for the transposase, it was possible to stably integrate the transposon, including the target DNA, randomly into the genome. Unfortunately, the P-element turned out to rely on *D. melanogaster*-specific host factors and could not be used for other species (Handler et al. 1993). In the meantime, several transposons have been used for transgenesis in insects that do not rely on host factors, such as the piggyback, Minos and Mariner transposons (Berghammer et al. 1999, O'Brochta and Atkinson 1996, Pavlopoulos et al. 2004, Robertson 1993). In addition, a universal marker system was developed that allowed identification of the few positive transformants in hundreds of non-transformed animals. Essentially, an artificial enhancer sequence was used that is activated by a highly conserved gene active in the eyes of all animals. This artificial enhancer controls the expression of a fluorescent protein such that the transformed animals are marked by fluorescent eyes (Berghammer et al. 1999, Horn et al. 2003, Lorenzen et al. 2003). Based on this system, foreign DNA has been inserted into a variety of insect taxa. Unfortunately, DNA does not cross cell boundaries and therefore needs to be injected into eggs shortly after fertilization. Both the availability of large numbers of freshly fertilized eggs (hundreds or even thousands) and their injectability restrict the number of arthropod taxa amenable to transgenesis.

2.4 Precise Genome Editing via CRISPR/Cas9

An entire new cosmos of possibilities has been opened up by the recent development of the CRISPR/Cas9 technique, which enables high-precision genome editing. The process is

adopted from a bacterial defence system that leads to the destruction of viral DNA (Barrangou et al. 2007, Doudna and Charpentier 2014a, Garneau et al. 2010, Gasiunas et al. 2014). Harnessed to biotechnology, the process allows the cutting of genomic DNA at specific sites, which induces different endogenous DNA repair pathways (Doudna and Charpentier 2014b, Gilles and Averof 2014, Richardson et al. 2016, Sander and Joung 2014). The non-homologous end joining (NHEJ) mechanism fuses the cut DNA ends but frequently leads to small deletions or to insertions that generate mutations at the targeted locus. Also, insertion of linear foreign DNA is possible with NHEJ allowing for CRISPR/Ca9-mediated double strand breaks. In the homology directed repair mechanism (HDR), foreign DNA with stretches of homology is used to repair the targeted site (Gratz et al. 2014, Richardson et al. 2016). Depending on the repair DNA, the genome can be edited even at the single nucleotide level. Hence, specific mutants can be generated, fluorescent marker proteins can be introduced, misexpression constructs can be inserted, and so forth. As with transposon-mediated transgenesis, it is the availability and injectability of freshly fertilized eggs that may restrict the application to certain taxa.

2.5 The New Model Organism Diversity – Spearheaded by the Red Flour Beetle

A growing number of arthropod species have been established for functional genetics approaches based on genomic sequence, RNAi-mediated gene knockdown, and CRISPR/Cas9 genome editing. Of course, the practical and historical reasons outlined above remain important criteria for model system choice. Therefore, not many models will be used by large scientific communities. Rather, key biological questions that cannot be answered in *D. melanogaster* will be approached through a number of alternative model systems established for the respective purposes. The red flour beetle *T. castaneum* has been spearheading this development and within arthropods builds on the most versatile genetic and transgenic tool kit. Its main strength is its RNAi response, which is environmental and is specifically strong, leading to null phenotypes – at least in the handful of cases where the genetic mutation could be compared to the RNAi phenotype (Brown et al. 1999, Bucher et al. 2002, Peel et al. 2013, Shippy et al. 2006, Tomoyasu and Denell 2004). Essentially, the injection of dsRNA into the hemolymph leads to knockdown of the gene function in all cells of the injected animal (Miller et al. 2012, Tomoyasu and Denell 2004) and in case of females to their offspring (Bucher et al. 2002). The efficiency of generating transgenic beetles compares to *D. melanogaster*, and both heat-shock mediated misexpression and binary expression systems have been established (Berghammer et al. 1999, 2009, Lorenzen et al. 2003, Pavlopoulos et al. 2004, Schinko et al. 2012, 2010). A large-scale insertional mutagenesis has been performed, leading to a collection of enhancer traps and mutants (Trauner et al. 2009). Hence, *T. castaneum* has become the main model for Coleoptera and is being used by a growing scientific community and for diverse topics ranging from evolution and development to physiology and pest control (Brown et al. 2009, Klingler 2004, Schröder et al. 2008).

3. Limitations of the Candidate Gene Approach Using RNAi

As mentioned above, much of the success of *D. melanogaster* is based on its amenability to large-scale screenings that identify all genes involved in a certain process based on their mu-

Tab. 1 Amenability of model systems to techniques relevant for functional genetics

	Drosophila melanogaster	***Tribolium castanaeum***	**Species with injection into freshly laid eggs possible**	**Species with dsRNA uptake from hemolymph**	**Wherever dsRNA can be delivered into cells**	**All**
Identification of relevant genes						
Genome wide phenotypic screening	■	■				
Differential expression*	■	■	■	■	■	■
Genomic sequence	■	■	■	■	■	■
* many differentially expressed genes prove not to be essential while essential genes might be expressed ubiquitously or in other tissues						
Tools for functional genetics						
Extensive genome wide resources	■					
Some transgenic lines and resources	■	■				
Transgenesis / CRISPR/Cas9	■	■	■	■		
RNAi (systemic/ environmental)	■	■	■	■		
RNAi (non environmental)	■	■	■	■	■	
Transgenesis	■	■	■			
Strength of well established model systems versus pest and vector species						
Model systems	Relevance for pest or vector control / Technical amenability & speed of research					Pest and vector species

tant phenotypes (saturated phenotypic screen). Unfortunately, this approach is not realistic for most emerging model organisms, for technical reasons. Hence, most of the research in other model systems has been based on the candidate gene approach. Here, previous knowledge – mainly based on research in *D. melanogaster* – is used to identify a number of genes that might be involved in a certain process. These candidate genes are then tested for a function

and studied in more detail (Arakane et al. 2009, Bolognesi et al. 2008, Brown et al. 2000, 2009, Bucher and Klingler 2004, Choe et al. 2006, Posnien et al. 2011, van der Zee et al. 2005). This approach has been very fruitful, but it has an important limitation: as it is based on previous knowledge, it is unlikely to detect entirely novel or unexpected gene functions.

RNA-seq has been successfully used to detect genes involved in a process in an unbiased way. Here, the expression of all genes in a certain tissue or under a certain condition is measured and compared to controls. Statistical analysis subsequently reveals the differentially expressed genes that are usually involved in the respective process. This approach has turned out to be very effective at detecting differentially expressed genes. However, differential expression does not yet mean that the gene is essential – so the knockdown might not lead to any detectable phenotype. Usually, a couple of hundred of differentially expressed genes need to be screened by RNAi for their relevance in order to detect a few important components. Another drawback is that many genes are important but are not differentially expressed and will therefore not be identified by RNA-seq (Li et al. 2013, Oberhofer et al. 2014, Schmitt-Engel et al. 2015).

Is there a way to perform saturated genetic mutagenesis screens in other model systems than *D. melanogaster*? Five labs have been cooperating for five years to perform a classical large-scale screen in the red flour beetle (Trauner et al. 2009). However, despite this major effort, the screening remained far from saturation, and stock keeping has remained a significant burden for the involved labs. It is therefore unlikely that such screenings will be performed in other arthropods. However, large-scale or even genome wide RNAi screenings represent alternatives to genetic screens. Here, the candidate gene bias is eliminated by choosing the genes randomly or by knocking down all genes of an organism. The prerequisites for a model system to be amenable to large-scale RNAi screening are that it can be bred in large amounts and that its RNAi response is strong and environmental.

4. The iBeetle Project – Generating a Resource for Unbiased Large-scale Screenings

We have established the red flour beetle *T. castaneum* as an alternative insect model for unbiased phenotypic screening. Building on its strength, we have been performing an unbiased large-scale RNAi screening (iBeetle screen) with the aim of genome-wide coverage. This effort was funded by the *Deutsche Forschungsgemeinschaft* (Schmitt-Engel et al. 2015). Essentially, randomly picked genes are being knocked down *via* RNAi, and the resulting phenotypes are documented in the iBeetle database (Dönitz et al. 2015, Schmitt-Engel et al. 2015). There, the resulting phenotypes are stored and remain searchable online. For approximately one third of the genes, we performed two screenings in parallel. *First*, we injected female pupae in order to detect the phenotypes in the offspring. *Second*, we injected the same dsRNAs into mid-stage larvae in order to detect phenotypes during metamorphosis. Both parts generated scores for cuticle phenotypes, fertility, alterations of fluorescently marked muscles, and stink glands. For the remaining genes only the first screening will be continued. We hope to have screened all 16,000 *T. castaneum* genes by the end of 2019.

We were able to identify novel and unexpected gene functions. For instance, we found novel components required for epithelial adhesion, despite the fact that this process had been extensively screened for in *D. melanogaster*. Likewise, we were able to identify genes relevant for stink gland physiology, which were not identified by a previous differential expression analysis

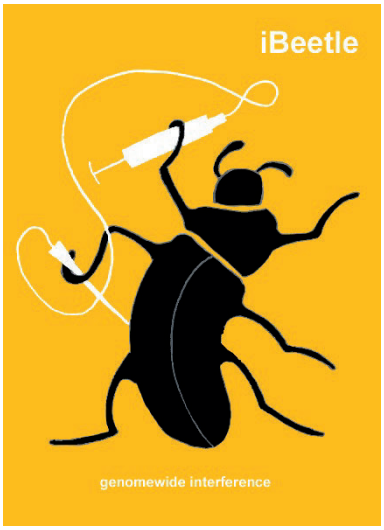

Fig. 1 The genome-wide RNAi screening *iBeetle* was funded by the *Deutsche Forschungsgemeinschaft.*

(Li et al. 2013, Schmitt-Engel et al. 2015). Not unexpectedly, we also missed genes identified by the other approaches. Apparently, the different dynamics of gene knockdown and sensitivity for certain phenotypes depending on species and technique require a combination of several techniques in order to identify all relevant genes. The *iBeetle* project provides the community with several resources that may assist in the study of pest and vector arthropods.

4.1 Resource 1: *Genome-wide Information on Gene Function*

The RNAi phenotypes stored at the iBeetle-Base are linked with additional information on the knocked-down genes such as the nucleotide and protein sequences, and links to the closest fly orthologs and to the *T. castaneum* genome browser (Dönitz et al. 2015). Similar to FlyBase, this system can be used to find genes involved in certain processes. Second, the information gives an initial quick overview of the function of these genes. For instance, if RNA-seq approaches have revealed some hundreds of differentially expressed genes in *T. castaneum* or other species, a simple database search will identify those that do not lead to lethality upon systemic application and might therefore not be relevant for application.

4.2 Resource 2: *Genome-wide Collection of dsRNA Templates*

A major hurdle for large-scale RNAi screens is the difficulty of achieving reliable high throughput production of templates for the production of dsRNAs. This task is both expensive and time-consuming and in most cases not part of the core expertise of labs working on insect pests or vectors. However, the *iBeetle* project is generating a genome-wide collection of templates, which will be available to the community at production cost. Even more straightforward: the dsRNAs are available commercially, obviating the need for any large-scale production of reagents.

4.3 The Potential: Future Large-scale Screening in the Red Flour Beetle

The iBeetle screening has focused on a rather large number of different developmental phenotypes. For scientists interested in other types of phenotypes, additional large-scale screenings can now be performed rather easily. The only prerequisite is that a reliable phenotypic readout is developed. If a future screening focuses on one simple readout (instead of many and complex phenotypes as in the *iBeetle* screening), the throughput can increase significantly. For instance, a genome-wide screening (16,000 genes) for larval lethality can be performed in three to four person years. The cost of dsRNAs is about $10 per gene, which adds to the $160,000 for a genome-wide screening. In summary, both cost and time for future screens have a realistic order of magnitude.

5. The Contribution of Emerging Genetic Model Systems to Research on Pest Insects and Vectors

The scientific community working on emerging model organisms might contribute to research on arthropod pests and vectors in two ways. First, thanks to the ease of stock keeping and the technical amenability, the identification of the genetic basis of arthropod biology or the proof of principle of novel approaches to vector or pest control are most efficiently developed there. This basic knowledge is then transferred to the actual pest species or vectors, which are usually much harder to work with. For example, genetic improvements to the sterile insect technique have been developed in *D. melanogaster* and have then been transferred to pest species (Horn and Wimmer 2002, Schetelig et al. 2009). Likewise, proof of principle of gene drive systems have been developed in *D. melanogaster* (Chan et al. 2011, Gantz and Bier 2015, Gantz et al. 2015). In the past, this pioneering work was restricted to *D. melanogaster* but, depending on the question or the arthropod taxon, one of the emerging model systems might be an interesting alternative. For instance, the *iBeetle* screening has revealed a number of novel RNAi target genes for pest control that are currently being transferred to actual pest species (Ulrich et al. 2015).

The second contribution to the field of pest or vector control should be the transfer of knowledge required to genetically engineer pests or vectors for research or application. The tedious work into establishing novel genetic tools leaves the scientists involved with – alongside an elevated frustration tolerance – an extensive body of experience regarding the techniques that might be applied and their respective advantages or drawbacks, and ideas for how they might approach the transfer of techniques to other species. Hence, novel approaches to fighting pests and vectors will profit from researcher tandems – one with experience in vector biology and the other with experience in developing a genetic tool kit.

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Geist – Gehirn – Genom – Gesellschaft
Wie wurde ich zu der Person, die ich bin?

Vorträge anlässlich der Jahresversammlung
vom 20. bis 22. September 2013 in Halle (Saale)

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Die Frage „Wie wurde ich zu der Person, die ich bin?“ betrifft jeden ganz unmittelbar. Der Band zeigt die Bedingungen, Prozesse und Einflussfaktoren auf, die uns in der Interaktion mit unserer Umwelt zu einzigartigen Individuen werden lassen. Er behandelt unser gegenwärtiges Wissen über die natürlichen und kulturellen Wurzeln menschlicher Individualität aus verschiedenen Perspektiven, die von der Humangenetik und Neurobiologie über die Psychologie und die Verhaltens- bzw. Kognitionswissenschaften bis hin zu Philosophie, Wissenschaftsgeschichte und Ethik reichen. In der Sicht der klassischen Bio- und Gesellschaftswissenschaften determiniert die im Genom des Menschen gespeicherte Information im Laufe der frühen Ontogenese den Aufbau des Gehirns, das so entstandene Gehirn schafft den Geist, und durch die Interaktion von Individuen entstehen gesellschaftliche Strukturen. Diese lineare Kausalitätskette ist aber nach unseren heutigen Erkenntnissen keineswegs vollständig. Gesellschaftliche Strukturen wirken auf das Denken von Individuen zurück, sodass sich Geist und Gesellschaft reziprok beeinflussen. Unser Denken beeinflusst auch unser Gehirn. Neuronale Prozesse wirken auf die Aktivitätsmuster des Genoms zurück. Genom und Gesellschaft interagieren. Geist und Genom stehen ebenfalls in einem Wechselspiel. Der Komplexität dieses Netzwerks aus Geist – Gehirn – Genom – Gesellschaft spürt der Band in vielen Facetten auf aktuellem Wissensstand nach.

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Tungiasis – eine vernachlässigte tropische Zoonose mit vielen Facetten

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Zusammenfassung

Die Tungiasis ist eine infektiöse Hauterkrankung, die von weiblichen Sandflöhen hervorgerufen wird. Die Flohweibchen bohren sich in die Haut ihrer Wirte ein, insbesondere an den Füßen. Im Gegensatz zu anderen Tungidae verfügt *Tunga penetrans* über ein großes Verbreitungsgebiet und ein breites Wirtsspektrum. Neben dem Menschen werden vor allem Schweine und Hunde, aber auch Katzen sowie viele Wildsäugetiere befallen. Die Tungiasis gehört zu den vernachlässigten Tropenkrankheiten, und unser Wissen über die Biologie, Epidemiologie und die Möglichkeiten zur Bekämpfung weist erhebliche Lücken auf. In die Haut penetrierte Flöhe haben nach einigen Tagen etwa Erbsengröße erreicht und damit ihr Volumen um den Faktor 2000–3000 vergrößert. Die klinische Pathologie ist durch eine starke Entzündungsreaktion geprägt, die durch Sekundärinfektionen weiter verstärkt wird. Unbehandelt führt die Tungiasis zu einer Einschränkung der Mobilität und hat erhebliche soziale und ökonomische Auswirkungen. Die epidemiologische Bedeutung der *T. penetrans*-Infektionen bei Tieren für die Infektion bei Menschen sowie deren Auswirkungen auf die Tiergesundheit und die Produktivität sind bisher nur wenig untersucht. Da Tungiasis typischerweise die ärmsten Bevölkerungsgruppen betrifft, ist sie ein wichtiges Hindernis für die ökonomische Entwicklung ressourcenarmer Gebiete in Afrika und Südamerika. Die Entwicklung von *One-Health*-basierten Bekämpfungsstrategien ist ein wichtiger Punkt zukünftiger Forschung über die Tungiasis.

Abstract

Tungiasis is an infectious skin disease caused by female sand fleas. Female sand fleas penetrate the skin of their hosts, particularly at their feet. In contrast to other members of the Tungidae, *Tunga penetrans* has a wide geographic and host range. In addition to humans, pigs, dogs but also cats and many wild mammals get infected. Tungiasis belongs to the Neglected Tropical Diseases, and our knowledge regarding biology, epidemiology and possible control measures are very limited. After a few days, penetrated fleas in the skin have grown to about the size of a pea, thus increasing their volume by about 2000–3000-fold. The clinical pathology is characterized by a strong inflammation which is further intensified by secondary bacterial infections. Without treatment, tungiasis restricts mobility and has substantial social and economic effects. The epidemiological relevance of animal tungiasis for human infections as well as the effects on animal health and productivity have only rarely been investigated. Since tungiasis particularly affects the poorest communities, it is an important hindrance for development of resource poor regions in Africa and South America. The development of One-Health based control strategies is an important aspect of future research regarding tungiasis.

1. Einleitung

Tunga penetrans (Sandfloh, *jiggers* und zahlreiche andere lokale Bezeichnungen) wurde als erste Art der Gattung *Tunga* von Carl von Linné im Jahre 1758 als *Pulex penetrans* beschrieben (Linneaus 1758). Im Vergleich zu anderen Mitgliedern der Ordnung Flöhe (Siphonapte-

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ra) zeichnen sich *Tunga* spp. durch eine fundamental unterschiedliche Lebensweise und einen erheblich abgewandelten Lebenszyklus aus. Während die Spezies aller anderen Gattungen Ektoparasien sind, sind *Tunga* spp. Endoparasiten.

Zumindest die beiden humanpathogenen Arten der Gattung *Tunga* waren ursprünglich auf den amerikanischen Kontinent begrenzt. Dass die Tungiasis im präkolonialen Peru häufig war, zeigt z. B. eine Tonfigur aus der Maranga-Kultur (von ca. 150 bis 650 n. Chr.) (Maco et al. 2011). Für die spanischen und portugiesischen Konquistadoren wurde die Tungiasis zu einer Plage mit zum Teil lebensbedrohlichen Komplikationen, wie Aufzeichnungen des Sekretärs von Hernán Cortes, Francisco López de Gómara, belegen (de Gomara 1552).

Als einzige bedeutende Infektionskrankheit wurde die Tungiasis nicht von der Alten in die Neue Welt verschleppt, sondern in umgekehrter Richtung. Mehrere, nicht immer eindeutige Hinweise in Veröffentlichungen aus den Jahren 1732, 1759 und 1838 lassen vermuten, dass *T. penetrans* schon sehr frühzeitig in Westafrika eingeschleppt worden war (Hoeppli 1963). Offenbar kam es nach den ersten Einschleppungen aber nicht zu einer räumlichen Ausbreitung, vermutlich weil es wenig Austausch zwischen den verschiedenen Regionen des Kontinents gab. Historisch gesichert ist, dass im Jahr 1872 *T. penetrans* an Bord eines Schiffes von Rio de Janeiro nach Ambriz in Angola gelangte (Hoeppli 1963). Noch im gleichen Jahr breitete sich *T. penetrans* bis nach Loanda im südlichen Teil von Angola und im Norden bis in den Kongo aus. Bereits 1886 war die Tungiasis in Monrovia (Liberia) häufig, und im Jahr 1895 erreichte *T. penetrans* die Ostküste Afrikas und kurze Zeit später auch Madagaskar. Im Jahr 1898 wurde die Krankheit erstmalig auf Sansibar beschrieben (Hoeppli 1963). Vermutlich wurde *T. penetrans* durch heimkehrende indische Soldaten aus Ostafrika nach Goa (Westküste von Indien) importiert, konnte sich jedoch dort nicht auf Dauer festsetzen.

Heute ist die Tungiasis in Mittel- und Südamerika (von Mexiko bis nach Argentinien), auf einigen Inseln der Karibik und in nahezu allen Ländern Afrikas südlich der Sahara verbreitet (Feldmeier et al. 2014). Zunehmend bringen Reisende aus Endemiegebieten eine Tungiasis in ihr Heimatland; vereinzelt sind auch mitreisende oder im Endemiegebiet adoptierte Haustiere erkrankt (Feldmeier und Keysers 2013, Heukelbach et al. 2007).

Die Tungiasis zählt zu den vernachlässigten Tropenerkrankungen (Hotez et al. 2009). Sie wird in ihrer Bedeutung nach wie vor unterschätzt. Die deutsche Wikipedia-Webseite liefert dafür ein eindrucksvolles Beispiel. Dort heißt es: „Der Befall wird Tungiasis genannt und ist eine harmlose, wenn auch störende Erkrankung.“[4] Dazu wird allerdings das in Abbildung 1 gezeigte Bild von einem Patienten mit schwerer klinischer Pathologie gezeigt, ohne dass dieser offensichtliche Widerspruch im Text aufgeklärt wird.

Dieser Übersichtsartikel fasst einerseits den gegenwärtigen Stand des medizinisch und veterinärmedizinisch relevanten Wissens zur Tungiasis und zur Biologie von *T. penetrans* zusammen und zeigt andererseits dringenden Forschungsbedarf auf.

2. Die Gattung *Tunga*

Die Gattung *Tunga* umfaßt neben *T. penetrans* noch 13 weitere Arten, die zwei verschiedenen Untergattungen zugeordnet werden: *Tunga* (= *penetrans*-Gruppe) mit *T. (T.) penetrans*, *T. (T.) travassosi*, *T. (T.) bondari*, *T. (T.) terasma*, *T. (T.) perforans*, *T. (T.) trimamillata* und *T.*

4 https://de.wikipedia.org/wiki/Tunga_penetrans; zuletzt aufgerufen am 13. 1. 2016.

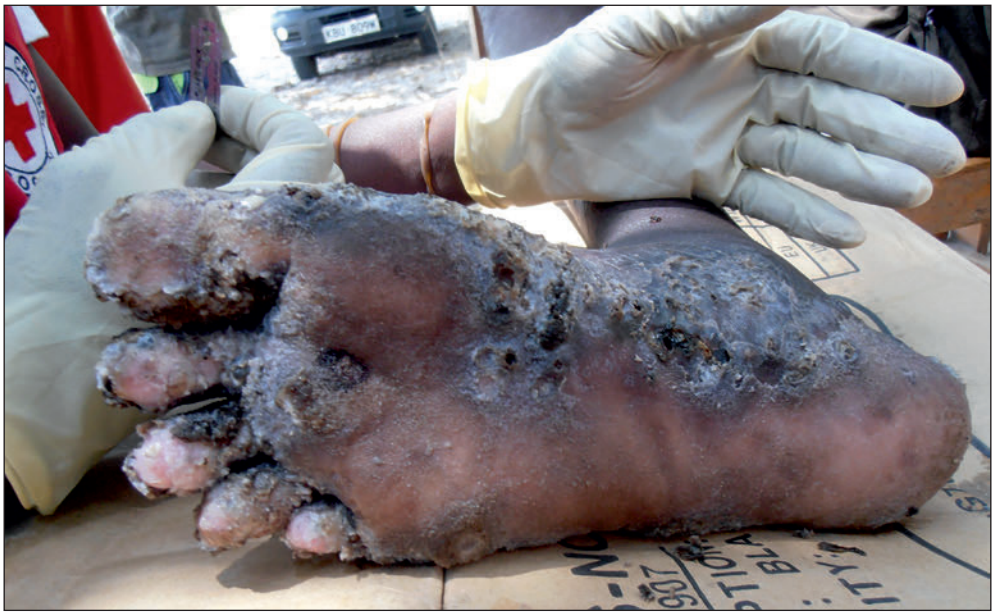

Abb. 1 Schwere Tungiasis. Der abgebildete Fuß mit zahlreichen nekrotisierten Läsionen ist auf der deutschen Wikipedia-Seite zusammen mit dem Satz „Der Befall wird Tungiasis genannt und ist eine harmlose, wenn auch störende Erkrankung." veröffentlicht. „Jigger infested foot (2)" von R. Schuster – Eigenes Werk. Lizenziert unter CC BY-SA 3.0 über Wikimedia Commons –https://commons.wikimedia.org/wiki/File:Jigger_infested_foot_(2).jpg#/media/File:Jigger_infested_foot_(2).jpg

(T.) hexalobulata; *Brevidigita* (= *caecata*-Gruppe) mit *T. (B.) caecata*, *T. (B.) caecigena*, *T. (B.) callida*, *T. (B.) libis*, *T. (B.) monositus*, *T. (B.) bossii*, and *T. (B.) bonneti* (De Avelar et al. 2013, Ezquiaga et al. 2015, Linardi et al. 2014). Die meisten Arten der Untergattung *Brevidigita* kommen vorwiegend bei Nagetieren, aber auch bei Insectivora und Didelphimorphia (Beutelratten) vor, haben ein kleines Wirtsspektrum und sind geographisch limitiert. Die Prädilektionsstellen von Flöhen bei dieser Untergattung sind artspezifisch und betreffen entweder das Ohr, die Anusregion oder den Schwanz. Im Vergleich dazu bevorzugen die Arten der Untergattung *Tunga* die Fußregion für die Penetration (De Avelar et al. 2013, Ezquiaga et al. 2015, Linardi et al. 2014).

Bis auf *T. (T.) hexalobulata* parasitieren die Arten der Untergattung *Tunga* in auf Südamerika beschränkten Nebengelenktieren (Xenarthra). Nur *T. (T.) hexalobulata* ist ausschließlich auf Paarhufern gefunden worden. Da *T. (T.) hexalobulata* erstmalig 2013 beschrieben wurde, sind die Kenntnisse allerdings nur als vorläufig einzustufen. Drei Spezies der Untergattung parasitieren außerdem domestizierte Huftiere, nämlich *T. (T.) hexalobulata*, *T. (T.) trimamillata* und *T. (T.) penetrans.* Die letzten beiden Arten finden sich auch beim Menschen. *T. penetrans* ist insofern außergewöhnlich, als dass dieser Floh nicht auf ein kleines geographisches Areal und wenige Wirtstierarten beschränkt ist (Linardi et al. 2014). Es werden zahlreiche Säugetierspezies (von Nagern über Affen bis hin zu Elefanten) parasitiert. Ob auch Vögel Wirte sein können, ist bislang unklar. Eine systematische Untersuchung von Geflügel in einem *T. (T.) penetrans*-Endemiegebiet in Uganda konnte allerdings keine Infektionen nachweisen (Mutebi et al. 2015).

3. Entwicklungszyklus

3.1 Die freilebenden Stadien

Der Entwicklungszyklus von *T. penetrans* ist in Abbildung 2 schematisch dargestellt. Wie bei allen Flöhen liegt ein holometaboler Entwicklungszyklus vor, der ein inaktives Puppenstadium einschließt (Peters 2003). Über die nicht-parasitischen, freilebenden Stadien von *T. penetrans* ist sehr wenig bekannt. Aus den von eingebetteten Flohweibchen produzierten

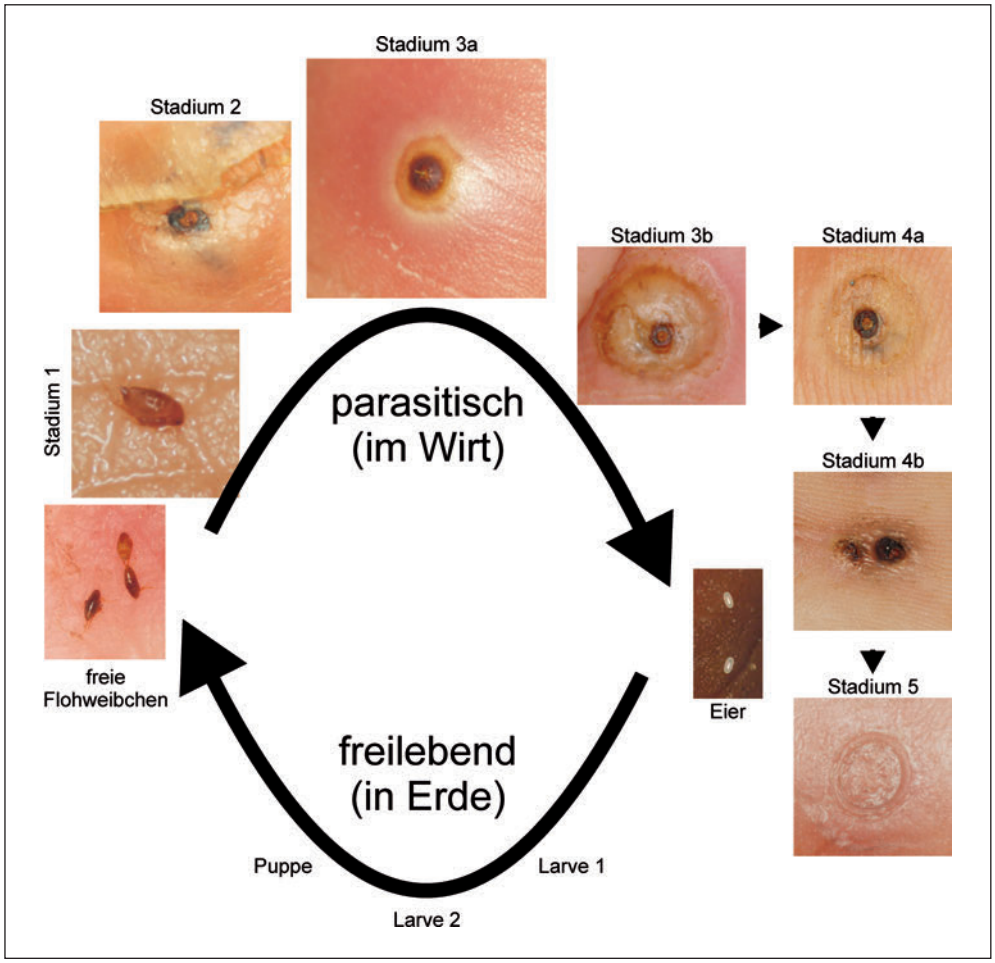

Abb. 2 Der Lebenszyklus von *T. penetrans*. Aus den von den weiblichen Tieren abgelegten Eiern schlüpfen typische Flohlarven, die im Boden leben. Nach einer Häutung zur zweiten Larve wird durch eine weitere Häutung die Puppe gebildet, die in einem klebrigen Seidenkokon eingesponnen ist. Aus der Puppe schlüpfen männliche und weibliche Imagines. Nur die weiblichen Tiere dringen permanent in die Haut ihrer Wirte ein (Stadium 1), wo sie von den Männchen begattet werden. Im Stadium 2 wachsen die Flöhe in erster Linie heran und ihre Ovarien hypertrophieren. Die Ausscheidung von Eiern erfolgt im Stadium 3. Stadium 3a ist durch eine starke Entzündungsreaktion gekennzeichnet. Schließlich sterben die Flöhe *in situ* im Stadium 4 und hinterlassen kreisrunde Impressionen im Stratum corneum (Stadium 5).

Eiern entwickeln sich Larven, die sich über nur zwei Larvenstadien zu einer Puppe weiterentwickeln (Nagy et al. 2007, Pampiglione et al. 2009). Die Eier sind gelblich-weiß, ovoid und haben eine Größe von ungefähr 600×330 µm (Nagy et al. 2007) (Abb. 2 und 3). Aus diesen schlüpft das erste Larvenstadium (L1) mit der typischen Morphologie von Flohlarven (Abb. 2). Die L1-Larven haben zunächst eine Länge von ca. 1500 µm, wachsen jedoch innerhalb von wenigen Tagen auf etwa 2900 µm heran. Die nach Häutung entstehenden L2-Larven haben einen größeren Durchmesser, sind jedoch mit ca. 1150 µm deutlich kürzer (Nagy et al. 2007).

Abb. 3 Eier von *T. penetrans* auf der Haut eines Patienten. Die Pfeile zeigen die Position der Eier an.

Während die meisten Flohspezies drei Larvenstadien haben (Bitam et al. 2010), scheinen es bei *T. penetrans* nur zwei zu sein (Nagy et al. 2007). Unter optimalen Bedingungen verpuppen sich L2-Larven sechs bis acht Tage nach dem Schlüpfen aus dem Ei. Sie beenden die Nahrungsaufnahme, und nachdem sich der Darm geleert hat, nehmen sie eine U-förmige Haltung an und beginnen einen Seidenkokon zu spinnen. Die Seidenfäden sind wie bei anderen Flöhen klebrig, so dass an der Außenseite Partikel, typischerweise kleine Sandkörner, haften (Nagy et al. 2007). Diese Beobachtungen sind bislang aber weder bestätigt noch widerlegt. Denkbar ist auch eine Entwicklung von L1 zu L2 bereits im Ei, gefolgt von einer Häutung von der L2 zur L3.

In Armensiedlungen in Brasilien wurden Larven in Gebäuden im Inneren von Häusern ohne festen Fußboden vor allem zwischen 2 und 5 cm Tiefe in einer lockeren Bodenschicht gefunden. Die zweithöchste Dichte von Larven fand sich in den oberen 2 cm des Bodens; nur sehr wenige Flohlarven waren unterhalb von 5 cm lokalisiert. Im Vergleich dazu hielten sich die Flohlarven im Freien tendenziell in etwas tieferen Bodenschichten auf (Nagy et al. 2007).

Wovon sich die Flohlarven in den relativ trockenen und nährstoffarmen Substraten ernähren, ist völlig unklar. Insbesondere weiß man nicht, ob sie ähnlich wie die Larven anderer Spezies, wie z. B. *Ctenocephalides* spp., den Kot von adulten Flöhen benötigen, der große Mengen von unvollständig oder nicht verdautem Blut enthält (Rust und Dryden 1997).

3.2 Entwicklung im Wirt

Adulte weibliche *T. penetrans* (Abb. 2) suchen sich einen Wirt und penetrieren innerhalb von wenigen Stunden die Haut, typischerweise im Fußbereich. Die männlichen *T. penetrans* saugen ebenfalls Blut, penetrieren den Wirt aber nicht dauerhaft (Nagy et al. 2007, Witt et al. 2004). Die Signale, die zur Findung und Erkennung geeigneter Wirte führen, sind unbekannt. Die Paarung findet offenbar erst nach Penetration des weiblichen Flohs statt (Nagy et al. 2007). Bei gemeinsam gehaltenen freilebenden weiblichen und männlichen *T. penetrans* konnten keine Kopulationen beobachtet werden (Nagy et al. 2007). In einem Selbstexperiment wurde beschrieben, dass penetrierte weibliche Flöhe, die nicht von männlichen Flöhen aufgesucht werden konnten, keine Eier produzieren und deutlich länger leben als befruchtete Weibchen (Thielecke und Feldmeier 2013). Sicher ist, dass eine vorherige Paarung keine Voraussetzung für eine Penetration des weiblichen Flohs ist, und die Kopulation von männlichen Flöhen mit eingebetteten Weibchen der Regelfall ist (Nagy et al. 2007).

Nach der Penetration des weiblichen Flohs kommt es, unabhängig von der Begattung (Thielecke und Feldmeier 2013), zu einer dramatischen Transformation der weiblichen Flöhe mit einem extremen Größenwachstum innerhalb von wenigen Tagen. Dabei wird das Köpervolumen ca. 2000–3000-fach vergrößert, es entwickelt sich also eine Neosomie.[5] Neosomie ist bei Arthropoden ein sehr seltener Vorgang und kommt insbesondere bei parasitisch lebenden Vertretern vor. Die Neosomie bei *T. penetrans* wird durch eine extreme Vergrößerung der Intersegmentalhaut zwischen zweitem und drittem Abdominalsegment erreicht.

Die folgende Beschreibung der Entwicklung der parasitischen Stadien von *T. penetrans* lehnt sich im Wesentlichen an die systematische Untersuchung von brasilianischen Patienten mit Tungiasis an (Eisele et al. 2003). Penetrierte Flöhe werden anhand der sogenannten Fortaleza-Klassifikation in Stadien eingeteilt (Tab. 1; Eisele et al. 2003).

Freilebende weibliche Flöhe sind mit nur maximal 1 mm Körperlänge erheblich kleiner als Flöhe, die nicht zur Gattung *Tunga* gehören. Der eigentliche Penetrationsprozess (Fortaleza-Stadium I) dauert in der Regel ca. drei Stunden, ab dem Zeitpunkt, zu dem das Sandflohweibchen einen geeigneten Ort für die Penetration gefunden hat. Dabei nimmt der Floh einen Winkel von 45–90° zur Hautoberfläche ein, das vordere Beinpaar wird nach hinten an den Körper angelegt, und der Floh startet den Penetrationsprozess mit dem Eindringen der Proboscis.

Schon sehr früh nach dem Eindringen kommt es zu morphologischen Veränderungen, insbesondere zur Separierung des zweiten und dritten Abdominalsegmentes. Die vier letzten Abdominalsegmente bleiben sichtbar. Hier befinden sich die Stigmata (Tracheenöffnungen), der Anus und der Genitalporus. Mit diesen Körperteilen bleibt der Floh für den Rest seines Lebens mit der Außenwelt in Kontakt.

Das Fortaleza-Stadium 2 (Abb. 2) umfasst die Tage eins bis zwei nach dem Eindringen des Flohs. Die vier letzten Abdominalsegmente sind noch sichtbar, die beiden letzten sind jedoch eingestülpt, so dass sich eine Art Miniatur-Krater bildet. In diesem bilden die vier Paare von Stigmata und der Genitalporus einen Konus mit einem basalen Durchmesser von bis zu 500 µm. Die hypertrophierte Zone zwischen dem zweiten und dritten Abdominalsegment ist bereits deutlich ausgebildet und hat eine an einen Rettungsring erinnernde Form. In der Haut

5 Neosomie wurde von Audy und Radovsky definiert als: „external transformation, with formation of new cuticle, during an active stadium in a group normally metamorphosing by molts“ (Audy et al. 1972).

Tab. 1 Die Fortaleza-Skala zur Klassifizierung der Entwicklungsstadien penetrierter Sandflöhe (Eisele et al. 2003)

Stadium	Zeitraum	Charakteristika	Symptome und Histopathologie
1	3–7 h Dauer	Penetration im 45–90° Winkel; Vorderbeine nach hinten an den Flohkörper angelegt; Abdominalseqmente 2 und 3 weichen auseinander	Leichte Rötung, selten Juckreiz; milde Infiltration von Neutrophilen und Eosinophilen
2	1–2 Tage nach vollständiger Penetration	Hypertrophierung der Intersegmentalhaut zwischen Abdominalseqment 2 und 3	Erythem um einen zentralen dunklen Punkt; Infiltration von Neutrophilen in Epidermis und von Neutrophilen, Lymphozyten und Plasmazellen in Dermis
3a	2–3 Tage nach Penetration für 2–3 weitere Tage	Kopf des Flohs an Grenze Epidermis/Dermis; sphärische Hypertrophiezone; Proboscis in subepithelialem Blutgefäß	Weißer Hof mit klarer Abgrenzung um einen dunklen Punkt herum; Läsion ist uhrglasartig erhoben; Ausscheidung von Faeces und einer wässerig bräunlichen Flüssigkeit; Hyperplasie, Hyper- und Parakeratosen; Ödeme, Erythem, pulsierende Schmerzen und starker Juckreiz; Infiltrationen verschiedener Zelltypen in Dermis und Epidermis; Mikroabszesse in Dermis und Epidermis
3b	6–7 Tage nach Penetration für ca. 2 Wochen	Verdickung des Exoskeletts in der Nähe der Hypertrophiezone Bildung eines wallförmigen Rings	Weißer Hof besteht fort; Läsion hat Caldera-Form; Ausscheidung von Faeces, wässerig bräunlicher Flüssigkeit und Eiern; Desquamation des Stratum corneum um Läsion herum; starke Schmerzen bei Berührung und beim Gehen; Hyperplasie, Hyper- und Parakeratose; Infiltrationen verschiedener Zelltypen in Dermis und Epidermis; Mikroabszesse in Dermis und Epidermis
4a	Wochen 3 bis 4 nach Penetration	Hypertrophiezone schrumpft; der Parasit stirbt oder ist bereits tot	Ausscheidung von Eiern und Faeces endet; Läsion verschrumpelt; Hyperplasie, Hyperkeratose; Infiltrationen verschiedener Zelltypen in Dermis und Epidermis; Mikro- und Makroabszesse, häufig eitrig
4b	Wochen 5 bis 6 nach Penetration	Nur ein toter Restkörper vom Floh übrig	Läsionen werden nekrotisch, eingetrocknet mit dunkler Verkrustung; Hyperplasie, Hyperkeratose; Abszesse; Beginn der Reorganisation der Epidermis
5	Ab Woche 6 bis 7 nach Penetration	Floh nicht mehr vorhanden	Kreisrunde, wie ausgestochene Vertiefungen in der Epidermis; Hyperplasie und abnehmende Zeichen von Entzündung

ist der Kopf des Flohs bis in das Stratum papillare der Dermis vorgedrungen, und er verschiebt dieses weiter nach innen, so dass eine U-förmige Einfaltung entsteht. Die Proboscis dringt in die Dermis ein und penetriert in kleine Blutgefäße (Abb. 4).

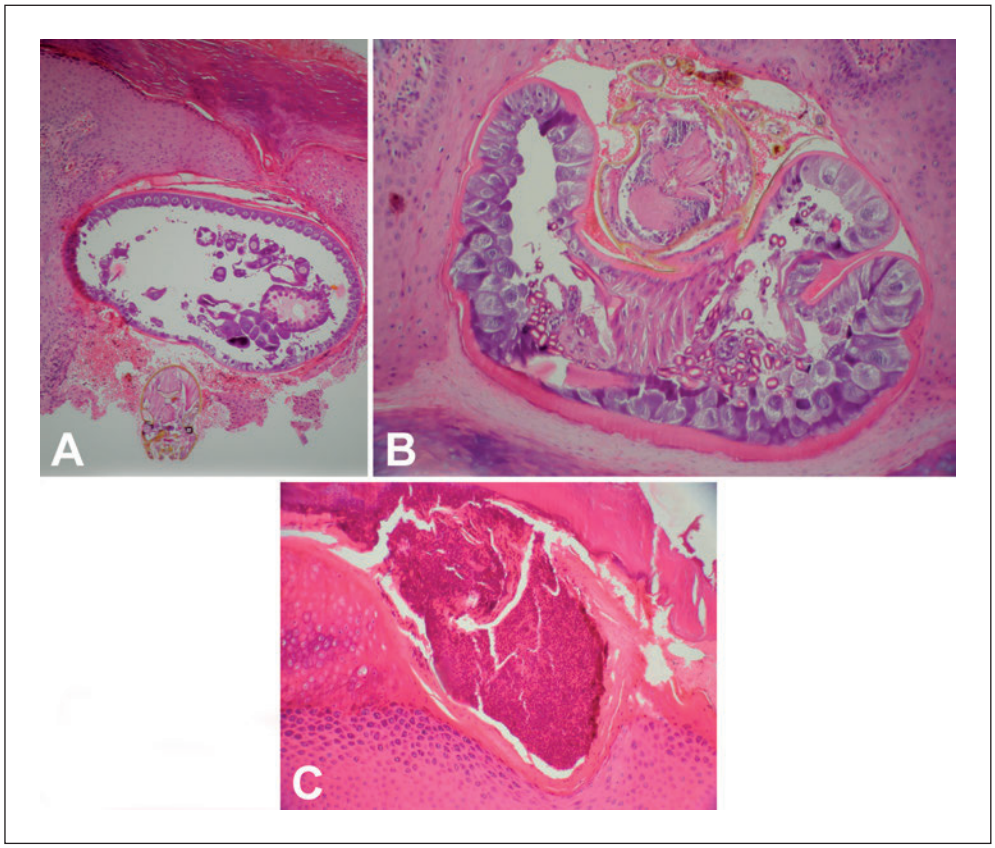

Abb. 4 Eosin-Hämatoxylin-Färbung einer Biopsie eines in die Haut eingebetteten *T. penetrans*-Weibchens. (*A*) Längsschnitt durch den gesamten Flohkörper. Die Proboscis liegt in der Dermis. (*B*) Querschnitt durch einen Flohkörper im Stadium 3b. (*C*) Mikroabszess zwischen Epidermis und Dermis.

Das Stadium 3 der Fortaleza-Klassifikation ist durch einen auffälligen gelblich-weißen Hof gekennzeichnet. Es wird in zwei Unterstadien gegliedert, Stadium 3a, das zwei bis drei Tage nach Penetration beginnt und weitere zwei bis drei Tage dauert, und Stadium 3b, das sechs bis sieben Tage nach Penetration beginnt und ca. zwei Wochen dauert (Abb. 2). Der im Stadium 3a sichtbare weißliche Hof entsteht dadurch, dass das Neosom sich so vergrößert hat, dass es makroskopisch sichtbar ist. Es hat eine sphärische Form und einen Durchmesser von 5–10 mm erreicht. Der Hof erhebt sich über das Hautniveau. Die über dem Neosom liegende Schicht des Stratum corneum wird dabei zum Teil durch die Volumenzunahme des Abdomens verdrängt und dadurch sehr dünn. Die gesamte Läsion hat beim Palpieren eine feste Beschaffenheit.

Charakteristisch für das Stadium 3 ist die Expulsion von Eiern und die Produktion von Faeces. Die Faeces werden als schraubige Schnur intermittierend ausgeschieden. Da der Kot klebrig ist, sammelt er sich in den umliegenden Hautpapillen an. Zusätzlich wird periodisch eine wässerig-bräunliche Flüssigkeit ausgeschieden, bei der es sich möglicherweise um verdautes Blut handelt.

Typisch für das Stadium 3 ist auch eine pulsierende Aktivität im Inneren des Flohs, die durch den weißen Hof hindurch erkennbar ist. Dabei sind im Bereich des weißen Hofes dunkle fadenförmige Strukturen (bis 1 mm breit) zu erkennen, die Veränderungen durchlaufen, die einer peristaltischen Bewegung ähneln. Diese pulsierende Aktivität steht in keinem erkennbaren zeitlichen Zusammenhang mit der Abgabe von Eiern oder Faeces. Außerdem sind spontane Bewegungen des Konus in vertikaler Richtung zu erkennen. Diese können auch von außen, z. B. durch Berührung, ausgelöst werden.

Histologisch finden sich um den penetrierenden Floh Infiltrate aus neutrophilen und eosinophilen Granulozyten, Lymphozyten und Mastzellen. Die kleinen Blutgefäße scheinen erweitert, insbesondere im Bereich des Stechrüssels. Es kommt bereits in dieser frühen Phase zu epidermalen Mikroabszessen (Feldmeier et al. 2004).

Im Stadium 3b nimmt die Eiausscheidung deutlich zu, die Ausscheidung von Faeces und wässeriger Flüssigkeit und das Pulsationsphänomen bleiben bestehen. Die Läsion verliert ihre feste Konsistenz, schrumpft ein, und von außen betrachtet entsteht die Form einer kleinen Caldera. Die Läsion färbt sich erst hellbraun, dann zunehmend dunkelbraun, und es bildet sich eine Kruste. Bei vielen Patienten kommt es zur Desquamation der Keratinschicht in der unmittelbaren Nachbarschaft der Läsion und zur Bildung von Fissuren. Histopathologisch sind Mikroabszesse fast immer vorhanden, und es finden sich eine Hyperplasie und Hyperkeratosen der Epidermis. Zellinfiltrate und Gefäßerweiterung in der Dermis bestehen fort.

Im Stadium 4 stirbt der Floh, und die Eliminierung des Restkörpers beginnt. Dieser Prozess beginnt etwa drei Wochen nach Penetration und dauert ca. zwei weitere Wochen. Das Stadium 4 wird in zwei Unterstadien gegliedert: Im Stadium 4a (Abb. 2) verliert der Parasit zunehmend Lebenszeichen, und im Stadium 4b sind keine Lebenszeichen mehr nachweisbar.

Im Stadium 4a schrumpft die hypertrophe Zone weiter zusammen, die Läsion ist tief schwarz-braun verfärbt, und im umliegenden Wirtsgewebe sind Hyperplasie und Hyperkeratose sehr deutlich ausgebildet. Mikro- und Makroabszesse sind nahezu konstant. Im Gewebe haben sich die histologischen Anzeichen einer Entzündungsreaktion weiter verstärkt, neutrophile Granulozyten dringen ins Innere des abgestorbenen Flohs vor.

Im Stadium 4b wird der Restkörper des toten Flohs aufgelöst und schließlich durch nachwachsende Epidermiszellen eliminiert. Der Restkörper des Flohs kann jedoch auch im Ganzen aus der Läsion herausfallen. Von außen ist die Läsion schwarz-braun verkrustet. Ausgehend vom Stratum germinativum kommt es zur Regeneration der Epidermis. Die Dilatation der Blutgefäße in der Dermis nimmt in dieser Phase stetig ab.

Im Stadium 5 bleibt eine kreisrunde, 5–10 mm große Vertiefung in der Hornhaut zurück, die über Monate persistiert und ein pathognomischer Hinweis auf eine durchgemachte Tungiasis ist. Auch in dieser Phase sind noch lymphozytäre Infiltrate in Stratum papillare und Stratum reticulare nachweisbar.

4. Klinisches Bild der Tungiasis

4.1 Tungiasis beim Menschen

T. penetrans penetriert beim Menschen vor allem in die Haut der Füße, und hier insbesondere an den Zehen (Feldmeier et al. 2013). Etwa 94 % der Läsionen befinden sich an den Füßen. Ektopische Lokalisationen finden sich am ganzen Körper, sind aber besonders häufig an den

Händen (etwa 5 %) (Heukelbach et al. 2002). Einige Menschen bemerken die Penetration als einen lokalisierten Schmerz, meist geschieht das Eindringen jedoch unbemerkt.

Im Stadium 2 treten Juckreiz und/oder Schmerzen schon bei mehr als 80 % der Patienten auf (Eisele et al. 2003). Klinisch ist das Stadium 2 durch ein Erythem charakterisiert, das den eingebetteten Flohkörper umgibt. Auf stark pigmentierter Haut ist das Erythem jedoch schwer zu erkennen (Eisele et al. 2003). Der Flohkörper ist als bräunlich-schwarzer Punkt mit einem Durchmesser von maximal 2 mm sichtbar.

Im Stadium 3a empfinden die Patienten den wachsenden Floh als druckausübenden Fremdkörper, der mit pulsierenden Schmerzen einhergeht. Schmerzen sind nachts stärker als tagsüber und werden durch Berührung der Penetrationsstelle verstärkt. Klinisch sind Erythem, Ödem, Überwärmung und Druckgefühl als Zeichen einer lokalen Entzündung charakteristisch. Die Klinik verstärkt sich im Stadium 3b weiter. Die Patienten haben mitunter sehr starke Schmerzen, insbesondere wenn beim Stehen oder Gehen Druck auf die Läsion ausgeübt wird. Es kommt zu einer Einschränkung der Mobilität (Eisele et al. 2003).

Im Stadium 4 kann der Juckreiz fortbestehen. Der Schmerz verstärkt sich in dieser Phase weiter, und es kommt zur Intensivierung der Entzündungsreaktion. Diese wird häufig durch die nahezu konstant vorhandene Superinfektion verstärkt (Eisele et al. 2003). Die starke Entzündungsreaktion geht mit einem charakteristischen Muster pro-inflammatorischer Zytokine einher (Feldmeier et al. 2004).

In den Läsionen lassen sich sowohl aerobe als auch anaerobe Keime nachweisen (Feldmeier et al. 2002). Möglicherweise sind diese Erreger auch die Ursache von Sepsis bzw. Gangrän, Komplikationen, die in historischen Berichten häufig erwähnt werden. Es gibt Hinweise, dass bei Personen ohne Impfschutz ein Tetanus als Folge einer Tungiasis entstand. In der Tat wurde *Clostridium tetani* wiederholt aus Läsionen isoliert (Pampiglione et al. 2009).

In aeroben Kulturen wurden in allen 99 untersuchten Proben Bakterien detektiert und 146 Isolate angezüchtet (Feldmeier et al. 2002). Am häufigsten kam hierbei *Staphylococcus aureus* (35,5 %) gefolgt von *Klebsiella pneumoniae* (17,8 %) und *Streptococcus pyogenes* (8,2 %) vor (Feldmeier et al. 2002). Daneben wurden Enterobacteriaceae sowie Mitglieder der Gattungen *Bacillus* und *Pseudomonas* gefunden. Anaerobe Erreger konnten nur aus etwa einem Viertel der Läsionen isoliert werden. Bei 40 % der anaeroben Erreger handelte es sich um Mitglieder der Gattung *Peptostreptococcus* und bei 35 % um *Clostridium* spp. (Brothers und Heckmann 1980, Feldmeier et al. 2002, 2014).

Ein weiteres, nicht zu unterschätzendes Gesundheitsrisiko ergibt sich aus der Tatsache, dass die Tungiasis eine Armut-assoziierte Krankheit ist. Das bedeutet, dass die betroffenen Personen über keine oder nicht ausreichende Ressourcen in Bezug auf Prävention und Therapie verfügen. In Endemiegebieten werden deshalb regelmäßig scharfe, unsterile Instrumente wie Messer, Scheren, Nadeln oder Dorne benutzt, um den eingebetteten Parasiten zu entfernen. Da diese Instrumente nacheinander bei mehreren Personen eingesetzt werden, und eine Extraktion eines eingebetteten Sandflohs immer zu einer Blutung führt, kann es durch die inadäquate Therapie zur Übertragung von humanen Papillomaviren, Hepatitis-B- und C-Virus und möglicherweise auch HIV kommen (Feldmeier et al. 2013).

4.2 Tungiasis bei Tieren

Tungiasis kommt in einem breiten Spektrum von Haus- und Wildtieren vor. Pampiglione et al. (2009) nennen als Haustierwirte Schweine, Rinder, Ziegen, Schafe, verschiedene Equi-

den, Lamas, Hunde und Katzen. Außerdem wurde *Tunga* spp. in Lateinamerika in der Haut diverser Wildtiere z. B. bei Nagetieren aus den Gruppen Murinae (Langschwanzmäuse, einschließlich Ratten) und Cavioidea (Meerschweinchen-Verwandte), bei Gürteltieren, Tapiren, Fledermäusen und Jaguaren nachgewiesen (Pampiglione et al. 2009, Widmer und Azevedo 2012). In Afrika sind Infektionen bei Elefanten, Gorillas und diversen Affenspezies beschrieben (Pampiglione et al. 2009).

Untersuchungen zur Pathologie und Pathogenese beziehungsweise zur Klinik der Tungiasis bei Tieren sind selten, systematische Studien fehlten bis vor kurzem völlig. Lediglich für Wistar-Laborraten (*Rattus norvegicus*) liegen detaillierte Angaben zur klinischen Pathologie einschließlich Beschreibung der histopathologischen Veränderungen vor (Feldmeier et al. 2007). Die Entwicklung der penetrierten Weibchen und das klinische Bild waren nahezu identisch wie beim Menschen (Abb. 5).

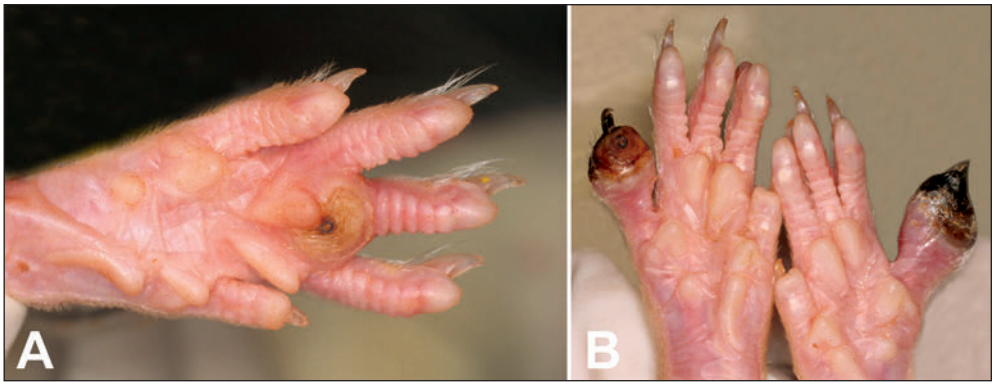

Abb. 5 *T. penetrans*-Läsionen im Stadium 3b (*A*) und 4 (*B*) am Fuß einer Wistar-Ratte. Auffällig sind die extrem starken Entzündungszeichen.

Allerdings zeigte sich histologisch ein wichtiger Unterschied zur humanen Tungiasis, da es bei der Ratte im Stadium 3a zu einem Durchbruch der Basalmembran der Epidermis kommt und deshalb nicht nur die Proboscis, sondern ein größerer Teil des Flohkörpers bis in die Dermis vordringt (Feldmeier et al. 2007). Dies führt zu einer extrem starken Entzündungsreaktion, möglicherweise auch deshalb, weil bakterielle Pathogene dadurch bis in tiefere Hautschichten vordringen können.

Kürzlich wurde erstmals die klinische Pathologie bei Schweinen und Hunden systematisch dokumentiert (Mutebi et al. 2016). Die Untersuchung von 121 mit *T. penetrans* infizierten Schweinen im ländlichen Uganda zeigte, dass bei Schweinen die Zehen noch ausgeprägtere Prädilektionsstellen sind als beim Menschen. Mehr als 99 % der Läsionen waren an den Füßen der Tiere lokalisiert. Bei Schweinen besteht eine erstaunliche – und bisher nicht näher erklärbare – Bevorzugung der Hinterbeine: Bei ca. 90 % der infizierten Tiere fanden sich Läsionen an den Hinterbeinen, während die Vorderbeine nur in etwas weniger als 60 % der Fälle betroffen waren. An den Zehen konzentrieren sich die Läsionen auf das Koronarband und die Basis der Klauen. Dabei sind die eigentlichen Zehen stärker betroffen als die Afterzehen. Die Häufigkeit der Läsionen an den Metatarsen ist bereits deutlich seltener (< 1 %) und vergleichbar mit der Häufigkeit ektopischer Läsionen beim Menschen (wenn Läsionen an den Händen

nicht als ektopisch betrachtet werden). Beim Schwein fanden sich ektopische Läsionen nur am Scrotum und am Perineum (Mutebi et al. 2016), nicht aber wie früher beschrieben an den Zitzen (Verhulst 1976). Klinisch sind Erosion der Hufwand, Nekrosen der Hufwand und der Haut um die Läsionen herum, Druckschmerz, Erytheme, Fissuren und Ödeme charakteristisch. Diese Symptome waren bei 40–70 % der infizierten Schweine vorhanden (Mutebi et al. 2016).

In extremen Fällen wurde eine Lockerung oder eine vollständige Ablösung der Hufe beobachtet, was zu einer starken Deformation, mitunter zum vollständigen Verlust der Afterzehe führte. Diese Komplikationen sind offenbar auf starkes Scheuern der betroffenen Stellen zurückzuführen. Das Scheuern wiederum ist ein guter Indikator für den starken Juckreiz und die Schmerzen, die die Tiere – insbesondere bei multiplen Läsionen – haben. Etwa ein Viertel der untersuchten Schweine hatte mehr als 30 eingebettete Sandflöhe, einige Tiere mehrere Hundert (Mutebi et al. 2016).

In dem Endemiegebiet in Uganda hatten Hunde nur sehr wenige Läsionen (maximal acht, meist nur ein bis vier) (Mutebi et al. 2016). Die Läsionen waren überwiegend in unmittelbarer Nähe zu den Krallen sowie in seitlichen Bereichen der Zehen lokalisiert, seltener direkt an der Sohle der Fußballen (das nur bei Welpen), in karpalen und metakarpalen Bereichen oder in den Zwischenzehenspalten. Häufige klinische Befunde waren Druckschmerz, Erythem und Ulcera. Trotz der deutlich niedrigeren Infektionsintensität im Vergleich zu Schweinen waren bei Hunden schmerzbedingte Veränderungen beim Gang häufiger zu beobachten als bei Schweinen.

In einer Studie in einer Armensiedlung in Brasilien wurde eine hohe Prävalenz bei Haus- und streunenden Katzen gefunden (Heukelbach et al. 2004b). Eine klare Beschreibung der Klinik fehlt allerdings.

Für Wiederkäuer liegen nur wenige Berichte vor. In Südamerika scheinen *T. trimamillata* und *T. hexalobulata* bei Rindern häufiger zu sein als *T. penetrans*. Zur Klinik der beiden erst genannten Arten ist bisher wenig bzw. nichts publiziert. Bei *T. trimamillata* ist das Neosom größer als bei *T. penetrans*, doch sind die histologischen Veränderungen der Läsion sehr ähnlich (Pampiglione et al. 2003). Da die Erstbeschreibungen von *T. trimamillata* und *T. hexalobulata* aus den Jahren 2003 bzw. 2013 stammen (De Avelar et al. 2013, Pampiglione et al. 2003), sind ältere Publikationen zum Vorkommen von *T. penetrans* bei Rindern mit Vorbehalt zu interpretieren, da in den meisten Fällen keine Abgrenzung zu anderen *Tunga*-Spezies vorgenommen wurde.

Die Tatsache, dass das Vorkommen von *T. trimamillata* ursprünglich als auf Ekuador beschränkt galt, der Parasit aber inzwischen auch in Peru und Brasilien identifiziert wurde (Fioravanti et al. 2006, Linardi et al. 2013), unterstreicht, dass systematische epidemiologische und klinische Untersuchungen überfällig sind. Insbesondere Studien, in die molekulare Analysen integriert sind, welche die Identifizierung etwaiger vorhandener kryptischer Spezies mittels Barcoding ermöglichen (z. B. mittels Sequenzierung von mitochondrialen Cytochromoxidase oder 16S-rRNA-Genen; Frank et al. 2012, Luchetti et al. 2005, 2007) sind dringend erforderlich.

In Uganda, wo wahrscheinlich nur *T. penetrans* vorkommt, wurde kürzlich die Infektion von zwei Jungziegen beschrieben (Mutebi et al. 2015). Hier wurde *T. penetrans* morphologisch von anderen Mitgliedern der Gattung abgegrenzt.

Völlig ungeklärt ist, welche ökonomischen Effekte die Tungiasis auf die Produktivität von Nutztieren hat. Hier ist eine Untersuchung von Schweinen von herausragender Bedeutung, da dieses Nutztier im ländlichen Afrika besonders häufig betroffen ist (Mutebi et al. 2015,

2016, Ugbomoiko et al. 2007) und denkbar ist, dass sich schwere Verlaufsformen aufgrund von Mobilitätseinschränkungen negativ auf die Gewichtszunahme auswirken. Da die Schweinezucht in einigen Ländern Afrikas zunehmend genutzt wird, um ein zusätzliches Einkommen für die Bevölkerung in ressourcenarmen Landgemeinden zu generieren, könnte eine erfolgreiche Bekämpfung der Tungiasis auch zur Verbesserung der wirtschaftlichen Entwicklung im ländlichen Raum beitragen.

5. Tungiasis als Zoonose

Die meisten Mitglieder der Ordnung Siphonaptera (Flöhe) weisen eine sehr geringe Wirtsspezifität auf und zeigen stattdessen eine Affinität für einen bestimmten Typ von Nest (Bitam et al. 2010, Peters 2003). Diese Spezies sind typischerweise nestassoziiert und suchen den Wirt nur zum Blutsaugen auf. Andere Spezies, die vorwiegend Tiere mit großem Bewegungsradius befallen, zeigen eine noch eingeschränktere Spezifität für bestimmte Wirte, verlassen aber ihren Wirt als Imagines nur selten.

Viele Flohspezies sind zoonotisch, so etwa Katzen- und Hundeflöhe (*Ctenocephalides felis* und *Ctenocephalides canis*), der Rattenfloh (*Xenopsylla cheopis*), der Geflügelfloh (*Ceratophyllus gallinae*) und auch der sogenannte Menschenfloh (*Pulex irritans*) (Bitam et al. 2010, Peters 2003).

Im Vergleich dazu sind die weiblichen Flöhe aus der Gattung *Tunga* durch ihre Penetration des Wirtes in der parasitischen Phase des Lebenszyklus stationär und nicht mehr zum Wechsel des Wirtes oder zur Rückkehr ins Nest befähigt. Es ist zu erwarten, dass durch die permanente Exposition gegenüber dem Wirt und das Eindringen ins Gewebe bei *Tunga* die Wirt-Parasit-Interaktionen deutlich stärker ausgeprägt und spezifischer sind als bei Spezies mit einer ektoparasitischen Lebensweise. Es ist ferner zu erwarten, dass stärker ausgeprägte Interaktionen auch zu einer Einschränkung der Wirtsspezifität führen. Tatsächlich trifft dies für die meisten *Tunga*-Spezies und insbesondere für die Untergattung *Brevidigitata* zu, allerdings nicht für *T. penetrans*. *T. trimamillata* und möglicherweise auch *T. hexalobulata* scheinen hinsichtlich der Breite ihres Wirtsspektrums eine Zwischenstellung einzunehmen.

Aus evolutionsbiologischer Sicht ist es wichtig zu wissen, ob ein enges oder breites Wirtsspektrum in der Gattung *Tunga* ein ursprüngliches oder ein abgeleitetes Merkmal darstellt. Bisher liegen allerdings nur für sehr wenige Arten der Tungidae Sequenzdaten vor, so dass die Phylogenie der Familie völlig unklar ist. Die Mitglieder der wahrscheinlich am nächsten verwandten Gattung von Flöhen, *Hectopsylla* (Zhu et al. 2015), weisen ein intermediäres Wirtsspektrum auf und kommen meist bei einer Reihe von Wirten aus der gleichen systematischen Gruppe vor (Hastriter und Méndez 2000). Mithin ist eine detaillierte phylogenetische Analyse beider Gattungen zusammen mit der Erhebung von verlässlichen Daten zum Wirtsspektrum notwendig, um ein grundlegendes phylogenetisches Verständnis der Evolution der Wirt-Parasit-Interaktionen zu erhalten.

Für die beiden zoonotischen Spezies, *T. penetrans* und *T. trimamillata*, ist offensichtlich, dass eine großräumige (oder gar vollständige) Eliminierung aufgrund der vorhandenen Reservoire derzeit kaum möglich erscheint. Das schließt aber eine deutliche Reduzierung des Infektionsdrucks durch gezielte Bekämpfungsmaßnahmen – basierend auf dem *One-Health*-Prinzip – nicht aus. Die folgenden Überlegungen beziehen sich ausschließlich auf *T. penetrans*, da für *T. trimamillata* nicht ausreichend Daten vorliegen.

In verschiedenen Studien wurden unterschiedliche Wirtsspezies als epidemiologisch relevante Reservoirwirte identifiziert. Am häufigsten wurden dabei das Schwein, der Hund und die Ratte als Reservoire im menschlichen Umfeld nachgewiesen (Cooper 1967, Heukelbach et al. 2004b, 2012, Mutebi et al. 2015, Pampiglione et al. 2009, Rietschel 1989, Verhulst 1976). In einer Studie wurde auch eine hohe Prävalenz bei Katzen gefunden (Heukelbach et al. 2004b). In Nigeria war die Präsenz von Katzen im Haushalt mit einem leicht erhöhten Risiko (Chancenverhältnis 1,91) für humane Tungiasis assoziiert (Ugbomoiko et al. 2007).

Auffällig ist, dass in Südamerika insbesondere die Relevanz von Hunden und Ratten als Tierreservoire hervorgehoben wurde. Die beiden einzigen systematischen Studien aus Afrika (Nigeria und Uganda) legen jedoch nahe, dass Schweine dort eine sehr viel wichtigere Rolle als Erregerreservoire spielen (Mutebi et al. 2015, Ugbomoiko et al. 2007). Da Tungiasis auch in Gebieten ohne Schweinehaltung (z. B. mit überwiegend muslimischer Bevölkerung) hoch prävalent sein kann, ist das epidemiologische Bild derzeit sicherlich inkomplett.

In den Untersuchungen, in denen Tungiasis bei Menschen und Tieren parallel untersucht wurde, bestand eine hoch signifikante Korrelation in Bezug auf Prävalenz und Intensität der Infektion zwischen humanen und tierischen Haushaltsmitgliedern. Es stellt sich daher in erster Linie die Frage, welches die wichtigsten Amplifikatoren für den Lebenszyklus von *T. penetrans* in unterschiedlichen Endemiegebieten sind.

In Anbetracht der in den meisten Regionen charakteristischen saisonalen Schwankung mit einem Prävalenzmaximum in der Trockenzeit und einer Prävalenzabnahme in der Regenzeit, muss es im Laufe einer Trockenzeit zu einer dramatischen Vermehrung der Parasiten und damit einem steigenden Infektionsdruck kommen. Für das Verständnis der Infektionsdynamik wäre entscheidend zu identifizieren, in welchen Wirten sich die Flöhe auch in der Regenzeit vermehren können und in welchen die Vermehrung zu Anfang der Trockenzeit stattfindet. Diese können durchaus verschieden von den Wirten sein, die in der Hochsaison besonders betroffen sind. Insbesondere wäre zu erwarten, dass im Zeitraum des höchsten Infektionsdrucks mehr Wirtsspezies betroffen sind als während der Regenzeit. Um also zu klären, in welchem saisonalen Kontext Haustiere und Ratten Risikofaktoren für Tungiasis beim Menschen sind, muss die Vermehrung der Flöhe in allen potentiellen Wirten im Laufe eines Jahres analysiert werden.

6. *Tunga penetrans* als Vektor

Bei der humanen Tungiasis ist eine bakterielle Superinfektion nahezu konstant vorhanden (Feldmeier et al. 2007). Der eingebettete Floh funktioniert dabei als „Leitungsbahn", entlang der Bakterien in tiefere Schichten der Epidermis eindringen können bzw. schon beim Penetrationsvorgang in die Haut eingeschleppt werden. Es wird auch diskutiert, ob die ballonartig aufgetriebene Intersegmentalhaut die Bildung eines mikrobiellen Biofilms induziert (Abb. 6). Die Vektorfunktion des Flohs bliebe gleichwohl „mechanisch" (siehe Abschnitt 4.1).

Flöhe sind bekannt als wichtige Vektoren, insbesondere für bakterielle Pathogene, aber auch für Nematoden und Cestoden. Die wichtigsten durch Flöhe übertragenen Pathogene sind *Yersinia pestis* sowie verschiedene Rickettsienspezies, wie u. a. *Rickettsia typhi*, *Rickettsia prowazekii* und *Rickettsia felis*. Für mehr als 30 Flohspezies ist eine Rolle als Vektor nachgewiesen, und *Y. pestis* kann wahrscheinlich von nahezu jeder Flohspezies übertragen werden (Bitam 2012, Eisen und Gage 2012, Perry und Fetherston 1997) und ist überdies in vielen Nagetierpopulationen prävalent, die Flöhen als Wirte dienen.

Abb. 6 *Streptococcus* sp. auf der Intersegmentalhaut eines Neosoms von *T. penetrans*

Yersinia pestis bleibt im Floh auf den Verdauungstrakt beschränkt und wird nicht transovariell auf die nächste Generation übertragen (Bitam et al. 2010). Deshalb setzt die Übertragung von *Y. pestis* durch Flöhe einen Wirtswechsel von adulten Flöhen voraus. Aufgrund der speziellen Lebensweise weiblicher Sandflöhe saugen diese grundsätzlich nur an einem einzelnen Wirt und kommen daher als Vektoren für *Y. pestis* nicht in Betracht. Die männlichen Sandflöhe saugen allerdings ebenfalls Blut (Witt et al. 2004) und können wahrscheinlich auch an mehreren Wirten nacheinander saugen. Dementsprechend ist eine Vektorfunktion theoretisch möglich. Bisher wurde lediglich einmal das Vorkommen von *Y. pestis* in *T. penetrans* beschrieben (Brumpt 1949, Pampiglione et al. 2009). Es gibt nur eine neuere Studie zum Vorkommen von *Y. pestis* in *T. penetrans* in ko-endemischen Gebieten: In der Demokratischen Republik Kongo waren alle PCRs zum Nachweis von *Y. pestis*-DNA in Sandflöhen negativ (Sackal et al. 2008). Bisher gibt es keine Laborexperimente zum Nachweis einer möglichen Vektorfunktion von *T. penetrans*.

Im Gegensatz zu *Y. pestis* werden Rickettsien transovariell übertragen, daher kommen auch weibliche Sandflöhe als Vektoren in Betracht. Tatsächlich wurden in der Demokratischen Republik Kongo *R. felis* in nicht eingebetteten *T. penetrans* nachgewiesen (Sackal et al. 2008). Da mindestens in einem Teil der untersuchten *Tunga*-Proben auch humane DNA nachweisbar war, besteht die Möglichkeit, dass die Flöhe an einem Menschen Blut gesaugt hatten, der mit *R. felis* infiziert war. Nachweise anderer *Rickettsia*-Spezies gibt es nicht (Bitam 2012).

Sackal et al. (2008) fanden außerdem auch DNA von zwei Arten der Gattung *Bartonella*, *Candidatus* Bartonella rochalimae und *Bartonella* sp. ED377. *Candidatus* B. rochalimae ist

zoonotisch und wird bei Hunden mit Endokarditis in Verbindung gebracht (Chomel et al. 2009). Über *Bartonella* sp. ED377 ist bisher außer einer Sequenz in GenBank nichts bekannt.

Bisher wurden Untersuchungen zu Mikroorganismen, die mit *T. penetrans* assoziert sind, entweder mit klassischen mikrobiologischen Methoden durchgeführt (Feldmeier et al. 2002) oder mit Polymerasekettenreaktionen (PCRs), die sich auf einzelne Erreger oder eine kleine Gruppe von Erregern konzentrierten, die von human-pathogener Bedeutung sind. Darüber hinaus sind *T. penetrans* und auch *T. trimamillata* auf das Vorhandensein von Bakterien der Gattung *Wolbachia* untersucht worden. Dabei wurden in zwei Studien für *T. penetrans* aus Südamerika Wolbachien nachgewiesen (Fischer et al. 2002, Heukelbach et al. 2004a), in einer anderen Studie allerdings nicht (Luchetti et al. 2004).

Überraschenderweise waren die bei *T. penetrans* gefundenen Wolbachien-Spezies eng verwandt mit denen von Filarien und weniger mit solchen von Insekten (Fischer et al. 2002). Im Gegensatz dazu weisen die bisher für *T. trimamillata* beschriebenen Gensequenzen von Wolbachien hohe Ähnlichkeiten zu denen anderer holometaboler Insekten (Mücken und Wespen) und auch von hemimetabolen Grillen auf (Luchetti et al. 2004). Diese zunächst erstaunlich erscheinenden Verwandtschaftsbeziehungen sind vor dem Hintergrund der Tatsache, dass Wolbachien bei Filarien zwar eindeutig Symbionten, bei Arthropoden aber eher infektiöse Agentien sind, eigentlich nicht verwunderlich, denn Wolbachien-positive und Wolbachien-negative Populationen sind bei der gleichen Spezies von Insekten nicht ungewöhnlich. Auch wenn vertikaler Transfer der wichtigste Übertragungsweg der Wolbachien ist, so ist horizontaler Transfer sowohl innerhalb einer Art als auch zwischen phylogenetisch weit voneinander entfernten Arthropoden möglich (Baldo et al. 2008, Chomel et al. 2009, Heath et al. 1999, Huigens et al. 2004, Le Clec'h et al. 2013, Narita et al. 2006, Pigeault et al. 2014, Shoemaker et al. 2002). Daten zu Effekten von Wolbachien auf die Physiologie von Sandflöhen – wie beispielsweise Verschiebung des Geschlechterverhältnisses zu weiblichen Tieren oder zytoplasmatische Inkompatibilität bei der Fortpflanzung – und zu der Frage, wie weitverbreitet Populationen verschiedener *Tunga*-Spezies mit und ohne Wolbachien sind, gibt es nicht.

Um einen umfassenden Einblick in die potenziellen Vektorfähigkeiten von *T. penetrans* bezüglich der Übertragung von Pathogenen zu erhalten, sind besonders moderne metagenomische Methoden geeignet. Eine 16S-rRNA-PCR mit anschließender Analyse der Amplikons mittels „next-generation-sequencing" ist hier ein möglicher Ansatz. Allerdings sind nicht-limitierte Methoden zu bevorzugen, da die vorgeschaltete 16S-PCR zu einer Verzerrung der Resultate führen kann. Die direkte Sequenzierung von DNA nicht penetrierter Sandflöhe könnte die assoziierten Pathogene ohne eine solche Verzerrung identifizieren. Dabei würden sowohl mechanisch als auch zyklisch-alimentär oder zyklisch-exkretorisch übertragene Erreger gefunden. Bisher unbekannte Pathogene könnten ebenfalls nachgewiesen werden.

7. Behandlungs- und Präventionsoptionen bei Mensch und Nutztieren

Es liegt nahe, bei der Therapie der Tungiasis an die gegen andere Ektoparasiten mit großem Erfolg eingesetzten Wirkstoffgruppen der makrozyklischen Laktone und Pyrethroide zu denken. Da in vielen Endemiegebieten in der Humanpopulation auch Läuse und Krätzemilben häufig sind, hätte ein solcher Ansatz den praktischen Vorteil, dass mit einer Substanz mehrere parasitäre Hauterkrankungen behandelt werden könnten. Allerdings haben bislang keine Ver-

suche, mehrere parasitäre Erkrankungen mit einem Medikament zu behandeln, zu zufriedenstellenden Ergebnissen hinsichtlich der Bekämpfung der Tungiasis geführt (Heukelbach et al. 2004d). Bei Therapiestudien zur Tungiasis muss berücksichtigt werden, dass die eingebetteten weiblichen Flöhe nach 4 – 6 Wochen ohnehin absterben. Es muss also stets das jeweilige Stadium der eingebetteten Flöhe und deren Weiterentwicklung berücksichtigt werden.

Vor einigen Jahren konnte gezeigt werden, dass die zweimalige topische Applikation von Ivermectin und Trichlorfon (= Metrifonat, ein Organophosphat), nicht aber von Thiabendazol (Benzimidazol), bei Patienten mit Tungiasis sieben Tage nach Applikation zu einer signifikant niedrigeren Anzahl von lebenden eingebetteten Flöhen im Vergleich zu einer Plazebo-Behandlung führte (Heukelbach et al. 2003). Die Unterschiede waren jedoch relativ klein, und Neuinfektionen konnten nicht ausgeschlossen werden. Im Gegensatz dazu hatte die orale Behandlung mit Ivermectin keinerlei Effekt auf die Entwicklung weiblicher Flöhe (Heukelbach et al. 2004c). Eine bevölkerungsbasierte Studie zeigte, dass einen Monat nach der ersten Behandlung mit Ivermectin die Prävalenz der Tungiasis unverändert war (Heukelbach et al. 2004d). Der Prävalenzabfall neun Monate nach der ersten Behandlung war vermutlich durch saisonale Schwankungen und nicht durch Ivermectin zu erklären (Heukelbach et al. 2004d).

Die zweimal tägliche Anwendung des Repellents Zanzarin®, einer Lotion auf der Basis von Kokosnussöl (*Cocos nucifera*), Jojoba-Öl (*Simmondsia chinesis*) und *Aloe vera*, reduzierte die Infektionsrate um mehr als 90 % und die mit Tungiasis einhergehende klinische Pathologie nahezu vollständig (Feldmeier et al. 2006). Da bestehende Läsionen innerhalb weniger Wochen – durch die Eliminierung des Residualkörpers der abgestorbenen Parasiten – abheilen, ist eine auf Repellentseffekten basierende Intervention im Prinzip ausreichend, um eine klinische Heilung herbeizuführen. In der Tat führte die Applikation bei Patienten mit schwerster klinischer Tungiasis innerhalb von wenigen Wochen zum völligen Abklingen der klinische Pathologie und war wesentlich effizienter als der Gebrauch von Schuhen (Schwalfenberg et al. 2004, Thielecke et al. 2013b). Die deutlich höhere Effizienz der wiederholten Anwendung von Zanzarin® gegenüber der Ausgabe von Schuhen konnte auch in einer großangelegten Interventionsstudie belegt werden (Thielecke et al. 2013a). Ferner konnte gezeigt werden, dass auch die intermittierende Anwendung von Zanzarin® (Behandlung in jeder zweiten Woche) noch einen signifikanten Schutz erzielt (Buckendahl et al. 2010). Allerdings ist Zanzarin® nicht mehr auf dem Markt. Die Wirkung anderer Repellentien wurde bisher nicht getestet.

Vor kurzem wurde gezeigt, dass NYDA®, ein zur Behandlung der Pediculosis capitis genutztes Therapeutikum, hervorragend gegen *T. penetrans* eingesetzt werden kann (Thielecke et al. 2014). NYDA® enthält ein Gemisch zweier niedrig-viskoser Dimeticone (Polydimethylsiloxilane). Diese Dimeticone sind oberflächenaktive Substanzen mit hervorragenden Kriecheigenschaften. Ihre Wirkung gegen Läuse, und wahrscheinlich auch gegen *T. penetrans*, beruht nach gegenwärtigem Kenntnisstand auf rein physikalischen Mechanismen. Die Polysiloxane dringen in kleinste Öffnungen ein und verschließen diese. Das trifft auch auf die Stigmata zu. Auf diese Weise wird wahrscheinlich nicht nur die Sauerstoffaufnahme blockiert, sondern auch die Abgabe von Wasserdampf. Dies führt während des Blutsaugens bei den Kopfläusen zu einem osmotischen Ungleichgewicht und schließlich zur Ruptur des Darms (Burgess 2009). Auch wenn die Wirkungsweise von NYDA® gegen *T. penetrans* nicht näher untersucht wurde, konnten Thielecke et al. (2014) zeigen, dass fünf Tage nach einer einmaligen Applikation auf frühe Flohstadien (Stadium 2a–3a) 90 % der Flöhe sich nicht mehr normal entwickelten. Bei 78 % konnten am Tag sieben nach Auftragen von NYDA®

keine Vitalitätsanzeichen mehr beobachtet werden. Zu diesem Zeitpunkt waren auch die Entzündungsreaktionen bereits signifikant reduziert. Eine zwischenzeitlich in Uganda durchgeführte Studie zeigte, dass eine gezielte Applikation des Dimeticons auf den Abdominalkonus des eingebetteten Sandflohweibchens die Heilungsrate auf 98 % erhöht (Feldmeier et al. Manuskript in Vorbereitung).

Rietschel (1989) beschrieb, dass Trichlorfon eine Tungiasis bei Hunden heilt. Diese Studie war allerdings nicht kontrolliert. Klimpel et al. (2005) verwendeten ein Kombinationspräparat aus Imidacloprid und Permethrin (Advantix®) zur Behandlung der Tungiasis bei Hunden. In der Behandlungsgruppe kam es zu einer 60 %igen Abnahme der Flöhe im Stadium 3 pro Hund am Tag sieben nach Behandlung und zu einer Reduktion um 97,5 % am Tag 14. Am Tag 21 war allerdings nur noch eine Reduktion der Flohzahl in Stadium 3 pro Hund um 89 % und am Tag 28 um 66 % festzustellen.

Eine systematische Bekämpfung der Tungiasis bei Tieren wird sich nur realisieren lassen, wenn man diese entweder ökonomisch begründen kann – der Produktivitätsgewinn also die Kosten der Behandlung deutlich übersteigt – oder wenn es einen klaren positiven Effekt der Bekämpfung beim Tierreservoir auf die Prävalenz und Infektionsintensität bei der menschlichen Bevölkerung gibt. Für beides gibt es derzeit keine Daten. Allerdings unterstützen die bisher existierenden Daten die Vermutung, dass ohne eine erfolgreiche Bekämpfung der *T. penetrans*-Infektion bei haushaltsnah gehaltenen Tieren die Tungiasis des Menschen nicht kontrolliert bekämpft werden kann.

8. Bekämpfungsmaßnahmen und *One-Health*-Prinzip

Bekämpfungsmaßnahmen sollten auf dem *One-Health*-Prinzip fußen, dabei evidenzbasierte Bekämpfungsstrategien verwenden und die wichtigsten Reservoirwirte sowie freie Flohstadien in der Umwelt mit einbeziehen. Dabei sollten Insektizide möglichst gezielt verwendet werden, um eine möglichst hohe strategische Effizienz der Behandlungsmaßnahme zu erreichen. Wichtig wäre auch eine langfristige Ausrichtung der Bekämpfungsmaßnahmen anstatt kurzfristiger Kampagnen bei saisonalen Prävalenzschwankungen. Für humane Tungiasis gibt es mittels Repellentien und Dimeticonen Optionen, schwere klinische Tungiasis zu verhindern. Problematisch ist nach wie vor die chemotherapeutische Therapie und Bekämpfung der Tungiasis bei den Reservoirwirten, weil kaum evidenzbasierte Ansätze zur Verfügung stehen.

Die erfolgreiche Bekämpfung der Larvenstadien in der Umgebung würde die Inzidenzen bei Menschen und Tieren deutlich herabsetzen. Allerdings ist unser Wissen über die Biologie der *T. penetrans*-Larvenstadien sehr gering. Es ist lediglich bekannt, dass die Entwicklung sowohl in den Häusern als auch im Freien stattfinden kann (Nagy et al. 2007). Im Inneren der Häuser kann die Zementierung von Böden verhindern, dass sich Larven und Puppen entwickeln. Im Zusammenhang mit der Bekämpfung von Anophelesmücken werden bereits heute in vielen *T. penetrans*-endemischen Regionen Insektizide in den Gebäuden versprüht. Hierbei könnten die Brutstätten von *T. penetrans* relativ leicht mit einbezogen werden. Für die Bekämpfung der Flohentwicklung werden in den Industriestaaten neben klassischen Insektiziden auch sogenannte „insect growth regulators“ (IGRs) verwendet. Hierbei handelt es sich meist um Juvenilhormon-Analoga (z. B. Methopren und Pyriproxyfen) oder Hemmer der Chitinsynthese (z. B. Diflubenzuron) (Henderson und Foil 1993, Kawada und Hirano

1996, Moser et al. 1992, Rajapakse et al. 2002), die meist weniger toxisch als konventionelle Insektizide sind und auch zur Bekämpfung der Tungiasis genutzt werden könnten.

Lediglich eine Studie wurde bislang publiziert, in der eine Intervention nach dem *One-Health*-Prinzip durchgeführt wurde (Pilger et al. 2008). Dabei wurden bei Menschen die Sandflöhe alle 2–3 Wochen mechanisch entfernt. Hunde und Katzen wurden mit Insektiziden behandelt. Diese Maßnahmen wurden für drei Monate implementiert. In den letzten zwei Wochen dieses ersten Teils der Interventionsphase und in den folgenden beiden Monaten wurde außerdem in den Gebäuden und an Ruheplätzen von Hunden und Katzen Deltamethrin versprüht. Diese Maßnahmen senkten die Prävalenz beim Menschen um ca. 75 % und bei Tieren um ca. 66 % (Pilger et al. 2008). Die Intensität der Infektion wurde ebenfalls sowohl für Menschen als auch Tiere deutlich reduziert. In einem Kontrolldorf wurden keine entsprechenden Veränderungen beobachtet. Wenige Monate nach dem Ende der Intervention waren die Unterschiede zwischen Interventions- und Kontrolldorf jedoch nicht mehr vorhanden, und ein Jahr nach der Intervention waren Prävalenz und Infektionsintensität auf das Niveau vor der Intervention zurückgekehrt (Pilger et al. 2008).

9. Experimentelle Tungiasis

Nagy et al. (2007) beschrieben die Etablierung des Lebenszyklus von *T. penetrans* im Labor, wobei Wistar-Ratten als Wirt genutzt wurden. Es wurde allerdings lediglich ein einziger Durchlauf des Lebenszyklus beschrieben, bei dem sich der weitaus größte Teil der Eier nicht bis zu Imagines entwickelte. In einer weiteren Studie wurden Wistar-Ratten genutzt, um den Zyklus zu etablieren und die klinische Pathologie bei Ratten zu dokumentieren (Feldmeier et al. 2007).

Die Wistar-Ratte hat als Modell für die Tungiasis allerdings zwei wesentliche Nachteile: Zum einen kommt es bei der Infektion von Ratten zu einem Durchbruch der Basalmembran der Epidermis durch den Parasiten, so dass die Flöhe und mitgeschleppte Keime viel tiefer in die Dermis eindringen als bei humaner Tungiasis. Zum anderen findet man aufgrund der kleinen Füße der Ratten immer nur sehr wenige eingebettete Flöhe je Tier, und die beim Menschen so typischen Läsionscluster kommen nicht vor. Gerade für die schweren Formen humaner Tungiasis kann die Ratte folglich kein gutes Modell sein. Zumindest in Bezug auf den zweiten Punkt wären *T. penetrans*-infizierte Schweine wesentlich besser geeignet.

Die Etablierung des Modells beim Schwein würde es erlauben, genügend Eier und Larven zu produzieren, um Parameter für eine optimale Entwicklung der freilebenden Stadien zu identifizieren und chemische sowie nicht-chemische Interventionsmöglichkeiten unter kontrollierten Laborbedingungen auszutesten. Solche Modelle können außerdem genutzt werden, um die Wirksamkeit verschiedener Insektizide zu erproben. Das Modell der porzinen Tungiasis könnte auch verwendet werden, um den Effekt von *T. penetrans*-Infektionen auf die Produktivität der Schweine zu quantifizieren. Im Labor können ferner Flöhe ohne assoziierte Keime oder mit definierten Keimen kontaminierte Flöhe gezüchtet werden. Diese können für kontrollierte Studien zu Parasit-Wirt-Interaktionen, einschließlich immunologischer und immunpathologischer Reaktionen, eingesetzt werden. Das Tungiasis-Ratten-Modell könnte andererseits zur Charakterisierung einer möglichen Vektorfunktion von *T. penetrans* verwendet werden, da viele Floh-übertragene Pathogene wie *Y. pestis* und *R. felis* Nagetiere als natürliche Reservoirs haben (Bitam et al. 2010, Perry und Fetherston 1997).

Infektionen unter kontrollierten Bedingungen sind außerdem die Voraussetzungen, den parasitischen Teil des Lebenszyklus besser zu verstehen. Dieser ist durch die endoparasitische Lebensweise des weiblichen Flohs und die damit verbundenen Entwicklungsprozesse auch aus grundlagenwissenschaftlicher Sicht von hohem Interesse.

10. *Tunga penetrans* als Model für Neosomie

Mit der Neosomie weist der Entwicklungszyklus von *Tunga* spp. ein für Insekten sehr ungewöhnliches Merkmal auf, das in Bezug auf das Ausmaß der Veränderungen einer Metamorphose ohne Häutung entspricht (Audy et al. 1972). Durch das starre Exoskelett ist bei den Adulten fast aller Arthropoden die Körperform und Größe nicht weiter veränderlich. Davon gibt es nur wenige Ausnahmen, wie z. B. die durch Eiproduktion massiv geweiteten Hinterleibe von Königinnen bei Termiten oder die extreme Dehnung des Abdomens bei blutsaugenden weiblichen Schildzecken (Ixodidae).

Die Vergrößerung der Intersegmentalhäute innerhalb der ersten Tage des parasitären Teils des Lebenszyklus von *T. penetrans* stellt ein ideales System dar, in dem solche Prozesse modellhaft studiert werden könnten. Da es während der Neosomie zur Bildung neuer Kutikula in Bereich der Abdominalsegmente kommt, sind viele der beteiligten Enzyme mit denen identisch, die für die Kutikulabildung während der Häutung benötigt werden. Das legt nahe, dass zumindest ein Teil der beteiligten Regulationsmechanismen ebenfalls an beiden Prozessen beteiligt ist. Parallel dazu werden aber während der Neosomie noch weitere Prozesse wie die Aktivierung der Speicheldrüsen durch den Beginn des Blutsaugens und die Differenzierung der Eierstöcke zur Produktion der Eier stattfinden. Die Etablierung eines Modellsystems würde die detaillierte Analyse dieser Prozesse auf molekularer Ebene erlauben und auch Untersuchungen ermöglichen, ob sich diese Prozesse als Ziel für Interventionsstrategien eignen.

In Bezug auf die Evolution der endoparasitischen Lebensweise wäre ein Vergleich mit den Mitgliedern des Schwestertaxons der Gattung *Hectopsylla* sinnvoll. Diese haben bereits Weibchen, die sich permanent am Wirt anheften, die allerdings nicht in die Haut eindringen und auch kein Neosom ausbilden (Hastriter und Méndez 2000). Vergleichende transkriptomische Analysen der Veränderungen in den ersten Stunden/Tagen nach Wirtsfindung könnten hier wertvolle Informationen liefern, die unser Verständnis dieser speziellen Anpassung einer Parasitengruppe erheblich vertiefen.

11. Schlussfolgerungen

Die Tungiasis ist eine wichtige und verbreitete Zoonose, die alle Kriterien einer Vernachlässigten Tropenkrankheit aufweist und außerdem hochgradig mit Armut in den betroffenen Bevölkerungsgruppen korreliert ist. Unsere Kenntnisse über Parasit und Parasit-Wirt-Interaktionen sind bislang jedoch äußerst gering – selbst im Vergleich zu anderen vernachlässigten Tropenkrankheiten. Eine konsequente Analyse der epidemiologisch relevanten human- und veterinärmedizinischen Faktoren sowie die systematische Evaluierung von durchführbaren und finanzierbaren Interventionsstrategien könnten zu einer deutlichen Verbesserung der gesundheitlichen und ökonomischen Situation der Bevölkerung in den Endemiegebieten beitragen.

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Mosquito Monitoring in Germany

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Abstract

Due to a dramatic decline in mosquito research following the eradication of malaria from Europe in the middle of the 20th century, up-to-date data on the indigenous culicid fauna have become scarce for Germany. However, monitoring programmes have recently been initiated in several European countries in consequence to increased frequencies of outbreaks and autochthonous cases of mosquito-borne diseases such as chikungunya, dengue, West Nile fever, malaria, Usutu fever and dirofilariases. In Germany, nation-wide mosquito collections, focussing on both the occurrence and geospatial distribution of mosquito species and the screening of mosquitoes for circulating pathogens, started in 2011, using active and passive approaches.

Between 2011 and 2014, a total of 39 of the 46 culicid species previously described for Germany were found, among them several species not reported for decades. In addition, three new, invasive species were detected including the potential vectors *Aedes japonicus* (Asian bush mosquito) and *Ae. albopictus* (Asian tiger mosquito). While the first has now become established in Germany with at least three separate populations, the latter is regularly introduced by vehicle transport from endemic regions in southern Europe.

With some species / species complexes, identification problems exist since they are highly similar not only morphologically but also genetically in their CO1 DNA barcoding region. In general, however, reliable data are accumulating, thus facilitating species distribution maps and future risk analyses for mosquito-borne diseases.

Zusammenfassung

Infolge eines dramatischen Einbruchs in der Stechmückenforschung nach der Auslöschung der Malaria zur Mitte des 20. Jahrhunderts sind aktuelle Daten zur einheimischen Stechmückenfauna in Deutschland selten geworden. Als Reaktion auf häufiger werdende Ausbrüche und autochthone Fälle Stechmücken-assoziierter Krankheiten, wie Chikungunya, Dengue, West-Nil-Fieber, Malaria, Usutu-Fieber und Dirofilariosen, wurden jedoch kürzlich Monitoringprogramme in mehreren europäischen Ländern initiiert. In Deutschland starteten 2011 unter Einsatz aktiver und passiver Strategien bundesweite Stechmückensammlungen, um sowohl das Vorkommen und das räumlich-zeitliche Auftreten der Mückenarten als auch in den Mücken zirkulierende Pathogene zu erfassen.

Zwischen 2011 und 2014 wurden insgesamt 39 der 46 für Deutschland beschriebenen Stechmückenarten gefangen, darunter mehrere, die seit Jahrzehnten nicht mehr dokumentiert worden waren. Außerdem wurden drei neue, invasive Arten entdeckt, einschließlich der potenziellen Vektoren *Aedes japonicus* (Asiatische Buschmücke) und *Ae. albopictus* (Asiatische Tigermücke). Während sich die erste Art inzwischen mit mindestens drei Populationen in Deutschland etabliert hat, wird die zweite regelmäßig über den Fernverkehr aus endemischen südeuropäischen Regionen eingeschleppt.

Bei einigen Arten/Artenkomplexen treten Identifizierungsprobleme auf, da sie sich nicht nur morphologisch, sondern auch genetisch in der CO1-DNA-Barcoding-Region extrem ähneln. Generell sammeln sich jedoch nach und nach zuverlässige Daten an, die die Erstellung von Verbreitungskarten von Stechmückenarten und Risikoanalysen für Stechmücken-übertragene Krankheiten erlauben.

1. Historical Background

Contrary to common knowledge, mosquito-borne diseases are not a new phenomenon to Europe. Yellow fever and dengue fever were widely distributed in the Mediterranean in the 19th century (Morillon et al. 2002, Schaffner and Mathis 2014), and malaria was already

documented in ancient times even for northern parts of the continent (Bruce-Chwatt and de Zulueta 1980). The occurrence of these diseases implies the former presence of the vectors of their etiological agents, which were represented by the yellow fever mosquito *Aedes aegypti* for the dengue and yellow fever viruses and by various *Anopheles* species for the malaria parasites. Dengue and yellow fever disappeared around the turn of the 19th to the 20th century as a consequence of the disappearance of *Ae. aegypti*, the reasons for which have remained obscure. Malaria had significantly declined already during the 19th century due to drainage of wetlands and improved sanitary conditions, but it was actively controlled in Europe from the early 20th century by the large-scale application of insecticides (DDT) and newly developed synthetic antimalarial drugs. It was only eradicated from the continent in the 1970s. With no mosquito-borne disease of significant relevance anymore, mosquito research became more and more neglected in Europe, and field data on the indigenous mosquito fauna outdated.

During the last two decades, mosquito-borne diseases have emerged and resurged in Europe. Autochthonous cases and even outbreaks of malaria, West Nile fever, chikungunya, dengue, Usutu fever and dirofilariasis have been reported with increasing frequency (Kampen et al. 2012a, Schaffner et al. 2013, Kampen and Werner 2015). At the same time, growing international trade has considerably supported the introduction of exotic mosquito species, including known vectors of disease agents. Some of the species have been quickly eliminated again, some have succeeded in getting established, and some are still struggling for survival. The most important invasive species in Europe are the Asian tiger mosquito *Ae. albopictus*, the yellow fever mosquito *Ae. aegypti*, the Asian bush mosquito *Ae. japonicus*, and *Ae. koreicus* (Medlock et al. 2015). These species are proven vectors of various disease agents, at least experimentally (Medlock et al. 2015). By contrast, few data exist on the vector competences of the mosquito species endemic to Germany (*ECDC* 2014), with hardly any study into indigenous populations.

Except for three cases of autochthonous malaria (Krüger et al. 2001, Zoller et al. 2009), Germany has not knowingly faced emerging and resurging mosquito-borne diseases recently. However, the unexpected outbreak of bluetongue, a biting-midge-borne disease of ruminants, in 2006 (Saegerman et al. 2008), was taken as an alarm signal that we once again need to scientifically approach potential insect vectors of disease. Both human and animal mosquito-borne pathogens, such as those of the diseases recently reported plus Rift Valley fever virus, which it is feared will cross the Mediterranean some day, have caused great concern and established a need for preparedness among health authorities.

As a first step towards a risk assessment on mosquito-borne diseases in Germany in the future, it was considered necessary to determine the occurrence and distribution of mosquito species (Diptera: Culicidae) in Germany. For this purpose, a monitoring programme was initiated on behalf of the German human and animal health authorities in 2011, including active and passive mosquito collections all over Germany. This contribution deals with the time period 2011 to 2014, although a follow-up project was started in 2015.

2. Materials and Methods

2.1 Active Mosquito Monitoring

Mosquitoes were actively collected in traps all over Germany during the vegetative seasons 2011 to 2014, i.e. from April to October each year. BG sentinel and EVS (encephalitis virus

surveillance) traps were located at 126 sites with a running period of one to three years. Further EVS traps, gravid traps and ovitraps were set up for a few weeks or months at about 300 additional collection sites. The trapping sites included natural, rural and urban areas and covered ecologically diverse settings.

In addition to trapping, mosquitoes were collected manually at close to 2,000 sites. Adults were netted and aspirated in the vegetation, in animal sheds and when flying outside during field work in the summer, as well as in cellars, dungeons, natural caves etc. during wintertime. Larvae and pupae were collected from breeding places by dipping and sieving.

2.2 Passive Mosquito Monitoring

A citizen science project called "Mueckenatlas" ("mosquito atlas") was launched in early 2012 to support community participation in mosquito collection (Kampen et al. 2015). Through press releases, TV appearances, newspaper articles, public talks and flyers, the general public were requested to capture mosquitoes in their private surroundings and to submit them. A website (www.mueckenatlas.de) was created providing general information on mosquitoes and on the project. It also describes how to collect and submit a mosquito. A questionnaire to be added to the submitted mosquito is available for download asking for details about its collection. Upon identification, the submitting person is informed about the collected mosquito species and its biology. On demand, contributors can be made visible by name or a pseudonym on an interactive website map showing date and site of the submission.

Until the end of 2014, more than 6,000 submissions by interested citizens were registered including more than 25,000 mosquitoes from more than 4,600 sites. Distribution maps of the indigenous mosquito species submitted are intended to be presented on the website.

2.3 Mosquito Identification and Registration

Since adults are easier to identify than eggs, larvae and pupae, immature mosquitoes were usually reared in the laboratory until adult emergence. Morphological identification was then done using determination keys by Schaffner et al. (2001) and Becker et al. (2010). Alternatively, in particular in the case of cryptic species or damaged/incomplete specimens, genetic techniques were applied, such as species-specific PCR assays (Proft et al. 1999, Rudolf et al. 2013, Kronefeld et al. 2014) or CO1 barcoding (Folmer et al. 1994, Hébert et al. 2003).

All information on the mosquitoes collected and identified was fed into the German mosquito database CULBASE, which had been constructed within the framework of the monitoring project.

3. Results and Discussion

Of the 46 mosquito species listed for Germany (Dahl et al. 1999), 39 were unambiguously identified morphologically or genetically by late 2014. These included several rare species not documented for decades, such as *Culiseta alaskaensis*, *Cs. glaphyroptera* and *Cs. ochroptera* (Kampen et al. 2013a).

In addition, four species previously not reported for Germany were demonstrated: three invasive ones (*Ae. albopictus*, *Ae. japonicus*, *Cs. longiareolata*) and a recently described cryptic

one (*Anopheles daciae*). *Aedes albopictus* was trapped repeatedly in the southwestern Upper Rhine Valley along a motorway entering Germany from the south, suggesting introduction of specimens from southern Europe, where this species is widely distributed (Werner et al. 2012, Kampen et al. 2013b). In 2014, reproduction of *Ae. albopictus* could be documented for three months in that same area (Werner and Kampen 2015).

Tab. 1 Culicid species found and not found although inventoried during the nation-wide monitoring programme in Germany (2011–2014)

Species found						Species listed, but not found
Genus *Aedes*	Genus *Anopheles*	Genus *Coquillettidia*	Genus *Culex*	Genus *Culiseta*	Genus *Ochlerotatus*	Various genera
Ae. albopictus	*An. atroparvus*	*Cq. richiardii*	*Cx. hortensis*	*Cs. alaskaensis*	*Oc. annulipes*	*An. algeriensis*
Ae. cinereus	*An. claviger*		*Cx. modestus*	*Cs. annulata*	*Oc. cantans*	*Cs. fumipennis*
Ae. geminus	*An. daciae*		*Cx. pipiens* (forms *pipiens* and *molestus*)	*Cs. glaphyroptera*	*Oc. caspius*	*Cs. subochrea*
Ae. japonicus	*An. maculipennis*		*Cx. molestus*	*Cs. longiareolata*	*Oc. cataphylla*	*Cx. martinii*
Ae. rossicus	*An. messeae*		*Cx. territans*	*Cs. morsitans*	*Oc. communis*	*Oc. cyprius*
Ae. vexans	*An. plumbeus*		*Cx. torrentium*	*Cs. ochroptera*	*Oc. detritus*	*Oc. nigrinus*
					Oc. diantaeus	*Oc. refiki*
					Oc. dorsalis	*Ur. unguiculata*
					Oc. excrucians	
					Oc. flavescens	
					Oc. geniculatus	
					Oc. intrudens	
					Oc. leucomelas	
					Oc. pullatus	
					Oc. punctor	
					Oc. riparius	
					Oc. rusticus	
					Oc. sticticus	

After submission of several individuals to the "Mueckenatlas", two new *Ae. japonicus* populations were detected in western and northern Germany in 2012 and 2013 respectively (Kampen et al. 2012b, Werner and Kampen 2013). While the West German population has since been expanding through active migration, the North German one seems to be remaining static so far, probably due to its still being in the founder phase (Zielke et al. 2015). Further specimens of *Ae. japonicus* were collected in southwestern Germany, where its occurrence had already been detected in 2008 (Schaffner et al. 2009).

Culiseta longiareolata, a third invasive species, although not considered a vector of pathogens of humans or livestock, was detected in the same, relatively warm southwestern region of Germany in 2011 (Werner et al. 2012, Kampen et al. 2013a).

Anopheles daciae, a member of the *An. maculipennis* complex, had probably been present in Germany as long as its indigenous sibling species but was only recently recognized and separated from its sibling *An. messeae* (Nicolescu et al. 2004). It was found to be distributed primarily in southwestern and northeastern parts of Germany (Kronefeld et al. 2014).

Seven mosquito species inventoried for Germany were not collected during the monitoring programme: *An. algeriensis*, *Cs. fumipennis*, *Cs. subochrea*, *Cx. martinii*, *Ochlerotatus cyprius*, *Oc. nigrinus*, *Oc. refiki* and *Uranotaenia unguiculata*. While the latter three species are thought to be rare and not widely distributed, *An. algeriensis* was only recently rediscovered in Germany after decades without reporting (Krüger and Tannich 2014). *Culex martinii* and *Oc. cyprius* probably do not exist anymore as they had only been demonstrated at a single place each in the 1920s and 1930s, respectively (Mohrig 1969).

It could not be verified whether *Cs. fumipennis* and *Cs. subochrea* were among the mosquitoes collected. These species are closely related to *Cs. morsitans* and *Cs. annulata*, respectively, and can be reliably distinguished from them by morphological features in the male gender (genitalia) only. While *Culiseta morsitans* and *Cs. annulata* males could be identified morphologically among the collections, this was not the case with *Cs. fumipennis* and *Cs. subochrea*. Interestingly, genetic identification by DNA barcoding could not help as CO1 nucleotide sequences were identical within these pairs of species. The same problem occurred with some other groups of species (*Ae. geminus/rossicus*, *Oc. annulipes/cantans/excrucians/riparius*, *Oc. cataphylla/leucomelas*, *Oc. intrudens/diantaeus*), even if they can be separated morphologically when intact specimens are available. When genetic identification was necessary, CO1 barcoding failed.

4. Summary and Conclusions

While some native mosquito species probably disappeared from Germany during recent decades, three others have recently been introduced. In South Germany, specimens of the invasive Asian tiger mosquito *Ae. albopictus* were regularly found. These have been imported from southern Europe, and reproduction has taken place over an extended period of time, raising fears of imminent establishment. By contrast, the Asian bush mosquito *Ae. japonicus* is already established, with three populations present in Germany by 2014. Continued expansion can be observed in at least the West German population.

Tendencies in the spatial distribution of several mosquito species can be recognized from the collected data, but more data, particularly of less frequent and invasive species, are needed. Also, the data need to be supplemented so that they display the seasonal dynamics of the species. A new government-funded programme that started in mid-2015 is intended to fill the gaps and continue monitoring.

Problems do occur with both morphological and genetic species identification since morphologically highly similar species and damaged specimens of some species that are morphologically distinguishable in principle, have been shown to possess identical CO1 barcodes. Species-specific genetic markers, alternative to the CO1 barcodes, therefore need to be identified and developed for reliable identification.

The “Mueckenatlas” has proved to be an efficient passive mosquito surveillance tool, particularly regarding invasive species, and thus is thought to be an appropriate early warning system for detecting changes in the mosquito fauna. It should be permanently used to supplement or even replace active monitoring.

A major factor hindering reliable risk assessments on mosquito-borne disease in Germany is the limited knowledge about the vector competences of the endemic mosquito species. Vector-competent species probably do occur, but it is not known which mosquito species is able to transmit which pathogen and under what conditions. Urgently needed infection and transmission studies are envisaged for the near future.

In addition to experimental work, a "mosquito task force" was appointed in late 2015 to provide advice, guidance and practical assistance to national and federal authorities on all matters connected to mosquitoes and mosquito-borne diseases.

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Wahrnehmen und Steuern

Sensorsysteme in Biologie und Technik

Vorträge anlässlich der Jahresversammlung
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Mensch und Tier sind für ihr Überleben in der Auseinandersetzung mit der sie umgebenden Umwelt auf die Wahrnehmung optischer, akustischer, olfaktorischer, gustatorischer und haptischer Eindrücke angewiesen. Mit ihren sensorischen Systemen können sie die vielfältigen chemischen und physikalischen Reize aufnehmen, die der Organismus verarbeitet und die schließlich das Verhalten und die Gefühlswelt beeinflussen. Für die Problematik der Sensorik und des Wahrnehmens des Menschen spielt die Begrenzung durch entsprechende Einschränkungen eine besondere Rolle. Diese kann durch die wissenschaftlichen und technischen Möglichkeiten über künstliche Sensorsysteme immer besser kompensiert werden. Schließlich kann sich wahrnehmendes Steuern vom Menschen gänzlich lösen und z. B. Robotern zugewiesen werden. Die Verbindung der Sinn- und Wahrnehmungsproblematik mit ästhetischen Fragestellungen und künstlerischen Herangehensweisen liefert ein weiteres interessantes Diskussionsfeld. Der Band behandelt auch die Themen „Biologische Kommunikation“, „Hören und Sehen“, „Sprache, Denken und Lernen“, „Medizintechnik, angewandte Biomechanik und Robotik“ sowie „Gesellschaft“.

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Dirofilariasis – A New Emerging Vector-Borne Zoonosis in Germany

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Abstract

The filariases are a group of rather diverse diseases resulting from infection with vector-borne nematodes called filariae. A broad range of filarial species can infect mammals including humans. The most important human filarial species are *Onchocerca volvulus*, *Wuchereria bancrofti*, *Brugia* ssp. and *Loa loa*, which are responsible for diseases such as onchocerciasis (river blindness), lymphatic filariasis (elephantiasis) and loiasis (Calabar swelling), respectively. However, in addition to classical human filariae, there are zoonotic filariae, such as *Dirofilaria repens*, which may accidentally infect humans. Dirofilariosis, caused by an infection with *Dirofilaria repens*, is considered an emerging zoonosis in Europe. The main reservoirs for the parasite are dogs and other carnivores. Mosquitoes transmit infectious L3 larvae which develop into fertile macrofilariae in their definitive vertebrate hosts. Humans may become infected as aberrant hosts and in most cases the worms remain infertil. Until recently, Central Europe, including Germany, was not expected to become endemic for *D. repens* due to local climatic conditions. All human cases diagnosed in Germany were associated with recent travel histories of the patients to countries known to be endemic for *D. repens*. An autochthonous human case of dirofilariasis was not reported in Germany until 2014. However, new projections suggest that climatic conditions in some parts of southwest and eastern Germany might be suitable for the development of infectious L3 larvae. To determine whether local transmission of *D. repens* is taking place in Germany, we analyzed a total of more than 350,000 mosquitoes for the presence of filarial DNA. This, together with our finding of mosquitoes carrying *D. repens* in Brandenburg in five successive years and the recurrent detection of infected dogs in this area strongly argues for constant local transmission of *D. repens* in this region. At present, however, the true burden of *D. repens* infections is unknown as data on parasite prevalence in both humans and dogs or other carnivores are not available. Our findings argue for the timely transmission of information to local physicians and veterinarians to implement control measures and to increase awareness of the disease.

Zusammenfassung

Die Filariosen sind eine Gruppe recht unterschiedlicher Krankheiten, die durch Infektionen mit vektorübertragenen Nematoden, den Filarien, hervorgerufen werden. Ein breites Spektrum von Filarien-Spezies kann Säugetiere, auch den Menschen, infizieren. Die wichtigsten Filarien-Spezies sind *Onchocerca volvulus*, *Wuchereria bancrofti*, *Brugia* ssp. und *Loa loa*, die für solche Erkrankungen wie Onchozerkose (Flussblindheit), Lymphatische Filariose (Elephantiasis) und Loiasis (Kamerunbeule oder Calabar-Schwellung) verantwortlich sind. Zusätzlich zu den klassischen humanpathogenen Filarien gibt es zoonotische Filarien, wie *Dirofilaria repens*, die eher zufällig auch den Menschen infizieren können. Die Dirofilariose, die durch Infektion mit *Dirofilaria repens* hervorgerufen wird, gilt in Europa als vordringende Zoonose. Die Hauptreservoire für die Parasiten sind Hunde und andere Raubtiere. Stechmücken übertragen die L3-Larven, die sich zu geschlechtsreifen Makrofilarien in ihren definitiven Wirbeltierwirten entwickeln. Menschen können als aberrante Wirte infiziert werden, die Würmer bleiben dann aber oft infertil. Bisher wurde aufgrund der lokalen klimatischen Verhältnisse nicht erwartet, dass Mitteleuropa, einschließlich Deutschland, endemisch für *D. repens* werden könnte. Alle in Deutschland diagnostizierten Fälle bei Menschen waren auf kürzlich absolvierte Reisen in als Endemiegebiete bekannte Länder zurückzuführen. Autochthone Fälle von humaner Dirofilariose wurden bis 2014 in Deutschland nicht berichtet. Neue Projektionen gehen jedoch davon aus, dass die klimatischen Bedingungen in Teilen von Südwest- und Ostdeutschland mittlerweile für die Entwicklung der L3-Larven als ausreichend angesehen werden können. Um festzustellen, ob eine lokale Übertragung von *D. repens* in Deutschland stattfindet, untersuchten wir 350 000 Stechmücken auf das Vorhandensein von Filarien-DNA. Das Auf-

finden *D. repens*-tragender Stechmücken in Brandenburg in fünf aufeinanderfolgenden Jahren sowie der wiederholte Nachweis infizierter Hunde in diesem Gebiet deuten auf eine konstante lokale Übertragung von *D. repens* in dieser Region hin. Gegenwärtig ist allerdings die reale Belastung durch *D. repens*-Infektionen unbekannt, da Daten über die Verbreitung des Parasiten sowohl in Menschen als auch Hunden und anderen Raubtieren fehlen. Unsere Resultate sprechen jedoch für eine frühzeitige Weitergabe von Informationen an Ärzte und Tierärzte, um Kontrollmaßnahmen einzuführen und die Aufmerksamkeit für die Krankheit zu erhöhen.

1. Introduction

The filariases are a group of rather diverse diseases resulting from infection with vector-borne tissue-dwelling nematodes called filariae. A broad range of filarial species can infect mammals including humans (Cook and Zumla 2009, pp. 1477–1513). Depending on the species, adult filariae may live in the lymphatic system, blood vessels, skin, connective tissues or serous membranes. Females produce stage 1 larvae (microfilaria), which live in the bloodstream or skin. Development of microfilariae into infective stage 3 (L3) larvae requires uptake by arthropods and, depending on temperature, a period of development within the insect. All filariae that infect humans are transmitted by dipteran vectors such as flies or mosquitoes. The most important human filarial species are *Onchocerca volvulus*, *Wuchereria bancrofti*, *Brugia* ssp. and *Loa loa*, which are responsible for diseases such as onchocerciasis (river blindness), lymphatic fialariasis (elephantiasis) and loiasis (Calabar swelling), respectively. These classical forms of filariasis are restricted to humans as the only relevant definite host of the parasites. They are mainly present in countries with a tropical climate since transmission of the various filarial species requires elevated temperatures for the development of infective L3 larvae within the vectors. However, in addition to classical human filariae, there are zoonotic filariae, such as *Dirofilaria repens*, which may accidentally infect humans.

2. *Dirofilaria repens* and Dirofilariosis as an Emerging Zoonosis in Germany

Dirofilariosis, caused by an infection with *Dirofilaria repens*, is considered an emerging zoonosis in Europe (Genchi et al. 2011). The main reservoirs for the parasite are dogs and other carnivores. As with other filarial species, mosquitoes transmit infectious L3 larvae which develop into fertile macrofilariae in their definitive vertebrate hosts. Humans may become infected as aberrant hosts, and in most cases the worms remain infertile (for a review see Genchi et al. 2011 and Simón et al. 2012). Infections in humans usually manifest as subcutaneous nodules which are caused by macrofilariae trapped by immune mechanisms (Pampiglione and Rivasi 2000). Subcutaneous migration of the worm may result in local swelling with changing localization (creeping eruption). In addition, severe organ manifestations have been reported, which may affect various organs including the brain, the lung, or the eye. The latter is particularly found during the migratory phase of the parasite (Poppert et al. 2009, Pampiglione et al. 2000). Transmission of *D. repens* is found in various regions of the "Old World" including Europe, Africa, and Asia. Until recently, the main endemic areas in Europe were countries in the Mediterranean region, where appropriately warm temperatures allow the infectious L3 larvae to develop in the mosquito. However, during the last decade, autochthonous cases of canine and human dirofilariasis have been reported from countries further

north, such as Austria, the Czech Republic and Poland (Svobodova et al. 2006, Cielecka et al. 2012, Auer and Susani 2008).

Until recently, Central Europe, including Germany, was not expected to become endemic for *D. repens* due to local climatic conditions (Genchi et al. 2009). All human cases diagnosed in Germany were associated with recent travel histories of the patients to countries known to be endemic for *D. repens* and an autochthonous human case of dirofilariasis was not reported in Germany until 2014 (Simón et al. 2012). However, new projections from 2011 and 2012 suggest that climatic conditions in some parts of southwest and eastern Germany might be suitable for the development of infectious L3 larvae (Genchi et al. 2011, Sassnau and Genchi 2013). In addition, a survey of hunting dogs from the Upper Rhine region revealed that three animals were positive for *D. repens* microfilariae, and in a kennel of sled dogs, located in the federal state of Brandenburg close to Berlin, five of the 29 animals were infected with the parasite. However, questions remain as to whether these dogs indeed acquired their infection in Germany (Pantchev et al. 2009, Sassnau et al. 2009).

To determine whether local transmission of *D. repens* is taking place in Germany, we analyzed a total of more than 350,000 mosquitoes from a large-scale German mosquito-borne virus surveillance programme for the presence of filarial DNA. Mosquitoes were collected during the main trapping seasons between May and September 2011 to 2015, respectively, using CO_2-baited encephalitis virus surveillance (EVS) or gravid traps (Jöst et al. 2011, Czajka et al. 2012). Mosquitoes from a total of 83 trapping sites located within 10 different federal states in the southwest and eastern part of the country were selected. Mosquitoes collected at the various study sites were classified morphologically based on the species level (Becker et al. 2010). Subsequently, up to 250 mosquitoes of the same species from individual collections were pooled together. These pools comprised a broad range of ornithophilic and mammalophilic mosquitoes common to Germany, with *Culex* ssp. and *Aedes vexans* being the most abundant ones. Nucleic acids were extracted from each pool, and the presence of *D. repens* DNA was determined by PCR using *D. repens*-specific primers and probes (Czajka et al. 2014). Interestingly, nine mosquito pools were positive for *D. repens*, all of which were from mammalophilic mosquitoes collected at six trapping sites (see Fig. 1).

Two of the sites were located in the federal state of Brandenburg in close proximity to the Oder Valley, one in each of the federal states of Saxony-Anhalt, Hesse, Baden-Wuerttemberg and Bavaria. Mosquito pools that were positive for *D. repens* included *Culiseta annulata*, *Aedes vexans*, *Anopheles maculipennis sensu latu* and *Anopheles claviger*. The identity of *D. repens* in all nine pools was confirmed through DNA sequencing. Consistent with the elevated temperature required for *D. repens* larval development in the mosquito, all positive pools were collected between mid-July and mid-August. Moreover, based on the mean daily temperatures recorded by weather stations closest to the collection sites of the mosquitoes, it was concluded that the mosquitoes were trapped in time periods that allowed for the completion of the developmental cycle of the worms in the mosquitoes and subsequent transmission to vertebrate hosts (Sassnau et al. 2014). Interestingly, during this study, the first autochthonous human case of *D. repens* infection was identified in Germany. The patient was a passionate angler who lived in the federal state of Saxony-Anhalt along the Elbe Valley in close proximity to the site where *D. repens*-positive mosquitoes were trapped (Tappe et al. 2014). The most intense infestation of mosquitoes with *D. repens* was detected in the federal state of Brandenburg along the Oder Valley. In this region, conditions for larval development were predicted to be sufficient in each of the last 15 years (Sassnau and Genchi 2013). This, together with

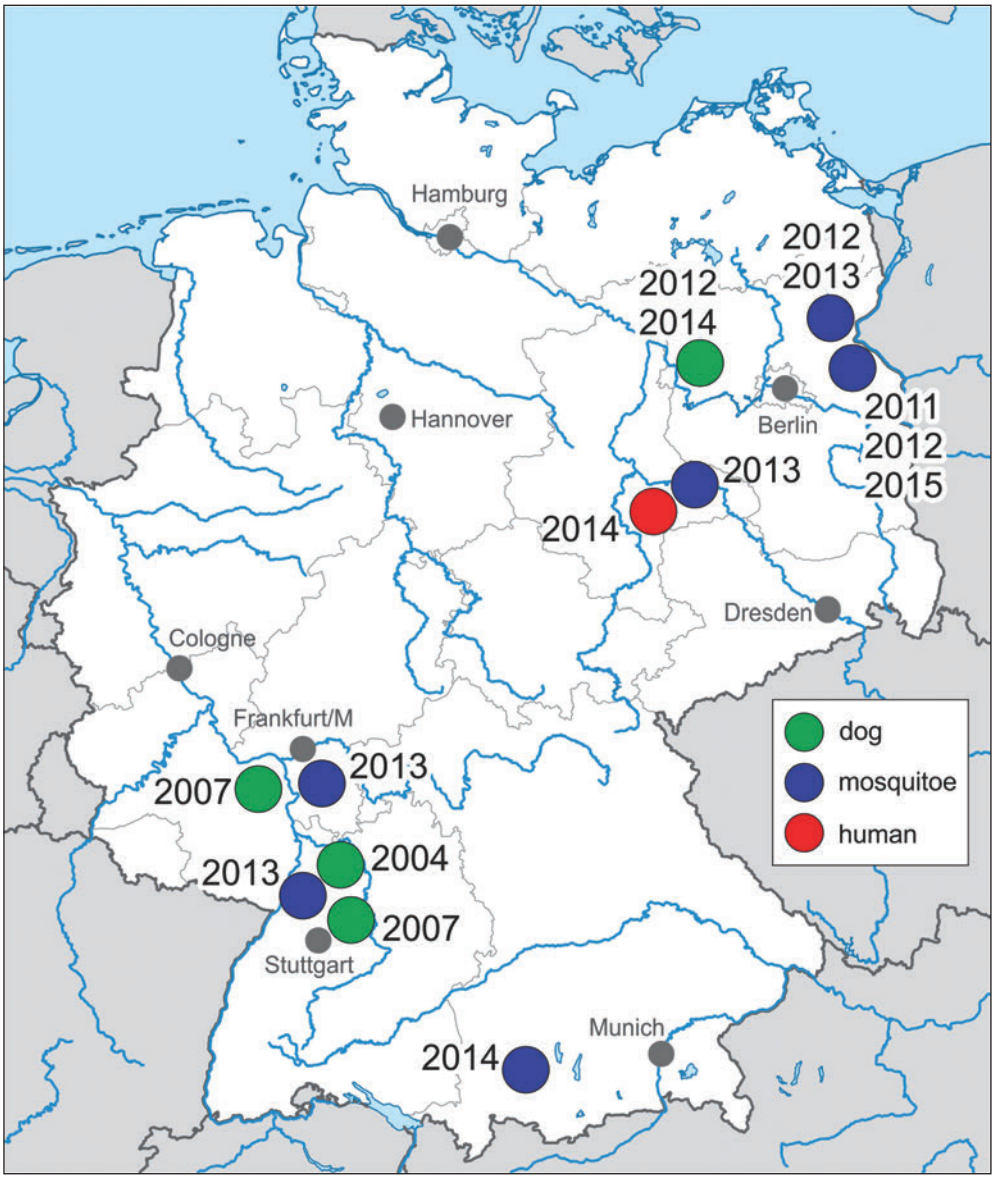

Fig. 1 Geographical distribution of autochthonous human and canine *Dirofilaria repens* infections and the origin of mosquitoes testing positive for *D. repens* DNA in Germany, as of October 2015.

our finding of mosquitoes carrying *D. repens* in Brandenburg in five successive years and the recurrent detection of infected dogs in this area (Sassnau et al. 2009, 2013, Härtwig et al. 2015), strongly argues for constant local transmission of *D. repens* in this region.

3. Conclusions

At present however, the true burden of *D. repens* infections in the federal state of Brandenburg is unknown as data on parasite prevalence in both humans and dogs or other carnivores are not available. However, our findings argue for the timely transmission of information to local physicians and veterinarians to implement control measures and to increase awareness of the disease. Due to the lack of awareness, respective clinical symptoms may not be attributed to an infection with *D. repens*. This will delay the initiation of appropriate antihelminthic treatment.

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The Mosquito *Aedes albopictus* and Chikungunya Emergence

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Abstract

The most medically important vectors of emerging and re-emerging infectious vector-borne diseases are mosquitoes. They transmit arthropod-borne viruses (arboviruses). In 2004, chikungunya fever emerged in Kenya and spread to the islands of the Indian Ocean including La Réunion where the mosquito *Aedes albopictus* is predominant. A single amino-acid change in the viral genome was selected for a more efficient transmission by *Ae. albopictus*. The worldwide dispersal of the mosquito *Ae. albopictus* through global commercial trade may have set the stage for the emergence of arboviruses in Europe. Chikungunya virus spread over most tropical regions has been facilitated by *Ae. albopictus*-adaptive mutations in the viral genome leading to a more efficient inter-human transmission, the same scenario may occur in temperate regions.

Zusammenfassung

Der medizinisch bedeutendste Vektor von erscheinenden und wiedererscheinenden vektorgebundenen Infektionskrankheiten sind (tropische) Stechmücken. Sie übertragen arthropodengebundene Viren (Arboviren). Im Jahr 2004 tauchte das Chikungunya-Fieber in Kenia auf und breitete sich auf die Inseln im Indischen Ozean, einschließlich Réunion, aus, wo die Stechmücke *Aedes albopictus* vorherrschend ist. Es selektierte sich ein einzelner Aminosäureaustausch im viralen Genom für eine effizientere Verbreitung des Chikungunya-Virus durch *Ae. albopictus* heraus. Die weltweite Verbreitung der Stechmücke *Ae. albopictus* im Gefolge des globalen kommerziellen Handels könnte die Voraussetzungen für das Erscheinen von Arboviren in Europa hervorgebracht haben. Das Chikungunya-Virus, zunächst in den meisten tropischen Regionen verbreitet, wurde durch an *Ae. albopictus*-angepasste Mutationen im viralen Genom in einer Weise gefördert, die eine effizientere Übertragung von Mensch zu Mensch erlaubt, das gleiche Szenario könnte nun auch in temperierten Regionen vorkommen.

Vector-borne diseases represent a significant proportion of emerging and re-emerging infectious diseases. The most medically important vectors of these diseases are mosquitoes, and among the most important pathogens they transmit are arthropod-borne viruses (arboviruses). For arboviruses to persist and to spread, mosquito vectors must encounter susceptible vertebrate hosts and favourable environments. Arboviruses are transmitted by the bites of competent arthropods that become infective after feeding on viremic hosts, and completing the extrinsic incubation period during which the virus replicates. In contrast to vertebrate hosts that are able to develop efficient immune responses clearing viral infection, mosquito vectors remain infective for life offering to the vector a central role as a viral reservoir. Disregarding the enzootic cycle where the virus circulates naturally between wild mosquitoes and non-human primates, the establishment of an epidemic is undoubtedly related to the introduction of a viremic host, principally vertebrates (humans, animals) acting as vehicles for importation in favourable environments.

In 2004, chikungunya emerged in Kenya and spread to the islands of the Indian Ocean including La Réunion where the mosquito *Aedes albopictus* is predominant. A single amino-acid change in the viral genome, at the position 226 in the E1 glycoprotein in a region promoting virus fusion within endosomes of target cells, was selected for a more efficient transmission by *Ae. albopictus* (Schuffenecker et al. 2006, Tsetsarkin et al. 2007, Vazeille et al. 2007).

This mosquito was able to deliver infectious viral particles in its saliva two days after ingesting an infectious blood-meal (Dubrulle et al. 2009). The epidemic variant E1-226V present at low levels in natural viral populations could rapidly emerge after being selected at the midgut level (Arias-Goeta et al. 2013). Strong genetic bottlenecks were detected at mosquito anatomical barriers, midgut and salivary glands, leading to the selection of variants with high epidemic potential in mosquito saliva (Stapleford et al. 2014). This mosquito was able to sustain a high level of replication with almost 10^9 viral particles in females from day 3 after infection. When infected, *Ae. albopictus* from La Réunion died 6–9 days earlier than non-infected females but not soon enough to interrupt transmission (Martin et. al. 2010). Besides, the primarily vector *Aedes aegypti* survived well to viral infection thanks to the antiviral immune response, RNA interference triggered just after infection (McFarlane et al. 2014).

The worldwide dispersal of the mosquito *Ae. albopictus* through commercial trade may have set the stage for the emergence of arboviruses in Europe. *Ae. albopictus* is now present in 20 European countries (Medlock et al. 2012) where it can survive through the winter. Chikungunya virus (CHIKV) spread over most tropical regions has been facilitated by *Ae. albopictus*-adaptive mutations in the viral genome (Tsetsarkin et al. 2014) leading to a more efficient inter-human transmission, the same scenario may occur in temperate regions. In southern Europe, CHIKV arrived in Italy in 2007 and France in 2010. French *Ae. albopictus* was as efficient as the typical tropical vector *Ae. aegypti* to experimentally transmit CHIKV emphasizing its potential responsibility for future outbreaks in Europe (Vega-Rua et al. 2013). Furthermore, *Ae. albopictus* populations from Europe are competent to transmit CHIKV even at low temperatures (Vega-Rua et al. 2014). Because CHIKV circulates in dengue virus (DENV)-endemic regions where *Ae. albopictus* can transmit both viruses, reports of co-infection in humans are increasing (Caron et al. 2012). *Ae. albopictus* orally infected with the two viruses in a single blood-meal is able to deliver concomitantly infectious particles of CHIK and DENV in saliva (Vazeille et al. 2010).

The European situation reflects the permissiveness of Western countries to "tropical" arboviruses. Even if all ingredients – the presence of competent vectors, annually-reported imported cases and environmental conditions suitable for transmission – should have led to CHIKV emergence in the Americas long time ago, it has happened only recently. Thus, CHIKV has been detected in the Caribbean island of Saint-Martin in late December 2013, and then spread in most islands reaching French Guiana in February 2014. The ability of American *Ae. aegypti* and *Ae. albopictus* has been examined for different CHIKV genotypes. While viral dissemination in all mosquito populations was high suggesting a weak role of the midgut as barrier, transmission was highly variable proposing an important role of salivary glands in selecting CHIKV for efficient transmission (Vega-Rua et al., 2015).

Vector-borne diseases such as chikungunya will continue to increase in incidence causing devastating public health consequences until effective vaccines are available or vector control improved. Today, the world is facing the global expansion of zika virus (ZIKV) whose pattern of emergence from Africa, throughout Asia, to its subsequent arrival in South America and the Caribbean closely resembles the emergence of CHIKV. In Europe, returning ZIKV-viremic travellers may become a source of local transmission in the presence of *Aedes* mosquitoes, exclusively *Ae. albopictus* (Chouin-Carneiro et al. 2016).

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The Impact of Biotic and Abiotic Factors on Vectorial Capacity of *Culex* Mosquitoes for West Nile Virus

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Abstract

West Nile virus (WNV) is a member of the Japanese encephalitis (JE) antigenic complex in the family *Flaviviridae*, genus *Flavivirus*. The virus perpetuates in nature by replication in alternating hosts, predominantly ornithophilic mosquitoes of the genus *Culex* and birds. Humans and horses become infected but do not mount a viremia sufficiently high to infect mosquitoes, so are considered dead end hosts. Vector competence of a mosquito population can vary over time; it is dependent on intrinsic and extrinsic influences, including environmental and genetic factors, as well as their interaction. This trait is one component of vectorial capacity, which also takes into account longevity, as well as blood feeding preferences and frequency. Climate changes forecast in the coming years are likely to result in substantial alterations to the distributions and populations of vectors of arthropod-borne pathogens. Characterization of the interaction of the complex factors comprising vectorial capacity is critical to our understanding of how climate changes could impact the epidemiological patterns of vector-borne disease. To this end, we determined the effect of temperatures ranging from 16 ºC to 32 ºC on development time, immature survival, adult survival, mosquito size, blood feeding, and fecundity of both field and colonized populations of the West Nile virus vectors, *Culex pipiens* L., *Culex quinquefasciatus* Say, and *Culex restuans* Theobald. Our results demonstrate that temperature significantly affects all of these traits, yet also that the extent of this effect is at times incongruent among temperatures, as well as being population and species-specific. Our results indicated that geographic region, as well as species and population differences, must be considered when measuring the effect of temperature on vector populations. Furthermore, exposure to West Nile virus itself has an impact on life history traits of *Cx. pipiens*.

Zusammenfassung

West-Nil-Virus (WNV) ist ein Teil des Japanischen Enzephalitis (JE)-Antigen-Komplexes der Familie *Flaviviridae*, Oberbegriff *Flavivirus*. Das Virus besteht in seiner Art durch Replikation in wechselnden Wirten fort, vornehmlich in ornithophilen Mücken der Gattung *Culex* und Vögeln. Menschen und Pferde können infiziert werden, zeigen aber keine Virämie, die ausreichend hoch ist, um Mücken zu infizieren, und werden somit als Fehlwirte bezeichnet. Die Vektorkompetenz eines Mückenbestands kann sich mit der Zeit ändern. Sie hängt von intrinsischen und extrinsischen Einflüssen ab, einschließlich umweltbedingter und genetischer Faktoren sowie deren Wechselwirkung. Dieses Merkmal ist eine Komponente der Vektorkompetenz, die auch die Lebensdauer sowie blutsaugende Vorlieben und Häufigkeiten in Betracht zieht. Die Vorhersagen bezüglich der Klimaveränderungen in den kommenden Jahren werden wahrscheinlich erhebliche Veränderungen hinsichtlich der Verteilung und des Bestands der Vektoren von durch Arthropoden übertragenen Krankheitserregern zur Folge haben. Die Kennzeichnung der Wechselwirkung von komplexen Faktoren, die die Vektorkompetenz beinhalten, ist wichtig, um zu verstehen, wie Klimaänderungen die epidemiologischen Muster von durch Vektoren übertragenen Krankheiten beeinflussen. Zu diesem Zweck haben wir die Auswirkungen von Temperaturen zwischen 16 ºC und 32 ºC auf die Entwicklungszeit, für das Überleben des Jungbestandes, für das Überleben des Altbestandes, für die Mückengröße, das Blutsaugen und die Fruchtbarkeit sowohl des Feldbestands als auch des kolonisierten Bestands der West-Nil-Virus-Vektoren, *Culex pipiens* L., *Culex quinquefasciatus* Say und *Culex restuans* Theobald, bestimmt. Unsere Ergebnisse zeigen, dass die Temperatur alle Merkmale erheblich beeinflusst, aber auch, dass das Ausmaß dieser Auswirkung immer inkongruent zwischen den Temperaturen sowie dem Bestand und den Arten ist. Unsere Resultate demonstrierten, dass Unterschiede in den geographischen Regionen sowie Arten und Beständen berücksichtigt werden müssen, wenn die Auswirkung der Temperatur auf den Vektorbestand gemessen wird. Des Weiteren hat die Einwirkung des West-Nil-Virus selbst eine Auswirkung auf die Lebensgeschichte von *Cx. pipiens*.

1. Introduction

West Nile virus (WNV) is a member of the Japanese encephalitis (JE) antigenic complex in the family *Flaviviridae*, genus *Flavivirus*. The virus is single stranded positive sense RNA, approximately 11 kb in length with 3 structural genes at the 5'-end and 7 nonstructural genes at the 3'-end. The enveloped virion is approximately 50 nm in diameter with a lipid bilayer derived from the host surrounding a nucleocapsid core containing the RNA complexed with multiple copies of the capsid protein (Brinton 2002).

West Nile virus perpetuates in nature by replication in alternating hosts, predominantly ornithophilic mosquitoes of the genus *Culex* and birds. Humans and horses become infected but do not mount a viremia sufficiently high to infect mosquitoes, thus are considered secondary or dead end hosts (Kramer and Bernard 2001). The population dynamics of the virus are therefore dependent on interaction between mosquitoes and birds. Passeriform birds appear to be the most important for virus amplification in the western hemisphere. American crows (*Corvus brachyrhynchos*) are highly susceptible to disease and consequently serve as sentinels for the presence of virus transmission, but may not be important in the enzootic cycle.

West Nile virus (WNV) was first detected in the West Nile district of Uganda, Africa, in 1937 (Smithburn et al. 1940) and since then has been isolated in Europe (Hubalek and Halouzka 1999), Asia (Zeller and Schuffenecker 2004), and Australia (MacKenzie et al. 1994). Beginning in the mid-1990s the incidence of West Nile neuroinvasive disease increased especially around the Mediterranean basin, and the geographic range of WNV expanded. Outbreaks occurred in Russia and Europe (Murgue et al. 2002), and the virus was introduced into the Americas in 1999 (Hayes and Gubler 2005, Lanciotti et al. 1999) where it is now well established. There are two predominant lineages of WNV, lineage 1 which contains 3 groups 1a, 1b limited to Australia, and 1c, found in India. Lineage 2 was historically found in sub-Saharan Africa and Madagascar, but in 2004 expanded its range to Europe, including Hungary, where it was first detected, Austria, Greece (2010), and Italy (2011) (Hernandez-Triana et al. 2014) (Fig. 1). WNV has not caused neurologic disease in South America, with the exception of equine cases in Argentina (Morales et al. 2006) and a single human in Brazil (Vieira et al. 2015).

Mosquito populations fluctuate in size due to temperature and rainfall patterns that vary within and between seasons. Similarly, bird populations vary in abundance due to several factors, including seasonal migration and roosting behaviours. This variability is taken into account either directly or indirectly by vectorial capacity (VC) of mosquito populations and a particular virus. Biotic and abiotic factors have an impact on VC, which is essentially an entomological restatement of the "basic reproductive rate" (R_0) of a pathogen, i.e., the number of secondary infections expected to occur from the introduction of a single infection in a naïve population. An equation formalizing VC was described (Macdonald 1961) and later modified by others. One of these equations (Black and Moore 1996) provides a useful platform for rational examination of selective forces that may shape WNV (and other arboviruses). This formula is:

$$VC = ma^2(I^*T)p^n/-\ln p \qquad [1]$$

where VC is vectorial capacity (R_0); *m* is vector density in relation to the host; *a* is the probability that a vector feeds on a host in 1 day (i.e. the host preference index[1] x feeding frequency);

1 The host preference index is the proportion of blood meals taken from the competent vertebrate host of interest. It is multiplied times feeding frequency to arrive at "a".

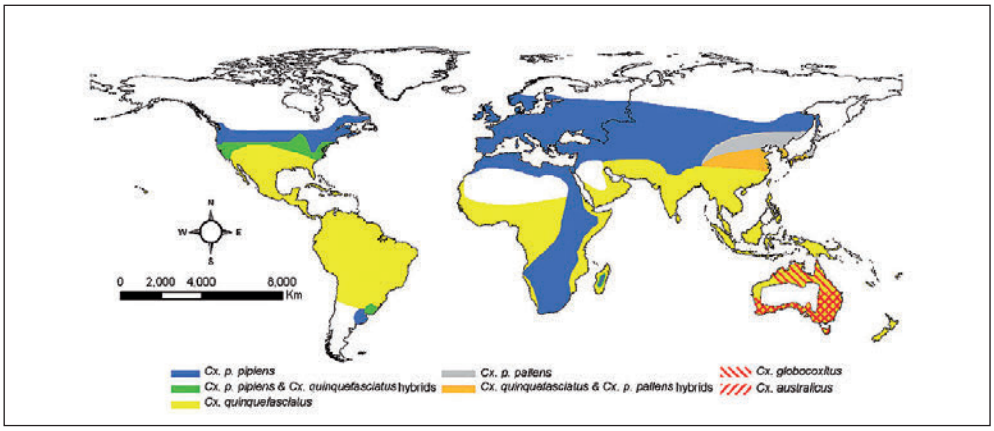

Fig. 1 Global distribution of *Cx. pipiens* complex mosquitoes. Geographic range for *Cx. p.* pipiens includes both forms (pipiens and molestus). *Cx. australicus* and *Cx. globocoxitus* are restricted to Australia. (From Farajollahi et al. 2011)

p is the probability that a vector survives one day; *n* is the duration of the extrinsic incubation period (EIP) in days; I is the infection rate * T the transmission rate = vector competence (the proportion of vectors ingesting an infective meal that are later able to transmit the infection; and 1/(–ln(*p*) is the duration of the vector's life in days after surviving the EIP. This equation demonstrates that the abundance (*m*) and vector competence (*b*) of mosquito populations would impact the reproductive rate of WNV linearly, and thus relatively weakly. In contrast, host feeding (*a*), vector longevity (*p*) and EIP (*n*) would impact R_0 much more powerfully (e.g. as a square or exponent). It seems to follow that virus infectivity for mosquitoes, which would be incorporated into VC as *b*, would be of relatively minor importance relative to viral factors such as the speed of dissemination from the midgut that would impact the duration of the EIP, which would influence VC as *n* (Kramer and Ciota 2015). Thus, natural selection might favour a poorly infectious but rapidly disseminating virus over a highly infectious virus that disseminates slowly. Similar predictions might be made about viral influences on other mosquito-associated factors such as host preference, survivorship, etc.

Complex biotic or intrinsic factors and abiotic factors affect vectorial capacity. Examples of the former include vector and viral genetics, vector and host competence, vector life-history traits; and of the latter abiotic factors, temperature, rainfall, and human land use. Vertebrate factors including host competence, population dynamics, and immune status also affect exacerbated by the fact that not only can divergent hosts differentially alter the virus, but the virus also can affect both vertebrate and invertebrate hosts in ways that significantly alter patterns of virus transmission.

2. Biotic Factors

2.1 Mosquito Genetics

The *Cx. pipiens* complex includes *Cx. pipiens* (Linnaeus 1758), *Cx. quinquefasciatus* Say, *Cx. australicus* Dobrotworsky and Drummond, and *Cx. globocoxitus* Dobrotworsky,

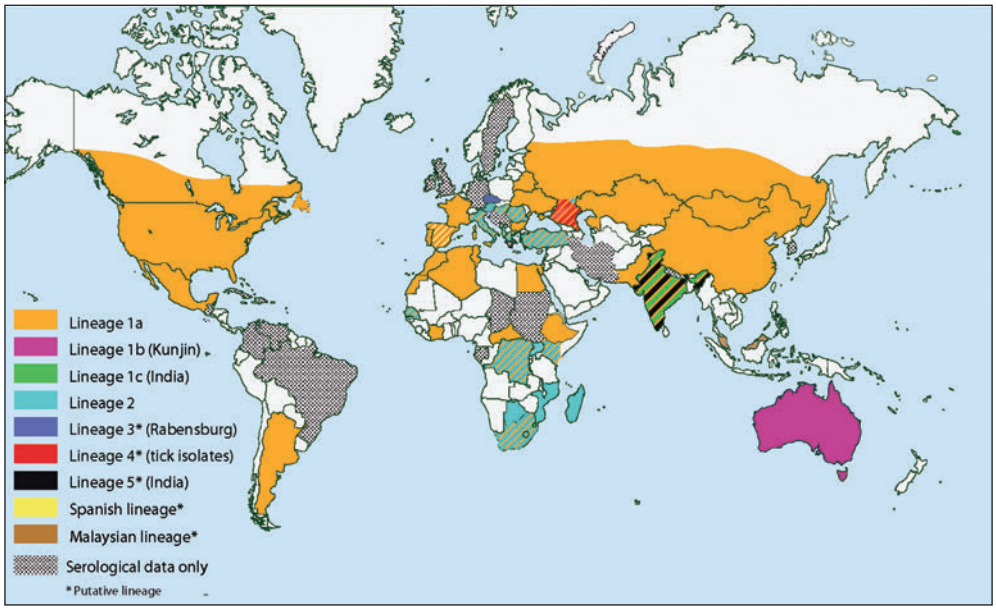

Fig. 2 Worldwide distribution of West Nile virus (from Ciota and Kramer 2013)

mosquito species that have a worldwide distribution (Fig. 2), are evolutionarily closely related, and difficult to separate morphologically (Farajollahi et al. 2011).

There are two recognized subspecies of *Culex pipiens*, i.e., *Cx. pipiens pipiens*, an Old World taxa distributed from Northern Europe to the highlands of South Africa, and *Cx. p. pallens*, distributed east of the Urals across temperate Asia (Fonseca et al. 2009). Furthermore, *Cx. pipiens* has two bioforms – molestus and pipiens – which differ in a number of biological characteristics including autogeny, stenogamy, ability to diapause, and feeding preference (Farajollahi et al. 2011). The sibling species *Cx. quinquefasciatus* differs from *Cx. pipiens* in that it does not undergo diapause and thrives in tropical and sub-tropical regions south of 36° N latitude (Edillo et al. 2009) but, like *Cx. pipiens*, is predominantly ornithophilic, preferring to feed on birds, although both also will feed on humans. Hybrids of *Cx. pipiens* and *Cx. quinquefasciatus* are found in a zone where the two overlap extending from approximately 30° N to 40° N latitude in North America (Huang et al. 2011). Studies of hybrid populations of *Cx. pipiens* complex mosquitoes including *Cx. pipiens* form pipiens, *Cx. pipiens* form molestus, and *Cx. quinquefasciatus* indicate hybridization has a significant effect on WNV infection, dissemination, and, particularly, transmission. Specifically, presence of *Cx. quinquefasciatus* signature in the hybrid mosquitoes increased susceptibility to infection; and the percent of infected hybrid populations transmitting by day 13/14 was found to be significantly higher than one or both parental populations (Ciota et al. 2013). Therefore, extrinsic factors such as land use and urbanization in particular, which likely increase the potential for hybridization between bioforms (Kilpatrick 2011), may have an impact on WNV activity. Extensive discussion of the *Cx. pipiens* species complex can be found elsewhere (Andreadis 2012, Farajollahi et al. 2011).

2.2 Vector Competence

Vector competence of a mosquito population can vary over time and appears to be dependent on intrinsic and extrinsic influences, including environmental and genetic factors, as well as their interaction. Although vector competence is not the most important determinant of vectorial capacity, and relatively incompetent populations are capable of sustaining arbovirus outbreaks (Miller et al. 1989), capacity does fluctuate directly with competence and is therefore represented as a linear term in expressions of vectorial capacity. Viral genetics and temperature (discussed below) appeared to play a role in the displacement of the originally introduced strain of WNV to the US (NY99) by the mutated strain (WN02) with a valine replaced by an alanine at position 159 in the envelope protein. *Cx pipiens* and *Cx tarsalis*, two important vector species, were able to transmit WNV02 2–4 days earlier than NY99 (Kilpatrick et al. 2008).

3. Abiotic Factors

3.1 Climate and Landscape

Studies with *Cx. pipiens* demonstrated an accelerating effect in ability to transmit virus with increasing temperatures, suggesting degree day models are likely to underestimate the effects of temperature (Kilpatrick et al. 2008). Even modest increases in EIP will exponentially increase vectorial capacity independent of intrinsic transmissibility (competence), so temperature, viral genotype, and other factors altering the pace of viral infections in mosquitoes are likely to be of primary importance in governing activity. Vector competence for WNV also has been shown to vary among mosquito populations of the same species (Goddard et al. 2003, Reisen et al. 2008), with evidence of a genetic basis (Hayes et al. 1984), and may vary seasonally (Vaidyanathan and Scott 2006).

Arboviruses that exist in temperate environments also must adapt to the seasonal activity of their hosts. WNV, which is generally believed to have originated in Sub-Saharan Africa, readily survives the harsh winters in the northeastern and north central US. This seems to be facilitated by vertically infected, diapausing adult female mosquitoes (Nasci et al. 2001). Experimental studies with flaviviruses demonstrate that the rates of successful vertical transmission are extremely low (reviewed in Rosen 1987). Although some studies of WNV using intrathoracically inoculated *Cx. tarsalis* indicate minimum filial infection rates as high as 6.9 (Goddard et al. 2003), the relevance of these findings to the natural transmission cycle, where mosquitoes are infected perorally, is unclear. While it is conceivable that overwintering following vertical transmission could create a population bottleneck wherein genetic drift might become important, the success of WNV over several years in temperate North America suggests that bottlenecks may in fact be rare and demonstrates the phenotypic robustness of this virus.

High precipitation in late winter/early spring, high summer temperatures, summer drought, irrigated crops, and highly fragmented forests are positively correlated with increased WN risk (Marcantonio et al. 2015). Crop land cover and water catchment depressions in the semi-arid environment of Texas led to increased incidence of WN cases (Warner et al. 2006). But rain-fed crop lands in Europe showed no significant association with WNV, possibly because the more variable water supply is less favourable to mosquito populations

(Marcantonio et al. 2015). In the US, drought conditions appear to lead to increased transmission of WNV, perhaps because of more favourable larval habitat for *Cx. pipens* which prefer organically rich water, or as a result of limited water availability bringing avian host and mosquitoes in closer proximity.

3.2 Impact of Temperature of Immature Stages on Life History Traits

The effect of temperature on immature development of *Cx. pipiens*, *Cx. quinquefasciatus*, and a potentially important early spring/fall vector, *Cx. restuans*, were examined in order to quantify the specific relationships between temperature and life history traits as well as direct comparison of the relative importance of temperature among medically important mosquito vectors. Numerous studies have evaluated the impact of temperature on adult mosquitoes in vector competence assays, but have ignored rearing temperature. The study demonstrated temperature significantly affects rates of immature development, survival of immature stages, adult size, adult longevity, blood feeding, and fecundity of *Culex* mosquitoes, and that both species and population are additional factors significantly altering these life history traits and their susceptibility to temperature shifts (Ciota et al. 2014).

3.3 Impact of Virus on the Arthropod Vector

Arboviruses were originally considered to cause little if any damage to their arthropod hosts. But it is now clear, infection may lead to cellular pathology in the vector. It was demonstrated that Eastern equine encephalitis virus (EEEV) caused apoptosis in the midgut of infected *Aedes*. Another study demonstrated apoptosis and other cytopathologic changes occurred more frequently in WNV-infected mosquitoes than uninfected controls (Girard et al. 2007). Earlier, it had been shown that with dengue virus (DENV) infected *Ae. aegypti*, the mean of the total time required for feeding by infected mosquitoes was significantly longer than that for uninfected mosquitoes. Similarly, the mean of the time spent probing was significantly longer in infected mosquitoes than in uninfected mosquitoes (Platt et al. 1997). Besides affecting feeding behaviour, infection with arboviruses may have an impact on mosquito fecundity and survivorship (Ciota et al. 2011, Styer et al. 2007). The latter study demonstrated that a WNV strain that was selected for increased replication and transmission in *Cx. pipiens* nonetheless had a diminished vectorial capacity because of decreased survivorship and changes in fecundity. This highlights the importance of vectorial capacity in understanding the force of transmission of a virus in the environment.

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Evolutionary and Ecological Insights into the Emergence of Arthropod-Borne Viruses

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Abstract

The prevalence of vector-borne diseases is increasing worldwide. A large part of these diseases are arthropod-borne viral diseases (arboviroses) that undergo cyclic transmission between arthropod and vertebrate hosts. In order to understand the emergence of arboviroses, we need more insight into the biodiversity and ecology of viruses that are carried by arthropods but do not, or not yet, undergo arboviral dual-host transmission.

In studying the biodiversity of mainly insect-associated viruses, we have detected an extremely wide spectrum of previously unknown RNA viruses. These viruses establish novel genera within known virus families, or even novel virus families such as the first insect-associated nidovirus Cavally virus, the type species of the family *Mesoniviridae* (order *Nidovirales*). Within the family *Bunyaviridae*, which is considered the most diversified family of RNA viruses, we identified four novel viral genera that all seem to be restricted to insects and are in a sister relationship to major pathogenic arboviral genera. Because we have been able to isolate several novel insect-related viruses in cell culture, we obtained further insight into phenotypic properties, such as host cell tropism and sensitivity of replication to temperature. Combining phenotypic and phylogenetic studies of live insect-specific bunyaviruses, we found evidence for the hypothesis that arboviruses within the family *Bunyaviridae* have evolved from arthropod-specific progenitors.

Arbovirus evasion from primary habitat may be driven by ecological change. Analyses of virus diversity and infection rates in mosquitoes studied along disturbance gradients in Ivory Coast and Mexico showed a decrease in genetic diversity of viruses from natural to modified habitat types. In contrast, infection rates of mosquitoes with specific viruses from several different families increased with the level of disturbance. Such virus species could inherit a generally higher ability to spread to new geographic regions, driven by anthropogenic changes.

Zusammenfassung

Die Prävalenz vektorübertragener Erkrankungen nimmt weltweit zu. Ein Großteil dieser Erkrankungen gehört zu den arthropodenübertragenen viralen Infektionskrankheiten (Arbovirosen), die zyklisch zwischen Arthropoden und Vertebraten übertragen werden. Um das Auftreten und die Ausbreitung von Arbovirosen besser verstehen zu können, sind Untersuchungen zur genetischen Diversität und Ökologie von Viren aufschlussreich, die ausschließlich in Arthropoden replizieren, aber mit Arboviren entweder verwandt sind oder einen nah verwandten Vektor teilen.

Innerhalb von Studien zur Biodiversität von insektenassoziierten Viren haben wir ein breites Spektrum an bisher unbekannten RNA-Viren entdeckt. Diese neuartigen Viren haben zur Definition neuer Genera innerhalb bekannter Familien und in mindestens einem Fall zur Entdeckung einer neuen Virusfamilie geführt (Cavally-Virus, das erste insektenassoziierte Nidovirus in der Familie *Mesoniviridae* [Ordnung *Nidovirales*]). Innerhalb einer der am stärksten diversifizierten RNA-Virusfamilien, der Familie *Bunyaviridae*, haben wir vier bisher unbekannte Virusgruppen entdeckt, die vier neue Genera etablieren. Diese Viren scheinen alle auf Insekten als Wirte beschränkt zu sein, bilden aber unmittelbare phylogenetische Schwesterkladen zu bekannten pathogenen Bunyaviren. Durch die Isolation dieser Viren in Zellkultur konnten wir wichtige phänotypische Eigenschaften bestimmen. Hierzu gehörten beispielsweise die Permissivität von Vertebratenzellen und die Temperaturabhängigkeit der Replikation. Phänotypische und phylogenetische Analysen der insektenspezifischen Bunyaviren legten einen wahrscheinlichen Ursprung von Arbo-Bunyaviren in arthropodenspezifischen Vorläuferviren nahe.

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Die Ausbreitung von Arboviren aus ursprünglichen Habitaten wird vermutlich durch ökologische Veränderungen verursacht. Untersuchungen von Virusdiversität und Infektionsraten bei Moskitos entlang anthropogener Störungsgradienten an der Elfenbeinküste und in Mexiko haben eine Abnahme der genetischen Virusdiversität mit zunehmender Habitatstörung ergeben. Im Gegensatz dazu nahmen bei einigen Virusarten die Infektionsraten in Richtung der Habitatstörung zu. Diese Arten könnten eine grundsätzlich erhöhte Fähigkeit zur anthropogen bedingten geographischen Ausbreitung haben.

1. Taxonomic Diversity and Host Associations of Arboviruses

Arthropod-borne viruses (arboviruses) are maintained in transmission cycles in which the virus replicates in hematophagous arthropods and is passed on to non-immune vertebrate hosts during blood feeding. This dual host tropism of arboviruses is unique among RNA viruses. Arboviruses can be found in a few genera of six RNA-virus families, *Bunyaviridae* (genera *Phlebovirus*, *Orthobunyavirus*, *Nairovirus*), *Flaviviridae* (genus *Flavivirus*), *Togaviridae* (genus *Alphavirus*), *Reoviridae* (genera *Coltivirus*, *Orbivirus*), *Rhabdoviridae* (genera *Ephemerovirus*, *Vesilulovirus*), and in a single DNA-virus family, *Asfarviridae* (genus *Asfarvirus*), suggesting that the ability to infect invertebrates and vertebrates evolved through convergence in several viral families. Viruses within the other genera of the families have a monotropism for either vertebrates or arthropods. The dual host tropism of arboviruses is a paraphyletic property within viral families and, consequently, it is unknown whether arboviruses evolved from viruses with a monotropism for arthropods or vertebrates. The genus *Flavivirus* of the family *Flaviviridae* contains insect-specific viruses in basal phylogenetic relationship to the pathogenic arboviruses and it was suggested that the latter may have evolved from insect-specific viruses (Cook and Holmes 2006, Cook et al. 2012). However, a much more complex picture arises when recent findings of new clades of insect-specific flaviviruses that are grouped within the mosquito-borne viruses (Blitvich and Firth 2015, Huhtamo et al. 2009, Junglen et al. 2009a), and host associations of the other three flavivirus genera (genera *Hepacivirus*, *Pegivirus* and *Pestivirus*), which have a monotropism for vertebrates, are included in hypothetical evolutionary scenarios for the family *Flaviviridae*. We studied the case for one of the most genetically diversified RNA virus families, the family *Bunyaviridae*, in which four of the five established genera are transmitted by arthropods (Plyusnin et al. 2012, Schmaljohn and Nichol 2007).

2. The Family *Bunyaviridae*

The family *Bunyaviridae* contains significant viruses that are pathogenic for humans, animals and plants (Plyusnin et al. 2012, Schmaljohn and Nichol 2007). Infections can cause, for example, encephalitis, hepatitis, hemorrhagic fever, abortions, and malformations of offspring. Mutual characteristics of bunyaviruses are enveloped spherical virions of about 100 nm in diameter, a tri-segmented genome of negative sense single-stranded RNA, and intracellular budding at the Golgi complex. Mature virions contain two glycoproteins called Gn and Gc that are embedded in the viral envelope, a nucleocapsid (N) protein that forms a complex with the RNA genome segments called ribonucleoprotein complex (RNP), and an RNA-dependent RNA polymerase called L protein that contains an N-terminal endonuclease domain.

Bunyaviruses are divided into the genera *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus* based on genome organization and phylogenetic relationship. Dis-

tinguishing features among members of the five genera include differences in the size of genome segments and proteins, different consensus terminal sequences, and different coding strategies for the additional non-structural proteins NSs and NSm. Additionally, host and vector associations vary among the genera. Tospoviruses are the only bunyaviruses that infect plants and are transmitted by thrips. Viruses of the other genera infect vertebrates and are transmitted by blood-feeding arthropods – with the exception of Hantaviruses. Nairoviruses are transmitted by ticks, orthobunyaviruses mainly by mosquitoes, and phleboviruses by sandflies, mosquitoes and ticks. Hantaviruses infect rodents, insectivores and bats, and are transmitted by excreta.

2.1 Insect-specific Bunyaviruses

In addition to the five established genera, we have recently described four new deep rooting bunyavirus lineages. It has been suggested that they define four distinct genera named *Goukovirus*, *Herbevirus*, *Feravirus* and *Jonvirus* (Marklewitz et al. 2015, 2011, 2013). Notably, the viruses represent the first insect-specific bunyaviruses.

The *Goukovirus* clade is defined by the type species Gouléako virus (GOLV) that was isolated mainly in *Culex nebulosus* mosquitoes sampled in Ivory Coast (Marklewitz et al. 2011) and by Cumuto virus (CUMV) that was found in *Culex* sp. mosquitoes from Trinidad (Auguste et al. 2014). Goukoviruses show genetic distance of between 70 – 80 % to members of the genus *Phlebovirus* and share a common ancestor with members of the genus *Phlebovirus* (Fig. 1). The enveloped virions were pleomorph of approximately 120 nm in diameter. The Goukoviruses contain the shortest bunyavirus genomes known so far. The genomes consist of S, M and L segments of about 1.1, 3.2 and 6.4 kb, respectively. Notably, no coding regions for NSs and NSm proteins, as encoded in other bunyaviruses, were identified.

The second clade, *Herbevirus*, consists of three viruses called the Herbert virus (HEBV), Tai virus (TAIV) and Kibale virus (KIBV). These were mainly identified in *Culex* mosquitoes from Ivory Coast, Ghana and Uganda (Marklewitz et al. 2013). The viruses show similar genetic distances to members of the genus *Orthobunyavirus* and *Tospovirus* in all genes, and form a monophyletic clade in sister relationship to the genus *Orthobunyavirus* (Fig. 1). Virions were enveloped and 90–110 nm in diameter. The S, M and L genome segments of herbeviruses were around 1.1, 2.7 and 7.4 kb, respectively. The 5'-non-coding region of the TAIV M segment was 500 nt longer than has been observed for HEBV and KIBV. As with the goukoviruses, herbeviruses also do not appear to encode NSs and NSm proteins on their S and M segments, respectively. Interestingly, the L protein of herbeviruses contains an additional region of about 150 amino acids, which may fulfil similar functions as the non-structural proteins found in other bunyaviruses.

The clade of *Jonvirus*, defined by the type species Jonchet virus (JONV), is equally distant from all genera and branches from a deep node in the family *Bunyaviridae* in basal phylogenetic relationship to the genera *Hantavirus*, *Orthobunyavirus*, *Tospovirus* and to the clade of herbeviruses (Fig. 1; Marklewitz et al. 2015). JONV was isolated from *Culex* mosquitoes collected in Ivory Coast. The enveloped virions showed two types of morphologies, one with atypical morphology for bunyaviruses, represented by tubular forms measuring 60 nm in diameter and up to 600 nm in length, and one with spherical particles measuring 80 nm in diameter. The L segment of JONV was 6.9 kb in length, and the L protein was predicted to contain a putative endonuclease domain at its N-terminus. JONV had the longest M segment

ever observed in bunyaviruses measuring 5.5 kb. The M segment is predicted to encode a glycoprotein precursor protein that is posttranslationally cleaved into NSm, Gn and Gc proteins. The S segment was 1.7 kb in size and encoded a nucleoprotein and an NSs protein that is encoded upstream from the nucleoprotein ORF. This coding strategy has not been observed in any of the established bunyavirus genera before.

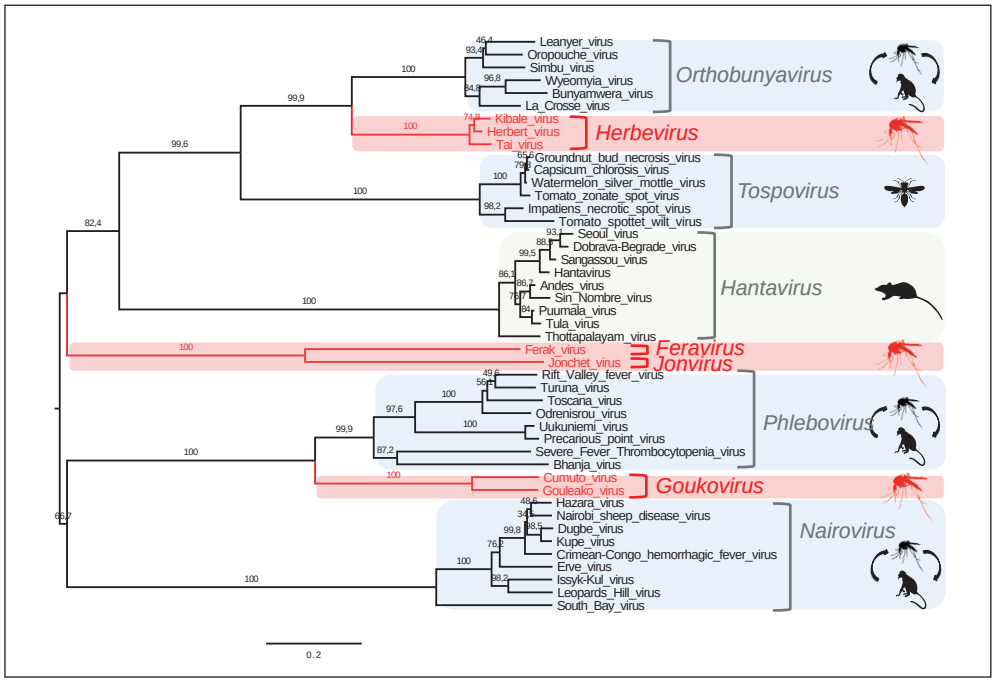

Fig. 1 Phylogenetic relationship and host associations of bunyaviruses. Maximum likelihood analyses based on bunyavirus L proteins.

The fourth clade, *Feravirus*, is defined by Ferak virus (FERV) that was isolated from mosquitoes sampled in Ivory Coast (Marklewitz et al. 2015). FERV shares a most common recent ancestor (MRCA) with JONV (Fig. 1) but the genetic distance as well as the differences in morphology and genome organization suggests that both viruses belong to different new putative genera. FERV virions were pleomorph and showed a similar morphology to Goukoviruses. The FERV genome consists of S, M and L segments of 1.5, 4.3 and 6.9 kb, respectively. The S segment encodes a putative NSs ORF that precedes the nucleoprotein ORF, a coding strategy that was also found in JONV and phasmaviruses. Interestingly, the FERV M segment also encodes an ORF that is upstream from the glycoprotein precursor gene and may code for an NSm protein. Whether the additional non-structural proteins detected in JONV and FERV have similar functions to those of other bunyaviruses, e.g. inhibition of the RNA interference pathway or suppression of the interferon response, remains to be studied (Bird et al. 2008, Bouloy et al. 2001, Bridgen et al. 2001, Takeda et al. 2002).

3. Ancestral Reconstruction of Bunyavirus Host Associations

Phylogenetic ancestral host reconstructions have shown that *Goukoviruses*, *Herbeviruses*, *Feraviruses* and *Jonviruses* have inherited the property of being restricted to insect hosts from a common ancestor (Marklewitz et al. 2015). The analyses were based on a parsimony-based algorithm that calculates the minimum number of host changes along the phylogenetic tree to explain the present host traits at the tree tips (Maddison and Maddison 2014). Confirmation of the hypothesis was based on probabilistic hypothesis testing in a maximum likelihood based framework that determines the most likely trait change matrix along the phylogeny (Pagel et al. 2004). The parsimony-based analyses reconstructed an arthropod host at the bunyavirus root with 100 % certainty (Marklewitz et al. 2015). The hypothesis-based maximum likelihood reconstructions showed that only exclusive arthropod associations at the bunyavirus root and at all deep tree nodes left the overall likelihood unaffected (Marklewitz et al. 2015). These findings suggest that the vertebrate-pathogenic dual host bunyaviruses evolved from viruses that exclusively infect arthropods. Similar evolutionary scenarios may be applicable to other viral families containing arboviruses, e.g. in the families *Flaviviridae* and *Togaviridae.*

4. The Interplay between Biodiversity and Infection Rates

Vector-borne diseases are increasing worldwide, driving the need to understand the mechanisms that foster their emergence and spread. The unprecedented loss of biodiversity we are experiencing right now may alter pathogen transmission patterns with consequences for human and animal health (Altizer et al. 2013, Johnson et al. 2015, Keesing et al. 2010).

We have studied the diversity and abundance patterns of mosquitoes and their viruses along anthropogenic disturbance gradients in tropical Africa. We found an extremely high diversity of previously unknown RNA viruses belonging to the families *Bunyaviridae*, *Flaviviridae*, *Mesoniviridae*, *Nodaviridae*, *Reoviridae*, *Rhabdoviridae*, and *Togaviridae*, as well as to the unclassified taxon Negevirus (Hermanns et al. 2014, Junglen et al. 2009a, Kallies et al. 2014, Marklewitz et al. 2015, 2011, 2013, Quan et al. 2010, Schuster et al. 2014, Zirkel et al. 2013, 2011). Phylogenetic analyses have indicated distant relationships of the novel viruses to established taxa. This suggests that tropical ecosystems may contain a larger spectrum of viruses than currently known from epidemic isolates. Virus diversity and abundance patterns suggest a decrease in diversity from natural to modified habitat types (Fig. 2; Junglen et al. 2009b, Zirkel et al. 2011 and unpublished data).

The prevalence of three bunyaviruses, one rhabdovirus and one mesonivirus has increased in highly disturbed habitats, congruent with the dilution effect hypotheses. Notably, a low prevalence of the other viruses (88 %) was found in specific mosquito species and specific habitat types. These data suggest that some viruses benefit from ecosystem disturbance, explaining the emergence of widespread arboviruses from ancestral sylvatic cycles (Kopp et al. 2013, Zirkel et al. 2011). Such virus species could inherit a generally higher ability to spread to new geographic regions driven by anthropogenic changes.

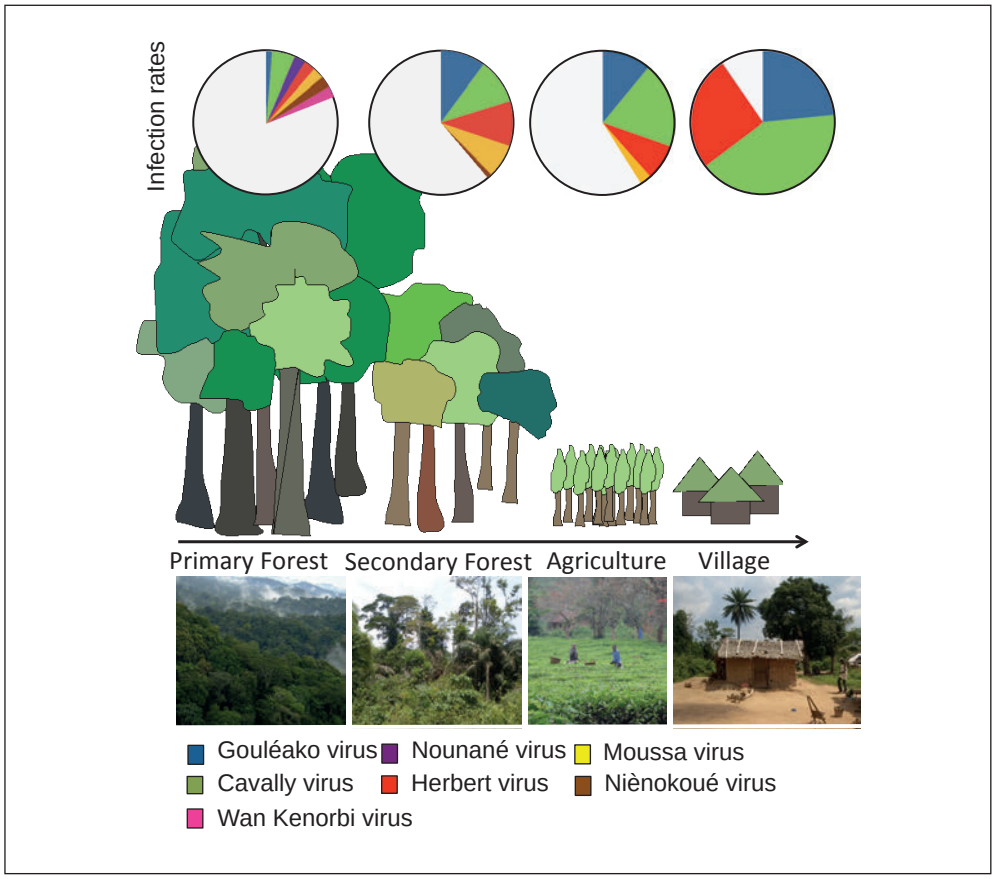

Fig. 2 Viral infection rates in mosquitoes in ecologically different habitat types. Mosquitoes were sampled along an anthropogenic disturbance gradient at the edge of the Taï National Park in Ivory Coast and tested for virus infection using RT-PCR.

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Culicoides Biting Midges and their Relevance as Vectors: A European Perspective

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Abstract

Culicoides spp. (Diptera: Ceratopogonidae) are globally distributed vectors of many viruses, some of which are of paramount veterinary relevance (e.g. bluetongue and African horse sickness). In EU countries, they had a devastating economic impact with the bluetongue epidemics that started in Italy in 2000 and then moved to central Europe in 2006. The effects of those epidemics are ongoing and have impacted most parts of Europe up to the southern UK. At present, nine BT serotypes have been recorded in Europe. In 2011, a new virus transmitted by *Culicoides* – Schmallenberg virus, which causes another economically relevant disease of ruminants – first appeared in Germany and the Netherlands, quickly spreading to the UK, France, Italy, Luxembourg, Spain, Denmark and Switzerland.

In this scenario, *Culicoides* study in Europe merits the growing level of interest it has experienced in the last two decades; such studies focus on all aspects of *Culicoides* taxonomy, biology, ecology, distribution and possible control strategies. Until the Italian epidemic, *C. imicola* was considered the only old world bluetongue vector; nowadays it is well known that the species responsible for virus circulation in Europe mainly belong to the *C. obsoletus* complex, and many studies have been carried out aimed at clarifying this complex taxonomy and the different role of its species as vectors. The same species group was shown to be the vector of Schmallenberg virus, too.

Notwithstanding this remarkable study effort, involving researchers all over the EU, many questions about the biology and ecology of *Culicoides* spp. and their role as vectors have still to be addressed.

Zusammenfassung

Culicoides spp. (Diptera: Ceratopogonidae) sind weltweit verbreitete Vektoren vieler Viren, von denen einige von höchster veterinärmedizinischer Bedeutung sind (z. B. Blauzungenkrankheit und Afrikanische Pferdepest). In EU-Ländern wurden die wirtschaftlichen Auswirkungen mit dem Beginn der Blauzungenkrankheit in Italien im Jahre 2000 und dann in Mitteleuropa im Jahre 2006 verheerend; Epidemien, deren Auswirkungen noch immer anhalten und den größten Teil Europas bis in den Süden des Vereinigten Königreiches einbeziehen. Derzeit sind 9 BZ-Serotypen in Europa verzeichnet worden. Im Jahre 2011 übertrug eine neue Art von *Culicoides* ein Virus, das Schmallenberg-Virus, das eine weitere, wirtschaftlich bedeutende Erkrankung der Wiederkäuer hervorrief, die zunächst in Deutschland und den Niederlanden auftrat und sich schnell in das Vereinigte Königreich, nach Frankreich, Italien, Luxemburg, Spanien, Dänemark und in die Schweiz ausbreitete.

In diesem Szenario verdienten Studien über *Culicoides* in Europa ein wachsendes Interesse in den letzten zwei Jahrzehnten. Diese Studien konzentrierten sich auf alle Aspekte ihrer Klassifizierung, Biologie, Ökologie, Verbreitung und möglichen Bekämpfungsstrategien. Bis zu der Epidemie in Italien wurde *C. imicola* als der einzige alte, weltweite Überträger der Blauzungenkrankheit angesehen, während heute bekannt ist, dass die für die Viruszirkulation verantwortlichen Arten in Europa hauptsächlich Arten sind, die zu dem Komplex *C. obsoletus* gehören. Viele Studien zielten darauf ab, diese komplexe Klassifizierung und die unterschiedlichen Rollen der Arten als Vektoren zu klären. Die gleiche Artengruppe wurde auch als Vektor des Schmallenberg-Virus nachgewiesen.

Ungeachtet dieser bemerkenswerten Arbeit im Zusammenhang mit den Studien, die Forscher in der ganzen EU einbezogen, müssen noch viele Fragen hinsichtlich der Biologie, Ökologie und Rolle von *Culicoides* spp. als Vektoren angegangen werden.

1. *Culicoides* Biting Midges

Culicoides spp. (Diptera, Ceratopogonidae) are vectors of 66 viruses, 15 protozoans, and 26 filarial nematodes. With their direct biting activity they can be a nuisance to people and animals, at times provoking allergic reactions (Borkent 2004). Species of this genus have been implicated in the transmission of viruses of the families Bunyaviridae, Reoviridae, and Rhabdoviridae (Mullen 2009), with the most relevant ones being the Reoviridae African horse sickness virus (AHSV), bluetongue virus (BTV), and epizootic hemorrhagic disease virus. In Europe they were recently shown to be vectors of Schmallenberg virus, within the Bunyaviridae family. Despite their small size (1–3 mm), *Culicoides* biting midges are of paramount relevance in veterinary medicine, causing devastating economic losses worldwide (Mellor et al. 2000).

Before the bluetongue (BT) epidemic started in Sardinia in 2000 and arrived in mainland Italy in 2001, the economic relevance of *Culicoides* in Europe had been quite limited; until that moment, only transient, sporadic incursions of BT and AHS viruses had been recorded on our continent (Mellor 1993, Mellor and Boorman 1995). From 2000 everything changed, with what was to become the most extensive, prolonged and costly period of BTV incursions into Europe ever recorded (Mellor and Wittman 2002, Purse et al. 2005) – a period that is still ongoing. After an initial wave in Mediterranean countries, where BT circulation has been almost continuous since 1998, in 2006 the virus was unexpectedly recorded in central Europe for the first time (Carpenter et al. 2009), rapidly spreading to northern countries. As a matter of fact, BT could nowadays be considered an established disease in some areas of southern Europe (southern Italy and some Greek islands), with clinical outbreak distribution fluctuating from southern Italy to the central UK and from Spain to Eastern Europe.

Within this scenario, in 2011 a new *Culicoides*-transmitted virus first appeared in Europe – Schmallenberg virus. It rapidly spread to the UK, France, Italy, Luxembourg, Spain, Denmark and Switzerland, and provoked economic losses alongside those caused by BTV.

1.1 Taxonomy

The systematic arrangement of the genus *Culicoides* is as follows:

Phylum: Arthropoda
- Class: Insecta
- Order: Diptera
- Suborder: Nematocera
- Family: Ceratopogonidae
- Genus: Culicoides

The genus is composed of 1,343 species worldwide (Borkent 2014), divided among 31 subgenera; in the subgenera *Avaritia* and *Culicoides* these include the vectors of BTV in Europe. Taxonomic studies of the genus have been driven largely by medical and veterinary concerns. Most of the identification keys are written for females, despite the fact that males have more diagnostic characteristics for distinguishing the species. Until recently, taxonomic studies relied almost exclusively on morphology, but the increasing interest in *Culicoides* due to the arrival and spread of BT in Europe has renewed interest in phylogenetic relationships among *Culicoides.* Molecular systematic taxonomy has provided an effective alternative to classical

taxonomy, in some cases allowing rapid characterization of the local fauna in the search for answers to epidemiological questions.

Regarding the European vector species: after the Italian epidemic started in 2000, many questions arose regarding group taxonomy of *C. obsoletus* and *C. pulicaris*, as epidemiological evidence immediately pointed to their probable role as BT vectors, a role later confirmed by virus isolations obtained from both species groups (Caracappa et al. 2003, De Liberato et al. 2005, Ferrari et al. 2005). Until that moment, those groups presented many taxonomical issues (Meiswinkel et al. 2004), which have only partially been resolved in recent years thanks to molecular biology.

The *C. obsoletus* group is known to consist of five species: *C. obsoletus* s. s., *C. scoticus, C. chiopterus, C. montanus* and *C. dewulfi* (Boorman et al. 1995), with female specimens hardly identifiable morphologically (Carpenter et al. 2008). The five species can be easily distinguished through observing the morphology of the males' genitalia, but males are usually caught in small numbers in light trap collections. Recent phylogenetic studies on members of this group questioned its composition, revealing the presence of different complexes inside the group (Meiswinkel et al. 2004, Gomulski et al. 2005) and possibly placing *C. dewulfi* outside the group.

Eleven species are usually considered as forming the *C. pulicaris* group, but there is a great amount of uncertainty and disagreement regarding its exact composition. Synonymies and species hiding different taxa are thought to exist in this group (Meiswinkel et al. 2004).

1.2 Biology and Ecology

Culicoides biting midges are among the smallest-known haematophagus insects, rarely exceeding 3 mm in size but usually less than 2 mm. The most particular trait of their morphology is the presence, in most of the species, of a well-defined pattern of dark and light markings on the wings. Thanks to their size and to the presence of this wing pattern, they can be easily distinguished from other biting Nematocera, such as those belonging to the genera *Leptoconops* (Ceratopogonidae), *Simulium* (Simuliidae) and *Phlebotomus* (Psychodidae). They are distributed worldwide, with the exception of Antarctica and New Zealand, feeding mainly on mammals and birds (Mellor et al. 2000). Males do not blood-feed. Mammalophilic and ornitophilic species can be distinguished by the distribution of a kind of receptor, sensilla coeloconica, on antennal segments (flagellomera): the species with sensilla coeloconica present on flagellomera 3–15 are ornitophilic and those with sensilla coeloconica on flagellomera 3, 12–15 are mammalophilic (Braverman and Hulley 1979).

Culicoides are characterized by a holometabolous life cycle, including eggs, four larval stages, pupa and adults. Females typically require a blood meal for development of eggs, but those of a few species are capable of producing an initial batch of eggs without feeding (autogeny). Larval stages need a certain amount of free water or moisture and are found in very diverse habitats, such as pools, streams, marshes, bogs, beaches, swamps, tree holes, irrigation pipe leaks, saturated soil, animal dung, and rotting fruit. Availability of these environments is a key factor determining their distribution, abundance and seasonal occurrence (Carpenter et al. 2013). The larvae of *Culicoides* species that are biting pests of livestock develop in the saturated soil of wastewater ponds and the overflow from watering troughs, both of which are typically enriched with livestock manure. Thus, these species are often facilitated precisely by animal husbandry, providing larval breeding sites that are very rich in

organic matter and permanently wet, sometimes in a general context of aridity (e.g. *C. imicola* in Sardinia).

Culicoides spp. are mainly crepuscular, with peaks of activity at sunset and sunrise and, to a lesser extent, through the night. Their active flight range is usually short, only a few hundred meters or at most two to three kilometers from their breeding sites (Lillie et al. 1981, Kettle 1984). However, they can be dispersed passively by the wind over great distances (up to 700 km) as aerial plankton (Braverman and Chechik 1996).

At our latitudes, many species have a population peak in spring followed by a secondary peak in the fall (Kline and Axtell 1976). In Central Italy, *C. obsoletus* have this kind of seasonality, with a relative peak in May/June and a higher peak in mid-October (De Liberato et al. 2010). In temperate countries, most species overwinter as fourth-stage larvae in diapause. However, in relatively warm areas, such as many Mediterranean countries, active adults of the *C. obsoletus* and of the *C. pulicaris* groups can be found all year round.

During the BT epidemic that started in Central Europe in 2006, the relevant authorities recommended keeping animals indoors as a means of protecting them, because of presumed exophily and exophagy of biting midges. On the other hand, *Culicoides* indoor winter activity was considered one of the ways BTV might have overwintered in Central and Northern Europe. These conflicting ideas probably originated from the lack of data on European *Culicoides* species endophily, and from the assumption that they had the same exophilic and exophagic tendencies as *C. imicola*. Nevertheless, studies carried out after the European epidemic demonstrated that midges of the *C. obsoletus* group promptly enter buildings and feed indoors (Baldet et al. 2008, Meiswinkel et al. 2008, Baylis et al. 2010). In France, females of the *C. obsoletus* group were more abundant indoors than outdoors in autumn, with high percentages of parous and freshly blood-fed specimens (Baldet et al. 2008) being found. The endophagic tendencies of the *C. obsoletus* group would be influenced by external temperatures, with the cold driving more specimens inside animal sheds. Hence, of the two extremes of protecting cattle by keeping them indoors or of BTV overwintering thanks to *C. obsoletus* indoor winter activity, the second would seem to give the more accurate portrayal of the situation.

2. Transmitted Viruses

In veterinary medicine, *Culicoides* biting midges are vectors of paramount importance, causing huge economic losses worldwide every year. Few other vector groups have the same relevance on a global scale. Several factors make *Culicoides* spp. such devastating vectors of animal viral diseases, including:

– Population size: Under favourable climatic conditions, their populations can reach incredibly high numbers, even in temperate regions. De Liberato et al. (2010) in Central Italy recorded a catch of *C. obsoletus* exceeding 34,000 specimens in one night with a single Onderstepoort black light trap and catches of >1,000 *C. obsoletus* per night in 50 trapping sites distributed across the whole territory of the Lazio and Tuscany regions, even in trap sites situated higher than 1,000 m a. s. l.
– Wind dispersal: Although not capable of flying actively over long distances, *Culicoides* spp. have been shown to be transported up to 700 km by prevailing winds, spreading viruses

downwind (Braverman and Chechik 1996, Bishop et al. 2000). Infected *Culicoides* moving as aerial plankton from Algeria and Tunisia to Sardinia in 2000 could have been the way that BT arrived in Italy (Maclachlan and Mayo 2013). The following year, in the same way infected *Culicoides* could have brought the virus from Sardinia to mainland Italy across the Tyrrhenian Sea, given that west winds prevail in those areas. To allow for their dispersal, a suitable wind is needed, offering sufficient warmth and moisture.

– Adults overwintering: *Culicoides* spp. do not vertically transmit viruses to progeny; hence, in temperate regions characterized by cold winters the problem arises of virus overwintering, given the short viremia, for example, of BTV in vertebrate hosts. In Southern Europe, biting midges of the *C. obsoletus* group can be active all year round, although at a reduced rate during the cold season; a number of adults can survive the winter months, even taking blood meals during relatively warm periods of a few days. In Central and Northern Europe, adults are absent for many months, and overwintering probably only occurs at larval stage. However, in these areas their indoor winter activity in animal sheds was postulated as a possible way that BTV was overwintering. In autumn 2006, overwintering of BTV in Central Europe was considered unlikely, due to the short BTV viremia in ruminants and to the absence of adult *Culicoides* for many months during winter. But the virus reappeared and spread again in 2007. Studies carried out during this epidemic highlighted the presence of active *C. obsoletus* adults in animal enclosures throughout the winter, allowing two possibly co-existing mechanisms of overwintering to be hypothesized: infected adult midges surviving the winter and allowing the virus to bridge the winter and/or active adult indoor midge populations causing continuous BT circulation throughout the winter, albeit at very low rates. If *C. imicola* were the European vector, given its exophily, BT would probably not have been able to overwinter in these areas.
– Breeding sites: Because of their nature, *Culicoides* sp. breeding sites are often very difficult to identify and circumscribe, as they vary in size from a few square meters in a farm to a whole forest of leaf litters. Even in the presence of huge adult populations, it is often impossible to identify their breeding site, which can be very scattered. Moreover, some species, such as *C. imicola* in Sardinia, for example, take advantage of manmade breeding sites, and other species such as *C. obsoletus* can breed in very diverse sites like wet soil enriched with cattle manure and forest leaf litter.
– Control: Due to the biological traits of *Culicoides* spp., with special reference to their high numbers and larval breeding sites, at present no valuable methods and strategies for their control have been developed, and their effective control has very rarely been achieved. Breeding sites are usually difficult to manage, unless we accept a dramatic modification of the environment. Also, animal protection through repellents has not yet given satisfying results.

2.1 Bluetongue

By far the most relevant disease transmitted by *Culicoides* spp. is BT, a globally distributed disease of domestic and wild ruminants (Taylor 1986). BTV belongs to the family Reoviridae, genus *Orbivirus*. At present, 24 serotypes have been described, distributed almost everywhere their vectors are present. In the last 15 years, BT global distribution has changed dramatically, with repeated incursions of the virus in Europe. Until 1998 only transient incursions of single BT serotypes were recorded in Europe (Mellor et al. 2008). Since 2000,

many BTV serotypes have invaded and spread throughout the continent, and the virus is now probably established in some southern parts of the region (Calistri et al. 2004).

BT provokes clinical disease mainly in sheep, with mortality levels up to 75 % (Mullen 2009). Cattle and goats are usually sub-clinically infected. While cattle are normally considered to be the virus reservoir (Mellor et al. 2000), during the BTV-8 European epidemic that started in 2006, the clinical disease also severely affected this species. In areas where the virus is endemic, clinical BT is a quite uncommon occurrence, while the disease can be devastating when the virus spreads to areas where ruminant populations have never met the virus before. BT is among the more economically devastating vector-borne animal diseases. Economic losses are due less to animal mortality than to indirect causes such as movement and trade restrictions, and surveillance and vaccination costs.

Following its first appearance in Sardinia in 2000, in September 2001 BT arrived in mainland Italy. An entomological surveillance system was immediately set up. This provided the information that while in Sardinia the vector was *C. imicola*, the main Old World BT vector, in Central Italy the situation was different. *C. imicola* was present in just 30 % of affected farms, only along the coast (while BT was also spreading inland) and always in very low numbers, with few specimens per night (De Liberato et al. 2003). On the other hand, *C. obsoletus* was always present, often in very high numbers. In 2002, BTV isolation was attempted from a pool of *C. obsoletus* caught 100 km south of Rome in a BT-affected farm. BTV was isolated (De Liberato et al. 2005), and from then on it was re-isolated or detected by PCR many times during the Italian epidemic. Thus, a new BT vector was identified. At the same time in Sicily, BT was isolated from *C. pulicaris*, another very common species in Europe (Caracappa et al. 2003). So, two new vectors had been identified. As a matter of fact, after the Italian epidemic, BT epidemiology was totally rewritten, with the identification of two new vectors, distributed throughout and often very abundant in the whole of Europe, from Sicily to Scandinavia. Until 2001, *C. imicola* was considered the old world BT vector, and areas that were "imicola-free" (that is to say, the main part of Europe) were also considered risk-free. After 2001, the risk seemed to be present in the whole of Europe, with the forecast dramatically confirmed in 2006 and the most economically devastating BT epidemic ever recorded occurring in Central and Northern Europe. *C. obsletus* can be considered the "European BT vector," with the role of *C. imicola* limited to very circumscribed areas of Spain, Sardinia and coastal mainland Italy. *C obsoletus* can be found from the southern regions of Italy as far as the UK, and it is present throughout the year in the southern part of its distribution (Rawlings and Mellor 1994, Jennings and Mellor 1988).

Following BT's arrival in Italy in 2000 and 2001, many authors claimed that *C. imicola* might spread geographically due to climate change. However, it must be said that until 2000 no one was studying *Culicoides* fauna in Italy and in most parts of Europe. So, there are no data for comparison and actually there is no real evidence of a *C. imicola* geographical spread. Moreover, with the definition of the role of *C. obsoletus* in BT circulation, there is no justification for positing a *C. imicola* spread as the reason for the presence of BT in Europe.

2.2 African Horse Sickness

African horse sickness virus (AHSV) is a member of the genus *Orbivirus* in the family Reoviridae, morphologically similar to BTV (Stanley 1981). AHSV infect equids: while zebra and donkeys usually exhibit no clinical signs, in susceptible populations of horses this virus

can be devastating, with mortality rates exceeding 90 % (Mellor and Hamblin 2004). AHS is endemic in sub-Saharan Africa (Mellor and Boorman 1995). With regard to Europe, AHS outbreaks were recorded twice in Spain, in 1966 and 1987. In 1966, the outbreak was rapidly stopped, also thanks to vaccination and slaughter policies. In 1987, the virus arrived within sub-clinically infected zebras imported from Namibia to a safari park (Lubroth 1988) and circulated again for the following three years, extending to Portugal and overwintering in the area for at least three cold seasons.

During the outbreaks of AHS in Spain and Portugal, as expected *C. imicola* was shown to be the major vector (Mellor et al. 1990). Areas where AHS was able to persist for three years were characterized by mild winters, and *C. imicola* was active year-round, thus allowing the virus to overwinter. However, the virus was isolated from mixed pools of *C. obsoletus* and *C. pulicaris*, suggesting that one or both of these species could be involved in the transmission of AHSV in Europe. Hence, it is possible that, as happened with BT, in regions where *C. obsoletus* and *C. pulicaris* are abundant, future incursions of the virus could also extend well beyond the distribution of *C. imicola* (Mellor and Hamblin 2004).

2.3 Schmallenberg Virus

In November 2011, a previously unrecorded virus made an appearance in Germany and the Netherlands, provoking economic losses due to teratogenic effects and milk yield reduction in sheep, cattle and goats. The virus was called Schmallenberg virus (SBV), after the village where it was first described (Hoffmann et al. 2012). The virus quickly spread to the UK, France, Italy, Luxembourg, Spain, Denmark and Switzerland (Beer et al. 2013) and was located in the genus *Orthobunyavirus*, family Bunyaviridae.

As many members of the *Orthobunyavirus* genus are transmitted by *Culicoides* (Doceul et al. 2013), and SBV seasonality and spread resembled those of BTV-8 in Europe in 2006 (Doceul et al. 2013), attention focused on *Culicoides* as probable vectors of the new diseases. In fact, SBV DNA was detected by PCR in pools of *Culicoides* of the *obsoletus* complex in Belgium, Denmark, Italy, the Netherlands and Germany (De Regge et al. 2012, Rasmussen et al. 2012, Elbers et al. 2013, Goffredo et al. 2013). In north-eastern Italy, SBV DNA was detected in *C. obsoletus* pools caught for the BT surveillance in September 2011 – that is, two months prior to the first description of the virus (Goffredo et al. 2013). The same researchers reported a prevalence of SBV in *C. obsoletus* ten times higher than those reported in the literature for BTV, thus perhaps evidencing a higher susceptibility of these midges to SBV than to BT.

As for BT, the question arises as to whether the virus overwinters as a result of the vector populations surviving the cold season or of the virus persisting in the cattle population or in other reservoirs. In this case also, indoor winter activity of *Culicoides* spp. was postulated as a possible way the virus overwinters.

3. Conclusions

From an entomological point of view, it is interesting to compare what happened in Europe with AHS and with BT, depending on the vectors involved. AHS, although introduced at least twice, was not able to spread, remaining limited to areas characterized by mild climates and

being quite "easy" to eradicate. Its vector, *C. imicola*, is present in Europe only in limited, milder areas; it is usually not abundant; it is able to overwinter at the adult stage only during especially warm winters; and, for these reasons, it does not offer good overwintering prospects for the virus. BT, on the other hand, once introduced, was able to spread and persist for many years in Europe. BT's presence has been almost continuous since 2000, and the virus has also been able to overwinter in areas with very long, cold winters. This is because its vector, *C. obsoletus*, is ubiquitous and very abundant almost everywhere from Sicily to the UK; it possibly allowed virus overwintering thanks to its indoor winter activity.

It must be emphasized that, despite the huge amount of work that researchers from all over Europe have dedicated to *Culicoides* and *Culicoides*-transmitted diseases – to BT in particular in the last 15 years – at present many hugely relevant factors remain unknown. Among other things, we do not know how so many BT serotypes entered Europe, and from where. Particularly puzzling is the matter of the appearance of BTV-8 in central Europe, thousands of kilometers from areas where this serotype was circulating. Another question is why all these serotypes arrived almost simultaneously in the few years after 2000. Can climate change alone explain this mass? How did SBV suddenly appear in the middle of Europe and where did it come from? How did BT and SBV overwinter? What is the role of the different species of the *obsoletus* complex as BT and SBV vectors? Our knowledge with regards to all these questions is just at the hypothesis level, so clearly much remains to be done!

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Threats and Risks of Phlebotomine Sand Fly-Borne Diseases Becoming Established in Germany and Northern Europe: Preparedness for Integrated Control and Prevention

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Abstract

This report considers whether phlebotomine sand flies (Diptera: Psychodidae, Phlebotominae) are spreading into northern Europe and establishing transmission cycles of the parasites and pathogens they regularly transmit in Mediterranean Europe, namely protozoan *Leishmania* species (Kinetoplastida, Trypanosomatidae) causing leishmaniasis in humans and reservoir hosts and arboviruses (Bunyaviridae) causing periodic fevers or neurological diseases. There is no documented spread of sand flies into northern Europe, but limited emergence of vectors in central France is suggested by new reports. Much excitement has been generated by the potential role of *Phlebotomus mascittii* in the transmission of *Leishmania infantum* in Germany and some neighbouring countries, but this sand fly has not been demonstrated to be a competent vector and its low human biting rates and autogeny are unlikely to give it a high vectorial capacity. There is clearly a threat of sand fly-borne diseases spreading into northern Europe, but there have been no detailed risk assessments, and autochthonous leishmaniasis cases have not associated with vectors.

Zusammenfassung

Dieser Bericht untersucht, ob sich Sandmücken der Gattung *Phlebotomus* (Diptera: Psychodidae, Phlebotominae) nach Nordeuropa ausbreiten und Übertragungszyklen von Schädlingen und Krankheitserregern schaffen, die sie regelmäßig im europäischen Mittelmeerraum übertragen, nämlich Protozoenarten von *Leishmania* (Kinetoplastida, Trypanosomatidae), die Leishmaniose bei Menschen und Reservoirwirten hervorrufen, und Arboviren (Bunyaviridae), die regelmäßig wiederkehrendes Fieber oder neurologische Erkrankungen hervorrufen. Es gibt keine dokumentierte Ausbreitung von Sandmücken in Nordeuropa, aber ein begrenztes Auftreten von Vektoren in Mittelfrankreich wird durch neue Berichte vorgebracht. Viel Aufregung wurde erzeugt durch die mögliche Rolle von *Phlebotomus mascittii* bei der Übertragung von *Leishmania infantum* in Deutschland und einigen angrenzenden Ländern, aber diese Sandfliege hat nicht bewiesen, dass sie ein kompetenter Vektor ist, und ihre niedrige menschliche Beißquote und Autogenität werden ihr wahrscheinlich keine hohe Vektorkompetenz geben. Es besteht eine klare Gefahr, dass durch Sandfliegen übertragene Krankheiten nach Nordeuropa verbreitet werden, aber es gibt keine detaillierte Risikoeinschätzung dazu.

1. Introduction

There is heightened awareness that climate warming could provoke the northward spread of "tropical diseases" of humans and animals caused by parasites and pathogens transmitted by arthropod vectors (Parham et al. 2015). This scenario is supported by the arrival of chikungunya (Chikv) and dengue (Denv) viruses in southern Europe and the spread of their mosquito vectors, *Aedes aegypti* and *Aedes albopictus*, as far north as the Netherlands during some warmer months of the year (Scholte et al. 2010). However, it should not be assumed that all insect and tick vectors in southern Europe are capable of responding to climate warming by spreading northwards and establishing new transmission cycles at higher latitudes. This report considers whether phlebotomine sand flies (Diptera: Psychodidae, Phlebotominae) are spreading into

northern Europe and establishing transmission cycles of the parasites and pathogens they regularly transmit in Mediterranean Europe, namely protozoan *Leishmania* species (Kinetoplastida, Trypanosomatidae) causing leishmaniasis in humans and reservoir hosts (Alvar et al. 2012, *Eurosurveillance Editorial Team* 2013, Ready 2013) and arboviruses (Bunyaviridae) causing periodic fevers or neurological diseases (Depaquit et al. 2010, Bichaud et al. 2014).

For the current report, northern Europe is defined as the geographical region between latitudes 47° and 55° N and longitudes 6° W to 23° E that contains all or parts of the United Kingdom, Netherlands, Germany, Poland, Belgium, Luxembourg, France, Switzerland, Austria, Czech Republic, Slovakia and Hungary. It contains no parts of Denmark, Lithuania, Russia, Belarus and western Ukraine, all far from the known ranges of phlebotomine sand flies (Ready 2013, *Vectornet* 2016). Therefore, this report focuses on the potential for natural northward spread of sand flies and their transmission cycles along two main routes: from endemic disease foci in Spain, Italy and southern France to northwest Europe *via* the plains, valleys and uplands of central France and the foothills of the Alps in western Switzerland; and, from endemic disease foci in the Balkans to the North European Plain south of the Baltic Sea, *via* the Danube valley to Bavaria (Germany) or *via* Ostrava (Czech Republic) across to the River Odra (Poland). An assessment of the potential for natural spread from the Black Sea region to the North European Plain *via* routes east of the Carpathian Mountains requires a dedicated study of the extensive literature from the former USSR, which is beyond the resources for the current report.

The incriminated sand fly vectors in the source Mediterranean regions being considered are *Phlebotomus* (*Larroussius*) species for zoonotic visceral and cutaneous leishmaniasis of humans and companion dogs (the main reservoir hosts) caused by *Leishmania infantum*, *Phlebotomus* (*Paraphlebotomus*) *sergenti sensu lato* for anthroponotic cutaneous leishmaniasis caused by *Leishmania tropica*, *Phlebotomus* (*Phlebotomus*) *papatasi* for periodic fevers of humans caused by *Phlebovirus* species, and *Phlebotomus* (*Larroussius*) *perniciosus* and *Phlebotomus* (*Larroussius*) *perfiliewi* for summer meningitis and other neurological diseases of humans caused by Toscana virus (Tosv) (Ready 2010, Charrel et al. 2012, Ready 2013).

The aims of the current report would be informed by any evidence for the natural spread or emergence of sand fly-borne diseases within the endemic Mediterranean regions, and so such evidence is now considered briefly, based on the extensive reviews and findings of Ready (2010), *Vbornet* (2012), *Eurosurveillance Editorial Team* (2013), Medlock et al. (2014) and *Vectornet* (2016). In summary, since 1980, there have been many discoveries of sand flies in new locations, including incriminated vectors associated with canine leishmaniasis in some cooler climes of Spain and Andorra (e.g. *Phlebotomus* (*Larroussius*) *ariasi* in Alava [Aransay et al. 2004] and at higher altitudes in the eastern Pyrenees Mountains [Ballart et al. 2012a,b]), either side of the boundary of the Mediterranean and temperate bioclimatic regions in southwest France (e.g. *P. perniciosus* and *P. ariasi* in the triangular area bounded by Perpignan, Tarbes and Cahors [Dereure et al. 2009, Hartemink et al. 2011, Mahamdallie et al. 2011, Mahamdallie and Ready 2012]), and near the northern limit of the Mediterranean region in Croatia, Serbia and southern Hungary (e.g. *P. perfiliewi*, *Phlebotomus* (*Larroussius*) *neglectus* and *Phlebotomus* (*Larroussius*) *tobbi* [Bosníc et al. 2006, Farkas et al. 2011, Tánczos et al. 2012]). However, new records provide evidence of spread or emergence only if associated with earlier absence records based on equivalent surveillance effort (Ready 2010, 2013). Such evidence is only available for one European region south of latitude 47° N, namely northern Italy, although pre-1980 (Biocca et al. 1977), and more recent surveillance efforts were not compared in detail by Maroli et al. (2008) when they concluded that canine leishmaniasis

and two of its sand fly vectors, *P. neglectus* and *P. perniciosus*, had spread into the pre-Alpine region or increased in abundance there. In contrast, there is evidence against any major changes in sand fly diversity and abundance in the Cévennes Mountains of southern France, at the northern edge of the Mediterranean region in Gard department, where the same locations were sampled using sticky-paper traps in 1977 and 2011–2014 (Prudhomme et al. 2015).

Any cut-off date for comparing changes in the distributions of sand fly-borne diseases before and after substantial climate warming will be somewhat arbitrary, but 1980 has been chosen because there was relatively more surveillance in Europe before then (Rioux and Golvan 1969, Biocca et al. 1977, Maroli et al. 2008, Rioux et al. 2013, Prudhomme et al. 2015) compared with the following two decades, when molecular research was the focus for many "leishmaniacs" (Schönian et al. 2008, Ready 2010). It is also an appropriate cut-off because 1951–1980 is the base period in the Goddard Institute for Space Studies global temperature analysis (Gistemp) (Hansen et al. 2010), and global surface temperature in 2015 was warmer by 0.87 °C relative to the mean for 1951–1980 but warmer by just 1.13 °C relative to the mean for 1880–1920 (Hansen et al. 2016). The choice of 47 °N for the southern boundary of northern Europe was not entirely arbitrary, because sand flies have long been recorded just below this latitude, in central France (Rioux and Golvan 1969), western Switzerland (Gaschen 1956) and southern Hungary (Lőrincz and Szentkirályiet al. 1933).

2. Methods

2.1 Literature Searches

PubMed (http://www.ncbi.nlm.nih.gov) was searched in the period December 2015 – March 2016 using one term from the set "Phlebotominae, sand fly, sandfly, *Phlebotomus*, *Phlebotomus mascittii*, leishmaniasis, *Leishmania*, *Leishmania infantum*, arbovirus, *Phlebovirus*, Toscana virus" AND one term from the set "Europe, United Kingdom, Netherlands, Germany, Poland, Belgium, Luxembourg, France, Switzerland, Austria, Czech Republic, Slovakia and Hungary", and all pairings were tried.

2.2 Assessment Criteria for Literature Findings

Records for sand flies, the parasites and pathogens they transmit and the resulting diseases were not considered if unsubstantiated or second-hand. Many reviews uphold the spread of sand flies and sand fly-borne diseases by quoting references that provide no substantiated new distribution records.

3. Results and Discussion

3.1 Evidence for any Recent Spread of Phlebotomine Sand Flies into Northern Europe

The articles found by searching PubMed included those reported by Medlock et al. (2014). Articles did not always mention if the first discoveries of sand flies in countries and regions were made in localities where sand flies had previously been demonstrated to be absent.

Considering possible western routes of spread above latitude 47° N, there are few presence records (*Vectornet* 2016) for *P. ariasi* to the northwest of the upland Massif Central in France, where the most northerly record is from Sarthe department (c. 48° N) and pre-1980 (Rioux and Golvan 1969), and none to the northeast of the Massif Central. There are more recent records for the other incriminated vector in France, *P. perniciosus*, both northwest and northeast of the Massif Central (*Vectornet* 2016), but not further north than the Paris region, where it was found pre-1980 (see Rioux and Golvan 1969). Neither vector is abundant in northern France, except for *P. perniciosus* near Tours (Houin et al. 1975, B. Pesson and P. D. Ready unpublished observations). Both vectors remain unrecorded in Belgium (Depaquit et al. 2005), the Netherlands, Luxembourg and western Switzerland. The only sand fly record from the United Kingdom remains the report of one male *P. perniciosus* on the island of Jersey (c. 49° 15' N), close to France (Marett 1923).

In contrast to these two sand fly species, *Phlebotomus* (*Transphlebotomus*) *mascittii* has not been incriminated as a vector of any *Leishmania* species or arbovirus (Ready 2013), but it has long been known to have an extensive range in temperate regions, including western Switzerland (Canton Vaud, south of latitude 47° N) (Gaschen 1956), central and northern France to Beauvais (c. 49° 26' N) (Rioux and Golvan 1969), and more recently to Reims (49° 16' N) in northeast France and to Sainte-Cécile (Florenville, c. 49° 43' N) just in Belgium (Depaquit et al. 2005).

In Germany, *P. perniciosus* was detected for the first time in 2001, when one male and three females were collected in a miniature CDC (Centers for Disease Control) light trap placed throughout the summer in the village of Gehrweiler, in Rhineland-Palatinate state, where in "[...] 1998/1999, a suspected case of autochthonous leishmaniasis in a dog was reported to local veterinarians [...]" (Naucke et al. 2008). This is the only record of *P. perniciosus* in Germany, despite widespread sampling by the same team (Naucke et al. 2008), using light traps that captured a total of 237 specimens of *P. mascittii* from one locality in Rhineland-Palatinate state (c. 50° 20' N) and 16 localities in Baden-Württemberg state to the south, including the first records of any sand fly in Germany from three localities along the Rhine valley (c. 47° 40' to 47° 55' N) (Naucke and Pesson 2000). During an entomological survey by a second German team in July 2013, one female of *P. mascittii* was caught a little further north in Giessen (50° 35' N) within the adjoining Hesse state (Melaun et al. 2014). In contrast, a third German team failed to capture a single sand fly in Bavaria state, which adjoins Baden-Württemberg state on its eastern border, even though 202 CDC light traps were set over 38 warm nights (202 trap-nights) in 155 localities from mid-June to late August 2009–2010 (Haeberlein et al. 2013).

Sand flies might also reach northern Europe along eastern routes, but there are no pre-1980 or more recent reports providing evidence for this. *Phlebotomus neglectus* and *P. mascittii* have been detected recently in Hungary, more abundantly in the south (c. 45° 48' to 46° 57' N) where the presence of *P. perfiliewi* was confirmed, and also in small numbers (two males and one female, respectively) just south of Budapest (c. 47° 26') (Farkas et al. 2011). However, there are no records of any sand flies north of Budapest, on the possible routes to Bavaria *via* the Danube valley or to the North European Plain *via* the northeast of the Czech Republic. Sand flies have been detected recently in Austria (Naucke et al. 2011), but only the non-vectorial *P. mascittii* (4 males and 22 females) and only in the southern foothills of the Alps (c. 46° 39' to 46° 51' N), in Carinthia province bordering Mediterranean Slovenia, not on a route to northern Europe.

3.2 Evidence for any Recent Increase in Leishmaniasis Transmission in Northern Europe

There is considerable evidence for an increase in the number of cases of human and canine leishmaniasis caused by *L. infantum* reported from Germany, the United Kingdom, and other northern European countries since 1980, and most of these can be explained by travel infections acquired in the Mediterranean region and the importation of unscreened reservoir hosts (usually companion dogs) from that region (Harms et al. 2003, Menn et al. 2010, Ready 2010, Mencke 2011, Ehehalt et al. 2014). However, autochthonous infections of *L. infantum* are not infrequent in dogs and have also been found in horses, especially in southern Germany (Bogdan et al. 2001, Koehler et al. 2002, Naucke et al. 2008, Müller et al. 2009, Mencke 2011). Congenital and other non-vectorial transmission of *L. infantum* among dogs could be widespread in Europe, as it might be in North America (Duprey et al. 2006), and there is at least one proven case in Germany (Naucke and Lorentz 2012).

3.3 Evidence for any Recent Establishment of Leishmaniasis and Arbovirus Transmission by Sand Flies in Northern Europe

Leishmaniasis is not usually a notifiable disease in northern Europe, but reference laboratories in many countries use microscopical, serological and molecular techniques of adequate specificity and sensitivity, which have routinely detected a diversity of exotic *Leishmania* species causing human cutaneous and visceral leishmaniasis as well as strains of *L. infantum* of European origin in humans and dogs (Schönian et al. 2008, Menn et al. 2010, Solano-Gallego et al. 2011, Wall et al. 2012, Lachaud et al. 2013, Poeppl et al. 2013, Wolf et al. 2014, Gebhardt et al. 2015). Central France is the only region where there is evidence that canine leishmaniasis is spreading northwards (P. Bourdeau and G. Bourdoiseau, personal communication), based on questionnaires answered by veterinary practices, such as those reported by Bourdeau et al. (2014), but it is unclear whether any transmission involves sand flies.

There appears to be no autochthonous infections of sand fly arboviruses in northern Europe and, compared with leishmaniasis, much less surveillance of humans and companion animals in regions of possible spread such as central France and Hungary (Depaquit et al. 2010, Charrel et al. 2012, Bichaud et al. 2014). Sand fly storage and screening protocols are being optimised (Remoli et al. 2015), but they are rarely used outside the Mediterranean region. One early serosurvey found that all 8 acute infections of Tosv in German tourists were acquired in Italy or Portugal (Schwarz et al. 1995).

Presence of a sand fly vector in an emerging focus (Medlock et al. 2014), such as those of canine leishmaniasis in central France, is necessary but not sufficient for the establishment and maintenance of vector-borne transmission, which depends not only on vector competence but also on poorly understood transmission dynamics (Hartemink et al. 2011, Ready 2013, Bates et al. 2015, Cameron et al. 2016). Therefore, it is insufficient to produce only climate change models that predict the presence of a competent vector, such as the models for Germany and Europe (Fischer et al. 2010, 2011, Haeberlein et al. 2013). The reliance on temperature as the main determinant of sand fly presence (Naucke et al. 2008, Fischer et al. 2010) is certainly unwise, because not all species or populations have the facultative diapause that permits *P. perniciosus* (origin Marseilles, France) to delay larval hibernation and extend its adult season in response to higher temperatures; in contrast, the larval diapause of *P. ariasi* (origin Cévennes, France) can be induced by photoperiod that is not over-ridden by higher temperatures (Ready and Croset 1980).

3.4 Preparedness for Integrated Control of Sand Fly-Borne Diseases and Prevention of their Establishment in Northern Europe

Integrated control of sand fly-borne diseases might be undertaken as part of the control of other vector-borne diseases (Bates et al. 2015). Either way, it should include adequate surveillance for infections in humans and reservoir hosts (possibly including cats as well as dogs for *L. infantum* [Solano-Gallego et al. 2011]), rapid specific identification (Bichaud et al. 2014, Solano-Gallego et al. 2014, Wolf et al. 2014, Gebhardt et al. 2015) followed by rapid treatment for anthroponotic and syringe-transmitted human infections (Alvar et al. 2012), and contingency plans for reducing the number of sand fly bites by the use of well-chosen repellents, indoor residual spraying (IRS), area spraying and deltamethrin-impregnated dog collars (Quinnell and Courtenay 2009, Bates et al. 2015). Vector control would be aided by adequate local surveillance for sand flies, and this will require not only an awareness of the available trapping methods (Alten et al. 2015) but also a statistically robust sampling strategy for stratified habitats.

It is uncertain whether sand fly surveys will be cost-effective for monitoring the geographical spread of vectors and the establishment of sand fly-dependent transmission cycles, because of the short-term temporal changes in sand fly densities (Rioux et al. 2013, Prudhomme et al. 2015) and the complexity of transmission modelling (Quinnell and Courtenay 2009, Hartemink et al. 2011, Espejo et al. 2015).

The culling of dogs does not provide community protection against leishmaniasis transmission to dogs and humans (Quinnell and Courtenay 2009), and this may also apply to deltamethrin-impregnated dog collars and other methods of dog prophylaxis (Wylie et al. 2014b). Therefore, the best method of prevention will be vaccination, which may soon be routinely available for dogs (Wylie et al. 2014a) but not for humans (Alvar et al. 2013).

4. Conclusions

There has been no documented spread of sand flies into northern Europe, but limited emergence in central France is suggested by increases in observed densities and new reports. Much excitement has been generated by the potential role of *P. mascittii* in the transmission of *L. infantum* in Germany, Austria and elsewhere (Naucke et al. 2008, 2011, Aspöck et al. 2008, Melaun et al. 2014), but this sand fly has not been demonstrated to be a competent vector despite laboratory colonization (Naucke et al. 2006), and its low human biting rates and autogeny are unlikely to give it a high vectorial capacity (Ready 2010, 2013).

There is phylogenetic and population genetic evidence that the two most important western European sand fly vectors, *P. perniciosus* (Aransay et al. 2003) and *P. ariasi* (Mahamdallie et al. 2011, Mahamdallie and Ready 2012), have made one or more natural northward invasions of France since the last glacial period, c. 11,000 years ago. However, the great land cover changes made subsequently by humans may not have permitted extensive or rapid range extensions of sand flies during the recent decades of climate change, and this could remain the case in the near future. Therefore, there is clearly a threat of sand fly-borne diseases spreading into northern Europe, but there have been no detailed risk assessments (Ready 2010). These require better knowledge of associations between sand fly vectorial capacity and land cover (Hartemink et al. 2011, Ready 2013).

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Infections with Spotted Fever Group *Rickettsia* in Man and Animals

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Abstract

Rickettsiae of the Spotted Fever Group (SFG) can cause febrile diseases with or without a rash in humans worldwide. Globally they are considered to be emerging disease pathogens based on the fact that there is an ongoing discovery of new *Rickettsia* species and new vectors for known *Rickettsia* species, as well as the fact that *Rickettsia* species previously thought to be non-pathogenic are now known to cause disease in humans. Nevertheless, this group of pathogens has been neglected, and knowledge of the distribution, vector- and host-association, abundance and transmission patterns is scarce. For most of the *Rickettsia* spp. we have a rough idea about their geographical distribution based on their primary vector, mostly a particular species of tick. *Rickettsia felis* is the only known SFG *Rickettsia* where the cat flea (*Ctenocephalides felis*) was long thought to be the sole arthropod vector for transmitting this pathogen, but frequent findings in ticks as well as recent studies demonstrating that *Anopheles* mosquitoes function as biological vectors show that we have a great deal to learn about the epidemiology of this group of bacteria. Using serology to detect antibodies against Rickettsiae is only of limited value in deriving transmission patterns since serological tests usually only distinguish between SFG and Typhus Group (TG) Rickettsiae because of strong cross-reactivities within the groups. Nevertheless, pets may be of use as surrogates for the human exposure to the *Rickettsia* species. Attempts to differentiate between the antibody reaction, together with seroprevalence rates in such animals, could serve as possible sentinels and indicators for the distribution of different *Rickettsia* species.

Zusammenfassung

Rickettsien der Fleckfiebergruppe können beim Menschen zu fieberhaften Erkrankungen mit oder ohne Erythem führen. Sie gelten weltweit als sogenannte „emerging pathogens“, wobei dies im Wesentlichen auf drei Dingen beruht: Zum einen werden ständig neue Rickettsienarten entdeckt und als Pathogen beschrieben, zum anderen tauchen bekannte Rickettsienarten in neuen Regionen auf, in denen sie vorher noch nicht gefunden wurden, oder sie nutzen neue Vektoren, um übertragen zu werden, und schließlich entpuppen sich schon länger bekannte und für apathogen gehaltene Arten als Krankheitsverursacher beim Menschen. Obwohl diese drei Entwicklungen offensichtlich sind, gehören Rickettsien nach wie vor zu den sogenannten *neglected*, d. h. den vernachlässigten, Erregern. Zu vielen Arten haben wir nur ein lückenhaftes Wissen, was die geographische Verbreitung, die Vektor- oder Wirtsassoziation, ihre Häufigkeit und die natürlichen Übertragungswege angeht. Für einige Rickettsienarten haben wir für diese Eigenschaften eine gewisse Vorstellung, jedoch in den meisten Fällen rein deskriptiv. Während die meisten Fleckfieberrickettsien Zecken als Vektoren nutzen, dachte man lange, dass *Rickettsia felis* die einzige Ausnahme darstellt und ausschließlich den Katzenfloh (*Ctenocephalides felis*) nutzt. Aber vermehrte Funde in Zecken und eine neue Studie aus Afrika, bei der gezeigt werden konnte, dass *Anopheles*-Stechmücken *R. felis* übertragen können, demonstrieren, dass wir noch viel über diese Gruppe von Bakterien zu lernen haben. Serologische Untersuchungen sind dabei nur von begrenzter Hilfe, da über die ausgeprägte Kreuzreaktivität innerhalb dieser Gruppe so keine Übertragungswege für die einzelnen Rickettsienarten nachvollzogen werden können. Mit serologischen Untersuchungen von Haus- oder Wildtieren kann aber sehr wohl die generelle Exposition der Menschen abgeschätzt werden. Neue Entwicklungen zur Differenzierung der Antikörperantwort könnten hier helfen, die Verbreitung und Übertragungswege der Rickettsienarten besser untersuchen zu können.

1. A Concise History of Rickettsioses

The first contemporary account of a disease, which is nowadays known as epidemic typhus caused by louse-borne *Rickettsia prowazekii*, comes from the conquest of Granada. In 1490 this last Arabian enclave on the Iberian Peninsula was reconquered by Christian troops from Spain during which an estimated 17,000 people died of a typhus-like disease (Quintal 1996). Only a few decades later, in 1528, around 30,000 French soldiers succumbed to typhus in Naples and 10,000 died in Metz in 1552. By then it had been formally described as a disease by Fracastorius in Italy. Toward the end of the 16th century, typhus killed more than 2 million native Indians in the Mexican highlands (Harden 1993). In the following centuries the incidence of epidemic typhus increased in the New World and in Europe. Around 2 million people died between 1813 and 1819 when, after the battle of the nations in Leipzig, soldiers returning home brought with them deadly louse-borne typhus. During World War I and the Russian Revolution a further 25 million cases were ascribed to epidemic typhus with about 3 million cases being fatal (Quintal 1996). Meanwhile Howard Ricketts described the etiology of Rocky Mountain Spotted Fever and incriminated the wood tick as being the vector responsible (Ricketts 1907). The Nobel Prize was given to Charles Nicolle in 1928 who had proven in 1909 that epidemic typhus was transmitted by lice. This was also suggested shortly thereafter by Ricketts during his investigations in Mexico in the same year and was further substantiated by the Austrian Stanislav von Prowazek who regularly found the disease-causing organisms in lice taken from typhus patients in Serbia in 1913. Ricketts died in 1910 and von Prowazek died in 1915, ironically of typhus, a disease caused by an organism that was later given both their names: *Rickettsia prowazekii* (Gross and Schäfer 2011). The years that followed saw the description of the "tache noir", the eschar that is typically found at the site of the tick bite in spotted fever *Rickettsia*, in 1923, the first cultivation in guinea pigs in 1930 and in embryonated eggs in 1938, and the first typhus vaccine in 1939. With the dawn of the era of antibiotics in the late 1940s, typhus and other rickettsial diseases lost most of what made them so terrifying. This lasted until around thirty years ago when, particularly, the tick-borne spotted fever group Rickettsiae regained its importance as a group of emerging pathogens.

2. Emergence of the *Rickettsia* Species

Three aspects of the epidemiology of *Rickettsia* spp. have led to a global emergence of this group of bacteria over the last three decades. The first aspect is the continuing discovery of new *Rickettsia* species. As outlined earlier, some well-known members of the species have been infecting humankind for centuries. With improvements in molecular techniques, new *Rickettsia* species and putative new members of this genus are now identified almost every year. Since many of them have yet to be cultivated *in vitro*, they still have the status of "Candidatus", however, many of them will eventually turn out to be human pathogens (Parola et al. 2005). Due to the mostly similar and unspecific clinical presentation of a fever, rash, headache, and sometimes myalgia, and changes in the blood including anemia, thrombocytopenia and elevated liver transaminases, differential diagnoses include a long list of microorganisms that cause febrile illness. In addition, specific diagnostic tools are not available in most instances. As serological tests cross-react within the spotted fever group *Rickettsia*, including the transitional group

Rickettsia, it is not possible to assign them to a particular *Rickettsia* species (Fig. 1). Some of the new *Rickettsia* species were found to be responsible for human disease at the same time that they were discovered. Examples here include *R. japonica*, which caused a febrile illness (Japanese spotted fever) in three patients in Japan in 1984 and was isolated from another patient a year later (Uchida et al. 1986). Another example is Flinders Island spotted fever, which was described in 26 patients from the tiny island close to Tasmania south of Australia in 1991 (Stewart 1991). The causative agent, later identified as being *R. honei*, was isolated from two patients from Flinders Island the following year (Baird et al. 1996).

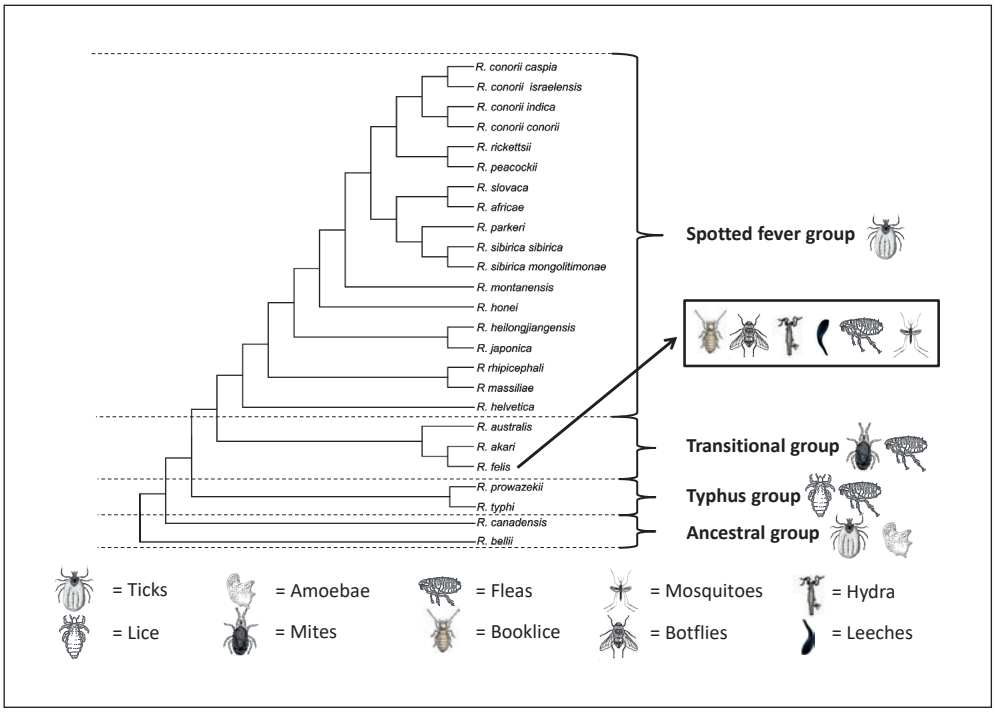

Fig. 1 Cladogramme of the most important *Rickettsia* spp. showing the genetic relationship between the members of the genus *Rickettsia* together with the known vectors for each group or species. Please note that, serologically, members of the spotted fever group and the transitional group do cross react, however, they can be distinguished from typhus group Rickettsiae. While most *Rickettsia* spp. use only one vector species or taxon, *R. felis* is an extreme exception with a wide variety of arthropods, terrestrial leeches (*Haemadipsida* spp.) or *Hydra* spp. (Hydridae).

In addition to the identification of new pathogenic *Rickettsia* spp., a series of spotted fever group *Rickettsia*, previously thought to be nonpathogenic, has turned out to be responsible for human diseases. The reason for this may lie in the globally growing number of immunocompromised people who are more susceptible to developing illness from infections that are otherwise subclinical. But it is more likely that the Rickettsiae that were isolated from ticks were initially regarded as being endosymbionts or were not linked to individual, previously unrecognized, rickettsial-like diseases in the same area. Likewise, when a particular *Rickettsia* species was known to occur in a particular region, new findings were not thoroughly investi-

gated as they were taken to be already known. Examples for rickettsial pathogens that emerged in this way are *R. slovaca*, described as early as 1968 (Rehacek 1984) and recognized as a human pathogen in 1997 (Raoult et al. 1997), *R. helvetica*, described in 1977 (Burgdorfer et al. 1979) and recognized as a human pathogen twenty years later in 1999 (Nilsson et al. 1999), *R. aeschlimannii*, which was described in 1997 (Beati et al. 1997) but first recognized as a pathogen in 2002 (Raoult et al. 2002), or *R. massiliae*, described in *Rhipicephalus sanguineus* ticks from France in 1992 (Beati et al. 1992) and found to be responsible for spotted fever in a 45-year-old patient from Italy in 2005. Interestingly, this diagnosis was made from the patient's serum that was taken 20 years earlier in 1985 (Vitale et al. 2006). The longest known time span between the initial description and isolation of a spotted fever group *Rickettsia* and its demonstration as a human pathogen was 65 years. In 1939, *Rickettsia* sp., later named *R. parkeri*, was among the first *Rickettsia* to be cultivated from *Amblyomma maculatum* ticks taken from cows in Texas (Parker et al. 1939). In 2004 this particular *Rickettsia* was shown to cause spotted fever in a 40-year-old man, and *R. parkeri* was isolated from the eschar of this patient (Paddock et al. 2004). In a recent review of tick-borne spotted rickettsioses, six more *Rickettsia* species were listed here for Europe alone (Oteo and Portillo 2012), and even more were found worldwide (Parola et al. 2013; Fig. 2).

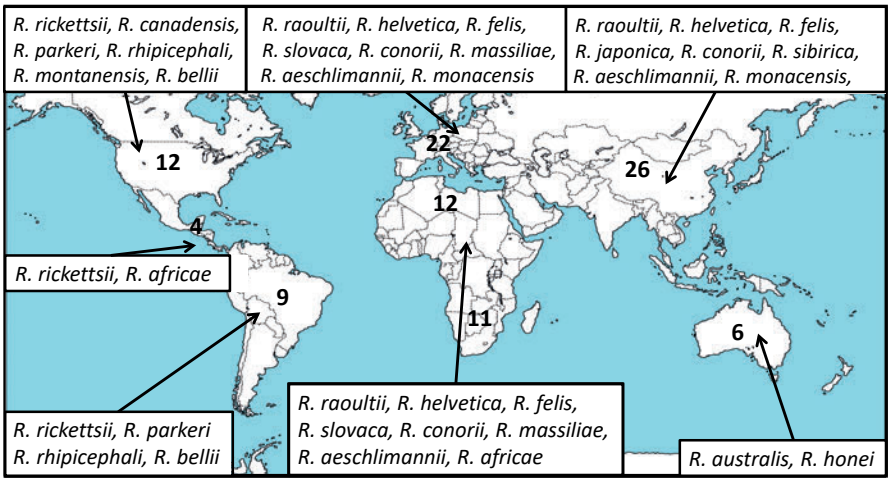

Fig. 2 Global distribution of known *Rickettsia* spp. (adapted from Parola et al. 2013). Only the most medically important and recognized species are listed, Candidatus *Rickettsia* spp. are not given. Numbers on the continents (Africa is divided into north and south of the Sahara) do include them and thus provide an account of the species richness per continent. Typhus group Rickettsiae (*R. prowazekii* and *R. typhi*) are considered to have a global distribution.

A very recent article has taken a closer look at the national surveillance data of spotted fever group rickettsioses in the United States (Drexler et al. 2016). This paper describes an incidence increase from 1.7 cases of spotted fever group rickettsioses per million person-years

in 2000 to 14.3 cases per million person-years in 2012. The authors nicely discuss that this tremendous increase may not only be ascribed to highly pathogenic *R. rickettsii*, but also to the less pathogenic *Rickettsia* species. This is a dilemma that is always faced when the laboratory diagnosis of rickettsioses is based on serology alone. Although the overall geographical distribution of Rocky Mountain spotted fever has not changed since the mid-1990s, an ecological change has led to local clusters of Rocky Mountain spotted fever in Arizona where the brown dog tick (*Rhipicephalus sanguineus*) was shown to efficiently vector *R. rickettsii* (Demma et al. 2005, Regan et al. 2015). *Rhipicephalur sanguineus* is a cosmopolitan tick species present in warm regions where dogs are present. It is thus an efficient vector tick when it comes to the dispersal of tick-borne pathogens like *R. rickettsii*.

3. The Case of *Rickettsia felis*

While transmission of the spotted fever group Rickettsiae is confined to a particular arthropod taxon, its geographic distribution and epidemiology is consequently linked to the ecology of the vector (Fig. 3). *Rickettsia felis* from the transitional group Rickettsiae, however, behaves fundamentally differently.

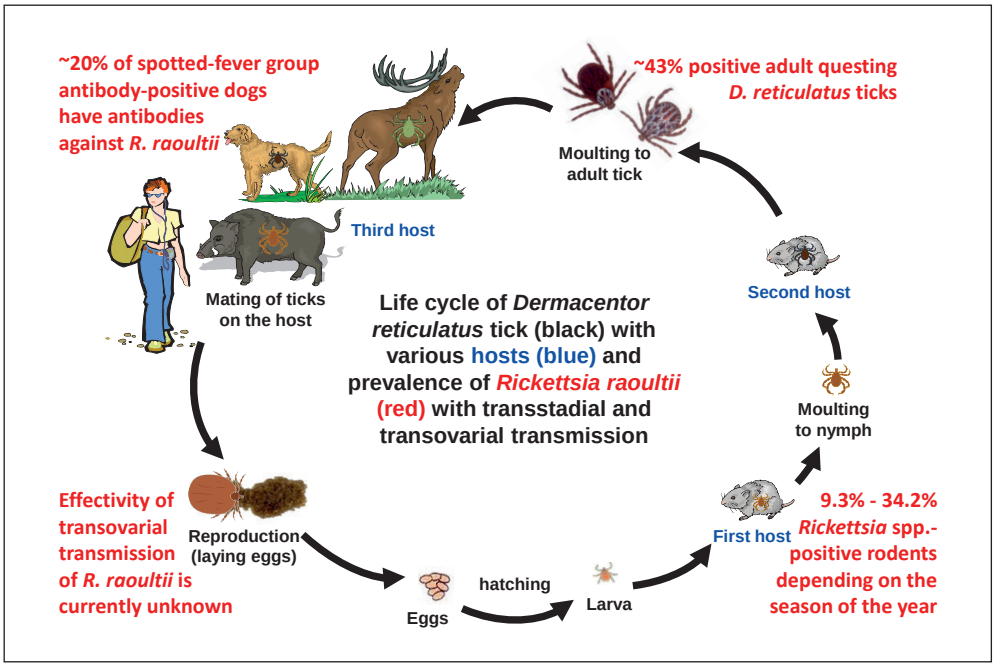

Fig. 3 Example of a transmission cycle of *R. raoultii*, which mainly uses *Dermacentor reticulatus* ticks as vectors between rodents (mainly bank voles) and larger mammals. Prevalence in rodents is 9 % and 35 % depending on the season, while in adult ticks, *R. raoultii*-DNA is found in about 43 % of flagged ticks. This provides some evidence for an efficient transstadial transmission from the vector ticks to the rodents who serve as reservoir hosts. Transovarial transmission of *R. raoultii* in *D. reticulatus* ticks is suspected but its extent and, thus, its epidemiological importance is unknown (data from Wächter et al. 2015, Obiegala and Pfeffer, unpublished).

First identified in the cat flea (*Ctenocephalides felis*) in 1990 (Adams et al. 1990), this arthropod was considered to be the only vector and *R. felis* to be nonpathogenic (see above) for almost two decades. Meanwhile more than 70 human cases of *R. felis* infections had been recorded worldwide (Dieme et al. 2015) with about 10 in Europe (Portillo et al. 2015). Attempts to identify a mammalian reservoir host have failed thus far which is why vertical and horizontal transmission paths have been intensively investigated. Results were not conclusive as some researchers described a vertical transmission for up to 12 generations without any evidence for a horizontal transmission (Wedincamp and Foil 2002), while others reported the exact opposite with no vertical transmission, only horizontal (Hirunkanokpun et al. 2011). Nevertheless, *R. felis* was documented in many institutional and commercial flea colonies as well as in wild fleas worldwide (Reif and Macaluso 2009). Rarely, and most likely without epidemiological importance, *R. felis* was isolated from hard ticks of the genera *Rhipicephalus*, *Haemaphysalis* and *Ixodes*. These findings may be the result of bacterial take-up during the last blood meal on a rickettsiaemic host. This may also explain why *R. felis* is detected in botflies, but is not in the case of hydra, leeches or booklice (Fig. 1). For the latter, non-hematophagous insect, dust samples in beds from *R. felis*-infected patients were positive for *R. felis* and live booklice were found within these dust samples (*Liposcelis bostrychophila*) which were also found on the human skin (Parola et al. 2015). In-depth genetic analyses showed a unique plasmid (pLbaR) found in *R. felis* strains from booklice which may mediate a specialization to this host making *R. felis* an obligate mutualist of *L. bostrychophila* (Gillespie et al. 2014). The lack of this plasmid, together with other genetic changes, may keep *R. felis* a facultative parasite of fleas and other arthropods and a putative pathogen for the hosts they feed on. The latest discovery was that *Anopheles gambiae* mosquitoes were found to be a competent vector for *R. felis* (Dieme et al. 2015). Although *R. felis* has previously been detected in other *Anopheles*, *Aedes*, and one *Monsonia* mosquito species, this was initially thought to be due to remnants of a previous blood meal (Dieme et al. 2015). The host- and vector-promiscuous nature of *R. felis* contributes to its global emergence and future work has to focus on the genetic mechanisms and plasmid configuration that make *R. felis* so successful.

4. Concluding Remarks

In the emerging field of rickettsioses, many aspects need more research in order to gain a better understanding of how to combat spotted fever rickettsioses. Two particular areas of research should be addressed. *Firstly*, there is a need for better (and discriminatory) laboratory methods, a greater awareness by physicians so that they consider *Rickettsia* spp. to be a possible diagnosis and thus ask for appropriate diagnostic testing. Notification of such cases should be mandatory and may lead to active surveillance systems in regions with particular rickettsioses. Once more public health related aspects have improved, recommendations would help to reduce incidence rates. *Secondly*, we have to learn a great deal about the ecology of almost all *Rickettsia* spp. Examples given in this short article demonstrate that we lack a detailed understanding of the mechanisms or circumstances driving the emergence of most of the *Rickettsia* spp. known today, not to mention the many Candidatus *Rickettsia* spp. that we have not been able to cultivate *in vitro* and for which we currently only have nucleotide sequences from, e.g., tick samples. This area of research would also greatly benefit from improvements in the laboratory methods mentioned above.

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Tick-Borne Viruses

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Abstract

Ticks are among the most important vectors of pathogens. They can cause diseases by producing toxins and allergic reactions. Ticks can transmit metazoa, protozoa, bacteria, rickettsiae and viruses. The review discusses ecology and epidemiology of tick-borne viruses, especially the tick-borne encephalitis virus. The natural transmission cycle of tick-borne viruses is complex. It is basically understood although a lot of details are unknown, for instance many of the ecological, climatic, geological, biological and virological factors of the natural transmission cycles. However, understanding the transmission cycles is a pre-requisite for improving surveillance and forecasting the emergence of tick-borne viruses in a changing world.

Zusammenfassung

Zecken gehören zu den wichtigsten Vektoren für Pathogene. Sie können Erkrankungen durch die Bildung von Toxinen oder das Hervorrufen allergischer Reaktionen auslösen. Zecken können Metazoen, Protozoen, Bakterien, Rickettsien und Viren übertragen. Der Beitrag diskutiert die Ökologie und Epidemiologie durch Zecken übertragener Viren, vor allem des Zeckenenzephalitis-Virus. Der natürliche Übertragungszyklus von Zeckenviren ist komplex. Er ist in den Grundlagen verstanden, obwohl noch eine große Anzahl von Details unbekannt ist, z. B. viele der ökologischen, klimatischen, geologischen, biologischen und virologischen Faktoren der natürlichen Übertragungszyklen. Das Verständnis der Übertragungszyklen ist aber eine Voraussetzung der Überwachung und Voraussage des Auftretens von zeckenübertragenen Viren in einer sich verändernden Welt.

1. Introduction

Ticks are among the most important vectors of pathogens. Ticks can cause diseases by producing toxins and allergic reactions to their saliva, and they can transmit metazoa, protozoa, bacteria, rickettsiae and viruses. A total of more than 170 viruses from six virus families are transmitted by ticks: Flaviviridae, Bunyaviridae, Rhabdoviridae, Orthomyxoviridae, Reoviridae and Asfiviridae. All tick-borne viruses are classified as arboviruses. This ecological definition means that these viruses are exclusively, or to a large extent, transmitted as part of a natural transmission cycle of arthropods (ticks) and vertebrates. Humans are a dead-end host for all tick-borne viruses, which means that humans play no role in maintaining the natural transmission cycle (*WHO* 1967).

2. Distribution of Tick-borne Viruses

Tick-borne viruses are distributed all over the world, however, they are found more frequently in the northern hemisphere than in the southern. This may be because the search for tick-borne viruses is more intensive in some countries in the northern hemisphere. New tick-borne

viruses have been identified recently, e.g. the Heartland virus and the Bourbon virus in North America, and the Huayiangshan virus in China (Savage et al. 2013, Kosoy et al. 2015, Yu et al. 2011). Currently more than 25 tick-borne viruses are known to cause diseases in humans. Clinical symptoms caused by tick-borne viruses include fever, meningitis, encephalitis and hemorrhagic fever. They include some of the most dangerous and fatal viral diseases currently known, e.g. Crimean-Congo Hemorrhagic Fever (CCHF), the Huayiangshan virus, Powassan virus and Alkhumra virus.

3. Transmission Cycle of Tick-borne Viruses

Some mosquito-borne viruses, like the yellow fever virus, dengue virus or zika virus, circulate in nature in a (proposed) sylvatic cycle and in an epidemiologically more important urban cycle. Because tick-borne viruses cannot be transmitted back to ticks by viraemic humans, no urban cycles for tick-borne viruses are known to exist. In contrast, for some tick-borne arboviruses, e.g. CCHF, the virus can be transmitted directly from human to human and, therefore, nosocomial infections and micro-epidemics occur in hospitals and within families.

The natural transmission cycle of tick-borne viruses is complex. It is basically understood although a lot of details are unknown or have yet to be understood. The three main components of the transmission cycle are the virus, the vector and the vertebrate host. Many factors contribute to the natural transmission cycle. These include viral factors as well as the geological factors, climatic factors, and ecological factors of the natural hosts and the ticks (Fig. 1).

For most tick-borne viruses, little is known about this complex biocenosis, and therefore the transmission dynamics of the virus among the tick vectors and the vertebrate hosts are only poorly understood.

4. Example: Tick-borne Encephalitis Virus

The tick-borne encephalitis virus is the medically most important tick-borne arbovirus in Europe and Asia. *Ixodes ricinus*, the sheep tick, is its most important vector and reservoir in Europe. The TBE virus is a member of the tick-borne group of flaviviruses. It circulates among ticks and small rodents (*Apodemus* spp., *Clethrionomys* spp., *Microtus* spp.). The virus is transmitted by the infected tick to the rodent during the blood feeding and is also taken up by uninfected ticks from small infected mammals. There are two proposed mechanisms of transmission from the vertebrate host to the tick: viraemic transmission and co-feeding of infected and non-infected ticks at the same time close together on one animal. Both proposed mechanisms seem to play a role in the transmission cycle (Fig. 2).

Since the detection of the co-feeding mechanism it is now generally accepted that this mechanism is what circulates and maintains the TBE virus. However, it is yet to be proven that co-feeding plays an important role in the transmission of the TBE virus. Several older field studies from Austria were able to show that viraemic rodents may be sufficient in maintaining the natural transmission cycle (Pretzmann et al. 1963, 1964).

Currently the spatial structure of the foci of the TBE virus is unclear. Also no studies on the ecological composition of the natural foci of the TBE virus exist. Therefore, it is unclear whether the natural foci of TBE may be established at any place with sufficient tick and small

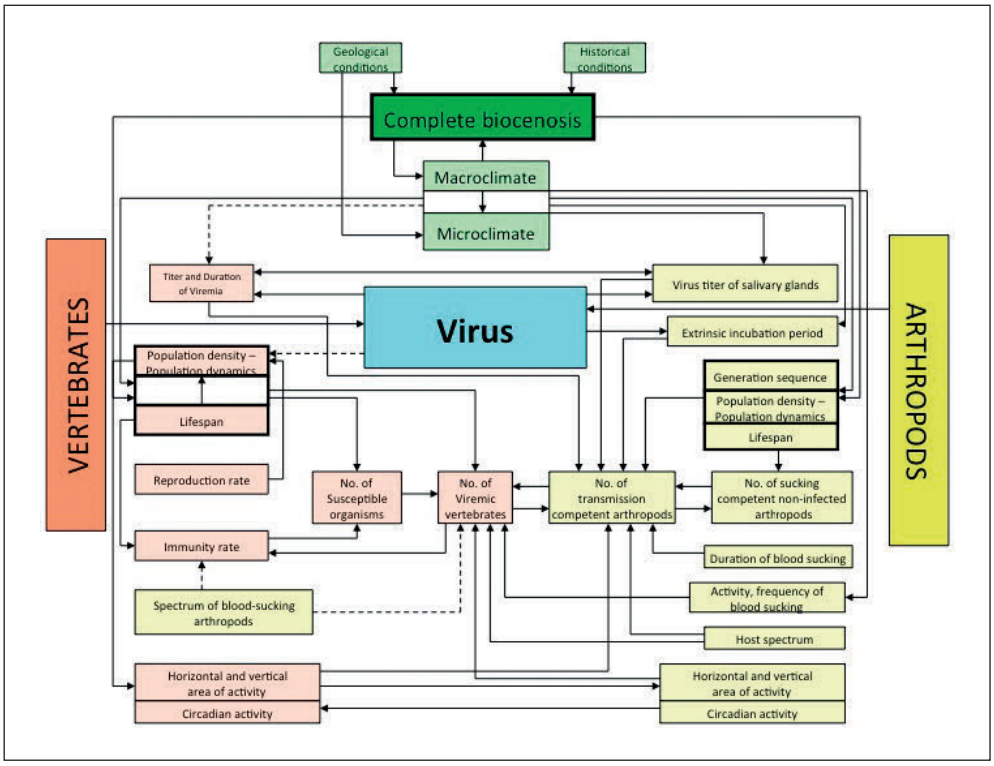

Fig. 1 Main factors for the transmission of tick-borne viruses in a biocenosis. Modified after Aspöck 1970.

mammal populations or whether other ecological conditions are needed to establish a natural TBE virus focus in addition to the pure number of vectors and hosts. Recently, information on the patchy distribution of TBE foci in Southern Germany may favour the second hypothesis whereby additional, currently unknown ecological and/or climatic factors may contribute to the establishment of the natural foci of TBE.

In our own studies on natural foci, conducted over more than seven years in different landscape structures, the prevalence of the TBE virus in ticks in one particular natural focus varied from 0.7 % to more than 8 % in some years. Also, the host feeding patterns of different tick stages seems to impact the transmission cycle. In a natural lowland cycle, it seems that nymphs are mainly infected, while in a natural cycle in the Alps (Zillertal) only adult stages were found to be infected with the TBE virus. This might mean that there is no single universal natural cycle for the TBE virus and that, possibly, the natural TBE virus cycle can be adapted to the particular landscape and ecological conditions respectively.

There is a lot of speculation about the influence of short and long-term weather and climate on the transmission cycle of TBE viruses. In the past few years a number of different models were introduced using defined assumptions and developing models up to 2050 and later. These models show a clear change in the distribution of tick species and therefore also in the natural transmission cycles of the TBE virus (Randolph et al. 2000). There is a lot of speculation and work showing that there has been a shift in latitude and altitude.

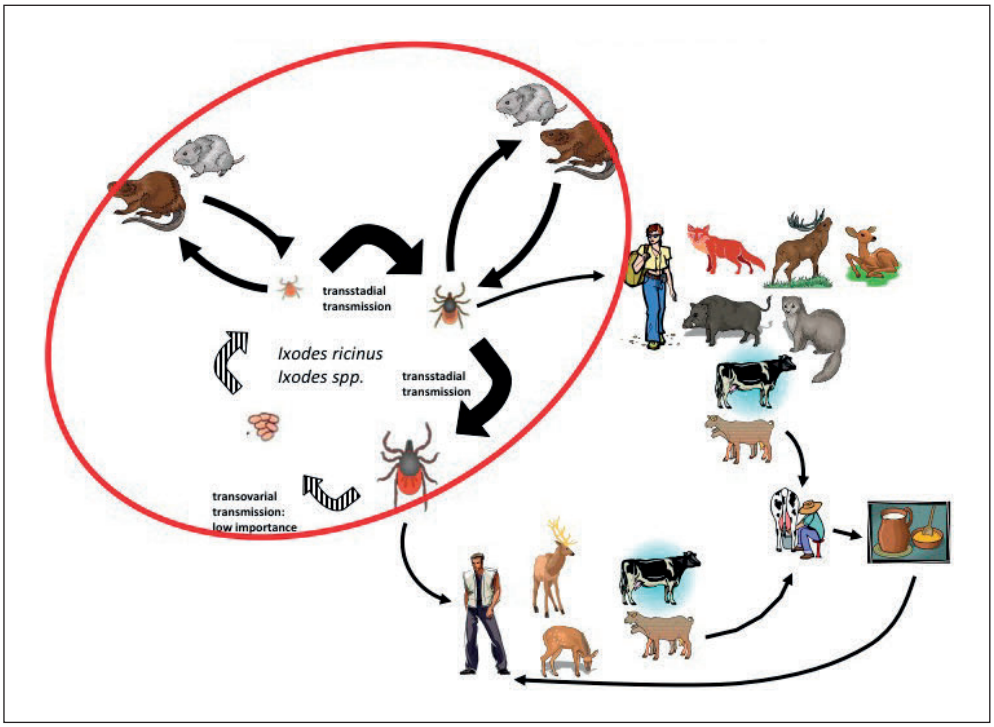

Fig. 2 The proposed simplified natural transmission cycle of TBE virus; an example of a tick-borne arbovirus

Russian scientists present the only convincing data so far on a shift in latitude. They were able to show that epidemiologically important human TBE was occurring farther north, in the District of Archangelsk (Tokarevic et al. 2011). In contrast, data from Sweden show that the area of distribution of *Ixodes ricinus* is spreading northward, however, this spread has no impact on the increasing number of human cases in Sweden from an epidemiological perspective. This increase seems to be mainly due to a spread of human TBE cases from the eastern coast to inland and not due to a spread to more northern areas (Jaenson et al. 2012). Czech reports that the TBE virus is climbing to higher altitudes currently cannot be verified for permanent natural cycles (Daniel et al. 2009). Other spectacular findings, like the oral infection of a group of people through goat milk ("Bianca" in the Federal State of Vorarlberg in Austria at an altitude of around 1,500 m) cannot disguise the fact that these are unique events and, so far, the TBE virus has not been detected in the location of infection after such events.

One aspect, which so far has not received sufficient attention, is the question of whether the higher temperatures could select specific TBE virus mutants with differing pathogenicity for humans and animals. Another question, which has not yet to be addressed, is, whether higher temperatures influence the amount of TBE virus in the tick and, therefore, also the amount of infectious virus that is transmitted (Elvång et al. 2011). It is not yet clear whether tick bites from different tick stages may cause different clinical forms of severity of the disease due to different amounts of virus injected during the blood meal. Studies in Scandinavia

show that the duration of the blood meal might also have some influence on the amount of transmitted virus and therefore possibly on the severity of the clinical outcome (Jaenson, personal communication 2015).

Many questions remain unresolved in terms of the vectors of tick-borne diseases. How does the microbiome in a tick influence the tick's potential to transmit the TBE virus (Qiu et al. 2014)? It is also unclear whether different sub-populations of ticks have different sensitivities to infection with the TBE virus (or other tick-borne viruses) or whether they have different transmission capabilities for the respective viruses or even virus strains of a virus (Roed et al. 2006). The genetic comparison of TBE viruses from different natural foci of the TBE virus shows that each TBE focus has its own virus differing by up to 3 % in its sequence from TBE viruses from other natural foci. The virus sub-population seems to adapt to the respective focus and its ecological conditions. So far it is unclear which factors contribute to the adaptation of TBE virus strains.

Furthermore, the role of the vertebrate hosts is far from resolved. The current hypothesis is that only different rodents and insectivora, as the maintaining vertebrate hosts, can contribute to the natural transmission cycle. It is so far unclear what the role of the particular species is in maintaining the virus within the life cycle. Experimental data show that the yellow-necked mouse (*Apodemus flavicollis*) might develop higher and longer lasting viremias than bank voles (*Clethrionomys glareolus*). It is not clear whether this might have any effect on the TBE virus itself. For example, could a TBE virus strain coming from a bank vole be more or less pathogenic than a virus strain taken up by a tick with a blood meal from the yellow-necked mouse? What is the importance of the overwintering of the TBE virus in the brain of *Microtus arvalis* as described in a Scandinavian group (Tonteri et al. 2011) and also found in Germany (unpublished observation)?

Statistical data show that human hantavirus infections and TBE infections have an inverse trend in Germany. This could mean that the incidence of TBE virus, and therefore the risk of infection for humans, might be much higher than previously thought, depending on the populations of rodents. In order to better understand the natural cycle of TBE and to estimate the risk of infection, the question might be: Are we looking in the right organism? Is the vector more important or is the natural vertebrate host more important for the total amount of virus circulating in a natural cycle?

Finally, there are many unanswered questions regarding the spread of the TBE virus. It is commonly accepted that the TBE virus evolved from an ancient tick-borne flavivirus in the Siberian taiga thousands of years ago (Heinze et al. 2012). Several thousand years ago a subgroup of this ancient flavivirus started to migrate westward and finally reached the current area of distribution. There seems to be a tendency towards westward migration with new human cases in the western part of Germany (North Rhine-Westphalia) and initial evidence of TBE seropositive cattle in Belgium (Roelandt et al. 2014). The way the virus is spread is unclear. While Russian researchers find evidence of an anthropogenic spread of TBE virus (corridors built for railways and highways), such evidence does not exist at the moment for Central Europe (Kovalev et al. 2009). One possible explanation is that the TBE virus is transported through migrating birds. There is evidence that this might happen in Scandinavia as the virus is found on islands where birds are most probably the only way for the virus to be transported (Jääskeläinen, personal communication, 2015). The available genetic information on more than 200 TBE virus strains from Central Europe also favours the distribution of TBE virus by migrating birds.

However, the question now is how migrating birds might transport the TBE virus from one focus to a new area. Several options might be discussed. Is it only a passive transport of infected ticks as described by several authors (Waldenström et al. 2007)? Another option would be that birds remain viraemic for some time and could infect blood-sucking ticks in a new area. Also, the question of co-feeding on birds has yet to be studied. Birds (the grouse, *Lagopus lagopus*) play a major role in the transmission cycle of the Louping ill virus, the flavivirus most closely related to the TBE virus, however, the role of birds for the TBE virus is not clear.

5. Conclusion

Summarizing the available data, we have, at best, a rough overview of the natural transmission cycle of the TBE virus and also of other tick-borne viruses. Many of the geological, ecological, climatic, biological and virological factors of the natural transmission cycles are unknown, and many of the questions regarding the ecology and epidemiology of the transmission of tick-borne viruses have yet to be asked and, more importantly, answered. However, understanding the transmission cycles is a pre-requisite for improving surveillance and forecasting the emergence of tick-borne viruses in a changing world.

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Die Bedeutung von Bildung in einer Dienstleistungs- und Wissensgesellschaft.

Welchen Bildungsauftrag hat die Universität?

Symposium veranstaltet von der
Deutschen Akademie der Naturforscher Leopoldina – Nationale Akademie der Wissenschaften, der Carl von Ossietzky-Universität Oldenburg und der VolkswagenStiftung
am 19. Mai 2015 im Tagungszentrum Schloss Herrenhausen, Hannover

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Wie sieht die moderne Universität des 21. Jahrhunderts aus? Im Spannungsfeld des Bologna-Prozesses und der Exzellenzinitiative, die bereits starke Umbrüche in den deutschen Universitäten bewirkten und im Rahmen von vertikalen und funktionalen Differenzierungen die Voraussetzungen für die Entwicklung neuer Profile in Forschung und Lehre geschaffen haben, steht der Bildungsauftrag von Universitäten zur Diskussion.

Der tiefgreifende Veränderungsprozess von der Universität als Bildungsstätte einer kleinen Elite hin zur Öffnung der Hochschulen für fast jedes Gesellschaftsmitglied ist, bei gleichzeitiger explosionsartiger Zunahme der Komplexität einer global agierenden Gesellschaft, der Ausgangspunkt aktueller Entwicklungen im Hochschulsystem. Einem Diskurs zum Bildungsbegriff und der Neuinterpretation des klassischen Bildungsverständnisses folgen Überlegungen zu den Herausforderungen der Digitalisierung als einem möglichen zukünftigen Haupttreiber des Wandels im Bildungssystem sowie ein Gedankenaustausch über die Wissenschaftskultur und notwendige Freiräume für neue Lehr- und Lernformate.

Wissenschaftliche Verlagsgesellschaft Stuttgart

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Wolbachia Biocontrol of Dengue and Japanese Encephalitis

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Abstract

Arboviral diseases such as dengue and Japanese encephalitis cause significant human morbidity and mortality. Novel mosquito control strategies are needed, as current methods are ineffective or logistically demanding to implement. Significant advancements have recently been made using the bacterial endosymbiont *Wolbachia* for mosquito biocontrol. Transinfection of novel *Wolbachia* strains from *Drosophila* fruit flies into *Aedes* mosquitoes has resulted in dengue virus (DENV) refractory mosquito lines. *Wolbachia* strains have now been established in wild *Aedes aegypti* populations through open releases in dengue-endemic countries including Indonesia and Brazil. Japanese encephalitis is predominantly transmitted by *Culex tritaeniorhynchus* mosquitoes in Asia. Japanese encephalitis virus (JEV) exists in an enzootic transmission cycle with mosquitoes transmitting the virus between birds as reservoir hosts and pigs as amplifying hosts. Irrigated rice fields provide an ideal breeding ground for mosquitoes and attract migratory birds, maintaining the transmission. Current vector control includes intermittent irrigation of rice fields and space spraying of insecticides during outbreaks. However, *Cx. tritaeniorhynchus* is subject to heavy pesticide exposure in rice fields so insecticide resistance has developed. As JEV and DENV are closely related Flaviviruses, the successful establishment of *Wolbachia* strains in *Cx. tritaeniorhynchus* is predicted to significantly impact JEV transmission. The release of 'JEV-refractory' *Cx. tritaeniorhynchus* would likely provide inhibition of transmission for the enzootic transmission cycles in addition to any spillover transmission to humans. *Wolbachia*-based biocontrol may provide a safe, sustainable, environmentally friendly and effective long-term control option for arboviral diseases in which outbreaks are likely to increase in the future.

Zusammenfassung

Arbovirus-Erkrankungen, wie Denguefieber und Japanische Enzephalitis, verursachen erhebliche menschliche Morbidität und Mortalität. Neue Bekämpfungsstrategien für Mücken werden benötigt, da die derzeitigen Methoden ineffektiv oder logistisch schwer umsetzbar sind. Deutliche Verbesserungen sind kürzlich durch den Einsatz des bakteriellen Endosymbionten *Wolbachia* für die biologische Bekämpfung von Mücken erzielt worden. Transfektion von neuen *Wolbachia*-Stämmen von *Drosophila*-Fruchtfliegen in *Aedes*-Mücken hat zu gegen das Dengue-Virus (DENV) widerstandsfähigen Mückenstämmen geführt. *Wolbachia*-Stämme sind nun in wilden Beständen von *Aedes aegypti* durch offene Freilassung in Denguefieber endemischen Ländern, einschließlich Indonesien und Brasilien, angesiedelt worden. Japanische Enzephalitis wird hauptsächlich durch *Culex tritaeniorhynchus*-Mücken in Asien übertragen. Das Virus für Japanische Enzephalitis (JEV) existiert in einem enzootischen Übertragungszyklus mit Mücken, die das Virus zwischen Vögeln als Reservoirwirt und Schweinen als verstärkende Wirte übertragen. Bewässerte Reisfelder stellen eine ideale Brutstätte für Mücken dar und ziehen Wandervögel an, die die Übertragung aufrechterhalten. Derzeitige Vektorbekämpfung umfasst die periodische Bewässerung von Reisfeldern und das Besprühen der Fläche mit Insektenvertilgungsmitteln während der Ausbrüche. Da *Cx. tritaeniorhynchus* einem umfassenden Einsatz von Schädlingsbekämpfungsmitteln in Reisfeldern ausgesetzt ist, hat sich Resistenz gegen Schädlingsbekämpfungsmittel entwickelt. Weil JEV und DENV eng verbundene Flaviviren sind, wird prognostiziert, dass die erfolgreiche Ansiedlung von *Wolbachia*-Stämmen in *Cx. tritaeniorhynchus* erhebliche Auswirkungen auf die JEV-Übertragung hat. Die Freisetzung von ‚JEV-resistenten' *Cx. tritaeniorhynchus* würde wahrscheinlich die Unterbindung der Übertragung für die enzootischen Übertragungszyklen zusätzlich zu etwaigen Übertragungen auf Menschen bringen. *Wolbachia*-basierende biologische Bekämpfung kann eine sichere, nachhaltige, umweltfreundliche und effektive langfristige Bekämpfungsmöglichkeit für Arbovirus-Erkrankungen bieten, deren Ausbrüche wahrscheinlich in der Zukunft ansteigen werden.

1. Introduction

Arboviruses are transmitted principally by mosquitoes and ticks and cause human diseases such as dengue fever and encephalitis. Although there are over 80 different arboviruses that are known to cause human morbidity and mortality, dengue virus (DENV) infection results in the largest number of human cases. DENV is classified within the genus *Flavivirus* that also includes West Nile virus (WNV), yellow fever virus (YFV) and Japanese encephalitis virus (JEV). These medically important flaviviruses are predominantly transmitted by Culicine mosquitoes. Estimates of annual global DENV infections range from 100–390 million per year (Bhatt et al. 2013). Dengue is classified as a 're-emerging' disease partly because the geographical range of the mosquito vectors, *Aedes aegypti* and *Aedes albopictus*, is increasing due to factors including globalization and climate change (Kilpatrick and Randolph 2012).

Dengue outbreaks often take place in poor, tropical countries that lack the financial ability to prevent transmission (Guzman et al. 2010). As there are currently no vaccines or drugs available for dengue, mosquito vector control has been the only way to prevent transmission. Limited success has been achieved using mosquito control for dengue epidemics. One of the issues faced in dengue control programmes is that *Ae. aegypti* are anthropophilic (preferring to blood feed on humans) and are adapted to living in urban areas in close contact with large human populations. *Ae. aegypti* is also invasive and lays eggs in artificial containers (e.g. water tanks and unused tires), so widespread larval source reduction is often unrealistic. The use of insecticides, such as DDT and malathion, occurs during epidemics through outdoor spraying to kill adult mosquitoes.

2. Novel Mosquito Control Strategies

One strategy for dengue control is to replace the wild mosquito population with mosquitoes that are unable to transmit DENV. Although genetically modified mosquitoes that are 'refractory' to DENV transmission have been under development, the effects of GM on mosquito fitness, important if releasing to compete with wild mosquitoes, has prevented any major release programmes. An alternative approach is the use of endosymbiotic bacteria to prevent DENV from replicating within *Aedes* mosquitoes. Recently, *Wolbachia* biocontrol has shown the potential to be a control method that is environmentally benign, safe to humans and potentially economical. The 'eliminate dengue' project (www.eliminatedengue.com) has shown that *Wolbachia* bacteria can prevent DENV transmission in mosquitoes and can be established in *Ae. aegypti* wild mosquito populations.

3. *Wolbachia* and their Effects on Insect Hosts

Wolbachia reside naturally in more than 65 % of insect species (Hilgenboecker et al. 2008). These endosymbiotic bacteria manipulate host reproduction in order to enhance their own transmission through an insect population (Werren et al. 2008). In mosquitoes infected with *Wolbachia*, a reproductive phenotype termed cytoplasmic incompatibility (CI) occurs. CI results in the generation of unviable progeny when uninfected females mate with *Wolbachia*-in-

fected males. However, *Wolbachia*-infected females can produce viable offspring when they mate with both infected and uninfected males. This results in a reproductive advantage for *Wolbachia*-infected females over uninfected females. CI results in the maternally transmitted *Wolbachia* efficiently invading insect populations.

Although *Wolbachia* is present in some mosquitoes, such as *Culex pipiens* and *Aedes albopictus*, it is absent from some most major vectors including *Anopheles* spp. (transmit malaria), *Aedes aegypti* and *Culex tritaeniorhynchus* (principal vector of Japanese encephalitis virus). The first use of *Wolbachia* for mosquito control relied on the release of *Wolbachia*-infected males CI to 'crash' *Culex* mosquito populations (Laven 1967). The mating competitiveness of these released males prevented successful widespread suppression of mosquito populations. In the late 1990s, the discovery of a mutant *w*MelPop strain of *Wolbachia* in *Drosophila melanogaster* fruit flies, which dramatically lowered the lifespan of its host (Min and Benzer 1997), led to the idea that *Wolbachia* could reduce pathogen transmission. Mosquito-borne pathogens such as DENV require a significant extrinsic incubation period (EIP) in the female mosquito before the pathogen can migrate to the salivary glands and be transmitted to another vertebrate host. Therefore, mosquito longevity is an important factor in the intensity of pathogen transmission in human populations. In theory, the *w*MelPop strain could be used to shorten the longevity of adult female mosquitoes. The induction of CI would facilitate *w*MelPop to invade mosquito populations. This combination of phenotypic effects was predicted to have a large impact on DENV transmission by manipulation of the population age structure of the vector population (Brownstein et al. 2003).

Recent work in *Drosophila* fruit flies has provided further evidence that *Wolbachia* could be used to decrease mosquito-borne diseases. *Wolbachia* was shown to protect their native *Drosophila* hosts against infection by pathogenic RNA viruses (Teixeira et al. 2008, Hedges et al. 2008). *Wolbachia* strains closely related to the *w*Mel strain (including *w*MelPop) significantly reduce the density of a range of viruses in flies, which delays insect mortality (Osborne et al. 2009). In order to use *Wolbachia* for dengue biocontrol, the first step was to transinfect the major mosquito vector, *Ae. aegypti*, with strains from naturally infected insects such as *Drosophila*. The *w*AlbB strain was initially transferred into *Ae. aegypti* using embryo cytoplasm transfer from *Ae. albopictus* (Xi et al. 2005). Subsequently, the *Drosophila Wolbachia* strains *w*MelPop and *w*Mel were transinfected after adaptation to mosquito cell lines (McKeniman et al. 2009, Walker et al. 2011).

4. *Wolbachia* Prevents Transmission of DENV in Mosquitoes

Drosophila Wolbachia strains *w*Mel and *w*MelPop significantly reduce the vector competence of *Ae. aegypti* for DENV (Walker et al. 2011). High levels of bacteria stably infect tissues, such as the Salivary glands, that play a crucial role in DENV development within mosquitoes. The critical determinant of whether *Wolbachia* reduces the DENV vector competence in *Ae. aegypti* is the presence of infectious virus in mosquito saliva. Under laboratory conditions, complete blockage of DENV transmission was observed for both *w*Mel and *w*MelPop infected mosquitoes (Walker et al. 2011).

Wolbachia-based biocontrol of dengue would also require *Wolbachia* to successfully invade wild *Ae. aegypti* mosquito populations. *Wolbachia*-infected females need to pass on

bacteria to their offspring at a high frequency. In *Ae. aegypti*, transinfected *Wolbachia* strains show maternal transmission rates close to 100 % and induce high levels of CI (Walker et al. 2011, McMeniman et al. 2009, Xi et al. 2005). These phenotypes should allow rapid invasion of uninfected mosquito populations, as long as fitness costs are minimum. *Ae. aegypti* infected with the *w*MelPop strain have greater fitness costs than mosquitoes infected with the *w*Mel strain. For example, adult lifespan is reduced by 50 % by the *w*MelPop strain (McMeniman et al. 2009). Although this life-shortening effect would likely reduce DENV transmission, it is also predicted to inhibit the invasive potential of this virulent *Wolbachia* strain in wild mosquito populations. The avirulent *w*Mel strain only reduces adult lifespan by approximately 10 % (Walker et al. 2011), which could potentially remove older female mosquitoes but without significant effects on the ability of *w*Mel to invade mosquito populations. Another major measure of mosquito fitness is fecundity (number of eggs per female mosquito). The fecundity of *w*MelPop-infected female mosquitoes under semi-field conditions was reduced by ~ 60 % compared to uninfected wildtype and *w*Mel-infected mosquitoes (Walker et al. 2011). However, the fitness costs induced by infection with the *w*MelPop strain could actually be utilized as a control mechanism through mosquito population suppression (McMeniman and O'Neill 2010).

5. *Wolbachia* Invasion of Wild Mosquito Populations

Wolbachia invasion of wild *Ae. aegypti* mosquito populations will depend on a balance between negative selection imposed by mosquito fitness costs and positive selection associated with induction of the CI phenotype. The potential of *Drosophila Wolbachia* strains *w*Mel and *w*MelPop to invade *Ae. aegypti* mosquito populations were tested in a semi-field facility that replicated the natural habitat in north Queensland, Australia. Releasing *w*Mel-infected mosquitoes into the cages resulted in a rapid increase in frequency and this avirulent strain reached fixation in one cage within 30 days and within 80 days in the second cage (Walker et al. 2011). As predicted, the virulent *w*MelPop strain increased at a significantly slower rate, reaching fixation in the first cage after 40 days and reached approximately 80 % after 80 days in the second cage. Modeling the invasion of *Wolbachia* strains was very similar predicting a more rapid invasion rate for *w*Mel compared to *w*MelPop due to lower fitness costs (Walker et al. 2011).

Ae. aegypti mosquitoes infected with the *w*Mel strain were released into wild mosquito populations in two locations near Cairns in north Queensland, Australia (Hoffmann et al. 2011). Adults were released over a 10-week period during the wet season and the *w*Mel strain reached near-fixation (100 % of the population infected) in a few months following releases. Modeling also indicated that *w*Mel-infected *Ae. aegypti* mosquitoes were subject to relatively small fitness costs (Hoffmann et al. 2011). Preliminary field trials in Australia indicated that *Wolbachia* can be released and established in wild *Ae. aegypti* mosquito populations. Extensive community engagement in the release areas took place to determine local attitudes and levels of knowledge about dengue and mosquitoes. Experiments were performed to demonstrate that *Wolbachia* does not easily enter the food chain *via* organisms that prey on mosquitoes or infect non-target insects (Popovici et al. 2010). The success of these preliminary trials in Australia has led to further releases in dengue endemic countries such as Indonesia, Vietnam and Brazil (www.eliminatedengue.com).

6. Japanese Encephalitis

Japanese encephalitis (JE) is endemic in large parts of Asia and the Pacific, with an estimated three billion people at risk (Solomon 2006). JE is predominantly a disease of children in Eastern and Southern Asia with an annual incidence estimated to be in the range of 50,000–175,000 cases with 25–30 % cases resulting in mortality. Japanese encephalitis virus (JEV) is a flavivirus transmitted primarily by *Cx. tritaeniorhynchus* mosquitoes. Although first isolated in Japan in 1935, JEV appears to have evolved from its ancestral form to the present genotypic forms in South East Asia, over a relatively short period. Phylogenetic studies have classified JEV into five geographically and epidemiologically distinct genotypes: GI to GV (Solomon et al. 2003). Figure 1*A* highlights the geographical JEV transmission zones for the five genotypes.

JEV transmission occurs in an enzootic transmission cycle among mosquitoes and domestic pigs with the reservoir sylvatic bird hosts being primarily water birds from the *Ardeidae* family, including cattle egrets and pond herons (Solomon 2006). As pigs act as amplifying hosts, pork production is an important risk factor for human epidemics. Irrigated rice fields in Asia provide the optimal breeding environments for *Cx. tritaeniorhynchus* and attract migratory birds, maintaining sylvatic transmission. The JEV transmission zone spans the China-Russia border in the north to northern Australia in the south, and from the Western Pacific islands in the east to the India-Pakistan border region in the west (Endy and Nisalak 2002). JE epidemiology falls into two distinct patterns depending on the climate. In temperate northern areas of Asia, seasonal outbreaks occur during summer months with increased temperatures and rainfall. In tropical and subtropical countries in South East Asia, sporadic JE cases occur throughout the year with most cases occurring during the rainy season. JE incidence in Northern Asia (Japan and Korea) has declined mainly because of an increase in living standards and vaccination programmes (Erlanger et al. 2009). In contrast, JE cases are increasing in developing countries of South/South East Asia such as Bangladesh, Pakistan and Indonesia due to increased population growth, intensified rice cultivation and pig rearing. JEV also spread to the far north of Australia in the late 1990s (Hanna et al. 1996) highlighting the potential for an increase in the geographical transmission zone. Transmission of JEV is predominantly in rural areas where the presence of mosquito vectors breeding in rice fields is in close proximity to pig rearing. However, recent studies have shown that JEV is circulating in mosquitoes present in urban areas of South Vietnam (Lindahl et al. 2013) suggesting the possibility that JEV transmission may occur in urban areas with a much greater human population density (likely to lead to significantly greater epidemics in the future).

7. Control of JEV Mosquito Vectors

Mosquito vector control strategies represent a method more likely to eradicate JE, as mosquitoes play a key role in maintaining transmission in reservoir bird hosts in the sylvatic cycle. The principle mosquito vector of JEV, *Cx. tritaeniorhynchus*, has a wide distribution including parts of Africa, the Middle East, and Southern Europe in addition to the JE-endemic areas of Asia. The presence of this species was recently reported in rice fields of Western Greece (Lytra and Emmanouel 2014) demonstrating the potential risk of JEV transmission in non-endemic areas. Vector control has mainly focused on environmental management of

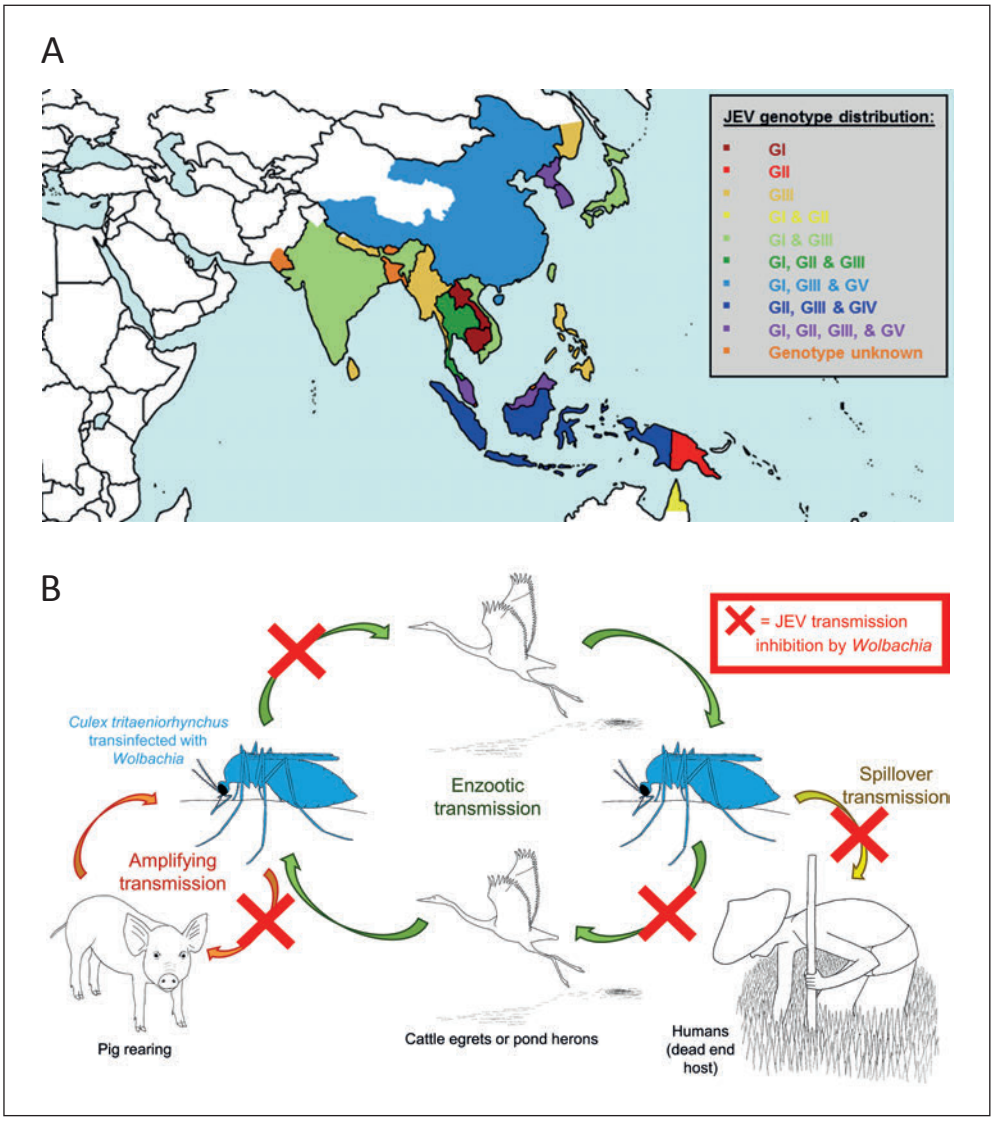

Fig. 1 JEV transmission and the potential inhibition using *Wolbachia*-based biocontrol strategies. (*A*) Genotypes of JEV (I–V) are shown in regions where the genotype (G) has been confirmed to be responsible for JE epidemics. (*B*) The JEV transmission cycle could be interrupted at various points using *Wolbachia* bacteria in which JEV-refractory mosquitoes are unable to maintain the enzootic transmission cycles in addition to preventing transmission to humans (adapted from Jeffries and Walker 2015 with permission).

rice fields. Alternative wetting and drying of rice fields (intermittent irrigation) has shown moderate success in reducing mosquito numbers (Keiser et al. 2005). Logistical difficulties with intermittent irrigation, including the need to apply this method to all rice fields over large areas with inadequate infrastructure (Rajendran et al. 1995), prevents this being widely used. The use of insecticides has also been undertaken using space spraying to target adult

mosquitoes during outbreaks of JE in densely populated areas. However, the prolonged use of pesticides in rice fields has led to significant levels of insecticide resistance building up in mosquitoes (Karunaratne and Hemingway 2000). Logistical difficulties in employing large-scale insecticide treatment of rice fields, often in isolated rural villages, are also problematic. Indoor residual spraying is also ineffective as *Cx. tritaeniorhynchus* is largely exophilic, resting outdoors (Reisen and Milby 1986).

8. Potential *Wolbachia* Biocontrol of JE

Wolbachia transinfection of *Cx. tritaeniorhynchus* could provide the basis for an environmentally friendly and cost-effective biocontrol strategy which could significantly impact JEV transmission (Jeffries and Walker 2015). As JEV is part of the same genus as DENV (*Flavivirus*), *Wolbachia* strains (particularly high density strains from *Drosophila* fruit flies) would likely provide similar inhibitory effects in transinfected mosquitoes. In laboratory experiments, *Wolbachia* results in strong inhibition of multiple DENV serotypes with similar efficacy (Frentiu et al. 2014). *Wolbachia* in *Ae. aegypti* has also been shown to reduce the transmission of chikungunya virus (Moreira et al. 2009) and yellow fever virus (van den Hurk et al. 2012). Several additional studies have also shown that *Wolbachia* has a wide range of inhibitory effects on mosquito-borne human parasites. For example, *Wolbachia* inhibits malaria *Plasmodium* parasites in *Anopheles stephensi* mosquitoes (Bian et al. 2013). Although the mechanism underlying viral interference is not fully known, the density of *Wolbachia* strains in particular insect tissues influences the extent of viral interference (Walker et al. 2011). *Drosophila Wolbachia* strains grow to high densities in their native and transinfected hosts and provide strong inhibition of both insect viruses in *Drosophila* (Hedges et al. 2008) and DENV in mosquitoes (Walker et al. 2011). Therefore, successful establishment of *Drosophila Wolbachia* strains in *Cx. tritaeniorhynchus* would likely have a significant impact on JEV transmission.

Another important consideration is that *Cx. tritaeniorhynchus* does not contain a natural *Wolbachia* infection (Tiawsirisup et al. 2008) and is responsible for the majority of JEV transmission. Secondary, JEV vectors in Asia (such as *Cx. gelidus*) and *Cx. annulirostris* in northern Australia are responsible for limited JEV transmission (Hall-Mendelin et al. 2012). Therefore, the replacement of wild *Cx. tritaeniorhynchus* with JEV-refractory populations would likely have significant impacts on JEV transmission. Interestingly, other species of *Culex* mosquitoes responsible for human disease transmission are infected with native strains of *Wolbachia.* The difference in arbovirus vector competence of members of the *Culex pipiens* complex may be due to the presence of these resident *Wolbachia* strains. For example, *Cx. quinquefasciatus* is infected with the *w*Pip strain of *Wolbachia* and is considered less susceptible to WNV infection than *Cx. tarsalis* (Goddard et al. 2002), which is not infected with *Wolbachia*. However, as resident *Wolbachia* infections in mosquitoes do not reach high densities in key tissues such as the Salivary glands, transinfected *Wolbachia* strains reduce arboviral transmission to a much greater extent.

As shown in Figure 1*B*, the transmission cycle of JEV would suggest the release of 'JEV-refractory' *Cx. tritaeniorhynchus* could be effective. Inhibiting transmission of JEV in the enzootic sylvatic cycle in reservoir bird hosts would also likely reduce the potential geographical expansion of JEV through bird migration. *Wolbachia*-infected *Cx. tritaeniorhyn-*

chus would reduce the enzootic amplification cycle in pigs, significantly reducing overall transmission. Any spillover JEV transmission to humans would also be inhibited by *Wolbachia*-infected *Cx. tritaeniorhynchus.*

9. The Potential Impact of Climate Change on JE

Rapid, unpredictable JE epidemics are difficult to control with traditional methods such as space spraying of insecticides having little impact. The impact of arboviral diseases such as dengue and Japanese encephalitis is expanding along with the geographical range of many mosquito species. Arboviral outbreaks often result from a rapid increase in mosquito populations resulting in significant human infection. Although climate change is likely to further increase the geographical range of JEV transmission, in a similar way as predicted for DENV (Naish et al. 2014, Colon-Gonzalez et al. 2013), the potential impact is yet to be determined. It is predicted that climate change will lead to increases in mosquito vector density, incursion of exotic mosquito species into novel areas, changes in agricultural practices and migration of host reservoir birds. In JEV endemic areas, rice fields will likely become more arid and the subsequent increase in flooding would provide optimal breeding conditions for *Cx. tritaeniorhynchus*. Climate change is likely to influence migration patterns of birds, which may result in long distance JEV dissemination in new areas (as demonstrated with WNV). WNV has a similar enzootic transmission cycle with reservoir migratory birds and introduction to novel areas has been strongly associated with bird migration (Roehring 2013, Reisen et al. 2010). As there is very little known about the particular migration patterns of the avian reservoirs for JEV, the likely impact of climate change remains unknown (Mackenzie et al. 2004).

10. Future Perspectives on *Wolbachia*-based Biocontrol

Novel vector control methods for dengue and Japanese encephalitis are needed, and the endosymbiotic bacterium *Wolbachia* may provide a safe, sustainable, environmentally friendly and effective long-term control option. Invasion and maintenance of *Wolbachia* in natural mosquito populations also provides a means of reducing arboviral transmission that may be relatively inexpensive to maintain if invaded populations are self-sustaining. *Wolbachia*-based biocontrol strategies are very much at the preliminary stages so a number of questions remain concerning implementation and effectiveness. In the case of dengue, current work is underway to determine the most appropriate *Wolbachia* strain (or combination of strains) to balance effects of DENV transmission and fitness costs to the mosquito. Mathematical models of DENV transmission incorporating the dynamics of viral infection in humans and mosquitoes predict that *w*Mel would reduce the basic reproduction number, R0, of DENV transmission by 66 to 75 % (Ferguson et al. 2015). Establishment of the virulent *w*MelPop strain at high frequency in a dengue-endemic setting would result in the complete prevention of DENV transmission. Better predictions of the evolutionary consequences of strong selection pressure on DENV are needed to fully understand the impact of *Wolbachia* on dengue epidemiology. Currently, a cluster-randomised trial is considered premature because the optimal *Wolbachia* strain for release and deployment strategies are still being determined (Lambrechts et al. 2015).

For JE, the successful transinfection of *Drosophila Wolbachia* strains into *Cx. tritaeniorhynchus* would likely result in JEV-refractory mosquito lines (Jeffries and Walker 2015). A major step to achieving this aim is to successfully colonise *Cx. tritaeniorhynchus*, a species that does tend to mate in confine spaces. The stability of the arboviral blocking phenotype, in wild *Ae. aegypti* mosquitoes (Frentiu et al. 2014) and in the long term evolutionary association between native *Wolbachia* strains in *Drosophila* flies (Hedges et al. 2008), suggests an inhibitory effect on JEV with transinfected *Cx. tritaeniorhynchus* will be present for the medium to long term. The potential ability of *Wolbachia* to prevent JEV transmission between reservoir hosts (egrets and herons), amplifying hosts (pigs) and ultimately to humans would suggest this biocontrol strategy could significantly reduce JE morbidity and mortality.

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Strahlenforschung in der Medizin – Relevanz und Perspektiven

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Die Strahlenmedizin mit ihren vielfältigen diagnostischen und therapeutischen Methoden hat in Deutschland einen im internationalen Vergleich hervorragenden Stand. Davon profitieren Patienten und die medizintechnische Industrie. Der Band gibt einen Überblick zu Fortschritten von bildgebender Diagnostik, interventioneller Radiologie und therapeutischer Strahlenmedizin. Neben allgemeinen Fragen der Strahlenanwendung in Krankenversorgung und medizinischer Forschung werden die aktuellen Entwicklungen der verschiedenen Verfahren (u. a. Magnetresonanztomographie, Computertomographie, Positronenemissionstomographie) und moderne Radiotherapien vorgestellt sowie der Einsatz von Radiopharmaka und *In-vivo*-Biomarkern behandelt. Ein besonderer Fokus liegt auf Anwendungen in Onkologie (Mammakarzinom, Prostatakarzinom, Neuroonkologie), Kardiologie und Neurologie.

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Analysis of Insecticide Resistance in Mosquito Disease Vectors: From Molecular Mechanisms to Management

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Abstract

The intense use of insecticides has resulted in a degree of insecticide resistance in major mosquito vectors to such an extent that controlling them has become challenging in several cases. The investigation of insecticide resistance mechanisms, using a variety of approaches including transcriptomics and functional proteomics, improves our understanding of the role and contribution of individual genes and mutations and the physiological basis of the phenomenon. As a result, molecular diagnostics have been developed to monitor the spread and distribution of resistance alleles in field populations and to support decision-making on resistance management strategies, as well as tools for screening novel active ingredients and resistance-breaking compounds. An example from among our most recent studies includes the analysis of insecticide resistance mechanisms in the major arbovirus vector *Aedes albopictus*.

Zusammenfassung

Der intensive Gebrauch von Schädlingsbekämpfungsmitteln hat zu einer Resistenz gegen diese Mittel bei großen Mückenvektoren geführt, und zwar in einem solchen Ausmaß, dass ihre Bekämpfung in mehreren Fällen schwierig wird. Die Untersuchung von Resistenzmechanismen gegen Schädlingsbekämpfungsmittel durch den Einsatz einer Reihe von Ansätzen, einschließlich Transkriptomik und funktioneller Proteomik, verbessert unser Verständnis für die Rolle und den Beitrag einzelner Gene und Mutationen und die physiologische Grundlage dieses Phänomens. Nachfolgend werden molekulare Diagnostiken entwickelt, um die Ausbreitung und Verteilung von Resistenzallelen im Feldbestand zu überwachen und Entscheidungen für Strategien im Resistenzumgang zu unterstützen sowie für Mittel zur Überprüfung neuer aktiver Inhaltsstoffe und resistenzbrechender Bestandteile. Ein Beispiel aus unseren jüngsten Studien beinhaltet die Analyse von Mechanismen der Schädlingsbekämpfungsresistenz in dem wichtigen Arbovirus-Vektor *Aedes albopictus*.

1. Mosquitoes as Vectors of Disease

Mosquitoes cause severe global health problems as vectors of several important human diseases. Malaria, dengue, zika and chikungunya are just some examples of these diseases that threaten almost half of the world's population and result every year in several thousand cases of mortality and morbidity, with a large socio-economic impact. Although the majority of these cases are reported in sub-Saharan Africa and in the tropical and sub-tropical areas of Latin America and Asia, Europe has also experienced recent outbreaks of mosquito-borne

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diseases. For example, West Nile Virus (WNV), almost never present or diagnosed until recently, has caused epidemics with a series of fatalities in several European countries (Dimou et al. 2013, Gabriel et al. 2013). Furthermore, diseases that presented no threat in Europe are now presenting a high risk, as for example the recent chikungunya (CHIK) epidemic in Italy (Tomasello and Schlagenhauf 2013) and the few cases of autochthonous malaria that appeared in certain prefectures of Greece in 2009 (Danis et al. 2013). Finally, although the last large dengue epidemic in Europe was observed during 1927–1928, cases were reported in southern France and Croatia in 2010, while an outbreak was also detected in 2012 in Portugal (Madeira; Tomasello and Schlagenhauf 2013). Although these outbreaks are still occasional and on a small scale, their appearance could be intensified in the future by factors such as climate change and colonization of invasive vector species, as it is the case with *Aedes albopictus*, which was directly associated with the chikungunya outbreaks in Italy (2007) and Croatia/France (2012) (Enserink 2006). Other factors that might also facilitate the occurrence of local disease transmission are the large numbers of tourists, going annually into endemic areas, and immigration (Danis et al. 2013).

The most effective way to prevent vector-borne disease outbreaks remains the use of insecticides. However, their intense use in both public health and agriculture has resulted in the emergence of resistant mosquito populations.

2. Insecticide Resistance: Scale of the Problem

Insecticide resistance is at a critical tipping point in public health. Some mosquito populations are now showing resistance to all insecticide classes, and the strength, and hence the impact, of this resistance is escalating every year. There has been a large number of publications (Ranson et al. 2010, Vontas et al. 2012) reporting resistant mosquito populations all over the world, while extreme resistance phenotypes have also been identified. For example, some *Anopheles* mosquito populations from West Africa are now showing high levels of resistance to all registered insecticide classes used in control programmes (Ranson et al. 2011). As only a limited number of different types of insecticides are available on the market, and new ones are developed very slowly, it is getting increasingly important to manage insecticide resistance in order to ensure the sustainability of current vector control programmes.

3. Insecticide Resistance Mechanisms

Strategies for Insecticide Resistance Management (IRM) and the integration of chemical and non-chemical means of control (in the context of Integrated Pest/Vector Management – IPM/IVM) must be evidence-based. This requires an understanding of the mechanisms and dynamics of insecticide resistance, including cross-resistance between different insecticide classes. Insecticide resistance mechanisms involve mutations at the target site of insecticides that render them less sensitive to inhibition by decreasing their affinity for the insecticide molecules. Among the most prominent target site resistance mutations are those of the para sodium channel; these have been correlated with pyrethroid and DDT resistance in several mosquito species (Davies et al. 2007). For *Anopheles gambiae* the best-studied mutation is at residue L1014 with two variants: L1014F and L1014S, also known as the west and east kdr.

More recently, an additional mutation, N1575Y (Jones et al. 2012), was found in *An. gambiae* haplotypes possessing the L1014F mutation, conferring high levels of pyrethroid resistance. Mutations at residues V1016 and F1534, either alone (Brengues et al. 2003, Kasai et al. 2014) or in combinations (Kawada et al. 2014), have been identified in pyrethroid-resistant *Aedes aegypti* mosquito populations, and they have been shown to alter the sensitivity of the sodium channel against pyrethroids in *in vitro* electrophysiology studies (Hirata et al. 2014). Mutations linked to resistance have also been detected in acetylcholinesterase, the molecular target of organophopshate and carbamate insecticides (Weill et al. 2004) and the γ-aminobutyric (GABA) receptor, the target of organoclorines (Du et al. 2005). In addition to mutations at the target site, resistance can also be conferred by over-expression of certain enzyme families like P450s, esterases and glutathione S-transferases, which metabolize or sequester the insecticide molecules, keeping them away from their target. P450s are the best-studied category of detoxification enzymes, including members that have largely been associated with pyrethroid resistance. For example, *Ae. aegypti CYP6BB2*, *CYP9J32* and *CYP9J28* have been found up-regulated in pyrethroid resistant strains and also shown in *in vitro* studies to be capable pyrethroid metabolizers (Kasai et al. 2014, Stevenson et al. 2012). The *An. gambiae* cytochrome P450s *CYP6M2* was shown to rapidly metabolize pyrethroids (Stevenson et al. 2011) and DDT (Mitchell et al. 2012), while *CYP6P3* is also a very efficient metabolizer of pyrethroids (Muller et al. 2008) as well as being capable of metabolizing the carbamate bendiocarb (Edi et al. 2014). The glutathione transferase *GSTe2* from *An. gambiae* (Ortelli et al. 2003) has very high DDT-dehydrochlorinase activity. Finally, esterases (CCEs) have been associated with resistance to organophosphates (OP), particularly in *Culex sp* mosquitoes. These enzymes usually do not act as quick metabolizers, but rather confer resistance through sequestration (Hemingway et al. 2004).

Despite the progress made in understanding insecticide resistance mechanisms, several questions remain to be answered in the future. Recent molecular studies demonstrated that the co-evolution of multiple mechanisms can lead to high levels of resistance; for example cuticular resistance may substantially contribute to the most striking resistance phenotypes recorded today (Balabanidou et al. 2016), while additional enzyme systems such as ABC transporters and UGTs may also dramatically enhance the phenotype (Ahn et al. 2012, Dermauw and Van Leeuwen 2014).

4. Insecticide Resistance in *Aedes albopictus*: Current Research

Our current work has focused on insecticide resistance in *Aedes albopictus* – one of the most invasive species (Bonizzoni et al. 2013) – which has recently also expanded in Europe and is a potent vector of several arboviral diseases like dengue, chikungunya, and zika. Despite its importance as both a vector of disease and a major pest, as it is an aggressive feeder, little is known about its specific molecular mechanisms of resistance. In a recent study (Grigoraki et al. 2015), we investigated the molecular basis for resistance against temephos in an *Ae. albopictus* population from Greece (Tem-GR). Temephos is an organophosphate larvicide being widely used in many parts of the world, especially in Asia and Latin America. In Europe, although it was being used for several decades, its use is currently banned. Nevertheless, it remains an important backup solution, in case of failure of the limited alternative larvicides, or of emergencies. Biochemical data indicated that resistance to temephos was associated

with elevated levels of esterase activity. In order to find which specific esterase genes were over-expressed, we followed a transcriptomic approach, comparing the transcript levels of the resistant strain to those of a standard susceptible laboratory colony, through next generation sequencing. Two CCE genes, *CCEae3a* and *CCEae6a*, were among the most highly up-regulated genes (27-fold and 12-fold respectively, compared to the reference susceptible strain) and this up-regulation was shown to be at least partially due to gene amplification. The link between *CCEae3a/CCEae6a* gene amplification and temephos resistance was further supported by genetic crosses, which demonstrated a strong association between survival to temephos exposure and gene copy numbers in the F2 generation.

Subsequently, we functionally expressed *CCEae3a* using the baculovirus expression system and investigated its interaction with temephos. Tissue localization analysis on paraffin sections and whole mounts of resistant *Aedes albopictus* larvae using a specific antibody for CCEae3a revealed the specific expression of those esterases in malpighian tubules and nerve tissue (Grigoraki et al. 2016). We are currently investigating the distribution of this temephos resistance mechanism in *Ae. albopictus* populations from several countries.

We have also screened *Ae. albopictus* populations from around the globe for target site pyrethroid resistance mutations. More specifically, we are searching for mutations on the para sodium channel at residues V1016 and F1534, associated with pyrethroid resistance, and also for mutation A302S (Tantely et al. 2010) on the GABA receptor, linked to dieldrin resistance. As yet we have not detected any known functionally characterized pyrethroid resistance mutation on the sodium channel, but individuals possessing the A302S mutation have been found in Europe and the United States.

5. New Tools to Tackle Insecticide Resistance

Understanding the mechanisms responsible for insecticide resistance can drive the development of new tools to overcome this resistance, such as new synergists and new formulations of insecticides. For example, the identification and characterization of detoxification enzymes that metabolize or sequester insecticides provide the basis alongside screening tools for the rational design of enzyme inhibitors (synergists) and/or improved active ingredients.

In addition, the identification and validation of markers that are associated with insecticide resistance allow the development of molecular diagnostics, such as PCR, Taqman (Ranson et al. 2000) and immunodiagnostic assays (Nauen et al. 2015), which are used to screen field populations for the presence of specific insecticide resistance alleles. This allows accurate monitoring of molecular mechanisms present in a specific area, which facilitates decision-making on the use of the most appropriate insecticides (Brengues et al. 2003, Mitchell et al. 2012, Nauen et al. 2013). However, the implementation of effective interventions also requires contemporary data on mosquito species composition and infection status, which can be achieved with additional separate diagnostic tools.

A fully automated platform (LabDisk) is now being explored for application with *An. gambiae* (www.dmc-malvec.eu), as well as other major arbovirus vectors subsequently, to simultaneously screen for mosquito species ID, pathogen infection and insecticide resistance alleles (including levels of detoxification genes), in a sample-to-answer approach (including nucleic acid extraction). Such automated multiplex diagnostics can be interfaced with Disease Data Management Systems (DDMS), i.e. custom-made data management software,

Fig. 1 Molecular analysis of insecticide resistance mechanisms drives the development of molecular diagnostics, to monitor the spread and distribution of resistance alleles in field populations and support decisions for resistance management strategies.

which will collate and manage data from routine entomological monitoring activities, providing information in a timely fashion based on user needs and in a standardized way (Chanda et al. 2012). Data generated using these tools can be readily integrated into existing alternative malaria decision-making support tools to facilitate the selection of the most appropriate combinations of interventions to control malaria vectors.

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Deglacial Changes in Ocean Dynamics and Atmospheric CO_2

Modern, Glacial, and Deglacial Carbon Transfer between Ocean, Atmosphere, and Land

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Vor 35 Jahren enthüllten Eiskernbohrungen erstmals die dramatischen Veränderungen des atmosphärischen CO_2-Gehaltes am Übergang vom Glazial zum Interglazial. Diese natürlichen Veränderungen gehören neben dem vom Menschen verursachten Anstieg des CO_2-Gehaltes in der Atmosphäre in der Gegenwart zu den herausragenden Themen der Erforschung des Kohlenstoffzyklus. Seit den 1980er Jahren wurde eine große Anzahl wissenschaftlicher Resultate und Modelle publiziert, die die Rolle des Kohlenstoffaustausches zwischen dem größten Reservoir auf der Erde, dem globalen Ozean, und dem in der Atmosphäre und auf dem Land aufdecken. Diese Studien verlangen Überblick und breite Synthese.

Der Band liefert die Basis, um Grenzen in den Modellen der Schlüsselprozesse des Kohlenstoffkreislaufs auszuloten, die den Austausch zwischen den vier Hauptkohlenstoffquellen auf der Erdoberfläche, dem Ozean, der Atmosphäre, der terrestrischen Biosphäre und den Böden, kontrollieren. Der vorliegende Band enthält fast 70 erweiterte Zusammenfassungen auf dem neuesten Wissensstand in den entsprechenden Gebieten der CO_2-Forschung. Die Beiträge untersuchen empirische und modell-basierte Forschungsresultate sowohl über sich verändernde Kohlenstoffvorräte der Vergangenheit als auch Formen, Lokalisierungen und Raten des Kohlenstofftransfers.

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Drosophila as a Model for Arbovirus Infection

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Abstract

Many pandemics caused by RNA viruses have been attributed to the ability of these agents for zooanthroponotic transmission. Athrophod-borne viruses (arboviruses), which are transmitted by insects to mammals and *vice versa*, are the causative agents of metazoonoses. They include a wide variety of RNA virus taxa, but the three major families are *Flaviviridae*, *Togaviridae* (genus Alphavirus) and *Bunyaviridae.* Arboviruses can efficiently replicate in evolutionary distinct hosts, such as mosquitoes and humans, yet they seem to depend on insect vectors for transmission. Competent vectors must not only allow virus infection and dissemination, but must also control the adverse effects of virus replication. Because of the variety of mosquito vectors and the paucity of genetic tools for those, studies of virus-vector interactions are sometimes difficult. The fruit fly *Drosophila melanogaster* can be a useful tool to overcome some problems since it is the best characterized insect with regard to genome and genetic manipulation possibilities. From studies in *Drosophila*, it is known that the insect's immune response to viruses mainly relies on the RNAi mechanism and that this mechanism is involved in the control of all viruses tested so far (*Flaviviridae*, *Togaviridae* and *Bunyaviridae*). In addition to RNAi, the Toll, IMD and Jak-STAT pathways as well as the induced Vago protein have been shown to act antiviral, but the range of viruses controlled by each pathway is more restricted. The insights gained with the *Drosophila* model can be used to inspire and guide new studies in vector insects and help to shed light on basic virus-vector interaction mechanisms.

Zusammenfassung

Stechmücken und andere Arthropoden übertragen eine große Zahl human- und tierpathogener Viren (Arboviren). Hierbei ist der Begriff als eine Sammelbezeichnung für alle von Arthropoden-übertragenen Viren und nicht als eine Angabe von phylogenetischer Verwandtschaft dieser Viren zu verstehen. Arboviren entstammen sehr diversen Virustaxa, wobei eine große Anzahl der Arboviren aus der Familie der Flaviviren, Togaviren und Bunyaviren entstammt. Eine faszinierende Eigenschaft der Arboviren ist ihre Fähigkeit, in zwei sehr unterschiedlichen Systemen, zum einen den Arthropoden und zum anderen Vögeln und Säugetieren, zu replizieren. Diese Fähigkeit erfordert spezifische Anpassung der Viren an evolutionär sehr unterschiedliche Systeme. Jedoch ist allen Arboviren gemein, dass sie zur Vervollständigung ihres Übertragungszyklus auf Arthropoden-Vektoren angewiesen sind. Kompetente Vektoren müssen hierbei nicht nur die Replikation des Virus erlauben, sondern auch die Virusinfektion kontrollieren, so dass es zu keiner Schädigung des Vektors durch die Infektion kommt. Im Gegensatz zu Säugern und Vögeln besitzen Arthropoden kein adaptives Immunsystem, sondern verfügen über verschiedene angeborene Immunmechanismen. Die Forschung an antiviraler Immunität in Arthropoden ist durch die große Vielfalt der möglichen Vektoren und das Fehlen fundierter genetischer Daten zu diesen Vektoren schwierig. Daher bietet es sich an, als erste Näherung bei der Erforschung von antiviralen Immunmechanismen in Arthropoden, zunächst gut etablierte Modellsysteme zu nutzen. Hierzu gehört die Fruchtfliege *Drosophila melanogaster*, welche seit über 100 Jahren für entwicklungsbiologische und evolutionsbiologische Fragestellungen genutzt wird. Durch Infektionsstudien in *Drosophila melanogaster* wurde zum Beispiel etabliert, dass das Immunsystem von Insekten virale Infektionen hauptsächlich mit einem als RNA-Interferenz (RNAi) bekannten Mechanismus bekämpft. Des Weiteren wurden auch für zwei NFkB-abhängige Signaltransduktionswege, den Toll- und IMD-Signalweg, eine Funktion bei der antiviralen Immunabwehr gefunden. Ebenso spielen in *Drosophila* der JAK-STAT-Signaltransduktionsweg und das Protein *Vago* eine Rolle bei der Bekämpfung verschiedener viraler Infektionen. Hierbei haben die in *Drosophila* generierten Daten nachfolgende Studien antiviraler Mechanismen in Stechmücken unterstützt.

1. Arboviruses

Arthropod-borne viruses (Arboviruses) transmitted by insects to mammals and *vice versa* are the causative agents of metazoonoses. Arboviruses include a wide variety of different RNA virus taxa, but the three major families are *Flaviviridae* (yellow fever virus, West Nile virus and zika virus), *Togaviridae* (genus Alphavirus, Sindbis virus and chikungunya virus) and *Bunyaviridae* (Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus and La Crosse virus). The recent spread of zika virus in Brazil and other parts of the Americas exemplifies the potential of arboviruses to rapidly move beyond their traditional geographical boundaries. In the absence of safe vaccines or treatments for most arbovirus infections, an understanding of how insect vectors control viral pathogens is urgently needed to devise new vector-based control strategies. Consequently, suitable model systems for virus infection in insects need to be developed.

One hallmark of Arbovirus transmission cycles is the efficient replication of these viruses in evolutionary distinct hosts, such as mosquitoes, midges or ticks, and mammals including humans. But most importantly, their spread depends on specific arthropod vectors. The factors that determine whether a specific vector can transmit a given virus (vector competence) often remain poorly understood. Major factors determining vector competence are (*i*) the ability of a virus to infect the mosquito, and (*ii*) control of viral replication to an extent that the vector itself is not affected by the virus. Like other multicellular organisms, arthropod vectors achieve control of intruding pathogens through specific immune mechanisms. In contrast to mammalian hosts, arthropods depend on innate immune mechanisms to control pathogen replication.

In recent years much research interest has been drawn to the specific mechanisms that allow arthropods to control viral pathogens. Because of the paucity of genetic tools and the lack of knowledge about mosquito, midge or tick genome organization, the insect model *Drosophila melanogaster* was used to study basic immune mechanisms. A small part of the antiviral mechanisms that have been described in recent years in *Drosophila melanogaster* will be discussed in the following chapters. The information presented in this article makes no claim to be complete and reflects more the personal experience of the author in the topic. Nevertheless, I hope to give the reader a limited overview of the fascinating subject of antiviral immune mechanisms in insects.

2. Antiviral Mechanisms in Insects

2.1 RNA Interference

Using the model insect *Drosophila melanogaster*, it has been demonstrated that RNA interference (RNAi) is crucial for controlling various *Drosophila* viruses and metazoonotic viruses such as Sindbis virus (SINV), West Nile virus (WNV), Vesicular Stomatitis virus (VSV) and dengue virus (Galiana-Arnoux et al. 2006, van Rij et al. 2006, Wang et al. 2006, Zambon et al. 2006, Chotkowski et al. 2008, Mueller et al. 2010, Mukherjee and Hanley 2010).

The RNAi mechanism is initiated by the recognition and cleavage of double-stranded (ds) RNA, such as viral replication intermediates, by the RNAseIII enzyme Dicer-2 (Dcr-2) and leads to the sequence-specific degradation of viral genomes or transcription products by the

RNA-induced silencing complex (RISC). The basic RNAi process can be divided into two major steps: the structure-dependent dicing and the sequence specific slicing (Kemp and Imler 2009). The Dcr-2 enzyme participates in both steps of RNAi. In the structure-dependent dicing step, long double-stranded RNA is cleaved by the activity of the RNaseIII domain of Dcr-2 into 21 bp small interfering (si)RNAs. In the second step, Dcr-2 interacts with the siRNA products and R2D2 protein to facilitate the assembly of the RISC complex (Liu et al. 2006). RNAseIII activity is not required for RISC assembly (Lee et al. 2004). To date it is not clear which domains of Dcr-2 participate in the binding of siRNA duplexes and the initiation of RISC assembly. Finally, the RISC complex will be guided by one strand of the siRNA duplex (guide strand) to the complementary target RNA and lead to the sequence-specific degradation of this RNA. The key component Dcr-2 is a multi-domain enzyme that contains a DEXD/H box (DexH and Hel-C) ATPase domain, a Domain of Unknown Function (DUF283), a PIWI Argonaute Zwille (PAZ) domain, two RNaseIII domains and a dsRNA Binding Domain (dsRBD). Biochemical, genetic and structural studies have established a model of the Dcr-2 core complex consisting of the two RNAseIII domains, the PAZ domain and the dsRBD playing the central role in siRNA production. This core complex shares similarities with other RNase IIIb domains of Dicers from human, *Drosophila* (Dm-Dcr-1), *C. elegans*, *S. pombe*, and *Giardia* as well as the corresponding domains of RNase III enzymes from *Aquifex* and *E. coli*. The role of the DEXD/H box remains unclear, and might vary among different species. The ATPase activity is needed for processive dicing in *Drosophila* (Cenik et al. 2011), but its role in antiviral RNAi is not known. In mammals, the ATPase activity is not required for dsRNA processing, and the DEXD/H box ATPase domain was shown to have inhibitory effects on dsRNA processing (Ma et al. 2008).

As stated above, RNAi has been demonstrated to act antiviral against a wide variety of different viruses. Mostly those viruses are classified as positive-strand RNA viruses. This term describes the nature of the viral messenger (m)RNA in infected host cells; in case of positive (+) strand RNA viruses, the viral genome is also used as mRNA, whereas viruses that use the antigenomic RNA or antigenomic subtranscrips as mRNA are named negative (–) strand RNA viruses. This is of crucial importance with regard to antiviral RNAi because (+) strand RNA viruses produce significant quantities of the *bona fide* trigger of RNAi, i.e. dsRNA, in the course of infection, whereas (–) strand RNA viruses do not produce detectable quantities of dsRNA et al. 2009, Sanchez-Vargas et al. 2009).

The lack of detectable amounts of dsRNA during (–) strand RNA virus infection was confirmed in the insect cells' system using VSV infection in *Drosophila* S2 cells. Interestingly, despite the absence of readily detectable dsRNA molecules, we were able to demonstrate that RNAi plays a major role in the host defence against VSV (Mueller et al. 2010). Flies lacking functional Dcr-2, R2D2, and AGO2 proteins are no longer able to control VSV replication and showed a more than two-fold increased viral titre compared to flies with an intact RNAi pathway. Furthermore, the recently described component of the RNAi pathway Ars2 is also required to control VSV replication in *Drosophila* cells (Sabin et al. 2009), strengthening the evidence that RNAi plays a major role in VSV control in insects. The uncontrolled viral replication in RNAi pathway mutants led to severely reduced survival of these flies, suggesting that indeed RNAi plays a major role in the control of (–)-strand RNA virus infection. Another hallmark of RNAi, the production of small interfering RNAs, was also of central interest in this study. Thus we compared small RNA profiles of wildtype and RNAi mutant flies upon VSV infection. The profiles of virus-derived siRNAs obtained from VSV-infected

AGO2$^{-/-}$ mutant flies had a high degree of similarity to the profiles from wildtype flies, but the number of reads was 100-fold higher in AGO2$^{-/-}$ flies. Regardless of the high number of produced small RNA in AGO2$^{-/-}$ mutant flies, they were as susceptible to VSV infection as Dcr-2$^{-/-}$ mutant flies. This indicates that dicing of viral dsRNA *per se* does not represent an efficient defence against viral infections. Furthermore, we can conclude that the similarities observed in WT and AGO2$^{-/-}$ profiles indicate that the absence of AGO2$^{-/-}$ does not influence Dcr-2 activity.

In another study, we investigated whether RNAi also participates in the control of DNA virus infections in invertebrates, as previously reported in plants (Blevins et al. 2006, Moissiard and Voinnet 2006) and, more generally, whether it represents a major host-defence pathway against all viruses. To do this, we addressed the question of whether RNAi-deficient flies are susceptible to infection with the DNA virus IIV-6 (*Iridoviridae*). Infection of Dcr-2 mutant flies led to a 20-fold increased replication of IIV6 compared to wildtype flies. The enhanced viral replication also led to a decreased survival rate of Dcr-2$^{-/-}$ mutants compared to the wildtype control. Similar results were obtained for different null allele of Dcr-2 or r2d2$^{-/-}$ and AGO2$^{-/-}$ null mutant flies (Kemp et al. 2013). Since IIV6 possess a DNA genome, the question arose of the nature of the substrate used by Dcr-2 in the context of this infection. Therefore, small RNA libraries from IIV-6-infected S2 cells or adult flies were sequenced. The resulting siRNA profiles were specific to IIV6-infected samples and showed a clear enrichment of virus-derived 21-nucleotide siRNAs. In contrast to small RNA profiles from the VSV study and other published data from RNA-virus-infected *Drosophila* cells, the IIV6-derived siRNAs were not uniformly distributed along the viral genome but rather showed several hotspots and strong symmetry of the peaks. These profiles suggest that these regions are transcribed on both strands and generate dsRNA. Indeed, a bidirectional transcription in the areas of the viral genome covered by the peaks was observed (Kemp et al. 2013).

Taken together, the results presented here and obtained by various other colleagues indicate that the siRNA pathway in *Drosophila* can also protect against virus infection, irrespective of the nature of the viral genome.

2.2 Inducible Immunity

2.2.1 JAK-STAT Pathway

Besides RNAi, a couple of inducible mechanisms have been described in *Drosophila* in recent years. The JAK-STAT pathway in *Drosophila* in antiviral defence came to public attention through a microarray study that showed that infection of wildtype flies with *Drosophila* C virus (DCV), a member of the *Dicistroviridae* family, leads to induction of some 130 genes (Dostert et al. 2005). The induction of some of these genes, for example *vir-1*, was dependent on the gene *hopscotch* (*hop*), which encodes the only JAK kinase in *Drosophila.* Furthermore, *hop* mutant flies succumb more rapidly than wildtype controls to DCV infection, and virus loads were increased in hop mutants (Dostert et al. 2005). To analyze whether the JAK-STAT pathway may constitute a general antiviral defense similar to the homologous JAK-STAT pathway in mammals, which is essential for induction of interferon-induced genes, we tested a panel of different viruses from different families in *hop* mutant flies. An unexpected finding was that *hop* mutant flies have a clear phenotype for DCV and Cricket Paralysis Virus (CrPV; *Dicistroviridae*), but not for SINV (*Togaviridae*), VSV (*Rhabdoviri-*

dae), Flock House virus (FHV, *Nodaviridae*), Drosophila X virus (DXV, *Birnaviridae*), and IIV-6 (*Iridoviridae*). This indicates that besides RNAi the JAK-STAT pathway is essential for controling Dicistrovirus infection in *Drosophila* but is dispensable for antiviral immunity against the other viruses tested.

The infection of flies with DCV induced the expression of the cytokines Upd2 and Upd3, which may lead to the subsequent activation of the JAK-STAT pathway similar to the activation of the pathway by the cytokine Upd during *Drosophila* embryogenesis (Harrison et al. 1998). As stated above, the activation of the JAK-STAT pathway by DCV infection induces the expression of several genes and flies mutant for *hopscotch* succumb to DCV infection 2–3 d before the controls. Altogether, the results highlight that the contribution of the inducible response to the control of DCV is similar to that of RNAi.

One key question is the nature of the receptor detection of *Dicistroviridae* infection, which triggers the inducible response. Several experiments with flies expressing the dsRNA-binding protein B2 and *Dcr-2* mutant flies clearly indicate that the *vir-1* induction does not depend on sensing of dsRNA (Deddouche et al. 2008). A possible inducer of the JAK-STAT pathway might be the sensing of tissue damage and/or cell death, which would match the observation that JAK-STAT-regulated genes are mostly induced by fast-killing viruses such as DCV, CrPV and FHV. Furthermore, a clear association of the JAK-STAT pathway with the cellular response to a variety of stresses has been reported before (Agaisse et al. 2003, Buchon et al. 2009, Croninet al. 2009, Jiang et al. 2009). Therefore, future efforts to uncover the nature of JAK-STAT induction upon Dicistrovirus and possibly other viral infections should focus on possible sensing of cellular stress and cell death.

In conclusion, the data available clearly demonstrate the presence of and importance of inducible antiviral responses in *Drosophila*. However, these responses are complex and not all factors have been identified as yet. Therefore, great care should be exercised before generalizing the results obtained from a single virus species. The complexity probably reflects the intricate association of viruses with their host cells in different tissues, their different strategies of replication or protein expression, or their acquisition of suppressors of host defence.

2.2.2 Toll and IMD

The Toll and immune deficiency (IMD) pathways, initially characterized for their role in the control of bacterial and fungal infections, are also associated with control of viral infections. Whereas the Toll pathway was associated with resistance to the *Drosophila* X virus (DXV) (Zambon et al. 2005), the IMD pathway was implicated in the control of SINV (Avadhanula et al. 2009) and CrPV (Costa et al. 2009).

In our study on inducible antiviral response, we also analysed the transcriptional response of *Drosophila* to DCV, FHV and SINV by microarray analysis (Kemp et al. 2013). One interesting aspect of the virus-induced transcriptomes was the upregulation of genes controlled by the Toll and IMD pathways. We found an enrichment of Toll pathway target genes induced in flies infected by DCV, but not FHV or SINV, suggesting that DCV infection triggers this pathway (Kemp et al. 2013). One of the genes induced by DCV is Ect4, the *Drosophila* orthologue of SARM, which was proposed to participate as a negative regulator of TLR-signalling in some antiviral defences (Carty et al. 2006). Two other interesting candidate genes found to be regulated by DCV are headcase, which was proposed to be a regulator of the siRNA pathway (Dorner et al. 2006), and CG9925, which encodes for a protein containing a zinc finger

and a Tudor domain. CG9925 has been identified in genome-wide RNAi screenings using the *Serratia marcescens* infection model and was found to be involved in the control of *S. marcescens* in the fly (Cronin et al. 2009). Furthermore, an enrichment of classical components of the Toll pathway such as the cytokine Spaetzle and the antifungal peptides Drosomycine and Metchnikowine was found after DCV infection (Kemp et al. 2013).

Regarding the IMD pathway, an enrichment of IMD-regulated genes, such as the antibacterial peptides Attacin-A and -C, Diptericin-B, and the transcription factor Relish was found in both DCV- and FHV-infected flies. However, when comparing the expression of diptericin and drosomycin – the two respective markers of activation of the IMD and Toll pathways – triggered by viruses to those triggered by bacterial and fungal infections, the expression after viral infection was weak and not significantly different to buffer injection. Therefore, the role of this weak induction in antiviral defence as well as the mode of activation of those antibacterial pathways needs to be clarified in future studies.

2.2.3 Vago

Within the frame of transcriptome analysis in DCV-infected flies, the gene Vago was identified as being specifically triggered during viral infection. Following the transcriptome studies, the regulation and function of Vago was analysed in more detail (Deddouche et al. 2008). Vago was found to be specifically induced upon viral infection in the fat body of flies, with expression profiles matching the detection of virus replication in this tissue. Furthermore, Vago was essential for controlling DCV virus replication in the fat body but not in other fly tissues.

Interestingly, the induction of Vago was dependent on Dcr-2, indicating that Dcr-2 is one of the sensors that trigger the antiviral inducible response in flies. These results are especially intriguing given the close phylogenetic relationship between the DExD/H-box helicase domains of Dicer and RIG-I-like helicases in mammals. As a result, one could speculate that an essential domain of a core molecule from the ancestral antiviral response, RNA silencing, was at some point recruited to sense viral RNAs in vertebrates and to subsequently activate a signalling pathway leading to production of IFNs.

Although the clear involvement of Vago in control of DCV infection in flies was shown, exact characterization of the function as either a antiviral molecule targeting the virions (Sheldonet al. 2007) or as a cytokine triggering an antiviral state in neighbouring cells (Kleinjung et al. 2004) is still missing. Unfortunately, most experiments addressing these questions were hampered by the poor stability of Vago protein in flies. The precise function of Vago in flies can only be speculated upon at this stage. In contrast to vir-1, the induction of Vago was not dependent on the JAK-STAT pathway and *vice versa*: vir-1 stayed fully inducible in in Vago-mutant flies, indicating that Vago is not involved in this cytokine-mediated response in flies. The strong tissue specificity of Vago in flies as well as the lack of conservation of this gene in several other insect species also challenges the idea of a circulating cytokine function for Vago. However, the *Culex* homologue of Vago (*CxVago*) was shown to activate the JAK-STAT pathway and thereby restrict West Niles virus replication in the mosquito (Paradkar et al. 2012). These oppositional data from flies and mosquitoes show that homologous genes might have different functions in different insect systems. Nevertheless, Vago was first described in flies and inspired targeted research in different mosquito species.

3. General Conclusion

In conclusion, all these data obtained through research using flies and mosquitoes show that *Drosophila* is a valuable model for uncovering general immune mechanisms in insects and inspiring research in arthropod vectors. However, one has to be careful when drawing conclusions about the antiviral function of molecules and pathways based solely on evidence from infection of one insect species with one specific virus. Functions of pathway and molecules might differ between insect species and within the same species upon infection with different viruses.

4. Outlook: Potential for Use of the *Drosophila* Model in Antiviral Screens

In addition to basic questions of antiviral immunity in insects, the *Drosophila* model could be used as an easy tool for antiviral screening. Thus far, *Drosophila* has been successfully used as a model for various bacterial (*Pseudomonas aeruginosa* [Campbell et al. 2008, Costa et al. 2009], *Mycobacterium marinum* [Daubney and Hudson 1931], *Listeria monocytogenes* [Faran et al. 1988], and *Salmonella* sp. [Brandt et al. 2004]) and viral infections (Galiana-Arnoux et al. 2006, van Rij et al. 2006, Zambon et al. 2006, Chotkowski et al. 2008, Mueller et al. 2010, Mukherjee and Hanley 2010, Wang et al. 2006). Recent studies suggest that this model system might also be useful for studying treatment options. For example, the *Drosophila* model has been used to study pathogenicity and response to antibiotic treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) (Gerdes 2004). Furthermore, preliminary drug screenings with a library of 1,280 small molecules, using *Drosophila* cells and RVFV infection, have shown that this model system is also suitable for antiviral screening (Filone et al. 2010). An *in vivo* model could be used to investigate the effects of antiviral drugs not only on viral replication but also on animal survival. For example, by using a feeding protocol for fly inoculation with drug candidates, along with a luciferase-expressing virus construct (Gunawardane et al. 2007), high-throughput screening using this *in vivo* model could be developed.

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Vorträge und Abhandlungen zur Wissenschaftsgeschichte 2013/2014

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Für die Leopoldina ist die Beschäftigung mit der Geschichte der Akademien und der Wissenschaften ein vordringliches Anliegen. Der Sammelband vereinigt aktualisierte Vorträge aus den wissenschaftshistorischen Veranstaltungen der Akademie und Abhandlungen zu ausgewählten Themen. Im Fokus stehen Gelehrte wie der Psychiater Helmut Rennert, die Biochemiker Otto Meyerhof und Otto Warburg, die Botaniker Kurt Mothes und Hans Stubbe, der Hethitologe Bedřich Hrozný oder der Wissenschaftshistoriker Olaf Breidbach, aber auch die Hof- und Leibärzte am Habsburger Kaiserhof. Behandelt werden Wettbewerb und Konkurrenzverhalten unter Wissenschaftlern sowie Auswirkungen von Fälschungen und Betrug in der Forschung, aber auch Fragen der Patentierung von Genen, der Geschichte des Interviews und der Authentizität von Gesichtern in der Wiederherstellungschirurgie. In die Frühgeschichte der Naturwissenschaften führen Beiträge zur Diskussion um Monstren in den Akademieschriften oder zur Missionspharmazie der Jesuiten.

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Vaccinomics Approach to the Development of Vaccines for the Control of Multiple Ectoparasite Infestations

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Abstract

Blood-feeding arthropod ectoparasites affect a variety of species and can transmit pathogens causing diseases in humans and animals worldwide. Ectoparasite-host-pathogen interactions have evolved through dynamic processes involving genetic traits of hosts, pathogens and ectoparasites that mediate their development and survival. New approaches to ectoparasite control are dependent on defining molecular interactions between hosts, ectoparasites and pathogens to allow for the discovery of key molecules that could be tested in vaccines for intervention of ectoparasite-pathogen cycles. Tick vaccines based on recombinant BM86/BM95 antigens are the only commercially available vaccines against ectoparasites. These vaccines offer the important advantages of being a cost-effective and environmentally friendly alternative, with a dual effect of reducing tick infestations and preventing ticks from transmitting disease-causing pathogens. Tick vaccine research may provide models for the development of vaccines against other arthropods, with the possibility of controlling multiple ectoparasite infestations. The challenge of developing improved vaccines against ticks and other ectoparasites arises from the need to understand the complex molecular relationship between vertebrate hosts, ectoparasites and pathogens, which requires a systems biology approach that allows the integrated analysis of metabolomics, transcriptomics, proteomics and other omics data for discovery of key pathway molecules that mediate ectoparasite-host-pathogen interactions. A vaccinomics approach could then be used to identify and fully characterize candidate protective antigens in different ectoparasites and to validate vaccine formulations. New candidate protective antigens will most likely be identified by focusing on abundant proteins with relevant biological functions in ectoparasite feeding, reproduction, development, immune response, subversion of host immunity, and pathogen infection and transmission. Additionally, the evolution of similar strategies for some pathogens to infect vertebrate and vector cells suggests the possibility of developing strategies to control pathogen infection in both vertebrate hosts and vectors. Vaccines based on antigen combinations or evolutionarily conserved ectoparasite protective antigens that affect both vector infestations and pathogen infection and transmission could then be developed and used to vaccinate at-risk human and animal populations in order to prevent disease, and reservoir host species in order to reduce vector infestations and their capacity to transmit pathogens that affect human and animal health.

Zusammenfassung

Ektoparasiten (Arthropoden) befallen eine Vielzahl von Arten und können Krankheitserreger übertragen, die weltweit Erkrankungen bei Menschen und Tieren hervorrufen. Die Ektoparasit-Wirt-Erreger-Wechselwirkungen haben sich durch dynamische Prozesse entwickelt, bedingt u. a. durch die genetischen Eigenschaften der Wirte, Krankheitserreger und Ektoparasiten, die ihre Entwicklung und ihr Überleben steuern. Neue Ansätze für die Bekämpfung von Ektoparasiten gehen von der Bestimmung der molekularen Wechselwirkungen zwischen Wirten, Ektoparasiten und Krankheitserregern aus, die die Ermittlung von Schlüsselmolekülen ermöglichen, die in Impfstoffen als Angriffspunkte im Ektoparasit-Krankheitserreger-Zyklus getestet werden könnten. Zeckenimpfungen, basierend auf re-

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kombinanten BM86/BM95-Antigenen, sind die einzigen kommerziell erhältlichen Impfstoffe gegen Ektoparasiten. Diese Impfstoffe bieten die wichtigen Vorteile, eine kostengünstige und umweltfreundliche Alternative darzustellen, mit dem doppelten Effekt, den Zeckenbefall zu reduzieren und Zecken daran zu hindern, krankheitsverursachende Erreger zu übertragen. Die Forschung im Bereich der Zeckenimpfung kann Modelle für die Entwicklung von Impfstoffen gegen andere Arthropoden liefern und ermöglicht, vielfältige Ektoparasitenplagen zu bekämpfen. Die Herausforderung für verbesserte Impfstoffe gegen Zecken und andere Ektoparasiten beruht auf dem Erfordernis, die komplexen Beziehungen zwischen Wirbeltierwirten, Ektoparasiten und Krankheitserregern auf molekularer Ebene zu verstehen. Dazu ist ein systembiologischer Ansatz notwendig, der durch die integrierte Analyse von Daten der Metabolomik, Transkriptomik, Proteomik und anderen Omiks die Identifizierung von wichtigen Schlüsselmolekülen in den Stoffwechselwegen ermöglicht, die die Ektoparasit-Wirt-Krankheitserreger-Wechselwirkungen steuern. Der Ansatz der Impfstoffherstellung könnte dann genutzt werden, um Kandidaten protektiver Antigene in verschiedenen Ektoparasiten zu bestimmen und zu charakterisieren sowie für entsprechende Impfstoffformulierungen zu validieren. Neue Kandidaten protektiver Antigene werden höchstwahrscheinlich gefunden werden bei der Fokussierung der Analyse auf die zahlreichen Proteine mit relevanten biologischen Funktionen in der Ernährung, Vermehrung und Entwicklung der Ektoparasiten, in der Immunantwort bzw. bei der Zerstörung der Immunabwehr des Wirts oder in der Erregerinfektion und -übertragung. Außerdem deutet die Evolution von ähnlichen Strategien bei einigen Krankheitserregern in der Infektion von Wirbeltier- und Vektorzellen auf die Möglichkeit hin, Strategien für die Bekämpfung von Erregerinfektionen sowohl für die Wirbeltierwirte als auch die Vektoren zu entwickeln. Impfstoffe, basierend auf Antigenkombinationen oder evolutionär konservierten ektoparasitprotektiven Antigenen, die sowohl den Vektorbefall und die Erregerinfektion als auch die Übertragung beeinträchtigen, könnten dann entwickelt und genutzt werden, um gefährdete Menschen und Tiere prophylaktisch zu impfen und damit den Vektorbefall und die Kapazität der Vektoren, Krankheiten zu übertragen und so die die Gesundheit von Mensch und Tier zu beeinträchtigen, zu reduzieren.

1. Introduction: Global Burden of Arthropod-Borne Diseases

Diseases caused by arthropod-borne pathogens account for over 20 % of all emerging infectious diseases recorded between 1940 and 2004 (Jones et al. 2008). Mosquitoes are the most important vectors of human diseases, while ticks are considered to be second worldwide to mosquitoes and the most important vectors of diseases that affect the cattle industry worldwide (de la Fuente et al. 2008, Peter et al. 2005). Furthermore, other ectoparasites are also relevant for human and animal health, and current research efforts are directed towards developing vaccines for their control (Hopla et al. 1994, Nisbet and Huntley 2006, McNair 2015) (Tab. 1).

2. Tick Vaccines: A Model for Development of Vaccines against Multiple Ectoparasite Infestations

2.1 Results and Possibilities for Tick Vaccines

The only commercially available vaccines against ectoparasites were developed and registered in the early 1990s for the control of cattle tick infestations (de la Furente et al. 2007). These vaccines are based on recombinant *Rhipicephalus microplus* BM86/BM95 antigens and demonstrate the important advantages of being a cost-effective and environmentally friendly alternative, with a dual effect of reducing tick infestations and preventing ticks from transmitting disease-causing pathogens (de la Fuente et al. 2007).

Research on tick vaccines is more advanced than that reported for other major ectoparasites and may provide models for development of vaccines for the control of other ectoparasites (Willadsen 2006, de la Fuente et al. 2011, 2013, Peter et al. 2005, Torres et al.

Tab. 1 Summary of reported vaccination experiments against ectoparasites different from ticks

Host	Ectoparasite antigen	Vector species	Pathogen species	Vaccine efficacy	References
Mice Monkeys	Pvs48/45	*Anopheles albimanus*	*Plasmodium vivax*	Full inhibition of parasite transmission to mosquitoes	Arévalo-Herrera et al. 2015
Mice	AKR	*A. gambiae*	*P. berghei*	Infection was reduced in more than 60% in mosquitoes fed on immunized mice	da Costa et al. 2014
Mice	Pfs25	*A. gambiae* *A. stephensi*	*P. falciparum*	100% transmission-blocking efficacy	Kumar et al. 2014
Mice	AnAPN1	*A. stephensi*	*P. berghei*	ND	Mathias et al. 2012
Mice	KSAC L110f	*Phlebotomus duboscqi*	*L. major*	ND	Gomes et al. 2012
Rhesus macaques	PdSP15 (salivary protein)	*P. duboscqi*	*Leishmania major*	ND	Oliveira et al. 2015
Mice	PpSP15	*P. papatasi*	*L. major*	ND	Katebi et al. 2015
Rabbit	rPc-act (actin)	*Psoroptes cuniculi*	NP	No statistically significant difference between vaccinated group and the controls	Zheng et al. 2013
Rabbit	SsTm (tropomyosin)	*Sarcoptes scabiei*	NP	No statistically significant difference between vaccinated group and the controls	Zhang et al. 2012
Mice	SUB AKR Q38[1] Q41[1]	*Aedes albopictus*	NP	92%[2] 97%[2] 28%[2] 99%[2]	Moreno-Cid et al. 2013.
Mice	SUB AKR Q38[1] Q41[1]	*P. perniciosus*	NP	72%[2] 27%[2] 42%[2] 32%[2]	Moreno-Cid et al. 2013.
Rabbit	*Dermatophagoides farinae* and *D. pteronyssinus* house dust mite 50:50 protein extract	*S. scabiei var. canis*	NP	71%[2]	Arlian et al. 1995
Rabbit	Ssag1 and Ssag2	*S. scabiei* var. *canis*	NP	No reduction in the numbers of mites	Haruma et al. 2003

Host	Ectoparasite antigen	Vector species	Pathogen species	Vaccine efficacy	References
Sheep	rPoGST, rPoCLP, rPoTRO, trPoPAR, Pso o 1 trDerp14	*P. ovis*	NP	7 of the 20 sheep in the trial became infested during the course of the experiment	Nisbet et al. 2008
Rabbits	Extracts of midguts from fleas	*Ctenocephalides felis*	NP	75 % mortality in fleas	Heath et al. 1994
Sheep	Peritrophin 95	*Lucilia cuprina*	NP	85 ± 2 % mean larval weight reduction.	Casu et al. 1997
Sheep	Peritrophin-55	*L. cuprina*	NP	Larval growth inhibitory activities of 51 and 66 % (2 trials)	Tellam et al. 2003
Salmon	AKR-2	*Lepeophtheirus salmonis*	NP	57 % inhibition of infestation in the vaccinated group	Carpio et al. 2011
Mice Fish	my32-Ls (AKR-2)	*L. salmonis*	NP	ND	Carpio et al. 2013
Hens	Deg-SRP-1 (serpin), Deg-VIT-1 (vitellogenin) Deg-HGP-1 (hemelipoglycoprotein) or Deg-PUF-1 (a protein of unknown function)	*Dermanyssus gallinae*	NP	Significantly increased risk of mite death (1.7–2.8-times higher than in mites fed blood from control hens immunised with adjuvant only, P<0.001)	Bartley et al. 2015
Hens	cathepsin D and L-like proteinases	*D. gallinae*	NP	Fitting a proportional hazards model to the time of death of mites, anti-Dg-CatD-1 and anti-Dg-CatL-1 IgY had 4·42 and 2·13 times higher risks of dying compared with controls (PF< 0.05).	Bartley et al. 2012
Hens	subolesin BM86	*D. gallinae*	NP	Mortality after feeding was 35.1 % higher (P = 0.009) in the subolesin group and 23 % higher (not significant) in the BM86 compared to the control group	Harrington et al. 2009
Rabbits	rDg-HRF-1(histamine release factor)	*D. gallinae*	NP	9.24 % (SD ± 1.63) mortality	Bartley et al. 2009

[1] Q38 and Q41 are tick subolesin and mosquito akirin chimeras.
[2] Vaccine efficacy was calculated considering the effect on the reduction of ectoparasite infestations, oviposition and fertility (de la Fuente and Contreras 2015) but in certain experiments only the effect on some of these parameters was considered.
Abbreviations: ND: not determined; NP: No pathogen included in the experiment.

2011, Marr et al. 2014, Sparagano et al. 2014, McNair 2015, da Costa et al. 2014). The protective mechanism characterized for BM86/BM95 tick vaccines is based on the development of antigen-specific antibodies in immunized hosts that interact and affect the function of the targeted antigen in ticks feeding on the immunized hosts (de la Fuente et al. 2007, Willadsen und Kemp 1988, Merino et al. 2011a). The application of the BM86/BM95-based vaccines results in a reduction in the number, weight and reproductive capacity of engorging female ticks, thereby reducing tick infestations in subsequent generations (de la Fuente and Kocan 2014, de la Fuente et al. 2007).

It is well documented that some tick species parasitize several vertebrate hosts and share habitat and hosts with other tick species (Estrada-Pena et al. 2015). Furthermore, ticks coexist with other ectoparasites that cause vector-borne and non-vector-borne diseases or serve as non-biological vectors of pathogens (Hopla et al. 1994, McNair 2015, Torres et al. 2012, Kocan et al. 2003). These facts stress the need to develop vaccines effective in different hosts and against several tick species and multiple ectoparasite infestations. However, a limited number of ectoparasite protective antigens have been characterized so far in different hosts and are cross-protective against multiple tick species or multiple ectoparasites (Nisbet and Huntley 2006, Parizi et al. 2012, Neelakanta and Sultana 2015, Rodriguez-Valle et al. 2012, Carreon et al. 2012, Moreno-Cid et al. 2013, de la Fuente et al. 2011, 2013, 2015, Torina et al. 2014, de la Fuente and Contreras 2015) (Tab. 1). However, despite these limited results, the use of similar strategies for the identification of protective antigens in different ectoparasite species may result in the development of vaccines for the control of multiple ectoparasite infestations.

2.2 Experience with Subolesin/Akirin-based Vaccines

Tick subolesin, the ortholog of insect and vertebrate akirin, was discovered in 2002 as a tick protective antigen in *Ixodes scapularis* by expression library immunization in a mouse model of tick infestations (Almazan et al. 2003). Then, in 2008 akirins were reported as highly conserved nuclear proteins required for NF-κB-dependent gene expression in fruit flies and mice (Goto et al. 2008). Only one subolesin/akirin gene has been identified in ticks and insects that is evolutionarily and functionally related to mammalian *akirin2* (de la Fuente et al. 2011, 2013).

In ticks, subolesin functions as a transcription factor required for NF-κB-dependent and independent gene expression and regulation of the innate immune response to pathogen infection (de la Fuente et al. 2011, 2013, Naranjo et al. 2013). The broad function of subolesin as a transcription factor explains the profound effect of gene knockdown on tick physiology and reproduction (de la Fuente et al. 2011, 2013) and as a protective antigen against infestation with multiple ectoparasite species and infection with vector-borne pathogens (Moreno-Cid et al. 2013, de la Fuente et al. 2011, 2013, Merino et al. 2013a). Vaccination with subolesin/akirin has shown a reducing effect on infestations by soft and hard ticks in their various developmental stages (*I. scapularis*, *I. ricinus*, *R. microplus*, *R. annulatus*, *R. sanguineus*, *Amblyomma americanum*, *Dermacentor variabilis*, *Ornithodoros erraticus*, *O. moubata*), mosquitoes (*Aedes albopictus*), poultry red mites (*Dermanyssus gallinae*), sand flies (*Phlebotomus perniciosus*), and sea lice (*Caligus rogercresseyi*) (de la Fuente et al. 2011a, 2013). Subolesin knockdown by RNA interference (RNAi) produces sterile female and male ticks (Merino et al. 2011, de la Fuente et al. 2011, 2013). Therefore, a sterile acarine technique for autocidal control of tick populations by release of subolesin-knock-

down ticks was proposed and proved effective for the control of *R. microplus* in combination with subolesin-based vaccination in cattle (Merino et al. 2011a).

Vaccination with tick subolesin reduces tick infection with *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Babesia bigemina*, and *Borrelia burgdorferi* (Merino et al. 2013a) and mosquito infection with malaria parasite *Plasmodium berghei* (da Costa et al. 2014). However, vaccination did not affect infection with tick-borne encephalitis virus (Merino et al. 2013a). Because of subolesin's role in tick innate immune response to pathogen infection (de la Fuente et al. 2011, 2013), targeting subolesin by vaccination or RNAi reduces tick immunity, thereby increasing pathogen infection levels. However, lower pathogen infection levels result from the effect on tissue structure and function and the expression of genes that are important for pathogen infection and multiplication (de la Fuente and Contreras 2015). Both effects of targeting subolesin result in lower tick infestations, feeding and fertility (Merino et al. 2013a, de la Fuente and Contreras 2015).

These results challenge the paradigm that intracellular proteins are not capable of inducing a protective response against ectoparasite infestations (de la Fuente et al. 2011). Host antibodies may interact with arthropod intracellular proteins through a process that has not been fully characterized, but results suggest that antibodies may be specifically transported across the midgut barrier into the hemolymph and then enter into cells to interact with these intracellular proteins (de la Fuente et al. 2011, Merino et al. 2013a). Nevertheless, other possibilities should be considered to explain the effect of the vaccination with subolesin, including the effect of a host-cell-mediated immune response and antibody responses that are cross-reactive with other proteins (Merino et al. 2013a, Trimmell et al. 2005, 2002).

These results obtained with subolesin/akirin antigens support the possibility of developing vaccines for the control of multiple ectoparasites and infection/transmission of vector-borne pathogens (de la Fuente and Contreras 2015).

3. Vaccinomics: Focusing on Ectoparasite-Host-Pathogen Interactions for Vaccine Development to Control both Ectoparasite Infestations and Pathogen Infection and Transmission

3.1 Vaccinomics Concept and Pipeline

Vaccinomics is a holistic approach based on the use of genome-scale or omics technologies and bioinformatics for the development of next-generation vaccines (Poland et al. 2011, de la Fuente and Merino 2013). The hypothesis behind vaccinomics is that ectoparasites, hosts and pathogens have evolved molecular interactions that affect their survival and life cycle, and that this co-evolution involves genetic traits of the ectoparasites, hosts and pathogens. Vaccinomics is thus translational research in which basic biological information on ectoparasite-host-pathogen interactions translates into the development of new generation vaccines. The pipeline and the methods proposed for tick vaccinomics have recently been described (de la Fuente and Merino 2013, Contreras et al. 2015b).

The systems biology approach to the characterization of the molecular mechanisms that mediate ectoparasite-host-pathogen interactions identifies host/ectoparasite response and infection-transmission factors and will likely provide new targets for the control of ectoparasite infestations and pathogen infection and transmission (de la Fuente and Merino 2013).

Omics data integration should be followed by the identification of candidate protective antigens using a combination of different screening platforms to formulate and test vaccines in controlled pen trials and under field conditions (de la Fuente and Merino 2013). Finally, the biological function and protective mechanisms elicited by vaccination should be characterized to provide additional information to design improved vaccines based on the combination of antigens with different functions to produce a synergistic effect upon vaccination (de la Fuente and Merino 2013). The integration of omics databases and the identification of candidate protective antigens are the major challenges in vaccinomics (de la Fuente and Merino 2013). However, recent results using the *I. scapularis*-host-*A. phagocytophilum* model illustrate the possibilities of these new technologies for the identification of key molecules and pathways involved in ectoparasite-host-pathogen interactions.

3.1.1 Systems Biology of *I. scapularis*-Host-*A. phagocytophilum* Interactions: the Tick/Host Side

The characterization of tick-*Anaplasma* molecular interactions using transcriptomics and proteomics has shown that *A. phagocytophilum* remodels the cytoskeleton to increase infection through actin alterations and spectrin-α chain or α-fodrin upregulation (Ayllon et al. 2013, Sultana et al. 2010). Cytoskeleton remodelling through actin reorganization has also been shown to facilitate *A. phagocytophilum* infection of vertebrate host cells (Severo et al. 2015).

Intracellular bacteria use different strategies to inhibit cell apoptosis in order to enhance their replication and survival. Recently, Ayllon et al. (2013, 2015) demonstrated that *A. phagocytophilum* infection inhibits cell apoptosis through the activation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway and inhibition of the mitochondrial intrinsic apoptosis pathway to establish infection in *I. scapularis* tick midguts and salivary glands, respectively. Furthermore, the integrated analysis of metabolomics, transcriptomics and proteomics data identified the effect of *A. phagocytophilum* infection on the protein processing in endoplasmic reticulum and glucose metabolic pathways, which represent additional mechanisms by which *A. phagocytophilum* infection inhibits cell apoptosis in order to increase tick hemocyte-type cell infection (Villar et al. 2015a). In vertebrate host cells, *A. phagocytophilum* infection also activates anti-apoptotic mechanisms to establish infection (Severo et al. 2015).

Pathogens subvert cellular immune response to favour infection and multiplication. Analyses of changes in tick cell transcriptome and proteome in response to *A. phagocytophilum* infection have demonstrated an effect of bacterial infection on the inhibition of cell innate immune responses (Ayllon et al. 2015, Pruneau et al. 2014). Additionally, tick saliva contains diverse pharmacologically active molecules that affect various immune cell populations in order to promote tick feeding in vertebrate hosts while also facilitating pathogen transmission (Kotal et al. 2015). In neutrophils, results suggest that *A. phagocytophilum* developed mechanisms to subvert the innate immune protective mechanisms while other pathways are activated to control infection (de la Fuente et al. 2005, Lee et al. 2008, Galindo et al. 2012, Pruneau et al. 2014). Menten-Dedoyart et al. (2011) recently demonstrated that tick feeding affects the host immune response by inhibiting differentiation of mature B cells into plasma cells at the feeding site while not altering the formation of memory B cells required for vaccine efficacy.

These studies suggest that *A. phagocytophilum* uses common strategies for infection of ticks and vertebrate hosts and provides candidate proteins and pathways to be targeted by vaccination affecting *A. phagocytophilum* infection and transmission.

3.1.2 Systems Biology of *I. scapularis*-Host-*A. phagocytophilum* Interactions: the Pathogen Side

The *Anaplasma* major surface proteins (MSP) such as MSP1, MSP2 (p44), MSP3 and MSP4 play a role in bacterial infection of tick and vertebrate host cells (Kocan et al. 2003, Pruneau et al. 2014, Dugat et al. 2015). Recently, Villar et al. (2015b) reported using a proteomics approach whereby *A. phagocytophilum* MSP4, heat shock protein (HSP) 60 (GroEL) and HSP70 proteins interact and bind to tick cells, thereby playing a role in pathogen-tick interactions. Furthermore, recent results support the role of *A. phagocytophilum* proteins secreted through the T4 secretion system (T4SS) in controlling host epigenetics and global DNA methylation (Rennoll-Bankert et al. 2015, Sinclair et al. 2015). These novel mechanisms are likely to occur in both tick and vertebrate host cells and allow nuclear effectors of intracellular pathogens to manipulate host chromatin and gene expression to establish infection.

These results provide candidate pathogen-derived antigens that could be used to control infection in the tick vector and, if conserved, these antigens could also be used to control pathogen transmission and host infection.

3.1.3 Systems Biology of Tick-Host Interactions

Vector-borne diseases challenge our understanding of emerging diseases. Recently, arthropod vectors have been involved in emerging anaphylactic diseases in America, Europe, Asia and Australia (Cabezas-Cruz et al. 2014, 2015). In particular, IgE antibody response to the carbohydrate α-gal (Galα1-3Galβ1-(3)4GlcNAc-R) after a tick bite was associated with allergies to red meat, cetuximab and gelatin (Cabezas-Cruz et al. 2014, 2015). In contrast, anti-α-gal IgM antibody response was shown to protect against mosquito-borne malaria (Yilmazy et al. 2014). Therefore, establishing the source of α-gal in ectoparasites and the immune response to vector bite and transmitted pathogens is essential for diagnosing, treating and ultimately preventing these emerging anaphylactic and other vector-borne diseases (Cabezas-Cruz et al. 2015).

Different proteomics approaches can then be used to characterize the anti-α-gal immunoglobulin content in patients with anaphylactic reactions to vector bite and the tick proteins that react with patients' serum when compared to an unrelated control individual with a history of tick bites but with no allergic reactions. The comparative proteomics analyses using sera from patients and matching healthy control individuals allow the identification of tick proteins that react with patient but not control sera. Tick proteins putatively present in the sialome (from the Greek *sialos* = saliva) and reacting with patient but not control sera could be used for diagnosis of a predisposing condition for allergy to tick bites. Furthermore, sialome proteins labelled with anti-α-gal antibodies and recognized by patient but not control sera could be selected as potential source of α-gal responsible for anaphylactic reactions to tick bites.

3.2 Vaccinomics Identification and Characterization of Candidate Tick Protective Antigens

The results of the systems biology studies of tick-host-*A. phagocytophilum* interactions suggest using a combination of *A. phagocytophilum*-derived ligands and/or tick/host receptors and other tick protective antigens in vaccines could be used to immunize vertebrate hosts to prevent pathogen infection while decreasing tick infestations and the probability for pathogen

infection in ticks feeding on immunized but already infected hosts (DE LA FUENTE and CONTERAS 2015). In this way, the vaccine containing vector and pathogen-derived antigens will protect vertebrate hosts against ectoparasite infestations and pathogen infection while also decreasing pathogen transmission and therefore the risk of disease in humans and animals.

As a proof of concept for the vaccinomics approach in ticks, a pipeline was designed based on proteins identified as involved in tick-pathogen interactions (DE LA FUENTE and MERINO 2013) (Fig. 1). These proteins were selected as candidate protective antigens based on their putative role in tick-*A. marginale* (silk, ZIVKOVIC et al. 2010), tick-*B. bigemina* (trospa, ANTUNES et al. 2012) and tick-pathogen (subolesin, MERINO et al. 2013b) interactions. First, *in vitro* capillary feeding was used to characterize their potential as antigens for the control of both cattle tick infestations and infection with *A. marginale* and *B. bigemina* (ANTUNES et al. 2014).

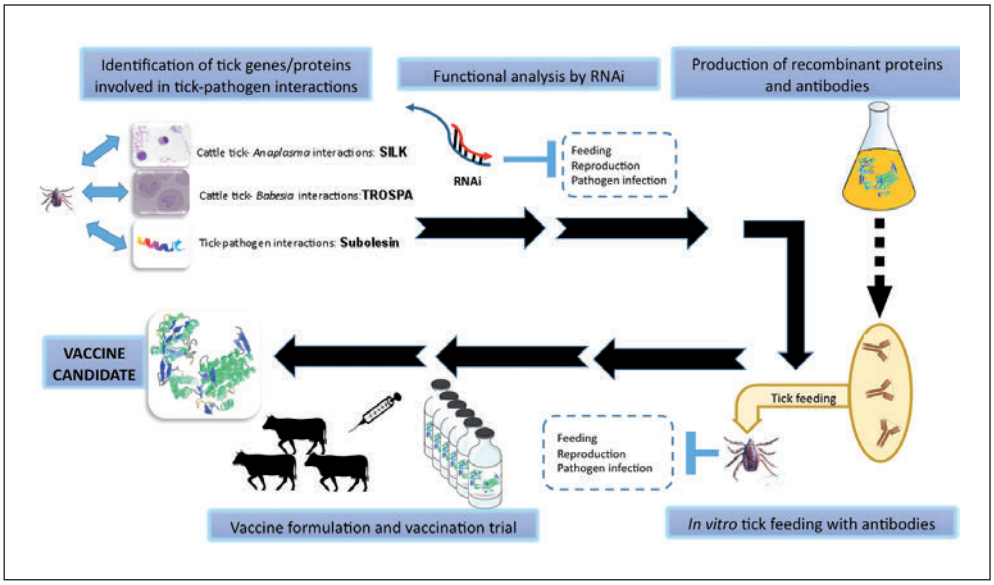

Fig. 1 Vaccinomics pipeline for the identification of ectoparasite-derived protective antigens

Recombinant proteins were produced in *Escherichia coli*, purified and used to generate antibodies in rabbits. Purified rabbit polyclonal antibodies were added to uninfected or infected bovine blood to capillary-fed female *R. microplus* ticks. Capillary-fed ticks ingested antibodies added to the blood meal, and a reduction in tick weight and/or oviposition was shown when compared to controls fed with blood alone (ANTUNES et al. 2014). However, no effect was observed on pathogen DNA levels in capillary-fed ticks (ANTUNES et al. 2014). Nevertheless, these results, along with previous results of functional studies by RNAi, suggest that these proteins are good candidate vaccine antigens for the control of *R. microplus* infestations and infection with *A. marginale* and *B. bigemina*. This supports the conducting of vaccine trials to validate these antigens (MERINO et al. 2011b). The results show that vaccination with Silk and Subolesin but not Trospa reduced tick infestations and oviposition with respect to

ticks that had fed on placebo-treated control cattle (Merino et al. 2011b). Furthermore, the results also showed that vaccination with Trospa and Subolesin reduced *B. bigemina* DNA levels in ticks while vaccination with Silk and Subolesin resulted in lower *A. marginal*e DNA levels when compared to ticks that had fed on placebo control cattle (Merino et al. 2011b).

These results prove the validity of the vaccinomics approach to selecting protective antigens and show that vaccines using tick proteins involved in vector-pathogen interactions could be used for the dual control of tick infestations and pathogen infection.

3.3 Characterization of the Biological Function of Protective Antigens and Vaccine Protective Mechanisms

The characterization of the biological function of protective antigens is important for explaining the protective mechanisms of the vaccine, which is essential for designing more efficient vaccine formulation with combined antigens (de la Fuente and Merino 2013, de la Fuente and Contreras 2015). If antigens protect through different mechanisms, then it may be possible to combine them to increase vaccine efficacy thanks to a synergistic effect between antigens.

Although not fully understood, the functions of BM86 and subolesin are likely to be different. While BM86 may be involved in tick feeding (Popara et al. 2013), subolesin is a transcription factor required for the regulation of the expression of genes involved in different processes such as tick feeding, reproduction and regulation of the innate immune response to pathogen infection (de la Fuente et al. 2011, 2013, Naranjo et al. 2013). Based on these results, a new vaccine formulation containing a combination of BM86 and subolesin was recently patented that claimed a synergy between these antigens resulting in higher vaccine efficacy against cattle tick infestations (Schetters and Jansen 2014, de la Fuente and Contreras 2015).

These results support the idea that a combination of protective antigens increases tick vaccine efficacy but also suggest the possibility of using a similar approach for the development of vaccines against multiple ectoparasite infestations.

4. Vaccinomics Approach to the Development of Vaccines for the Control of Multiple Ectoparasite Infestations

A vaccinomics approach could then be used to identify and fully characterize candidate protective antigens in different ectoparasites and to validate vaccine formulations. New candidate protective antigens will most likely be identified by focusing on abundant proteins with relevant biological function in ectoparasite feeding, reproduction, development, immune response, subversion of host immunity, and pathogen infection and transmission. Furthermore, focusing on evolutionarily conserved protective antigens across different ectoparasite species such as subolesin/akirin may allow the design of effective vaccines against multiple ectoparasite infestations. The application of experimental approaches previously used in ticks for the identification of candidate protective antigens could also be used in other ectoparasite species (Marr et al. 2014).

The efficacy of antigen combinations on ectoparasite infestations was first demonstrated by Allen and Humphreys (1979) using tick protein extracts. Recently, protective epitopes were characterized in tick subolesin and mosquito akirin (Prudencio et al. 2010). Then,

Moreno-Cid et al. (2013) used chimeric antigens composed of protective epitopes from tick subolesin and mosquito akirin for the control of tick, mosquito and sand fly infestations, and Merino et al. (2013a) obtained a higher vaccine efficacy with the chimeric antigen when compared to tick subolesin for the control of *R. microplus* infestations in cattle.

These results showed that it is possible to identify protective antigens in different ectoparasites and combine these antigens using chimeras containing conserved protective epitopes. Additionally, protective antigens from different ectoparasites or from pathogens and vectors could be combined to design vaccines against multiple ectoparasites and for the control of pathogen infection and transmission. These antigens could be produced using expression systems such as the antigen fusion to the N-terminal region of the *A. marginale* Major Surface Protein 1a (MSP1a) (Canales et al. 2008, Almazan et al. 2012, Contreras et al. 2015a). This system provides a novel, simple and cost-effective approach for the production of highly immunogenic protective antigens by surface displaying antigenic protein chimera on the *E. coli* membrane and demonstrates the possibility of using recombinant bacterial membrane fractions in vaccine preparations to protect cattle against tick infestations in controlled pen trials (Canales et al. 2008, 2009, 2010, Contreras et al. 2015a) and under field conditions (Torina et al. 2014).

5. Conclusions and Future Directions

The experience obtained from tick vaccine research may provide models for developing vaccines against other arthropods, with the possibility of controlling multiple ectoparasite infestations. The challenge of developing improved vaccines against ticks and other ectoparasites arises from the need to understand the complex molecular relationship between vertebrate hosts, ectoparasites and pathogens. This requires a systems biology approach that allows the integrated analysis of genomics, metabolomics, transcriptomics, proteomics, immunoproteomics and other omics data for discovery of key pathway molecules that mediate ectoparasite-host-pathogen interactions.

A vaccinomics approach could then be used to identify and fully characterize candidate protective antigens in different ectoparasites and validate vaccine formulations. New candidate protective antigens will most likely be identified by focusing on abundant proteins with relevant biological function in ectoparasite feeding, reproduction, development, immune response, subversion of host immunity, and pathogen infection and transmission. As previously demonstrated for tick vaccines (de la Fuente and Contreras 2015), ectoparasite antigens can have multiple impacts when used in a vaccine formulation, including reduction in ectoparasite infestation and fertility, pathogen infection, vector capacity for pathogen transmission, and response to pathogen infection.

Bioinformatics algorithms could be developed to compare data obtained from different ectoparasites to characterize similarities and differences in their response to different stimuli such as feeding or pathogen infection. In this way, it may be possible to identify evolutionarily conserved mechanisms that could be targeted for vaccine development against multiple ectoparasite infestations and/or pathogen infection and transmission. Finally, the role of the microbiome in the ectoparasite response to infestation or pathogen infection should also be considered in designing effective control strategies (Narasimhan and Fikrig 2015, Cabezas-Cruz et al. 2015).

Vaccines based on pathogen-derived and/or ectoparasite-derived antigen combinations or using evolutionarily conserved ectoparasite protective antigens that affect both vector infestations and pathogen infection and transmission could then be developed and used to vaccinate at-risk human and animal populations in order to prevent disease, and reservoir host species in order to reduce vector infestations and their capacity to transmit pathogens that affect human and animal health.

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The Small Hive Beetle in Italy

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Abstract

Aethina tumida Murray (small hive beetle, SHB) was first reported in Italy on 5 September 2014. Three nuclei containing honey bees (*Apis mellifera*) and located in a clementine (*Citrus*) orchard near the port of Gioia Tauro in the Calabria region (southern Italy) were heavily infested with adult and larval *A. tumida*. *A. tumida* infestation is a notifiable disease of honey bees in the European Union as well as an OIE (World Organization for Animal Health) listed disease. The importation of honey bees is strictly regulated in the European Union (Commission Regulation [EU] No. 206/2010) to prevent its introduction. Early reaction measures adopted in Italy require immediate notification of *A. tumida* discovery to the local veterinary services and the immobilization of colonies. Furthermore, a protection area (20 km radius) and surveillance (100 km radius) zone was established. The surveillance zone includes the entire Calabria and Sicily regions, following SHB detection in a single municipality in Sicily at the beginning of November 2014. Compulsory visits to all apiaries in the protection zone with georeferentiation and visual colony inspection according to 5 % expected prevalence (95 % CI) were applied. In the Calabria region, 60 SHB-positive sites were detected and destroyed. Destruction of infested apiaries is compulsory and the soil under the infested colonies must be ploughed and treated with pyrethroids. If apiaries in the protection zone are found to be negative, traps are placed. In the surveillance zone, apiaries are selected according to a risk analysis (migration in infested areas, honey bee or materials exchange) or randomly and colonies are inspected according to 2 % expected prevalence (95 % CI). Furthermore, a SHB surveillance programme has also been initiated at the national level. In September 2015, after almost nine months of epidemiological silence, SHB was detected again in apiaries in the Calabria region located within the protection zone.

Zusammenfassung

Aethina tumida Murray (Kleiner Beutenkäfer) wurde zum ersten Mal am 5. September 2014 in Italien gemeldet. Drei Beuten mit Honigbienen (*Apis mellifera*) in einer Klementinenplantage (*Citrus*) in der Nähe des Hafens von Gioia Tauroin in der Region Kalabrien (Süditalien) wurden schwer von Adulten und Larven von *A. tumida* befallen. Ein *A. tumida*-Befall ist in der Europäischen Union eine meldepflichtige Krankheit von Honigbienen sowie eine von der Weltorganisation für Tiergesundheit (OIE – *World Organization for Animal Health*) gelistete Krankheit. Der Import von Honigbienen ist in der Europäischen Union streng geregelt (Verordnung der Kommission [EU] Nr. 206/2010), um ihre unkontrollierte Einführung zu verhindern. Die ersten Schutzmaßnahmen in Italien betreffen eine sofortige Meldung der Entdeckung von *A. tumida* an den lokalen Veterinärdienst und ein Verbot der Umsiedlung betroffener Kolonien. Es wurde eine Schutzzone (20 km Radius) und ein Überwachungsgebiet (100 km Radius) festgesetzt. Nach dem Fund eines Kleinen Beutenkäfers in einer einzigen Gemeinde Siziliens Anfang November 2014 schließt die Überwachungszone das gesamte Gebiet Kalabriens und Siziliens ein. Obligatorisch wurden sämtliche Bienenstöcke in der Schutzzone nach Georeferenzierung aufgesucht und gemäß der erwarteten Prävalenz von 5 % (95 % CI) visueller Koloniekontrolle unterzogen. In der Region Kalabrien wurden 60 positive Stätten gefunden und zerstört. Die Vernichtung der befallenen Bienenstände ist verpflichtend vorgeschrieben, und der Boden unter den befallenen Stöcken muss gepflügt und mit Pyrethroiden behandelt werden. Erweisen sich die Bienenstände in der Schutzzone als negativ, dann werden Fallen aufgestellt. In der Überwachungszone werden Bienenbeuten nach einer Risikoanalyse (Abwanderung in befallene Gebiete, Honigbienen- oder Materialaustausch) oder beliebig ausgewählt und auf die erwartete Prävalenz von 2 % (95 % CI) überprüft. Außerdem wurde auf nationaler Ebene ein Überwachungsprogramm für den Kleinen Beutenkäfer eingeführt. Im September 2015, nachdem fast neun Monate epidemiologisch Ruhe herrschte, wurde der Kleine Beutenkäfer wieder in Bienenstöcken innerhalb der Schutzzone in der Region Kalabrien entdeckt.

1. Introduction

The first detection of *Aethina tumida* Murray, the small hive beetle (SHB, Cuthbertson et al. 2013), in Italy was made on 5 September 2014 (Palmeri et al. 2015). Three nucleus hives containing honey bees and located in a clementine (*Citrus*) orchard in the Calabria region (southern Italy) at the locality of Sovereto (N 38.45474 E 15.94110), Gioia Tauro municipality (Fig. 1) were found to be heavily infested with adult and larval beetles. Upon this discovery, the three bee nuclei were wrapped and brought back to the Università "Mediterranea" in Reggio Calabria, where they were killed and immediately deep frozen. A sample of approximately 15 adult and 15 larval beetles were taken for identification. The specimens were identified as *Aethina tumida* based on morphological characteristics. On 10 September 2014, the specimens were sent to the Italian National Reference Laboratory (NRL) (*Istituto Zooprofilattico Sperimentale delle Venezie*). The species was confirmed through morphological identification (*OIE* 2015a). Some adults and larvae were sent to the European Reference Laboratory (EU RL) in Sophia-Antipolis (France) where the species was subsequently confirmed through morphological identification on 15 September and through molecular techniques on 17 September 2014. On 18 September, *A. tumida* detection in Italy was reported to the OIE (World Organization for Animal Health) (Mutinelli 2014, Mutinelli et al. 2014, *OIE* 2015b).

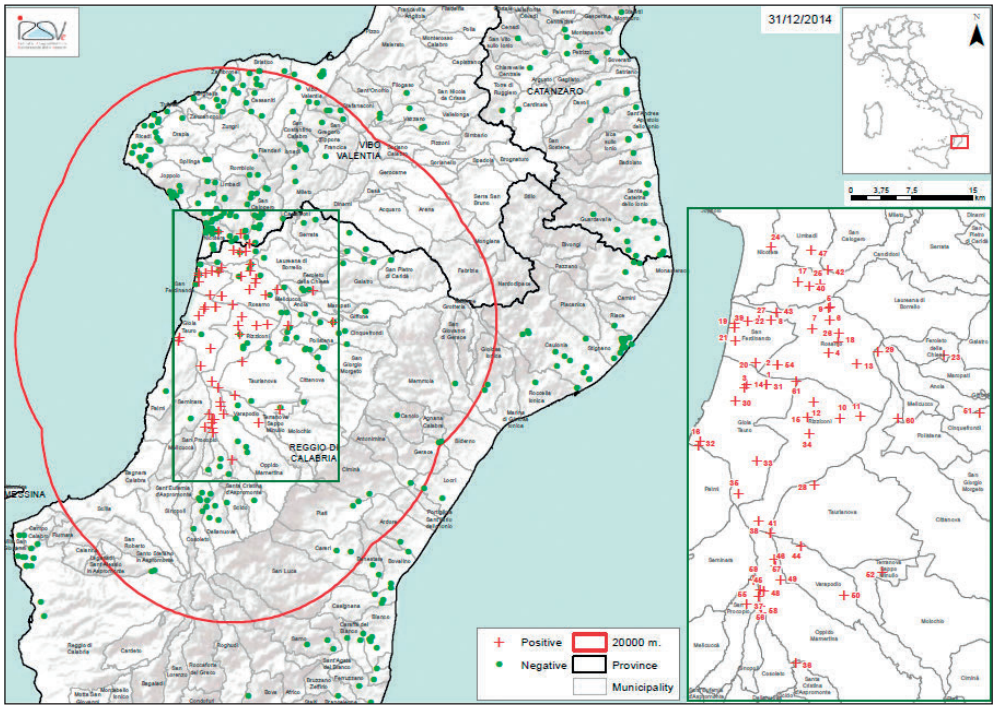


Fig. 1 Location of apiaries infested with SHB in the Calabria region (31 December 2014). *Red cross*: infested apiary. *Green dot*: visited apiary but no SHB detected. The control zone (20 km from infested apiaries) is delineated by the red circle.

The site where the beetles were discovered was treated with chlorpyriphos methyl on 5 September by the University team and on 11 September by both, the veterinary services and the University team. The pesticide was mixed with water and poured directly on the soil at 201 per 15 m² of soil surface. A third insecticide treatment was applied on the site by abundantly spraying a 1 % solution of cypermethrin and tetramethrin (6.85 % and 1.25 % concentration respectively) after having ploughed the soil on 17 September. Two new bee nuclei were installed on 17 September in close vicinity (50 m) of the initial nuclei site and were fitted with traps (E. H. Thorne [Beehives] Ltd; Rand, UK; Schäfer et al. 2008, 2010). The traps were reduced in size by two thirds so that they could fit through the nucleus hive entrances. Adult beetles were found in the nuclei on 10 October, so the nuclei were destroyed on 14 October. The soil under and around the hives was abundantly sprayed with a 1 % solution of cypermethrin and tetramethrin by the veterinary service team.

During October 2014, the three infested and frozen nuclei were thoroughly investigated at the Italian NRL in Legnaro (Padova) revealing the presence of 19 larvae; 12 recovered from adult bees and 7 from combs. Nucleus No. 1 contained 4 larvae, nucleus No. 2, 12 larvae, and nucleus No. 3, 3 larvae. No adult beetles were found.

2. Field Observations

On 17 September, the soil at the initial discovery site was excavated to search for SHB pupae. Five dipteran pupae and one larvae of a big coleopteran (according to the size and the morphological characteristics it was not *A. tumida* but probably a xylophagus species) were found. The soil was then ploughed and treated with a 1 % solution of cypermethrin and tetramethrin. On 18 September, traps established in the two newly installed nuclei were checked. No beetles were found. One nucleus was inspected visually, and no beetles were found.

From 16 to 17 September 2014, a team composed of beekeepers, biologists, and official veterinarians visited five apiaries, all located in the vicinity of the original discovery site. A proportion of the colonies (ranging from 20 to 50 %) in each apiary were inspected for SHB. Hive examination may provide an early diagnosis of infestation. This procedure is accomplished with two people, usually the beekeeper and the official veterinarian, one to work the colony and the second to collect the beetles. First they remove the lid from the colony and examine the inner part of the lid for the presence of adult beetles and place the lid at the side of the bee hive. Then they lightly smoke the colony, remove the outermost frame in the super and/or in the hive (Dadant-Blatt type), and quickly examine both faces of the comb. The outermost frame is then placed at the side of the hive and all the other frames undergo the same visual inspection one by one. Once inspected, each frame is reintroduced in the super or in the hive in the same order using the room left available by the outermost frame in order to prevent robbing. Then the inside faces of the hive and the bottom board are carefully examined. When all frames have been inspected, they are replaced in the original position as is the outermost frame and the hive is closed. When present, the frames of both the super and the hive should be thoroughly examined. This procedure allowed us to detect both beetle adults and larvae. In each apiary, 5 to 27 traps were installed during the visit.

Adult SHB were detected on 17 September 2014 through visual observation in one apiary located 2 km from the original discovery site in Collina locality, Rosarno municipality, Reggio Calabria province. Six colonies were examined out of 41 located in this apiary. SHB were observed in four colonies. In total, 7 adults were collected. A set of 27 colonies were equipped with

traps. On 18 September, all 41 colonies were inspected thoroughly. Eighteen adult beetles were collected from 12 hives. About six adult beetles escaped the sampling by flying away. Of the 27 traps, two hosted adult small hive beetles. No brood destruction was observed in any of the 41 colonies inspected. Out of the four positive colonies detected the previous day, two were positive again the next day. The entire apiary was destroyed according to the regulations in force.

Two weak nucleus bee colonies were placed in the same site mentioned above on 4 November, and they were found positive for SHB adults on 13 November 2014. Both nuclei were destroyed on 14 November 2014.

3. European and National Regulatory Framework

A. tumida was exotic to Europe until September 2014. Being a notifiable disease in the European Union (*European Commission* 1982, 1992, 2004) and an OIE listed disease (*OIE* 2015b), any identification of the pest must be reported to national competent authorities, to the European Commission, and to the OIE. Member states of the European Union must implement passive surveillance programmes specifically directed at this species. Assistance comes from the recent EU epidemiological study on honey bee colony losses (Chauzat et al. 2014). In case of detection, contaminated apiaries should be destroyed. In the present case, traps were installed in all the colonies located in apiaries with no signs of infestation (meaning the presence of SHB adults, larvae, or damaged frames).

The importation of honey bees is strictly regulated in the European Union. Only queen bees with attendants (20 maximum) should be imported in Europe according a stringent authorization pathway (*European Commission* 2010). Packages should be examined at the place of origin and at destination. Furthermore, queen bees should come from an area of at least 100 km radius that is not subject to any restrictions associated with the occurrence of SHB and where this infestation is absent (Mutinelli 2011). In 2004, the inspection carried out on queens and accompanying workers of *Apis mellifera ligustica* legally imported into the region of Alentejo (Portugal) from Texas (USA) prevented a potential introduction of *A. tumida* (Murilhas 2004, Neumann and Ellis 2008, Valério da Silva 2014). The queen bees, attendants, food and packaging, as well as the destination apiaries where the queens went before the check on the entire imported batch was completed, were destroyed.

Following the confirmed occurrences of the SHB in Italy, an implementing decision concerning certain protective measures was published on 16 December 2014 (*European Commission* 2014). Italy shall ensure the implementation of the ban on the dispatch of consignments of: (*i*.) honey bees; (*ii*.) bumble bees; (*iii*.) unprocessed apiculture by-products; (*iv*.) beekeeping equipment; and (*v*.) comb honey intended for human consumption from the areas listed in the Annex (the entire Calabria and Sicily regions) to other areas of the European Union, as well as the carrying out of immediate inspections and epidemiological investigations in the infested area. This requirement was due to expire on 31 May 2015 but was then extended until 30 November 2015 (*European Commission* 2015a). The period of application of the protective measures in relation to the SHB in Italy was then extended until 31 March 2017 by Commission Implementing Decision (EU) 2015/1943 of 27 October 2015 amending Implementing Decision 2014/909/EU (*European Commission* 2015b).

Furthermore, the Commission asked the European Food Safety Authority (EFSA) to provide scientific and technical assistance concerning: (*i*.) the currently employed diagnostic

methods for the detection of SHB and the risk-mitigation measures applied worldwide in relation to SHB in apiaries and in controlled establishments producing queens, as well as measures applied to domestic movements of colonies, queens, and other honey bee products and by-products; (*ii*.) the best practices or strategies to be applied in an infected area in order to respectively eradicate or control the spread of the SHB. The EFSA scientific report on small hive beetle diagnosis and risk management options was published on 17 March 2015 (*EFSA* 2015a). The EFSA scientific opinion on the survival, spread, and establishment of the small hive beetle (*A. tumida*) was published on 15 December 2015 (*EFSA* 2015b).

The EU Reference Laboratory for bee health – in cooperation with the NRLs of Germany, UK, and Italy – has produced a leaflet to inform veterinary services, practitioners, and beekeepers on the threat posed by SHB to honey bees in order to better identify any outbreak. This leaflet has been translated from English into other European languages and disseminated throughout the European Union and additional countries (*European Union Reference Laboratory* 2015).

Following the detection of SHB in Italy, a restriction zone with a 20 km radius from the initial infested site was established (Fig. 1). Any movement of colonies or beekeeping material within this zone and between this zone and other areas was forbidden. In this zone, all apiaries should be visited and a proportion of hives fully inspected. This proportion was deemed appropriate to detect SHB presence at an expected prevalence of 5 % with a 95 % confidence interval (CI). Therefore, the number of inspected colonies varied according to the number of colonies in the apiary. A surveillance zone within a 100 km radius from the initial infested site was established (Fig. 2). Apiaries in this zone were sampled according to two strategies: the presence of risk factors or, if none, a random selection of apiaries within the zone. This allowed us to detect SHB at a 2 % apiary level expected prevalence with a 95 % CI (i.e. at least 149 apiaries randomly selected). The traps (E. H. Thorne Beehives Ltd., Rand, UK; homemade corrugated plastic sheet; and Beetle Blaster®, Mann Lake, MN) were installed in all hives under surveillance. Figures 1 and 3 show the results of inspections as of 9 March 2015. SHB has been detected in 59 apiaries and one natural honey bee colony in the Calabria region and in eighteen municipalities belonging to two provinces (province with number of infested apiaries in parentheses): Reggio Calabria (56 and a natural colony) and Vibo Valentia (3). This represents an area of over 316 km^2. In most cases, with the exception of six sites, three in Gioia Tauro, one each in Rizziconi, Candidoni, and Cittanova municipalities where larvae have been detected (Fig. 1), only adult SHB were observed. In only one case, a single pupa was found (Gioia Tauro municipality) in the soil of an infested apiary that also contained adult and larval beetles. A single adult beetle was found in a natural honey bee colony (Gioia Tauro municipality). The colony and the combs were collected and the bees killed. Neither adult nor larval SHB were detected after a thorough investigation of bees and combs.

All the infested apiaries were destroyed by the veterinary service team according to the decision taken by the Italian Ministry of Health and shared with the National Beekeeping Associations during the meeting held in Lamezia Terme (Calabria) on 22 September 2014 and further confirmed in the meeting of the National Crisis Unit held in Rome on 11 December 2014. A Decree of the Italian Ministry of Health of 19 November 2014 guaranteed compensation for colonies and beekeeping equipment destroyed due to infestation by *A. tumida* according to Italian Law 218/1988.[1]

1 http://www.trovanorme.salute.gov.it/norme/dettaglioAtto?id=50871.

Apiary "stamping-out" was carried out according to a common protocol based on closure of the hives in the evening, killing the honey bees using sulphur dioxide spray, and burning the dead colonies on site. A soil treatment was abundantly applied in the apiary as 1 % solution of cypermethrin and tetramethrin of a commercially available product after soil ploughing.

The territory coverage where the infested apiaries were located is 38.98 % fruit trees and berry plantations, 25.42 % olive groves, 20.34 % annual crops associated with permanent crops, 5.08 % non-irrigated arable land, 3.40 % continuous urban fabric and complex cultivation patterns, 1.69 % construction sites and principally occupied by agriculture, with significant areas of natural vegetation (*Corine* 2006). The soil in the area where infested apiaries were detected is sandy, soft, warm, and dry (Province di Reggio Calabria e di Vibo Valentia 2013). The area is also well ventilated due to the vicinity to the sea.

For the 60 infested sites in Calabria, several other apiaries were visited, without reporting the presence of any SHB as of 7 July 2015: 222 apiaries within the protection zone (20 km radius from the infested sites); 452 apiaries within the surveillance zone (100 km radius); and 356 apiaries outside the two zones, for a total of 1,030 apiaries in 2014. From January to July 2015, 782 apiaries were visited throughout the territory of Calabria.

On 7 November 2014 SHB was detected in a 56 hives migratory apiary located in the municipality of Melilli, Siracusa province, Sicily region (Fig. 2). The epidemiological investigation carried out by the local veterinary service demonstrated that these hives had been in the area of the first detection of SHB (Gioia Tauro, Calabria region) from April to August 2014. As of 7 July 2015, no further detection of *A. tumida* in Sicily region has been reported according to the inspection carried out in 15 apiaries within the protection zone (10 km from the infested sites), 202 within the surveillance zone (100 km) and 11 outside the two zones for a total of 228 apiaries (Fig. 2) in 2014. Between January and July 2015, 318 apiaries have been visited throughout the Sicily region.

The results of field investigation are available on the website of the Italian National Reference Laboratory.[2]

A preliminary field investigation aiming at detection of SHB on rotten fruit in Calabria citrus and kiwi orchards was carried out between December 2014 and January 2015. Among collected samples, seven different non-SHB Nitidulid species were identified on rotten citrus, while no coleopters were detected on rotten kiwi. Furthermore, no SHB were detected on rotten citrus (Mutinelli et al. 2015). The same results were confirmed by field investigations carried out in July and November 2015 (Mutinelli and Federico, personal communication).

4. Hypothesis on the Introduction of SHB into Italy

As mentioned in the risk assessment performed by the EFSA there are several possible scenarios for the introduction of SHB into Europe (*EFSA* 2013). The port of Gioia Tauro is located near the initial infested apiary. About 2.5 million containers arrive each year in the 7.5 km long harbour. Being a transition platform, most of the containers undergo a document and seal check, and then are transferred from the mother ship to daughter ships or trucks and sent to their final destination. Before this, identity and physical controls are performed on a

2 http://www.izsvenezie.com/aethina-tumida-in-italy/.

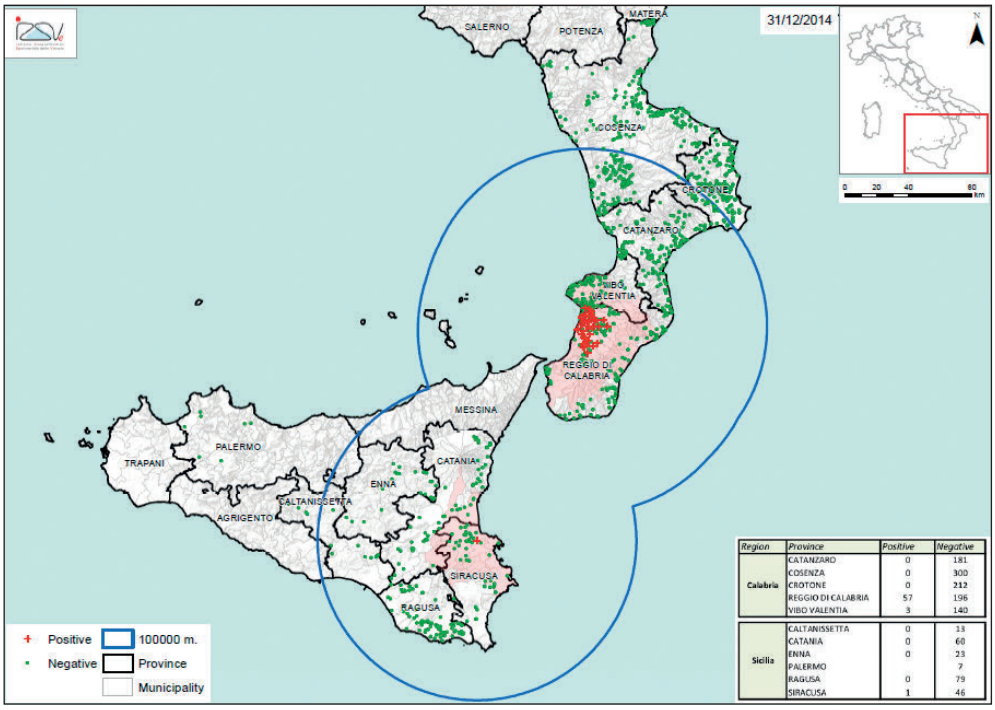


Fig. 2 Surveillance zone (100 km from the initial outbreaks in Calabria and Sicily) for SHB in Italy (December 31, 2014). *Red cross*: infested apiary. *Green dot*: visited apiary, but no SHB detected.

proportion of goods according to the EU Commission indications (custom inspections, plant health inspection, animal health inspection, i.e. Border Inspection Post [BIP], etc.). The list of ports of origin/loading of the containers that arrived in the port of Gioia Tauro between January and August 2014 was requested to the competent authority. The port of Gioia Tauro is not authorized for live animal introduction.

No honey bee swarm had been noticed in the harbour before the notification of the presence of SHB in Italy, although a wasp's nest had been observed on one occasion two years before the event.

The illegal importation of honey bees or bee products has also been considered. This much-trafficked area of southern Italy is subject to many movements of honey bee colonies as well as bee equipment. In the flat territory of Reggio Calabria province, the number of colonies usually doubles each year starting from April for the blooming of citrus, from 10,000 to 20,000 colonies. Extra colonies come mainly from Sicily and from other parts of Italy as well as from abroad to forage on the available plant resources, including citrus, eucalyptus, and chestnut. After the honey flow, colonies are returned to their place of origin, thus facilitating the possible spread of SHB. This area is a significant source of honey bees and queen bees for Italy, much of Europe, and for beekeepers on other continents, making honey production almost a secondary activity. From this point of view, the possible impact of SHB on the southern Italian beekeeping industry and honey bee production is devastating. Following the early reaction measures adopted by the Italian

Ministry of Health, regional veterinary service personnel were requested to investigate any risk movement of honey bees (i.e. migratory beekeeping during 2014), live bee materials (i.e. queen bees and package bees), and bee equipment (Order 0018842-P-12/09/2014 and 0020069-01/10/2014-DGSAF-COD_UO-P). Similar activity is also in progress in the other European countries. In addition to the measures adopted for the Calabria region (Regional Order No. 94 of 19/09/2014), a national surveillance programme for the detection of *A. tumida* was defined and further revised according to the EU Guidelines for the surveillance of SHB (*Aethina tumida*) infestation (Chauzat et al. 2015).

As the soil in this area is sandy, warm and dry (*Province di Reggio Calabria e Vibo Valentia* 2013) and the area well ventilated due to the vicinity to the sea, it can be hypothesized that the conditions are not very favourable for *A. tumida*. It could therefore have been present long before its detection, so the risk of accidental movement elsewhere is much greater. This has been confirmed by the detection of *A. tumida* in hives in the migratory apiary in Sicily that had been in Gioia Tauro from April to August 2014.

According to this consideration and risk analysis, regional veterinary services of all Italian regions are proceeding with apiary and colony inspections. Rapid actions are required to understand, as soon as possible, how far SHB has spread from its initial detection site. This information is needed in order to define the strategy for the possible eradication of the pest (the one presently adopted), to adjust this strategy if necessary or, in the worst case scenario

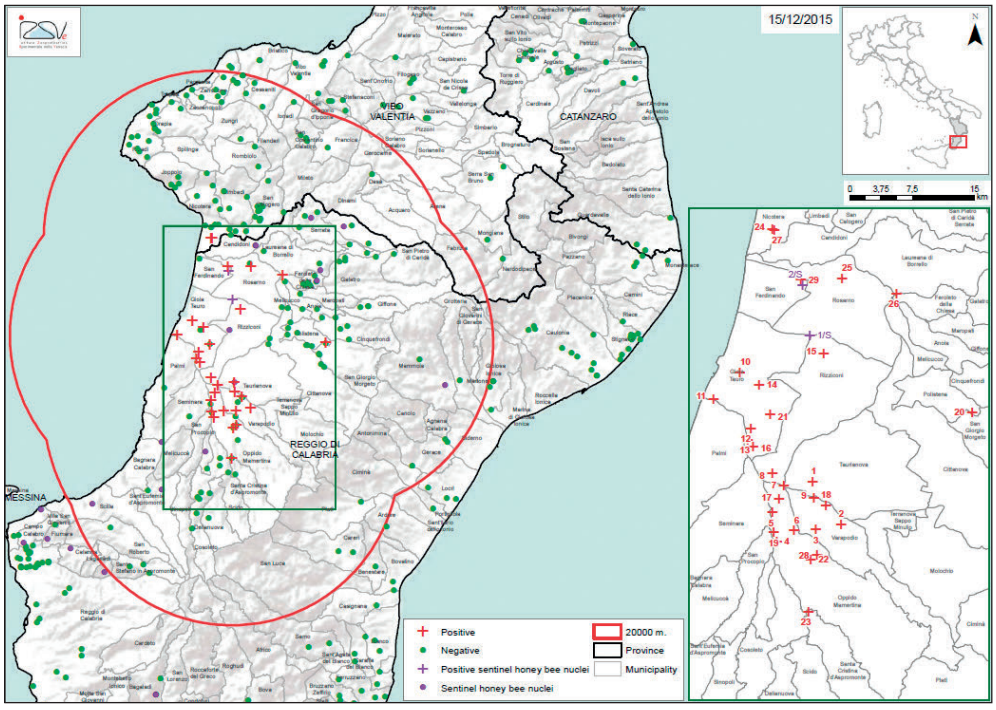


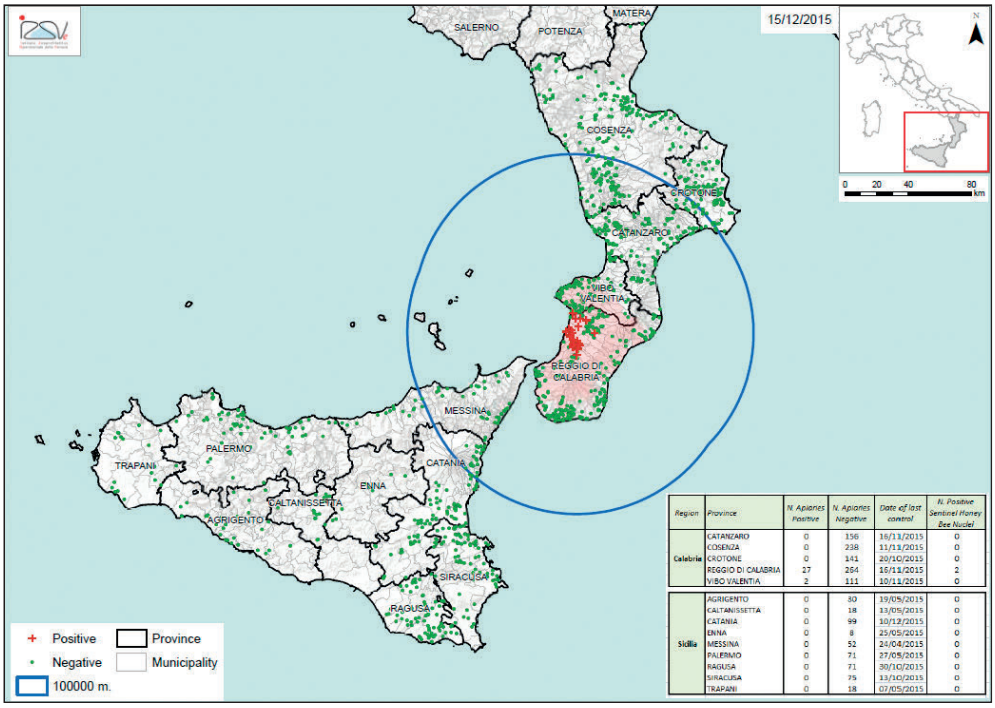
Fig. 3 Location of apiaries infested with SHB in the Calabria region in 2015 (15 December 2015). *Red cross*: infested apiary. *Green dot*: visited apiary but no SHB detected. The control zone (20 km from infested apiaries) is delineated by the red circle. Positive and negative sentinel honey bee nuclei are also included in the map.

(where SHB becomes established as an endemic pest), to replace the eradication protocol with one based on control.

5. Re-Emergence of SHB in Italy

On 16 September 2015, after almost nine months of epidemiological silence, SHB was detected again in an apiary of Taurianova municipality, Calabria region located within the protection zone (Fig. 3). As of 11 December of that year 29 apiaries located within the protection zone had been found infested with SHB (Fig. 3 and 4). All of them have been destroyed and the soil disinfested according with the rules in force. Interestingly, all apiaries infested with SHB in the Calabria region in 2015 presented almost the same location of those infested in 2014 (Fig. 5). No SHB has been detected outside the protection zone and in the rest of Italy (Fig. 4) according to the national surveillance programme.

Furthermore, sentinel honey bee nuclei have been placed within the protection zone and at the border between protection and surveillance zones to contribute to SHB early detection. Following the re-emergence of SHB in the Calabria region, the period of application of the protective measures in relation to the small hive beetle in Italy was extended until 31 March 2017 by Commission Implementing Decision (EU) 2015/1943 of 27 October 2015 amending Implementing Decision 2014/909/EU (*European Commission* 2015b).



Region	Province	N. Apiaries Positive	N. Apiaries Negative	Date of last control	N. Positive Sentinel Honey Bee Nuclei
Calabria	CATANZARO	0	156	16/11/2015	0
	COSENZA	0	238	11/11/2015	0
	CROTONE	0	141	20/10/2015	0
	REGGIO DI CALABRIA	27	264	16/11/2015	2
	VIBO VALENTIA	2	111	10/11/2015	0
Sicilia	AGRIGENTO	0	80	19/05/2015	0
	CALTANISSETTA	0	18	13/05/2015	0
	CATANIA	0	99	10/12/2015	0
	ENNA	0	8	25/05/2015	0
	MESSINA	0	52	24/04/2015	0
	PALERMO	0	71	27/05/2015	0
	RAGUSA	0	71	30/10/2015	0
	SIRACUSA	0	75	13/10/2015	0
	TRAPANI	0	18	07/05/2015	0

Fig. 4 Surveillance zone (100 km from the initial outbreaks in Calabria and Sicily) for SHB in Italy in 2015 (15 December 2015). *Red cross*: infested apiary. *Green dot*: visited apiary but no SHB detected.

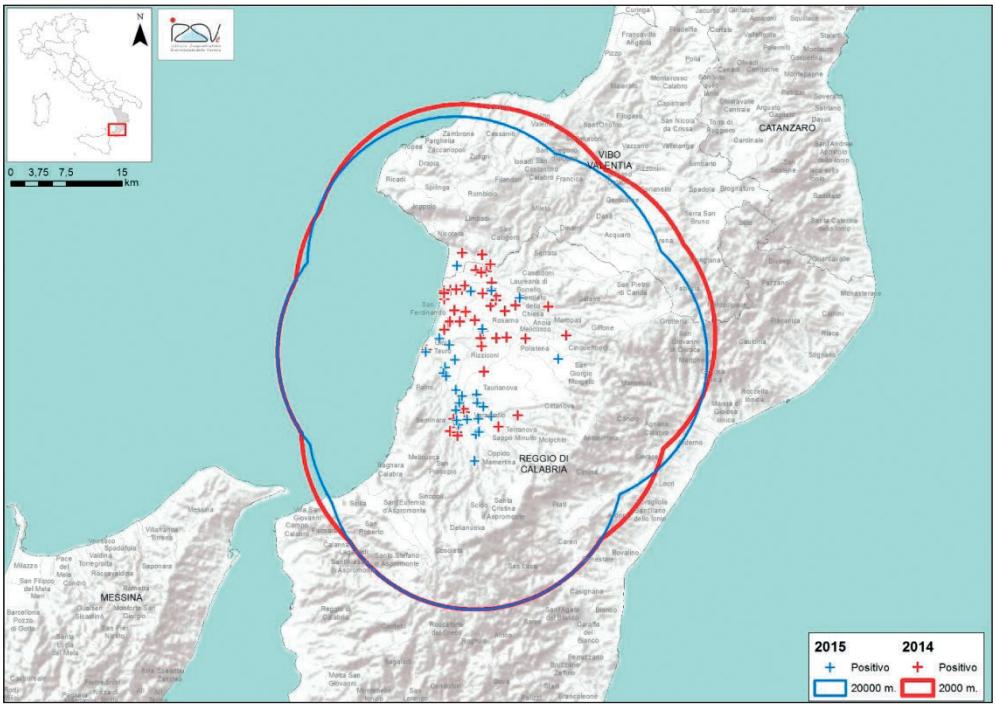


Fig. 5 Location of apiaries infested with SHB in the Calabria region in 2014 and 2015 (11 December 2015). *Red cross*: infested apiary in 2014; *blue cross*: infested apiary in 2015. The control zone (20 km from infested apiaries) is delineated by the red and blue circles for 2014 and 2015, respectively.

6. Conclusions

It is worth noting that visual inspection of the colonies proved to be much more sensitive than the use of traps. Detection of SHB was highly variable depending on the method (visual detection or traps), probably due to the low prevalence of adult beetles in the hives at the time of observation. It is recommended to leave the traps in the hive for at least 48 hours before examination (Neumann et al. 2013). However, until further data are available, we recommend performing a thorough visual inspection of colonies to detect the presence of SHB, using traps as a complimentary tool, as traps cannot replace visual inspection. Furthermore, after one year of surveillance, we realized that in general traps did not work. Only recently we found some beetles in the Better Beetle Blaster traps placed in the hive between two topbars. Diagnostic traps (corrugated plastic sheet, Schäfer type) were not able to capture beetles, probably due to the low level of infestation. Furthermore, this type of trap is very soon propolised by the bees losing any efficacy. Better Beetle Blaster traps are propolised too but last longer. In addition, the hot temperature of the summer of 2015 strongly affected Schäfer type traps; they became curved and could no longer adhere to the hive bottom board.

The early reaction measures adopted by the Italian Ministry of Health were aimed at containment and possible eradication of SHB in the Calabria and Sicily regions. According to the data provided by the active surveillance carried out in the infested regions, infestation

seems to be confined to a limited area of the Calabria region (protection zone) and a single outbreak in Sicily region. The 2014 winter season prevented the continuation of the intense surveillance activity carried out from September to December 2014. However, in spring 2015, the resumption of the surveillance activity according to the national programme provided new information until the re-emergence of SHB in the Calabria region in September 2015. Furthermore, apiaries infested with SHB in the Calabria region in 2015 presented in almost the same location of those infested in 2014 (Fig. 5). No SHB has been detected outside the restriction zone or in the rest of Italy.

The new picture of the situation that is now available implies appropriate evaluation and decisions about the current strategy applied.

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Varroa destructor: From an Invasive Parasite to a Permanent Threat

Peter Rosenkranz (Stuttgart)

Abstract

Varroa destructor was originally a hemolymph-sucking mite affecting the eastern honey bee *Apis cerana*. During the past 50 years the mite has spread around the world on its new host, the western honey bee *Apis mellifera*. As a result of a nearly unlimited population growth of the mite in the new host, and the transmission and activation of pathogenic honey bee viruses, *V. destructor* is now considered the main cause of honey bee colony losses in temperate regions. This paper will give an overview of the life cycle, population dynamics and pathogenesis of *V. destructor*, followed by a "historical" review on the *Varroa* treatments that have been performed by beekeepers in recent decades. The presentation will focus on the factors in the host-parasite relationships that are crucial for the extraordinary reproductive success of the parasite. Recent approaches have revealed new details on the activation and triggering of the reproduction of *Varroa* females. The paper will demonstrate how the disturbance of mite reproduction could be used (*i*) for selective breeding and (*ii*) for future control strategies.

Zusammenfassung

Ursprünglich war *Varroa destructor* eine hämolymphsaugende Milbe auf der östlichen Honigbiene *Apis cerana*. Auf ihrem neuen Wirt, der westlichen Honigbiene *Apis mellifera*, hat sich diese Bienenmilbe während der letzten 50 Jahre weltweit verbreitet. Inzwischen gilt *V. destructor* als weltweit größtes Problem für die Imkerei und als Hauptursache für die periodisch auftretenden Bienenvolkverluste („Bienensterben"). Die Ursachen für die dramatischen Wirtsschädigungen beim neuen Wirt sind ein nahezu ungebremstes Populationswachstum und die Übertragung und Aktivierung von Bienenviren. Nach einer kurzen Einführung in den Lebenszyklus, die Populationsdynamik und Schädigung durch den Parasiten folgt ein „historischer" Rückblick zur *Varroa*-Bekämpfung während der letzten Jahrzehnte. Der Schwerpunkt liegt in der Diskussion derjenigen Faktoren im Parasit-Wirt-Verhältnis, die für den hohen Reproduktionserfolg der *Varroa*-Weibchen verantwortlich sind. Neuere Untersuchungen zur Aktivierung und Steuerung der Varroa-Reproduktion eröffnen Möglichkeiten, durch (*i*) gezielte Selektion und/oder (*ii*) neue Bekämpfungsansätze die Milbenfortpflanzung zu stören.

1. Introduction

Honey bees (*Apis mellifera*) are now considered to be the third most important livestock in the world (Tautz and Heilmann 2008) because of the enormous contribution they make to the pollination of various crops and wild flowers. The global economic value of pollination is estimated to be between $150 billion (Gallai et al. 2008) and $265 billion (Lautenbach et al. 2012). This might explain why reports on honey bee colony losses – often reduced to "Bienensterben", the term now used internationally – have generated enormous media interest. In addition to their undoubtedly economic and ecological importance, honey bees and beekeeping have become a well-established part of society which has led to an emotionalizing of the discussion. In view of the still controversial discussion on the extent of the damages to and the reasons for colony losses, three points are currently widely accepted within the scientific

community and should be considered for further evaluation: (*i*) the number of managed honey bee hives has seen a clear increase worldwide in recent decades, mainly due to above-average growth in the southern hemisphere (Moritz and Erler 2016). (*ii*) The main reason for this seemingly stable balance is the fact that, as a huge eusocial entity, the honey bee colony is well buffered against environmental stress factors and is able to compensate for the temporal loss of individual bees (Rundlöf et al. 2015). Despite this, there is an increasing number of extraordinarily high losses of honey bee colonies in winter. In most cases, however, this is compensated by the practice of beekeeping (Van Endelsdorp et al. 2012, Van der Zee et al. 2015). (*iii*) Irrespective of the development of managed honey bee colonies, insect pollinators are, without a doubt, under a general pressure worldwide and measures to improve biodiversity are urgently needed (Potts et al. 2010).

In the case of honey bees, there is a complex mixture of factors – including other pathogens, pesticides and loss of nectar and pollen sources – that might contribute to these periodical high winter losses. Meanwhile it is widely accepted that the invasive parasitic mite *Varroa destructor* is the major threat to commercial apiculture worldwide (Le Conte et al. 2010, Genersch et al. 2010, Van Doormalen et al. 2012, Laurent et al. 2015).

2. Life Cycle of the *Varroa destructor*

V. destructor is an ectoparasite that sucks hemolymph in both adult bees and bees in larval/pupal stages. To reproduce, the female mite has to enter a brood cell briefly before cell capping. Here the mite starts feeding on the developing larval and pupal stages and lays one haploid male and up to five diploid female eggs. Both male and female offspring have to mature within the brood cell and, additionally, the male has to mate several times with its sister mites before the young host bee hatches. Only adult, mated daughter mites and the mother mite will become phoretic on adult bees whereas males and unmatured nymphal stages cannot survive outside the capped brood cell (Fig. 1). *Varroa* females are spread to other colonies on the adult worker and drone bees, or they enter new brood cells to reproduce within a few days (reviewed in Rosenkranz et al. 2010).

3. Reasons for the Particular Virulence of *Varroa* Mites

V. destructor was originally a parasite of the eastern honey bee, *Apis cerana*, and native distribution was, therefore, limited to Asia. Due to a balanced host-parasite relationship, the mite caused no economic damage to the original host. In the last century, commercial migration of *Apis mellifera* colonies to Asia led to a sympatric distribution of both honey bee species and a successful change in host by two haplotypes of *V. destructor* (reviewed in Rosenkranz et al. 2010). Meanwhile, *Varroa* mites have spread worldwide (with the exception of Australia), causing severe damage to the new host. Without periodic *Varroa* treatment, most managed honey bee colonies are expected to collapse within two to three years. However, the number of suitable acaricides is limited and nearly all *Varroa* treatments involve the risk of mite resistance and/or bee product residues (reviewed in Rosenkranz et al. 2010).

There seem to be three main reasons for the extremely unbalanced host-parasite relationship between *A. mellifera* and *Varroa* mites:

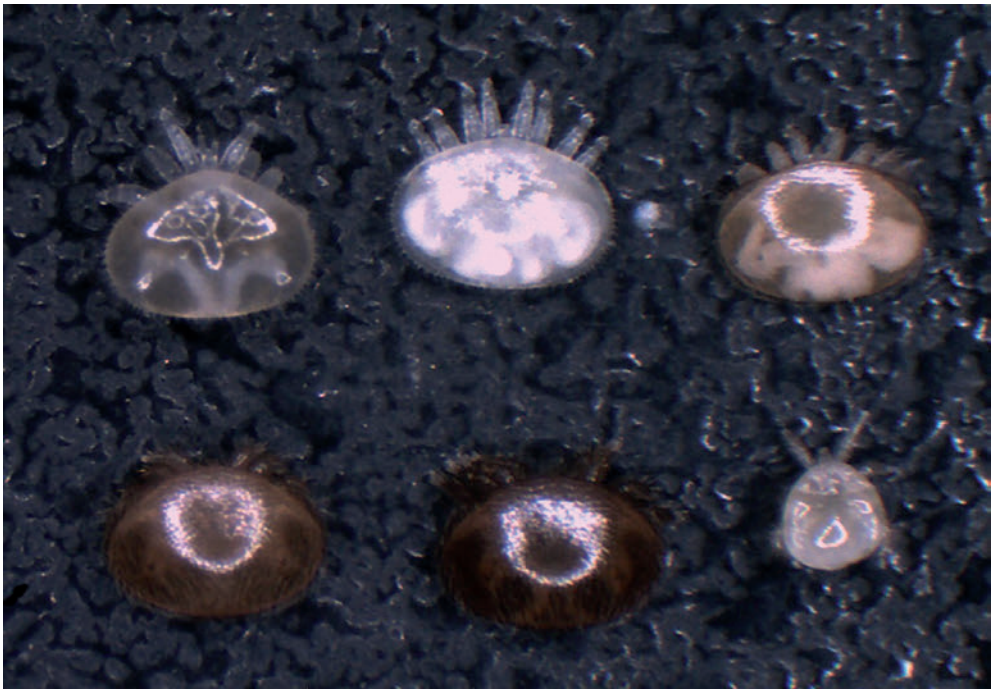

Fig. 1. The *Varroa* "family" within a sealed brood cell at the end of the reproductive cycle. Top row, from left to right: Deutonymph, deutochrysalis, freshly molted young female. Bottom row from left to right: elder daughter mite, mother mite, male (photo Bettina Ziegelmann).

(*1*.) An exponential increase in the *Varroa* population throughout the season with a doubling of population every three to four weeks. Thus, even with low infestation rates in spring, the mite population can increase to several thousand individuals by autumn. At this time of the year honey bee colonies are preparing for wintering: they reduce the brood amount and produce "winter bees" that need to be bred and maintained under optimal conditions in order to survive until the following spring (in contrast, "summer bees" only live for 3–4 weeks). The combination of an increasing *Varroa* infestation with a simultaneous decrease in the number of host brood cells impedes the production of healthy winter bees (Fig. 2).

(*2*.) *Varroa* mites harm their host by instigating a loss of hemolymph and, more importantly through the transmission and activation of certain bee viruses (Dainat et al. 2012, Francis et al. 2013, McMenamin and Genersch 2015). These viruses – including the most prominent variations of Deformed Wing Virus (DWV, Fig. 3) – are known to be more or less benign in the absence of *Varroa* mites (when transmitted among bees only by feeding) but become quite virulent when transmitted directly into the hemolymph by blood sucking *Varroa* mites. This mite-virus-honey bee interaction might also be responsible for the obviously increasing virulence of *V. destructor* compared to the first years after the introduction of the parasite into the respective countries (Le Conte et al. 2010).

(*3*.) *Varroa* mites (together with associated viruses) can spread rapidly from colony to colony by drifting and/or robbing. This is significantly exacerbated by the high density of honey bee colonies and migratory beekeeping (Frey and Rosenkranz 2014).

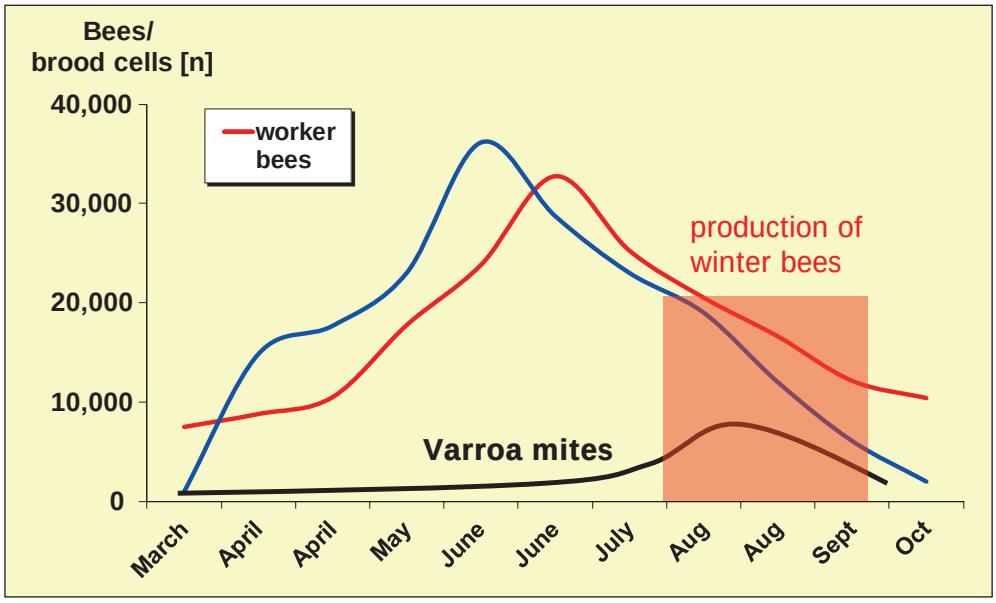

Fig. 2 Example of the population dynamic of the honey bee colony and the *Varroa* mite throughout the season. The peak of the mite infestation coincides with the decrease in the host population resulting in a dramatic increase in the relative infestation at a time when long-living winter bees should be produced.

V. destructor has been spreading through the western hemisphere for forty years now. However, this parasite still represents the most severe problem for global beekeeping and an "easy to apply" solution is not in sight.

4. "History" of *Varroa* Treatment in Germany over the Past 30 Years

When the *V. destructor* was introduced into Germany in 1978, no acaricide was available or even registered. The first effective acaricide was Folbex VA neu®, a time-consuming smoke application and a burden for both the honey bee colony and the beekeeper. Some years later the organophosphate Perizin®, whose active ingredient is Coumaphos, was registered as a trickling treatment. Until the early 1990s, one or two winter treatments with Perizin® were sufficient to control *Varroa* mite infestations (Boecking and Genersch 2008). Afterwards, an additional summer treatment with formic acid was recommended in order to reduce infestation levels before the production of winter bees. Now, a combination of biotechnic measures (drone brood removal), two formic acid treatments in late summer and a winter treatment with oxalic acid are crucial components of treatment concepts in Germany (see Baden Wurttemberg's control concept at https://bienenkunde.uni-hohenheim.de, Fig. 4).

This intensification of the *Varroa* treatment is a clear indication that the damage threshold has decreased over the years in infested honey bee colonies, presumably due to the more frequent viral infections vectored by *V. destructor* (Boecking and Genersch 2008, Rosenkranz et al. 2010). And despite all the efforts made by bee scientists and beekeep-

Fig. 3 Honey bee worker bees with several *Varroa* females and bees with crippled wings due to infections with the Deformed Wing Virus (DWV) which is transmitted by phoretic mites (photo Thomas Kustermann).

ers, varroosis still causes substantial damage and even colony losses. Therefore, sustainable solutions to the *Varroa* problem are urgently needed. Two avenues toward such a solution are presented below.

5. *Varroa* Resistance

Fortunately, there are several *A. mellifera* populations that have survived for many years without *Varroa* treatment. Most examples of such stable host-parasite relationships have been reported in tropical regions with honey bees of African origin (Rosenkranz 1999). However, in the temperate regions of Europe and the USA there are also promising cases of long-term survival of *Varroa*-infested honey bee colonies; most of these colonies, however, are part of small local populations (recently reviewed by Locke 2016). In particular, the so called Bond Test ("live and let die") on the island of Gotland confirmed that natural selection is possible and that *V. destructor* therefore will not exterminate our honey bees (Fries et al. 2006, Locke and Fries 2011). However, all these examples are the result of natural selection with presumably high colony losses in the course of this adaptation process. In most temperate regions there are hardly any feral honey bee populations with the consequence that natural selection does not exist. The bee-

Fig. 4 Examples of *Varroa* treatments that are currently recommended by the Apicultural State Institute at the University of Hohenheim. (*A*) Removal of drone brood which is about 10 times more highly infested with *Varroa* mites than the worker brood. (*B*) Evaporation of formic acid with a dispenser in order to kill phoretic mites on bees and brood mites within the sealed brood cells. (*C*) Trickling of an oxalic acid solution in winter when the bees are sitting in a close winter cluster without a brood.

keeping practice prevents any selection pressure through periodic treatment and, furthermore, distributes susceptible colonies through migratory beekeeping. To overcome this problem there are some promising approaches for the selective breeding of honey bees with *Varroa*-resistant traits, for example bees with particular hygienic activities that suppress mite reproduction (Danka et al. 2016). Currently, such "resilient" honey bees are expected to be able to reduce the number of required treatments as part of integrated pest management (IPM).

6. New Treatment Approaches

Surprisingly, no biological *Varroa* treatments are currently available. One reason might be that, despite 30 years of *Varroa* research, many details of the host-parasite interactions are still poor-

ly understood (Dietemann et al. 2012). Considering the exponential population growth of the host in spring and summer, and the fact that the most sensitive phase of the host colony (= the production of winter bees) coincides with the peak of this growth phase, it becomes clear that a suppression, or at least disturbance, of the mite reproduction is indispensable for sustainable control. This means that we need a better understanding of the complex interaction between host and parasite during the reproductive cycle within the sealed brood cell.

Reproduction of the *Varroa* females can only take place within the capped honey bee brood cell and the entire reproductive cycle is completely synchronized with the larval and pupal development of the host bee. A crucial limitation to successful reproduction is the duration of the capping period of the bee larva. During this period – which lasts approximately 11 to 12 days for worker broods depending on the honey bee subspecies – the mother mite has to lay at least two eggs (one male and one female), both have to reach sexual maturity and, finally, the male mite has to mate several times with its sister(s). Therefore, the reproductive rate of a mother mite in a worker brood is, on average, fewer than 1.5 viable daughter mites per reproductive cycle (reviewed in Rosenkranz et al. 2010). This means that even small disturbances might completely prevent the production of mated daughter mites and that the reproductive phase therefore offers a promising approach to biological control.

We have already shown that host factors provided by the freshly capped larvae are crucial in initiating the oogenesis of the invaded *Varroa* female (Frey et al. 2013) and that there is an obvious genetic basis for a host-dependent prevention of reproduction within the brood cell (Behrens et al. 2011). So far, little is known about the nature of these trigger factors; currently, a combination of cuticular volatiles and nutritional components of the hemolymph of the bee larva is under discussion (Cabrera et al. 2013, Frey et al. 2014). However, such compounds could be perfectly used to disturb the reproductive success of the mite during the season and we therefore urgently suggest that more effort be focused on this field of *Varroa* research.

Another approach for the disturbance of *Varroa* reproduction has already been successfully applied. Ziegelmann et al. (2013a, b) were able to identify the sex pheromone of the female *Varroa* mite consisting of three fatty acids and the respective ethyl esters. This sex pheromone elicits the final step in a behavioural cascade where the male mite moves to the ventral side of the female and transfers its spermatophore into the female's solenostomes (Fig. 5). The male uses a sensory pit organ on its front legs to perceive the sex pheromone released from the genital openings of freshly molted female mites (Häussermann et al. 2015). Unfortunately, the sex pheromone has hardly any repellent effect to the male mite even if applied in over-excessive amounts. However, applying one or several components of the sex pheromone into the sealed brood cell can significantly disturb mating behaviour. Under certain airborne concentrations the male mite is no longer able to distinguish between young, unmated female mites and the mother mite or nymphal stages. As a consequence, the *Varroa* male also copulates with immature and already mated female stages. This "waste of time" reduces successful mating with young daughter mites. Unmated daughter mites will not contribute to the mite's population dynamic because they cannot lay female (= fertilized) eggs during their next reproductive cycle.

We could indeed confirm that after an application of oleic acid (the main component of the sex pheromone) to brood combs, the rate of unmated *Varroa* females increased by up to 20 % (Ziegelmann and Rosenkranz 2014). This does not seem like very much, however, models of the *Varroa* population dynamic (Wilkinson et al. 2002) indicate that even a slight but permanent reduction in the reproductive rate will prevent the exponential growth of the

Fig. 5 Mating behaviour of *Varroa* mites. The smaller male is about to transfer a spermatophore with its spermatodactyles into the genital opening of a freshly molted female (photo Bettina Ziegelmann).

mite population. Such an application would provide at least two benefits: (*i*) a lower mite infestation rate and, therefore, fewer viral infections of the honey bee colony throughout the entire season, and (*ii*) no risk of exceeding the damage threshold in late summer. The exclusive use of such biological treatments might not be sufficient for the long-term control of the *Varroa* infestation, however, they could perfectly be used as an environmentally friendly and well-tolerated alternative as part of current treatment concepts.

An issue that has yet to be resolved for the use of this biological treatment in beekeeping practice is finding an easy and effective way of applying the respective compounds. We were able to show that components of the sex pheromone that were sprayed directly on comb foundations have a significant effect on the copulation success of the mites; however, the efficacy decreases after several cycles (Ziegelmann 2016, in preparation). In addition to this problem, there are currently two general restrictions for the development of such an application. First, all products used in *Varroa* treatment must be registered with the veterinary authorities, a process that can be extremely expensive. Secondly, the beekeeping business has only limited economic power which makes it difficult for companies to refinance the investments in the development of new treatments. Therefore, the development of a marketable product requires both a scientific resolution to the outstanding questions and the acquisition of companies that are prepared to invest in such alternative treatment strategies.

7. Conclusion

The *Varroa* problem is still far from finding a sustainable solution, and intensive and periodical treatments will continue to be necessary in the future. Therefore, the beekeeping practice urgently requires new control measures that neither increase the risk of residues in bee products nor promote the development of resistance in the treated *Varroa* mites. The reproductive biology of the *Varroa* mite within the honey bee brood cell offers such possibilities. The

disturbance of the reproductive success of the parasite by the application of the female sex pheromone represents the first biological approach for a *Varroa* treatment and will hopefully promote further research in this direction.

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Dermanyssus gallinae: A Never-Ending Story for the Poultry Industry and Public Health

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Abstract

The poultry red mite, *Dermanyssus gallinae*, is a blood-sucking mite of domestic, wild and synanthropic birds. It poses a significant threat to the poultry industry and hen health worldwide, particularly in Europe. *D. gallinae* is increasingly suspected of being a disease vector, and attacks on alternative hosts, like humans, are becoming more common. This is especially the case for poultry workers, but ordinary city residents living close to birds' nests are also coming under attack, meaning that *D. gallinae* is emerging as a public health problem. The economic importance of this pest has greatly increased for the poultry industry; although poultry production has moved from conventional cage systems towards a more welfare-oriented breeding system in many parts of the world, *D. gallinae* is likely to become more widespread and difficult to control. Synthetic acaricides are still the dominant means of control, although resistance and treatment failure are widely reported. Furthermore, there are also worrying reports of possible collateral effects on human health. Correct identification of the red mite is the first requirement before control methods are applied. A general updated overview of *D. gallinae* and the related problems are presented here.

Zusammenfassung

Die Rote Vogelmilbe, *Dermanyssus gallinae*, ist ein blutsaugender Ektoparasit von Nutzgeflügel, Wildvögeln und synanthropen Vögeln. Sie stellt weltweit, vor allem aber in Europa, eine bedeutende Gefahr für die Geflügelindustrie und die Gesundheit von Legehennen dar. *D. gallinae* steht zunehmend im Verdacht, ein Überträger von Krankheiten zu sein, und Angriffe auf alternative Wirte wie beispielsweise den Menschen sind immer häufiger zu verzeichnen. Dies ist insbesondere der Fall bei Beschäftigten in der Geflügelhaltung, aber auch normale Stadtbewohner, die in der Nähe von Vogelnestern leben, können betroffen sein. Daher entwickelt sich *D. gallinae* zu einem zunehmenden öffentlichen Gesundheitsproblem. Die wirtschaftliche Bedeutung dieses Schädlings hat für die Geflügelindustrie stark zugenommen; obwohl eine Umstellung von den konventionellen Käfigsystemen zu tierschutzfreundlicheren Haltungssystemen in der Geflügelproduktion in vielen Teilen der Welt erfolgte, wird sich *D. gallinae* wahrscheinlich zunehmend verbreiten und schwerer zu kontrollieren sein. Synthetische Akarizide sind immer noch das vorherrschende Mittel zur Bekämpfung, obwohl häufig über Resistenzen und Behandlungsfehler berichtet wird. Darüber hinaus gibt es auch besorgniserregende Berichte über mögliche Nebenwirkungen auf die menschliche Gesundheit. Vor der Anwendung von Bekämpfungsmaßnahmen ist die genaue Identifizierung der Roten Vogelmilbe die erste Voraussetzung. Eine allgemeine aktuelle Übersicht zu *D. gallinae* und den damit einhergehenden Problemen wird dargestellt.

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1. Introduction

The genus *Dermanyssus* Duges, 1834, recognizes at least 25 species of bloodsucking mites (Roy et al. 2009). The most represented and most important species for animal and human health is *Dermanyssus gallinae* De Geer, a ubiquitous species able to infest over 30 species of birds, including farmed birds (e.g. chickens, turkeys and ducks), and wild and synanthropic birds (e.g. canaries, sparrows, swallows and pigeons).

This mite species poses an economic problem to the poultry industry worldwide, particularly in Europe, Japan, and China, and also in the United States, where it is, however, less widespread (Chauve 1998, Sparagano et al. 2009). Economic costs associated with both control and production losses due to *D. gallinae* have been estimated at 130 million euros per year for the egg industry in the EU, with similarly large losses in other areas of the world (van Emous 2005).

D. gallinae is present in both industrial and rural poultry contexts, and in any type of system: cages, barns, free range, hobby farming, and in both classical and organic systems. The consequences of infestation, including the potential of *D. gallinae* to harbour and transmit pathogens, appear to be an important and emerging problem, also in connection with its role as a zoonotic agent. Major reviews on this pest have recently been published (Sparagano et al. 2014, George et al. 2015, Pritchard et al. 2015), while here we provide a general updated overview of *D. gallinae* and its economic, veterinary and public health significance, together with our most relevant and user-friendly original photographs.

2. Morphology and Biology

Dermanyssus gallinae (De Geer, 1778) (Acari: Gamasida: Dermanyssidae) is commonly known as the "red mite" and is a hematophagous ectoparasite. It is relatively small at the adult stage (0.5 × 1 mm in length) with long legs and a grayish-white body that becomes reddish-brown when engorged (Fig. 1).

It is very important to identify this species correctly, because effective control of this (or any) parasite depends on correct identification in both veterinary and human contexts. Confusion is possible with other mites that can also be present in poultry houses, mostly with those belonging to the *Ornithonyssus* genus (Acari: Gamasida: Macronyssidae). These mites resemble *Dermanyssus* but have a different relationship to their host. The most significant mites that are morphologically close to *D. gallinae* are: *Ornithonyssus sylviarum* (Canestrini and Fanzago 1877) (the northern fowl mite), a pest of chickens widespread in temperate regions; *Ornithonyssus bursa* (Berlese 1888) (the tropical fowl mite), a tropical and subtropical mite of wild and household birds less present in Europe (Varma 1993); and *Ornithonyssus bacoti* (Hirst 1913) (the tropical rat mite), a parasite of rodents that may occasionally infest birds (Taylor et al. 2007).

D. gallinae can be distinguished by the following characteristics: the buccal apparatus consists of two pedipalps with two long thin chelicera (Fig. 2*A*) and its dorsal surface has a shield with prominent lateral margins tapering towards the rear but which do not reach the distal end of the body and are truncated at the end (Fig. 2*B*).

On the ventral side, the sternal plate is wider than it is long (Fig. 3*A*) and has two pairs of bristles, while a third pair is placed posteriorly and is apart from the first two pairs. The genitoventral shield is posteriorly rounded (Fig. 3*B*) and bears one pair of setae. Finally, the anal plate is rounded and bears three characteristic setae (Fig. 3*C*).

Fig. 1 Fully engorged *Dermanyssus gallinae* female (original M. A. Cafiero)

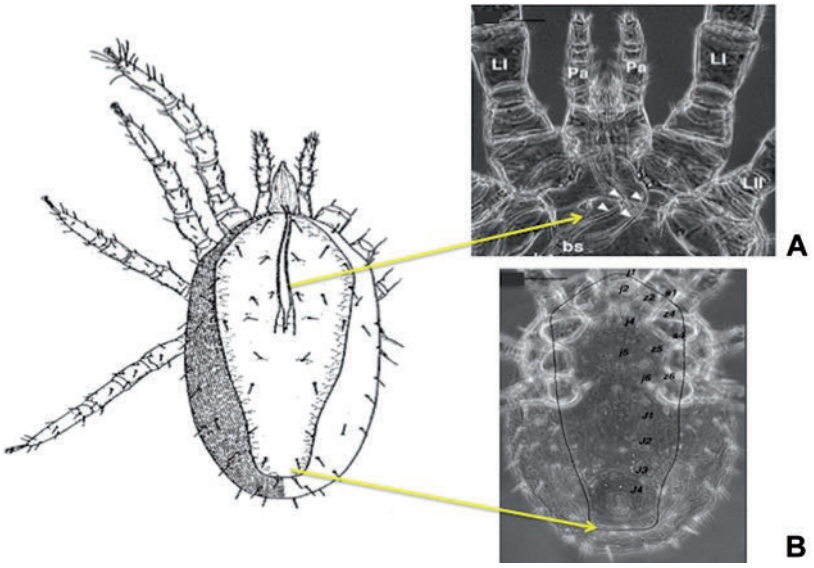

Fig. 2 Dorsal side of *Dermanyssus gallinae*: pedipalps with two long thin chelicera (*A*) and truncated end of the dorsal shield (*B*) (modified from Di Palma et al. 2012)

The *O. sylviarum* female has a single dorsal shield that narrows towards the rear. On the ventral side, the genitoventral (epigynal) shield is attenuated or narrowly rounded posteriorly

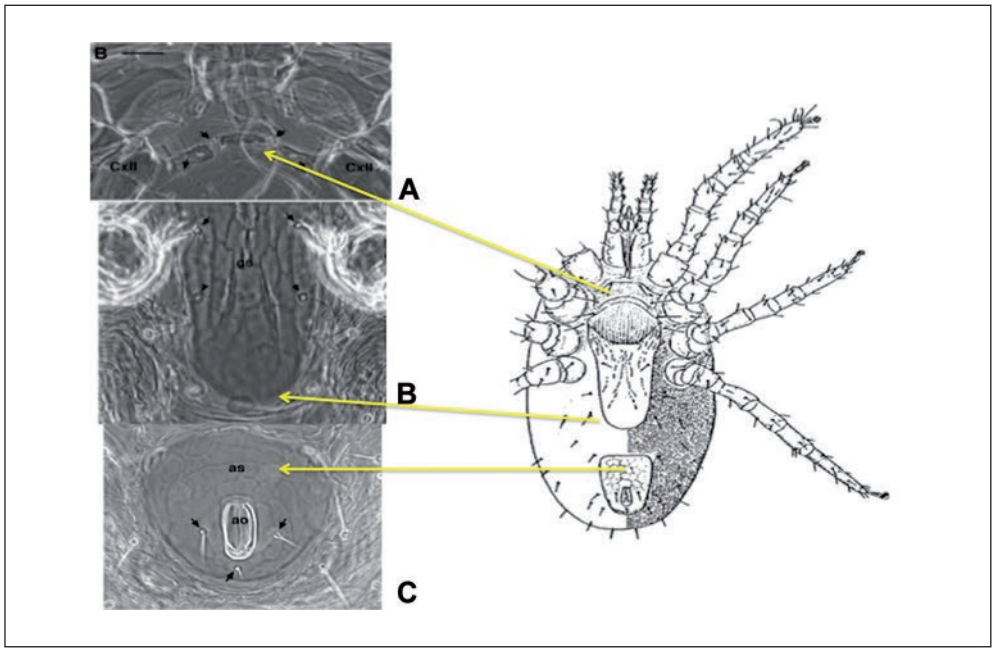

Fig. 3 Ventral side of *Dermanyssus gallinae*. (*A*) broad sternal plate; (*B*) posteriorly rounded genito-ventral shield; and (*C*) rounded anal plate with three characteristic setae (modified from Di Palma et al. 2012)

(Fig. 4). Differences concerning sternal shields can also be seen: in *D. gallinae* this is long and narrow (Fig. 3*A*), whereas in *O. sylviarum* it is broad (Fig. 4).

In *O. bursa* the sternal shield is almost rectangular, with three setae (Castelli et al. 2015) (Fig. 5). The general appearance of *O. bacoti* is quite peculiar, because it is very hairy compared with the other species (*D. gallinae* and *Ornithonyssus*) (Fig. 6).

In a typical caged-hen egg farm (Fig. 7), all stages of *D. gallinae* live near the host, concealed in all possible crevices below cages, in walls, floors, and host nests. They are easily seen under feeders, perches, conveyor belts, among dry bird droppings, as well as on egg trays, in the folds of cartons, in cages for transporting birds, and also in feed (Fig. 8*A* and 8*B*).

D. gallinae has five stages of development: egg, larva, protonymph, deutonymph, and adult. Most of its biological cycle usually takes place outside the host; it is on the host just to feed, and this mostly happens during the night, from sunset to the first hours of daylight. Mating occurs off the host and takes from 14 minutes to 1 hour, during which the male inserts its penis into the female spermatic orifice and transfers its spermatophor sack containing about 200 spermatozoa. Males can mate up to four times in four days, and the longest fertile period of females lasts three weeks (Hutcheson and Oliver 1988). After mating, the females reach the host to feed and then return to their hiding places to lay their eggs (four to eight per day) every two to four days. They lay a total of about 30 eggs during their fertile life, which lasts about three weeks. The males feed only rarely and die after mating (Chauve 1998). Hexapod larvae (0.42 mm long) hatch from the eggs (0.41 mm long and 0.28 mm wide) and are the only stage that do not feed; these molt firstly into octopode protonymphs, then into deutonymphs,

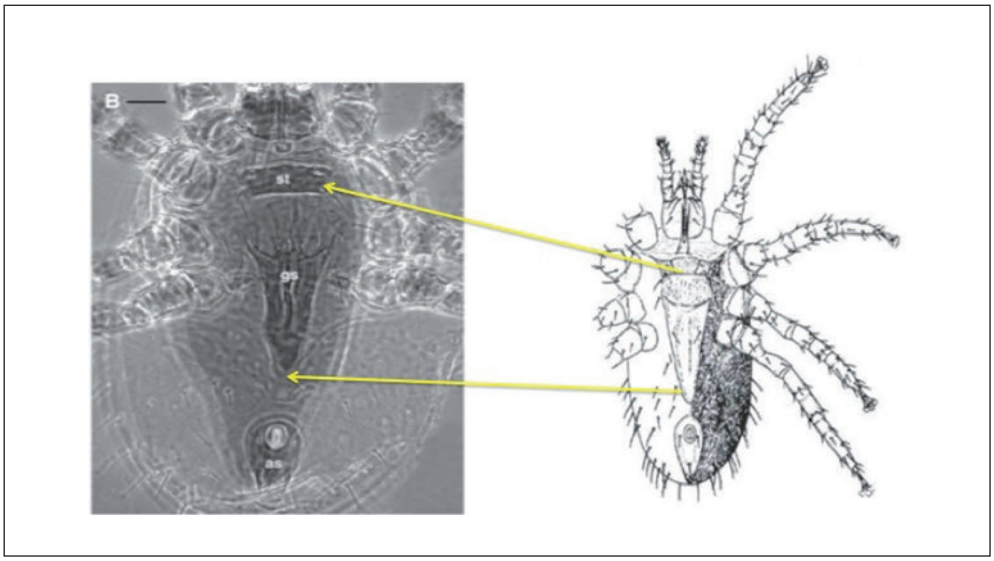

Fig. 4 Ventral side of *Ornithonyssus sylviarum*: thick sternal shield (st); posteriorly narrowing genito-ventral shield (gs) (modified from Di Palma et al. 2012)

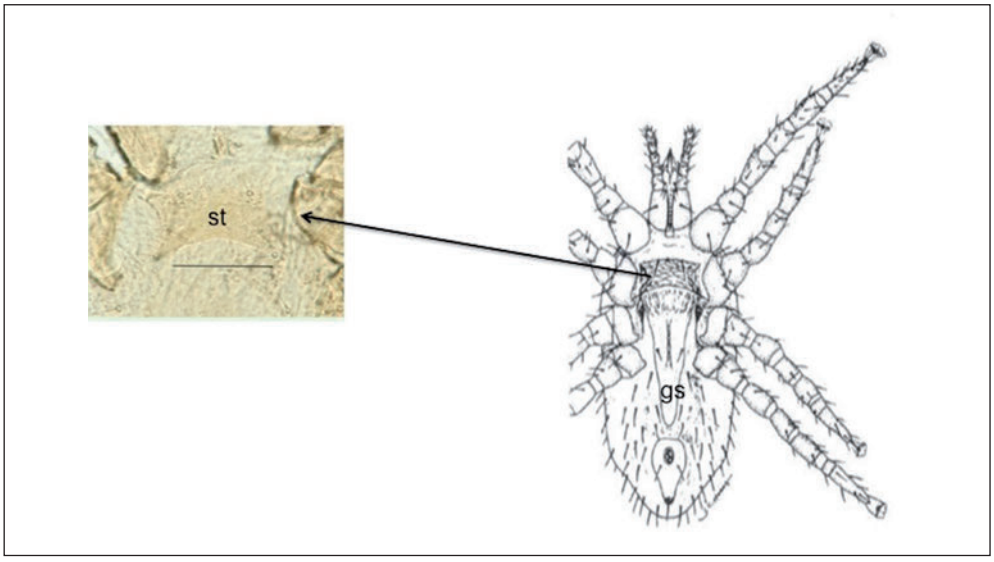

Fig. 5 Ventral side of *Ornithonyssus bursa*. A very thick sternal shield (st) with three setae (from Castelli et al. 2015); posteriorly narrowing genito-ventral shield (gs)

and finally into adults (males and females). In their hiding places, mites tend to form clusters by thigmokinesis (i.e. movement, or inhibition of movement, in response to contact stimuli); the larval stages usually stay in the center, with the females on the outside and the males on

Fig. 6 Engorged *Ornithonyssus bacoti* fernale: the dorsal shield (red arrow and long setae (black arrow) are visible (original M. A. Cafiero).

Fig. 7 Laying hens in enriched cage-system farms (original A. Giangaspero)

the top of the group (Entrekin and Oliver 1982). Thanks to specific receptors, the mite picks up chemical signals emitted (vibration, CO_2) by the host animal and reaches it to feed (Zeman 1988, Kilpinen et al. 2005, Kilpinen 2005). The mite prefers areas of the host's body with few feathers, where the skin is thin, and the veins are superficial, such as the neck, back, and under the wing; it uses its stylet-shaped chelicerae (Fig. 9) to bite its victims.

Feeds last up to an hour and take place every two to four days. One female can ingest about 200 µg of blood, while males suck blood only occasionally. However, stimuli captured

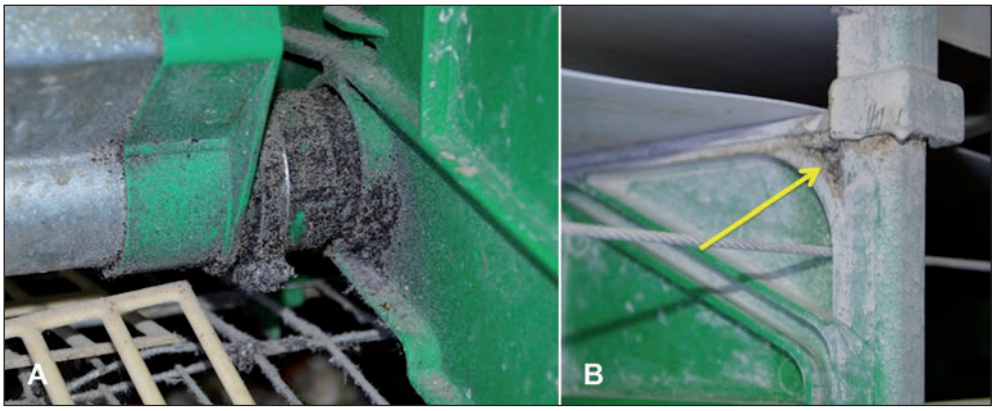

Fig. 8 (*A*) *Dermanyssus gallinae* clusters in the join of a supplementary feeder and (*B*) *Dermanyssus gallinae* hidden in clusters in cage structures (originals A. Giangaspero)

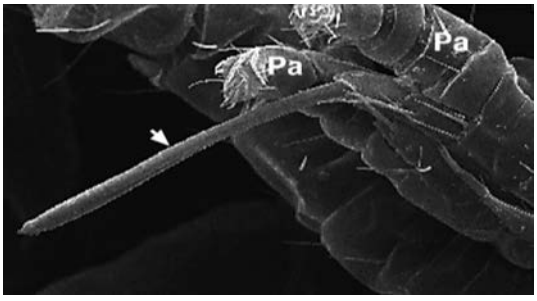

Fig. 9 *Dermanyssus gallinae* stylet (from Di Palma et al. 2012)

by the mite vary according to its nutritional status; if two or more days have passed since the last meal, the search for a host becomes more rapid and induces *D. gallinae* to feed more frequently. The search slows down if the starvation period lasts for over 15–20 days. This behaviour is justified by metabolic saving, which stops if a constant and prolonged thermal stimulus appears (Kilpinen and Mullens 2004).

Dermanyssus gallinae population growth is favoured by conditions inside poultry houses, where temperatures are typically kept at 18–24 °C, but might well rise above 28 °C during the summer. Temperature and high relative humidity (>70 %) facilitate *D. gallinae* reproduction and development, and poultry sheds provide good shelter, hiding places and the necessary food. In these conditions, the lifecycle of *D. gallinae* can be completed in seven to 17 days (Maurer and Baumgärtner 1994).

3. Epidemiology

Dermanyssus gallinae has spread throughout the world, but in Europe it is one of the major health problems of poultry farms, particularly in laying hens, because of their longer production

cycle (about 11 months) and the frequent lack of an interval between successive productive cycles. In broiler farms, the production cycle is shorter (about 55 days) and prevents the mite from spreading (Chauve 1998).

D. gallinae is present in all farming systems: cages, sheds and free range, both traditional and organic. Infestation rates vary in the different European countries; the most recent figures suggest that *D. gallinae* prevalence in laying hens exceeds 80 % in many EU countries, with an average prevalence of 83 % (Mul et al. 2013) (Fig. 10).

The changeover from conventional cages to enriched systems incorporating more complex environments that appear to favour *D. gallinae* is expected to worsen the problem. Mites can be introduced into the farm when new flocks arrive, and also in containers, crates and aeration pipes, as well as by wild birds, and also rodents (Mul and Koenraadt 2009). It can also be introduced by farmers, workers or visitors. If environmental conditions are suitable, the population increases progressively for four to six months until it reaches a plateau. Mite density commonly reaches 50,000 specimens per bird; however, in particularly severe cases of infestation, the number may be ten times higher (Kilpinen 2005). *D. gallinae* can survive very well even in the absence of a host: it can fast from five to six months under normal conditions, and up to eight months at low temperatures (Kirkwood 1963, Nordernfors et al. 1999). As mentioned above, *D. gallinae* is an obligatory but temporary blood feeder that affects not only chickens but also other farmed birds such as turkeys and ducks, and also wild birds such as pigeons and sparrows (Roy and Chauve 2007). Due to its low host specificity it has been found on dogs (Delclerq and Nachtegaele 1993), cats (Grant 1989), horses (Mignon and Losson 2008) and gerbils (Lucky et al. 2001), and – more importantly – it can also attack humans.

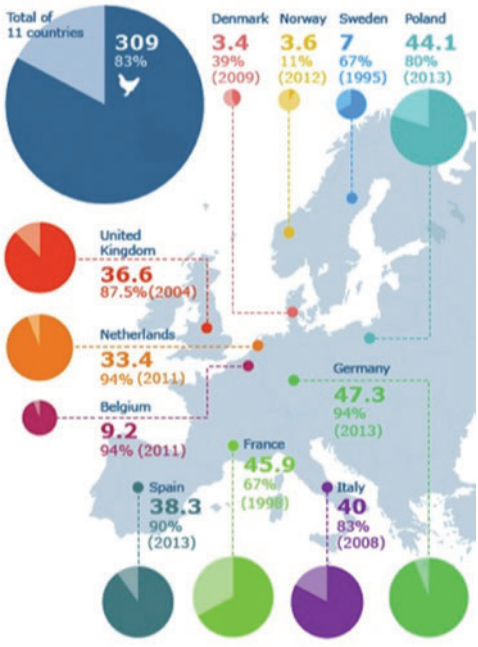

Fig. 10 Number of laying hens per country in millions (2012) and percentages of farms infested by *Dermanyssus gallinae* (from M. Mul, Wageningen UR Livestock Research)

Environmental conditions and acaricide/pesticide use can affect *D. gallinae* host population genetics. It is shown that red mite populations can fall into clades or lineages according to molecular phylogenic studies (Roy et al. 2009, Marangi et al. 2009b, 2014), and it may be this genetic plasticity that allows the red mite to adapt to new hosts.

4. Pathogenesis and Clinical Signs

The most obvious clinical sign of dermanyssosis in laying hens is dermatitis, which becomes very severe if the infestation is massive. The itching caused by the mite makes animals appear nervous, restless and irritable; they tend to peck each other and fight, causing wounds and increasing the likelihood of cannibalism within the group.

A female mite can withdraw about 200 µg of blood. If the number of mites remains constantly low, the bird's physiological system can compensate for the loss of blood by increasing haematopoiesis. In animals with infestations of over 50,000 mites (Fig. 11), haematopoiesis is insufficient to compensate for blood loss, and animals present a marked anaemia (with haematocrit reduced to 6.4 %) that is more pronounced in young subjects; the comb and wattles are pallid, and this anaemia can sometimes be a cause of death (Fig. 12) (Wojcik et al. 2000, Cosoroaba 2001, Kilpinen et al. 2005). A massive red mite infestation of birds is referred to as "red mange" and hyperkeratosis, parakeratosis and acanthosis have been experimentally observed on the skin after only 72 hours (Hobbenaghi et al. 2012).

Birds suffering from *D. gallinae*-induced stress have increased levels of corticosteron and adrenalin in the blood and reduced levels of beta- and gamma-globulins (Kowalski and

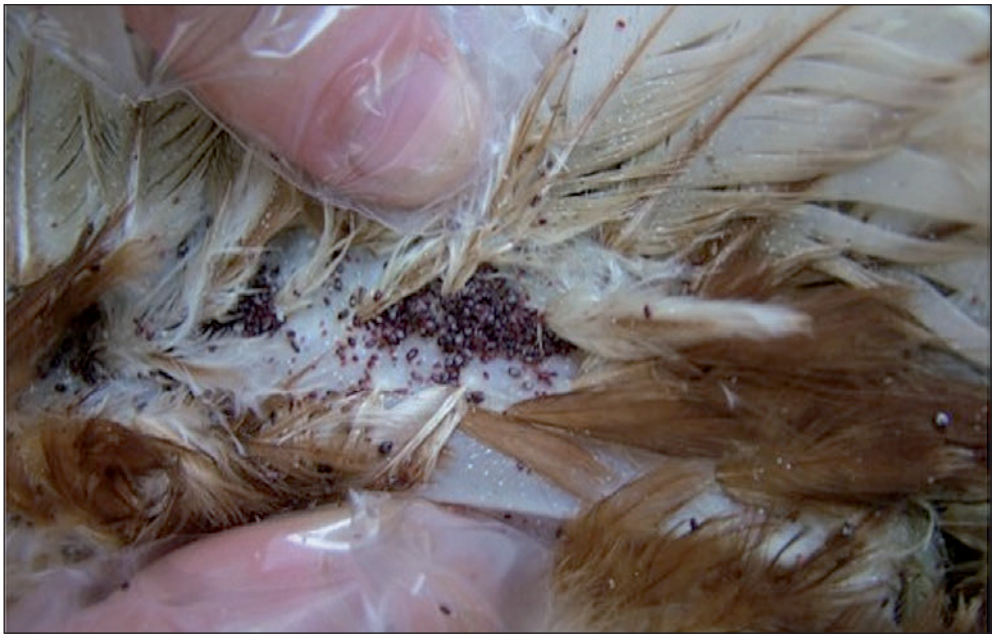

Fig. 11 Severe *Dermanyssus gallinae* infestation in a hen (original A. Camarda)

Fig. 12 Marked anaemia in a dead hen with severe *Dermanyssus gallinae* infestation (original A. Camarda)

Sokol 2009). Birds preen constantly, and their sleep patterns can be disrupted (Chauve 1998, Kilpinen et al. 2005, Mul et al. 2009). The increased aggressiveness of affected birds has a negative impact on weight gain, conversion index, and egg production. Egg quality is also affected, since egg weight falls, the shell becomes thinner, and the mechanical crushing of engorged mites leaves spots on the shell (Chauve 1998, Cosoroaba 2001).

Even small mite populations may have a significant impact on poultry health since *D. gallinae* may serve as a vector of several pathogens. Newcastle disease virus and poxvirus, but also zoonotic bacteria, such as *Salmonella* spp., *Pasteurella multocida*, *Erysipelothrix rhusiopathiae*, *Listeria*, *Coxiella* and *Chlamydia psittaci* have been isolated from the red mite. Scientific evidence of transmission has been documented only for *Salmonella enteritidis*, and the real vectorial competence of *D. gallinae* remains unconfirmed (Valiente Moro et al. 2005, 2009, Circella et al. 2011, Chu et al. 2015), but its potential should not be underestimated.

In addition, red mites may also limit hens' immunological responses to pathogens (Kowalski and Sokol 2009, Kaoud 2010). Heavy infestations are reported to reduce antibody titres to some viral vaccines or suppress antibody production by the host. Mites appear to use a feeding strategy that causes minimal interference or modulation of host immunity, which may corroborate these results. In any case, the birds' immune response does not seem sufficiently effective to *D. gallinae*, as shown by the lack of correlation between the levels of mite infestation and anti-*D. gallinae* IgY levels (Arkle et al. 2006).

5. Control Strategies

The application of hygiene regulations (cleaning and hygiene facilities, farmer and technician awareness of the problem, interruption of the production cycle and thorough disinfection before the arrival of new birds, careful checking of new animals) on poultry farms would prevent or limit infestation.

Monitoring of the entire production chain is an effective preventive measure. Since mites are present not only in closed systems but also in the open, all steps in the chain should be monitored, from putting the birds in cages to egg collection and transport. This can be done by placing simple traps made of cardboard or corrugated PVC, or pieces of knotted white cloth in cages or nearby; mites tend to hide in the folds in the cardboard or cloth and this makes observation easy (Mul et. al. 2015).

The fact that mites hide and survive for long periods in the absence of the host is a major obstacle to control on poultry farms. Worldwide, control of *D. gallinae* infestation is based almost exclusively on the use of acaricides. More than 35 molecules have been tested and proposed for use against mites: organophosphates, pyrethrins, pyrethroids, carbamates and amitraz use different mechanisms of action to cause paralysis and death. However, in practice, few products are licensed in the EU for use against *D. gallinae*: one is based on an organophosphorus compound (phoxim) and the other is a spinosad-based biopesticide. More importantly, several unlicensed or even banned (i.e. carbaryl) products are still widely used to fight infestations (Maurer et al. 2009, Marangi et al. 2012).

Farmers tend to increase the dose if treatment is not effective and lasting, as reported in a recent survey in Italy (Marangi et al. 2012), and it is no surprise that this complicates the situation. In fact, continuous use or incorrect concentrations may kill some sensitive mites but allow the development of new generations of resistant individuals. Reduced effectiveness of several chemicals has been reported in many countries (Marangi et al. 2009a, Sparagano et al. 2014); even for the recently marketed phoxim the data are variable, and resistance is suspected in some countries (Camarda et al. 2010, Zdybel et al. 2011).

Faced with this kind of problem, i.e. reduced effectiveness of the chemical, farmers are keen to increase the dose or treat their animals more often, as documented in Italy (Cafiero et al. 2008a). This exacerbates the appearance of resistant mites and, more importantly, can favour the accumulation of chemical residues in poultry, especially in laying hens.

Nonetheless, chemical control of poultry red mites using synthetic acaricides remains the dominant method in commercial premises, and this means that there is an urgent need to identify more specific control strategies. However, there are few safe alternative strategies (see Sparagano et al. 2014). The most hopeful and promising approach to controlling *D. gallinae* would be *via* vaccination, which offers the advantages of prolonged effectiveness, freedom from chemical residues and environmental pollution, and a reduced risk of resistance. The identification of suitable antigens for vaccine production is now a principal goal. Bartley et al. (2015) have used a new approach to develop an integrated methodology for identifying vaccine candidate molecules from *D. gallinae*, and have tested the potential of these molecules *in vitro* and *in vivo* (Bartley et. al 2015). Effective control of *D. gallinae* may be important not only for the poultry sector, but also for many other sectors, including human health.

6. Public Health

As mentioned above, *Dermanyssus gallinae* is a temporary blood feeder not only of chickens but also of other birds and even mammals, including humans, and its responsibility for human skin complaints is increasing. Dermanyssosis in humans is recorded most commonly among staff working in the poultry industry: farmers, workers who collect the eggs, technicians and

veterinarians are most at risk. In some countries, poultry workers demand three times the usual rate of pay before they are willing to work with *D. gallinae*-infested birds (Sahibi et al. 2008). In one case, after egg collection, workers claimed that they had been attacked by *D. gallinae*, and presented lesions on the arms and trunk, and a scabies-like dermatitis of tiny erythematous papules with severe itching (Rosen 2002). Another report of an affected poultry-farm worker involved unexpected sites such as the ear (auditory meatus) (Rossiter 1997), and a female poultry keeper had persistent infestations of the scalp (Pampiglione et al. 2001). In a questionnaire given to farmers in the southern Italian region of Apulia, 18 % (11/58) of poultry farm staff experienced irritating itchy skin eruptions at work after red mites had crawled on their skin. Of the 11 infected workers, two (18.18 %) reported dermatitis on just their arms and hands, with seven (63.63 %) reporting symptoms on their chests, and two (18.18 %) on their legs (Cafiero et al. 2011) (Fig. 13). It is important to note that *D. gallinae* may also be a potential vector of human diseases. Recent evidence supports acquisition of *Bartonella via Dermanyssus* spp. (Melter et al. 2012) and links attacks to Lyme disease, *Bartonella* and/or *Babesia* (George et al. 2015).

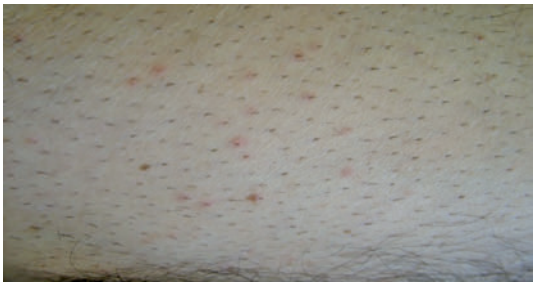

Fig. 13 *Dermanyssus gallinae* dermatitis in a poultry worker (original A. Camarda)

Bites on human skin should be regarded as an additional concern associated with this parasite, and this is the reason for the whole-hearted support of the red mite's inclusion as a zoonotic agent in all regulations regarding occupational safety, and of dermatitis by *D. gallinae* as an occupational hazard for individuals working with poultry (Cafiero et al. 2011). Reports of dermanyssosis have increased in frequency in recent years, particularly in residential settings, in association with common synanthropic birds such as pigeons, sparrows, etc. There are more complaints in towns, particularly from people living in older buildings. Infestation is mostly linked to pigeon nests in crevices and holes on the facades of houses or city buildings (behind external air-conditioners, in holes in walls of buildings, in the eaves, attics, etc.). Once the birds leave the nest, the mites go in search of alternative hosts: they enter homes through windows and from balconies and bite humans. Cases of *D. gallinae* infestation have been reported frequently in private homes, hospitals and public offices in Italy (Cafiero et. al. 2008b, 2013, Galante et al. 2011) and also in other countries (reviewed by George et al. 2013, Abdigoudarzi et. al. 2014). In humans, *D. gallinae* causes dermatitis with small erythematous itchy papules and an allergic reaction. In severe cases, dermatitis leads to eczema (papular itchy dermatitis). The rash can occur all over the body, but is most frequently reported on the arms, wrists, neck, chest and back (Cafiero et al. 2008b, 2009, 2013) (Fig. 14).

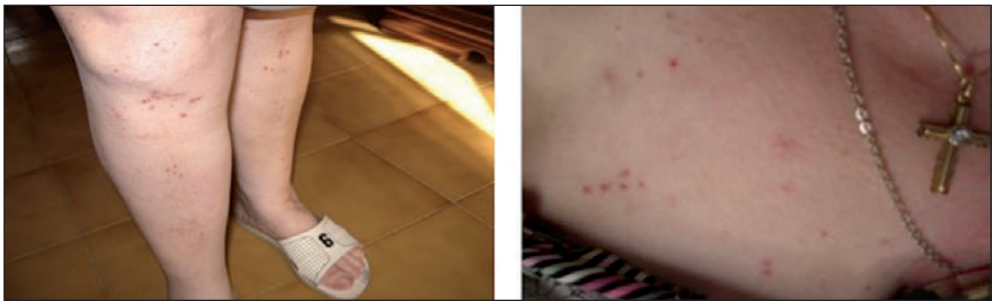

Fig. 14 *Dermanyssus gallinae* dermatitis in town residents living near abandoned pigeons' nests (original M. A. Cafiero)

The authors recommend that our parasitologist colleagues pay attention to their offices while they are working. If one day you see mites crawling over your laptop and on the desk, then check your air-conditioning. You will probably find that there is a recently abandoned pigeons' nest (Fig. 15*A*) so that the hungry mites are forced to feed on what is closest to them – you! This happened to the one of the authors of this contribution. As parasitologists, we have an advantage: we can suspect an infestation, collect the mites and look at them under the microscope. In that specific case, *D. gallinae* was identified as the culprit. Do not worry too much if when you go home from work you notice that you have a dermatosis with pruritus (Fig. 15*B*), because we are "hands-on" parasitologists! This means that you are having a "personal experience." Of course, we would recognize it, but red mite dermatitis can easily be misdiagnosed by dermatologists/doctors, who cannot do so, as we have ascertained in several dermanyssosis cases in Italy (Cafiero et. al. 2013), and not only there (Collgros et al. 2013). Firstly, we strongly recommend that the mite is correctly identified, as even recently some cases of dermatitis caused by *O. bursa* (Mentz et al. 2015, Castelli et al. 2015) or by *O. bacoti* (Cafiero et al. 2015) have been recorded in humans, and correct identification is the first requirement before applying control methods. Secondly, dermatologists should learn to work closely with veterinarian entomologists, who can help them in understanding some animal-related pathologies.

Fig. 15 (*A*) Abandoned pigeon nest in an air-conditioning unit in a private office. (*B*) *Dermanyssus gallinae* dermatitis on the chest (originals A. Giangaspero)

Another aspect of the public health problem comes from another experience in Italy. In addition to the appearance of acaricide-resistant mite populations, improper acaricide use by farmers to control *D. gallinae* can lead to the accumulation of pesticide residues in the organs and tissues of poultry, or in eggs, with consequent negative effects on product quality and inevitable risks to human health. In order to draw attention to the use of pesticides and their possible build-up, we investigated laying hen flocks in which resistant mites were detected. Tissues (fat, muscle and skin) and organs (liver, heart) of a batch of laying hens at the end of their productive cycle (about 11 months) were tested for carbaryl and permethrin. We found carbaryl residues, particularly in samples of fat and skin, and also permethrin, although in a more limited number of hens and organ/tissue samples (Marangi et al. 2012). It is clear that this misuse/abuse of acaricides can be dangerous for human health, and there is the additional problem that most farmers often work without personal protective equipment during treatment, out of ignorance or simply non-compliance with the regulations. Pesticides may be inhaled and can pass through the skin to accumulate in the human body.

7. Conclusions

Despite recent technological innovations in the poultry industry and the level of productivity achieved in recent years, *Dermanyssus gallinae* is still a very worrying problem. There is still a long way to go before we can control *D. gallinae*, but in the meantime the following simple considerations should be underlined. On farms, poor hygiene, lack of education among farmers, low standards of housing maintenance, inappropriate control measures, and the continuous presence of hens – often with no break between production cycles – are all factors that enhance and explain the high susceptibility to red mite infestation.

Alternative control measures are beginning to arrive on the market, although most candidate products are still at too premature a stage for commercialization. The red mite must be correctly identified before any control methods are applied. It is desirable for significant progress to be made in *D. gallinae* control *via* an integrated approach combining recent research with both existent and new strategies. Together with improved monitoring and modelling this will provide better information in support of treatment interventions (Sparagano et al. 2014, George et al. 2015, Pritchard et al. 2015). Numerous groups from research, public health, industry and poultry organizations throughout Europe have an interest in *D. gallinae*, but these are highly fragmented and nationally focused. For this reason, the EU is supporting a COST Action (FA1404) that aims to strengthen the existing networks and foster collaboration between 28 COST countries in order to improve our knowledge of this tiny but very important mite.

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„Krieg der Gelehrten“ und die Welt der Akademien 1914–1924

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Vor 100 Jahren erschütterte der Erste Weltkrieg Europa. Unter erheblichem Medieninteresse rückte das Gedenken an diese Katastrophe verstärkt bisher weniger beachtete Fragestellungen in den Fokus der historischen Analyse. Dazu gehört auch das Verhalten der europäischen Wissenschaftsakademien bei Kriegsausbruch und im Kriegsverlauf. Die Leopoldina und die französische *Académie des sciences* widmeten dem „Krieg der Gelehrten“ und den Positionen der Nationalakademien Europas in der Kriegs- und unmittelbaren Nachkriegszeit (1914–1924) ein Symposium, auf dem international renommierte Historiker und Wissenschaftshistoriker zusammen mit Vertretern der kriegsbeteiligten Nationalakademien bislang erarbeitete Ergebnisse zum Einfluss des Weltkrieges auf die großen Nationalakademien vortrugen und vergleichend diskutierten. Der Band versammelt Beiträge zum Forschungsstand zu den Akademien in Deutschland, Frankreich, Großbritannien und Russland und bildet den Auftakt zu einer Reihe weiterer Untersuchungen noch bestehender Forschungsdesiderate zur Gelehrtenwelt jener Jahre.

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Medical Entomology in the 21st Century: Retrospect and Challenges

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Abstract

Diseases caused by arthropods directly or indirectly – through the transmission of pathogens – have accompanied the Hominini throughout their entire evolution and have led to hundreds of millions of deaths. However, until the 19th century the striking and enormous medical importance of arthropods – particularly as vectors – was entirely underestimated or not even recognised at all. Only from about 1880 onwards, in quick succession, were vectors and the pathogens transmitted by them identified. The 20th century was characterised by pioneering discoveries in all fields of medical entomology.

Today we know most of the arthropods that parasitize on or in humans, cause toxic or allergic reactions, act as intermediate hosts or which, most importantly, transmit pathogens. Moreover, most life-cycles have been clarified. Nevertheless, new discoveries of relevant, undiscovered or neglected arthropods and pathogens are expected even in this century.

None of us knows what the next 85 years will bring. However, three events in this century, which are certain to happen and which are clearly linked to diseases caused by arthropods, include: (*1*.) Climate change and global warming. An increase in temperatures by 2 °C by the end of the century is unavoidable, an increase by 3 °C is likely and even higher values cannot be ruled out. This will lead to considerable changes in many ecosystems and to the spread of vectors and pathogens from tropical and subtropical regions and the establishment of these in temperate zones. (*2*.) The excessive increase in the world's population. Currently the world's population is ca. 7.4 billion, and it may reach 11 billion by 2100. Even if global population growth is lower, populations in the subtropical and tropical regions – where arthropod-linked diseases are particularly prevalent – will increase considerably. (*3*.) Further increase in numerous forms of globalization by voluntary or forced migrations of humans (e.g. more than one billion travellers per year and about 65 million refugees presently) and by the transport of animals, plants, goods etc.

All of these factors imply numerous ways of active and passive dispersal of arthropods and, specifically, vectors and pathogens. All of the pathogens presently known will remain important. Today we do have effective drugs against (almost) all bacteria, protozoa as well as all helminths transmitted by arthropods so that the number of fatalities due to such pathogens (Rickettsiales spp. and other bacterial infections, *Trypanosoma* spp., *Leishmania* spp., *Plasmodium* spp., ...) will gradually and continuously decrease. However, developments of new drugs will be necessary (not least as a result of drug resistance), and vaccines against Lyme borreliosis, leishmaniosis, and malaria may become available in our century.

A completely different situation arises in the case of arboviruses. There are about 130 arboviruses that are pathogenic for humans, and further ones will be detected. Unfortunately there are practically no effective drugs and only few vaccines (TBE, yellow fever, Japanese encephalitis). Vaccines against dengue fever may, however, be expected in the near future, possibly also against Chikungunya and Zika.

Another risk in terms of arbovirus infections comes from the importation, dispersal and amplification of exotic vectors, in particular mosquitoes, as has occurred in the recent past. Some of them have high vector capacities for a number of arboviruses that cause serious diseases. We have effective tools for monitoring, but currently no real satisfactory ways of specifically controlling mosquitoes. Most probably the effective control of certain mosquito vectors will have to be based on the genetic manipulation of mosquitoes (e.g. introduction of lethal genes into a population). Similar challenges may result from dispersal of other blood-sucking arthropods, particularly sandflies and ticks. Another largely open field of research is the search for viruses in arthropods which have not (not yet?) been transmitted to vertebrates.

The use of arthropods and arthropod-borne pathogens as biological weapons in the 21st century is unlikely. Nevertheless, global terrorism sounds a note of caution. It can hardly be argued against the fact that arboviruses and their vectors will represent the most important new challenges in medical entomology in the 21st century.

Zusammenfassung

Arthropoden beeinträchtigen in vielfältiger Weise die menschliche Gesundheit seit dem Auftauchen der Hominini in der Evolution: direkt, als Erreger von Krankheiten, und indirekt als Überträger von Krankheitserregern. Dennoch wurde die enorme Bedeutung von Arthropoden, vor allem ihre Rolle als Vektoren, bis zum Ende des 19. Jahrhunderts vollkommen unterschätzt. Erst ab etwa 1880 wurden in rascher Folge Erreger und Überträger identifiziert. Das 20. Jahrhundert war durch bahnbrechende Entdeckungen auf allen Gebieten der Medizinischen Entomologie geprägt.

Heute kennen wir die meisten Arthropoden, die (als Parasiten von Geweben oder Hohlorganen, als Erreger von toxischen und allergischen Reaktionen) Krankheiten hervorrufen, die als Zwischenwirte fungieren oder – und dieser Rolle kommt der weitaus größte Stellenwert zu – Krankheitserreger übertragen. Zudem sind die meisten Zyklen (weitgehend) geklärt. Trotzdem ist mit der Entdeckung weiterer medizinisch relevanter Arthropoden ebenso wie mit jener von bisher unbekannten Krankheitserregern zu rechnen.

Wir wissen nicht, was die nächsten 85 Jahre bringen werden. Wir kennen aber drei Entwicklungen in diesem Jahrhundert, die nicht mehr aufzuhalten sind und die mit intensiven Herausforderungen an die Medizinische Entomologie verknüpft sind: (*1*.) Klimawandel und globale Erwärmung. Ein Anstieg der globalen Temperatur um mindestens 2 °C ist unvermeidbar, eher ist dieser Wert zu niedrig, ein Anstieg um über 3 °C ist durchaus möglich. Das bedeutet steigende Risiken der Ausbreitung und Etablierung tropischer und subtropischer Arthropoden in gemäßigten Zonen. (*2*.) Anstieg der Weltbevölkerung von derzeit 7,4 Milliarden auf vermutlich ca. 11 Milliarden Menschen bis 2100. Selbst wenn sich das Bevölkerungswachstum verlangsamt, wird die Bevölkerung gerade in den tropischen und subtropischen Gebieten, wo mit Arthropoden verknüpfte Krankheiten einen besonders hohen Stellenwert haben, exzessiv zunehmen. (*3*.) Weiter eskalierende Globalisation in vielfältiger Form durch freiwillige oder erzwungene Migrationen (derzeit z. B. 1 Milliarde Reisende/Touristen pro Jahr; gegenwärtig ca. 65 Millionen Menschen auf der Flucht), durch Verfrachtung von Tieren, Pflanzen und Waren.

Alle diese Faktoren implizieren zahlreiche Wege der aktiven und passiven Verbreitung von Arthropoden, besonders von Vektoren, und von den durch Arthropoden übertragenen Erregern.

Alle heute bekannten Erreger werden auch in den nächsten Jahrzehnten von Bedeutung bleiben. Gegen alle bakteriellen Erreger, Protozoen und Helminthen stehen uns wirksame Medikamente zur Verfügung, wenngleich verbessernde Entwicklungen (nicht zuletzt wegen des immer bestehenden Resistenzproblems) notwendig sind. Weiter ist die Hoffnung durchaus berechtigt, dass in diesem Jahrhundert Impfungen gegen Lyme-Borreliose, gegen Leishmaniose und gegen Malaria verfügbar werden.

Mit einer vollkommen anderen Situation konfrontieren uns die Arboviren. Bisher kennt man ungefähr 130 Arboviren mit humanpathogenen Eigenschaften, die Entdeckung weiterer ist zu erwarten. Es gibt so gut wie keine Medikamente, und nur gegen drei (TBE, Gelbfieber, Japanische Enzephalitis) stehen Impfstoffe zur Verfügung. Die Verfügbarkeit eines Dengue-Impfstoffs – vielleicht auch von Vakzinen gegen Chikungunya und/oder Zika – darf in der nächsten Zukunft erwartet werden.

Ein wachsendes Risiko sind die Einschleppung und Etablierung von Arboviren einerseits und von Vektoren andererseits aus tropischen Gebieten in gemäßigte Zonen. Manche dieser Vektoren haben hohe Übertragungskapazität. Das Monitoring von (neobiotischen, oft als invasiv bezeichneten) Vektoren gewinnt zunehmend an Bedeutung, die Möglichkeiten spezifischer Bekämpfung von Stechmücken, Sandmücken, Zecken und anderen Vektoren sind allerdings unbefriedigend. Möglicherweise ist durch gentechnologische Eingriffe (z. B. Einbringen letaler Gene) ein wesentlicher Fortschritt zu erreichen. Ein weiteres, noch weithin offenes Forschungsfeld ist die Suche nach Viren in Arthropoden, die (noch nicht?) nicht auf Vertebraten übertragen werden können.

Der Einsatz von Arthropoden und durch Arthropoden übertragenen Erregern als Biowaffen im 21. Jahrhundert ist unwahrscheinlich, trotzdem mahnt der weltweite Terrorismus zur Beobachtung der Situation. Es kann kaum bezweifelt werden, dass Arboviren und deren Vektoren die weitaus größte neue Herausforderung an die Medizinische Entomologie im 21. Jahrhundert stellen.

1. Arthropods and Diseases in Humans

Arthropods have been affecting human health in many ways since the emergence of the Hominini, and they will remain an important and possibly even increasing risk in the future of *Homo sapiens*. Arthropods may afflict humans directly by causing diseases, indirectly as vectors that transmit pathogens – viruses, bacteria, protozoa, helminths – or as intermediate hosts of human parasites.

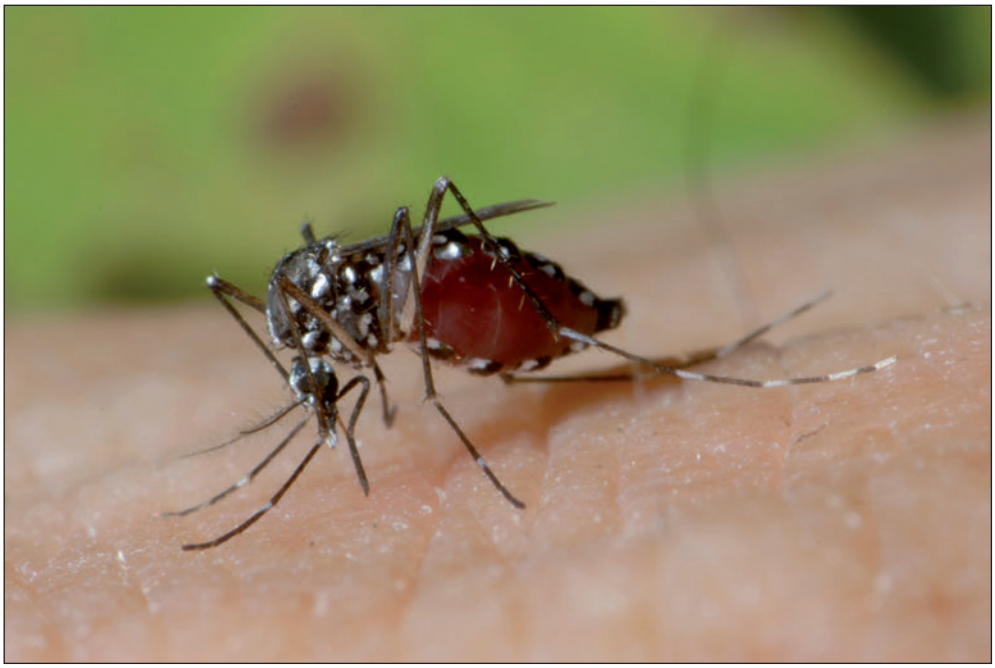

Fig. 1 *Aedes* (*Stegomyia*) *albopictus* (Skuse, 1895); Photo: R. Pospischil. From Aspöck 2010. This mosquito may be regarded as the symbol for the most significant challenges in medical entomology in this century. The original distribution of *Aedes albopictus* comprises tropical and subtropical parts of Southeast Asia. Due to globalization this mosquito species has been gradually introduced into other continents and has established large populations in many countries and particularly also in temperate zones. The species has a high vector capacity for many arboviruses. This symbolises the most urgent issues of medical entomology in the 21st century: Transmission of introduced pathogens, particularly arboviruses, by introduced vectors.

Table 1 lists the nine most important ways that arthropods may lead to diseases. Of these, the first 5 issues (*a–e*) are so obvious that the correlation between the arthropods and the diseases had already been made in ancient times. Issues (*f*) and (*g*) were not discovered until methodological conditions were right (particularly the introduction of microscopy).

The highest medical significance of arthropods is their capacity to transmit pathogens (issues *h* and *i*). Depending on the region, hundreds of millions of humans have been killed for centuries and millennia by viruses, bacteria, or protozoa transmitted by arthropods, but this remained unrecognised until the end of the 19th century.

2. Milestones in Medical Entomology

Arthropods have been the cause of disease and death throughout all of evolution and the history of mankind. Billions of humans have died of diseases caused directly or indirectly by arthropods. However, until the 19th century, the enormous importance of arthropods for the onset of so many diseases was entirely underestimated. Many of these diseases were known, but the aetiologies and particularly the correlations with arthropods remained unknown.

Tab. 1 Forms of medical significance of arthropods

(*a*)	Ektoparasites, usually in connection with blood-sucking: – ticks (Ixodidae, Argasidae); – lice (Anoplura: Pediculidae, Pthiridae); – bugs (Heteroptera: Cimicidae, Reduviidae); – fleas (Siphonaptera: Pulicidae, Ceratophyllidae, Tungidae); – midges and flies (Diptera: Culicidae, Simuliidae, Ceratopogonidae, Psychodidae / Phlebotominae, Rhagionidae, Tabanidae, Muscidae, Glossinidae, Hippoboscidae); – moths (Lepidoptera: Noctuidae)
(*b*)	Toxic / allergic reactions due to substances in the saliva of blood-sucking arthropods
(*c*)	Endoparasites in tissues and / or in body cavities (*Sarcoptes scabiei* and other mites living in – not on – the skin; Pentastomida; Diptera of various families causing myiasis)
(*d*)	Venomous arthropods Toxic substances of various origin can be injected by bites of arthropods (spiders, millipedes, but also certain insects like ants, some beetles) or by poisonous stings of scorpions and of bees and wasps or by blood-sucking arthropods
(*e*)	Psychic irritation, sometimes leading to disease, by (particularly mass occurrence) of arthropods of many taxa, e.g. cockroaches, bugs, beetles ...
(*f*)	Allergic reactions after inhalation of (dead) arthropods or parts of them, of excrements, secretions ... (house-dust mites; cockroaches; many other insects)
(*g*)	Intermediate hosts of helminths (Crustacea: Copepoda, Decapoda; Coleoptera, Siphonaptera)
(*h*)	Vectors of pathogens by mechanical transmission (cockroaches, flies, ... transmitting pathogens e.g. from excrements to food [viruses, bacteria, Protozoa])
(*i*)	Vectors of pathogens by cyclic transmission, i.e. after multiplication (or – in helminths – continuation of development) in the arthropod: – Viruses (Arboviruses); – Bacteria: Rickettsiales, Spirochaetales, Thiotrichales, Legionellales, Enterobacteriales; – "Protozoa": Euglenozoa, Apicomplexa; – Nematoda: Spirurida

The first confirmation of the transmission of a human pathogen by an arthropod was reported as late as in 1884, when Sir Patrick Manson (1844–1922) showed that *Wuchereria bancrofti* was transmitted by mosquitoes (Manson 1884, Grove 1990, Auer and Aspöck 2010).

In 1880 Charles Louis Alphonse Laveran (1845–1922) discovered *Plasmodium* in the blood of a patient suffering from malaria (Laveran 1880, Poser and Bruyn 1999), however, it was not until the end of the 19th and the beginning of the 20th century that the transmission

of the *Plasmodium* species by mosquitoes was uncovered in brilliant experiments by Ronald Ross (1857–1932), Giovanni Battista Grassi (1854–1925), Amico Bignami (1862–1929), Giuseppe Bastianelli (1862–1959) and others (Poser and Bruyn 1999).

In 1893 Theobald Smith (1859–1934) and Frederick Lucius Kilborne (1858–1936) were the first to show that ticks are able to transmit pathogens (*Babesia*) (Assadian and Stanek 2002).

Between 1894 and 1898, Alexandre Yersin (1863–1943) and Paul Louis Simond (1858–1947) discovered the causative agent of plague (today known as *Yersinia pestis*) and how it was transmitted (Winkle 2005, Pfeffer 2010).

In 1900 Walter Reed (1857–1902) confirmed the hypothesis of Carlos Finlay (1833–1915) that yellow fever was indeed transmitted by mosquitoes (Winkle 2005).

In 1909–1911 Charles Jules Henri Nicolle (1866–1936) discovered the vector (*Pediculus*) and the agent causing typhus (Dobler 2010).

In 1909 Carlos Justiniano Ribeiro Chagas (1879–1934) described the pathogen, the vector, and the clinical symptoms of the disease which is now known as Chagas disease.

Almost 30 years after the discovery of how yellow fever is transmitted, as late as in 1927, the causative agent of the disease was clarified; it was the first time that a virus pathogenic to humans had been isolated (Dobler and Aspöck 2010, Calisher 2013).

In the same year, Schneider (1931) described the disease which we now know as tick-borne encephalitis (TBE). Ten years later the first isolation of the virus causing this disease was reported (Hubálek and Halouzka 1996).

Although the clinical symptoms, particularly erythema chronicum migrans, had been known to be associated with tick bites for decades (since the beginning of the 20th century) *Borrelia* wasn't detected in hard ticks until 1982 by Willy Burgdorfer (1925–2014). Two years later, in 1984, this species was described as *Borrelia burgdorferi* by R. C. Johnson (Stanek 2010).

3. Global Arthropod-related Diseases Today

Table 2 provides a few figures to show the magnitude of the impact of arthropod-linked diseases. More than 5,000 humans die every year due to scorpion stings (Komposch 2010) and, according to Habermehl (1994), possibly 300,000 people die of toxic and allergic reactions to the stings of hymenoptera (bees, wasps, ...).[1] However, arthropod-borne infections are, by far, the most significant when it comes to arthropod-linked diseases.

Table 2 shows figures for three mosquito-borne infections (dengue, yellow fever, and malaria); leishmanioses, transmitted by sandflies; and African sleeping sickness, transmitted by tsetse flies. We do not really know how many individuals are afflicted by infections, clinical manifestations, or death due to one of these pathogens; the estimated figures vary greatly. It is estimated that more than 500 million humans are infected annually by arthropod-borne pathogens and probably one million (hardly less, possibly more) of these die of the disease.

1 It is hard to imagine that so many people die of stings from hymenoptera. However, Habermehl (1976) wrote in the 1st edition of his book that approximately 120,000 people die of them every year. Reliable evidence-based figures are not available.

Tab. 2 Arthropod-linked diseases worldwide today

Disease	Impact
Deaths after stings	
–	of scorpions > 5000
–	of hymenoptera 120,000 (?) (– 300,000 ?) (see footnote 1)
Dengue Fever	
–	– ca. 50 % of world population in endemic areas
–	– 50 – >500 million infections
	– 50–100 million cases of disease (among these 500,000 severe cases per year)
	– 25,000 deaths per year
Yellow Fever	
–	– 600 million people in endemic areas
–	– 200,000 cases of disease per year
–	– 30,000 deaths per year
African Sleeping Sickness	
–	– Prevalence: ca. 500,000 cases
–	– Incidence: 50,000–100,000 per year
Leishmanioses	
–	– Prevalence of infections: 12 million
–	– 350 million are living in risk areas
–	– Incidence: 2 million new infections per year
–	– ca. 60,000 deaths per year
Malaria	
–	– 240–300–500 million cases of Malaria per year
–	– 500,000 (– > 650,000 ?) deaths per year

4. Unavoidable Developments in the 21st Century Linked with Medical Entomology

No one knows what the next 85 years will bring – certainly many new discoveries, inventions, and methods. Unfortunately there are likely to also be many negative developments like wars and epidemics, though hopefully no terrestrial or extraterrestrial catastrophes. However, among the events to be expected with certainty there are three grave developments in this century which are clearly linked with problems resulting from diseases caused by arthropods.

4.1 Climate Change and Global Warming

Climate change and global warming are (have to be) accepted as facts; it is only a question of magnitude. An increase in temperatures by 2 °C by the end of this century is unavoidable, an increase by 3 °C is likely, and even higher values cannot be ruled out. Temperatures have increased globally by 0.9 °C since 1890 (natural and anthropogenic) and by 0.2 °C since 1990 (predominantly anthropogenic) (*IPCC* 2013, *APCC* 2014).

Over the past 400,000 years, concentrations of CO_2 in the atmosphere were never higher than 280 ppm. CO_2 concentrations have increased gradually since the start of the Industrial Revolution at the end of the 18th century. During the past 100 years there has been a rise from 300 to 400 ppm. If emissions of CO_2 continue to rise at this rate, they are expected to reach 650 ppm by 2100. This would lead to an increase in global temperatures by at least 4 °C to 5 °C.

To illustrate the effect of global warming: Since the latest glacial maximum (ca. 25,000 years BP) the increase in global mean temperatures was 4 °C – 7 °C. From 18,000 BP onwards the major glaciers (Scandinavia and Canada: up to 3,000 m high, in the Alps: 2,000 m) melted and largely disappeared. This led to a deep change in the flora and fauna of Europe and, moreover, of the northern hemisphere, and to an increase in sea levels by ca. 120 m (Aspöck 2008, 2010, Aspöck and Walochnik 2014). Without extensive additional measures to reduce emissions one can expect global average surface temperatures to rise by 3 °C – 5 °C by 2100 compared to the first decade of the 20th century (Aspöck and Walochnik 2014).

Apart from precipitation, temperature is the most significant factor for the composition of the biodiversity in a certain geographic area. Thus, fauna and flora in many parts of the world are already undergoing considerable changes, distribution areas of arthropods of medical importance will be altered, some species will extend their distribution to the north, on one hand, and to higher altitudes on the other. Neobiota will emerge and will become established, as has already been the case with exotic mosquitoes in Europe: *Aedes* (*Stegomyia*) *albopictus*, *Aedes* (*Stegomyia*) *aegypti, Ochlerotatus* (*O.*) *atroparvus*, *Ochlerotatus* (*O.*) *triseriatus*, *Ochlerotatus* (*Finlaya*) *japonicus*, and *Ochlerotatus* (*Finlaya*) *koreicus* (Kampen et al. 2012, Medlock et al. 2012, Schaffner et al. 2013, Kampen and Werner 2014).

On the other hand, the autochthonous tick species *Ixodes ricinus* has considerably extended its vertical distribution to higher altitudes, which has also left a vertical extension of transmission of tick-borne pathogens (Danielová et al. 2010, Daniel et al. 2011).

4.2 Increase in the World's Population

The present population of the world (at the beginning of 2016) is ca. 7.4 billion, however, it continues to increase and – according to estimations by the United Nations – will reach 11.2 billion by 2100. There are three main prognoses: the most probable one is ca. 11 billion, another estimation is about 16 billion, and a third prognosis foresees a decline in the world's population to 6 billion by the end of the century after an increase to more than 8 billion around the middle of the century. This last scenario seems highly unlikely.

Until the early modern age the world's population grew very slowly; in 1600 it reached about 500 million, then doubled within 200 years, and then began to increase rapidly. In 1930 the world's population was 2 billion, in 1960 it was 3 billion, in 1975 more than 4 billion, in 1987 5 billion, in 1999 6 billion, and in 2011 7 billion. (Detailed figures and graphs as well as references to further sources of information can be found in Wikipedia under "World population".) Even if the estimated number of ca. 11 billion at the end of the century may not be reached, a number below 10 billion seems very unlikely as this would imply a considerable decline in reproduction rates.

The population is expected to grow on all continents, but there are striking differences in the intensity with regard to certain geographic areas. The highest rates of growth are, and will be, in Africa, followed by Asia. The increase will most likely pertain to subtropical and

tropical regions, which have particularly favourable conditions for many vectors, especially mosquitoes, sandflies and blackflies.

4.3 Globalization and an Increase in All Forms of Migration

Humans have always carried pathogens and arthropods of medical importance over long distances directly or indirectly through migration and/or by transporting animals, plants, and goods. However, the intensity and, particularly, the duration of those events appear extremely modest compared to the situation we see today (see Tab. 3).

Tab. 3 Globalization, carry-over and dispersal of infectious diseases, of pathogens and of vectors

Carry-over and dispersal by
– Migrations of humans • for professional reasons (economics, politics, science, education, ...) • for recreation and pleasure (tourism) • for searching new possibilities of employment • by expulsion (refugees)
– Transport of goods on land, by ship, by airplane
– Transport of productive livestock and farm animals
– Transport of pets
– Transport of wild animals

Every day thousands of humans suffering from infections – estimations of the numbers vary – travel from one country to another and from one continent to another acting as a potential source of infection for other individuals. Momentous examples of infections transmitted from human to human are the Acquired Immune Deficiency Syndrome (AIDS), Influenza, the Severe Acute Respiratory Syndrome (SARS), ... However, pathogens transmitted by arthropods are also introduced by individuals who travel in the state of viremia or bacteremia or parasitemia, if these persons are bitten by a blood-sucking arthropod. Current examples are the chikungunya virus, dengue virus and, recently, the zika virus (Petersen and Powers 2016, Imperato 2016).

At first glance it seems surprising that more of these introduced pathogens have not become established. The explanation is that, in the majority of cases, a suitable vector is not present at the right time. A viremia usually lasts only a few days. However, the more people migrate, the higher the probability is that pathogens are introduced, become established and possibly cause epidemics.

Another aspect of the multiple forms of movements of humans and animals, and particularly of the transport of goods, is the introduction of arthropods with vector capacity which may get established in the new environment and may transmit a pathogen that has been introduced. The epidemics of the chikungunya virus transmitted by *Aedes albopictus* in Northern Italy is an impressive example (Watson 2007, Cavrini et al. 2009).

The magnitude of these enormous movements and migrations can be convincingly demonstrated by two figures: Presently about one billion tourists per year travel within or between

continents; in 2020 this number will increase to 1.6 billion. Realistic figures for the end of the 21st century are not available. A second impressive, but very sad figure: at the beginning of 2016, 60 million refugees were on the run. (Meanwhile, October 2016, the number of refugees has reached > 65 million.) A large percentage of them is migrating from tropical or subtropical regions in the north which means that pathogens from southern regions may be introduced into temperate zones. Nobody knows today how many refugees will be on the run in 20 or 30 years or at the end of the century, nor which regions they will be leaving and which regions they will be migrating to. However, the present political situation in many parts of the world is sad and depressing, and politicians are so far from finding any solutions that it is difficult to believe that the number of refugees in the world will decline.

There is no question that all forms of globalization including migrations will continue to increase in the course of the 21st century, thus continuously augmenting the risk that arthropod vectors (and the pathogens transmitted by them) will be transferred.

5. Arthropod-Related Diseases in the 21st Century

It is unlikely that any of the arthropod species of medical importance, or any human arthropod-borne pathogens will be eradicated in the next 85 years. Suffering from arthropod-related diseases will continue, hundreds of millions of humans will be seriously afflicted and many millions will die. Malaria will most probably remain the arthropod-borne infection with the highest number of fatalities per year. However, many arthropod-borne pathogens will be introduced into new regions and will disappear from others. This process has already begun.

All of the issues concerning the various forms of affliction of human health by arthropods listed in Table 1 will continue to apply, and no significant changes in magnitude are to be expected. The real and largely new problems will result from pathogens transmitted by vectors and particularly from pathogens that have been introduced by vectors that have been introduced. In Tables 4 and 5 we have attempted to estimate the probability of the emergence of non-autochthonous vectors and non-autochthonous pathogens transmitted by arthropod vectors.

Tab. 4 Probability of emergence of arthropods of medical importance in regions outside the present distribution areas in the 21st century

Arthropods of medical importance	Probability of emergence
– Culicidae (mosquitoes)	+++
– Phlebotominae (sandflies)	++
– Simuliidae (blackflies):	(+)
– Glossinidae (tsetse flies):	–
– Ceratopogonidae (gnats):	+
– Ixodoidea (ticks):	+(+)
– Others (Reduviidae, Pulicidae, Tabanidae)	(+)

+ probable, ++ in all probability, +++ certain, – improbable, () uncertain
Natural dispersal (per continuitatem or spread by birds) or anthropogenic by infected persons or by various human activities.

Tab. 5 Probability of emergence of arthropod-borne pathogens (Bacteria, Protozoa, Helminths, Arboviruses) in regions outside the present endemic areas in the 21st century

Arthropod-borne pathogens	Probability of emergence
Bacteria	
– *Borrelia* spp.	+
– *Yersinia* pestis	–
– Rickettsiales	+
– *Coxiella burnetii*	(+)
Apicomplexa	
– *Plasmodium* spp.	+
– *Babesia* spp.	–
Arboviruses	
– Chikungunya Virus	+++
– Eastern Equine Encephalitis Virus	(+)
– Western Equine Encephalitis Virus	(+)
– Venezuelan Encephalitis Virus	(+)
– Ross River Virus	+
– Phleboviruses	++
– Crimean Congo Hemorrhagic Fever Virus	+(+)
– Rift Valley Fever Virus	+
– Yellow Fever Virus	+
– Dengue Viruses	+++
– Japanese Encephalitis Virus	++
– West Nile Virus	+++
– Zika-Virus	+++
– Murray Valley Encephalitis Virus	+
– Other arboviruses	++(+)
Euglenozoa	
– *Trypanosoma brucei gambiense*	–
– *Trypanosoma brucei rhodesiense*	–
– *Trypanosoma cruzi*	–
– *Leishmania* spp.	++
Nematoda	
– *Wuchereria* spp.	–
– *Brugia* spp.	–
– *Mansonella* spp.	–
– Other rare Filarioidea	–
– *Onchocerca volvulus*	–

+ probable, ++ in all probability, +++ certain, – improbable, () uncertain

Table 5 lists only those pathogens which are cyclically transmitted by arthropods after multiplying (or – in Nematoda – after continuing to develop) in their hosts. Pathogens transmitted mechanically by arthropods – mainly intestinal viruses, bacteria or protozoa – from faeces to food play a considerable role in infectious diseases of the intestine, particularly diseases with severe forms of diarrhoea in children in poor countries in the tropics. They will continue to play a significant role in the future. However, no significant changes in the epidemiological situation and the magnitude of the problem are to be expected – except that, when the world's population is higher, the number of persons afflicted by these pathogens will also be higher.

Among the arthropods representing (potential) vectors, mosquitoes have the highest potential of being introduced into new regions and establishing stable populations, followed by Phlebotominae and Ixodidae. These arthropods are primarily Neobiota. They are often called "invasive", but this characterization should be confined to species that significantly impact the ecosystem of which they have become a part (Nentwig 2010, Aspöck and Walochnik 2014).

Among the bacteria, protozoa, and nematodes (Tab. 5) only very few pathogens may become established in new ecosystems. There is, however, one important exception: the *Leishmania* species. In Europe, leishmaniosis has been restricted to Mediterranean regions for a long time, but autochthonous cases of leishmaniosis have recently been identified (Bogdan et al. 2001, Walochnik and Aspöck 2010) and sandflies have also been recorded in Central Europe (Naucke and Pesson 2000, Naucke et al. 2011, Poeppl et al. 2013, Obwaller et al. 2014).

By far the most significant changes in distribution and epidemiology of pathogens in the 21st century are expected to be among the arboviruses (Tab. 5), several viruses (chikungunya, Phlebovirus, dengue, West Nile, Japanese Encephalitis, Usutu, zika) have emerged in regions, even in continents, that are not their original distribution areas (Weissenböck et al. 2009, Dobler and Aspöck 2010, Lourenço and Recker 2012, Paz and Semenza 2013), and undoubtedly these viruses will spread into more regions. Moreover, it is likely that other arboviruses will emerge in areas where they do not presently occur. This not only relates to mosquito-borne viruses, but also to sandfly-borne viruses (Depaquit et al. 2010, Ready 2013) and tick-borne viruses. Arbovirus infections will pose a great challenge for medical entomology in the 21st century.

6. Strategies against Arthropod-borne Infections in the 21st Century

Table 6 gives a condensed overview of the possible strategies for combatting arthropod-borne infections in the coming decades.

6.1 New Drugs

The development of new drugs against all bacteria (Rickettsiales; *Borellia* spp., *Francisella tularensis*, *Coxiella burnetii*, *Yersinia pestis*), Euglenozoa (*Trypanosoma* spp., *Leishmania* spp.), and against Apicomplexa (*Plasmodium* spp., *Babesia* spp.) may be expected. Presently we do have effective drugs against all bacteria transmitted by arthropods as well as against *Plasmodium* spp., however, drug resistance is a problem which requires permanent monitoring and efforts to develop new drugs. The medicines against *Trypanosoma* and *Leishmania* need improving.

Tab. 6 Strategies against arthropod-borne infections in the 21st century

Development of new drugs against – Bacteria (Rickettsiales, *Borrelia* spp., *Francisella tularensis*, *Coxiella burnetii*, *Yersinia pestis*) – Euglenozoa (*Trypanosoma* spp., *Leishmania* spp.) – Apicomplexa (*Plasmodium* spp., *Babesia* spp.) – Arboviruses?
Development of new vaccines
– Lyme-Borreliosis? – Leishmanoses? – Malaria? – Dengue Fever? – West Nile? – Chikungunya? – Zika? – Crimean Congo Hemorrhagic Fever (CCHF)? – Other arboviruses?
Control of vectors by a variety of methods, probably predominantly genetic engineering

With very few exceptions (Ribavirin against Crimean Congo Hemorrhagic Fever virus) no drugs against arboviruses are available. The search for substances to treat arbovirus infections will be an important challenge in our century.

6.2 Vaccines

Only a few vaccines against arthropod-borne pathogens are available. Finding a vaccine against *Rickettsia prowazekii* was of utmost importance in the past. Due to the effectiveness of tetracyclines, typhus has lost its fright (Dobler 2010).

A vaccine against *Yersinia pestis* is principally available, however, the disease lost its importance when effective drugs became available. Research into new vaccines against the plague has gained new significance in connection with international terrorism and the possible use of *Y. pestis* as a biological weapon.

No vaccine *ad usum humanum* against Lyme borreliosis is presently available. For a short time at the beginning of our century a vaccine (based on an unusual strategy: Stanek 2010) was used, but was removed from the market in 2002. Although we have effective drugs to treat Lyme borreliosis, a vaccine would be very useful.

We have no vaccines against leishmaniosis or malaria – despite decades of tremendous efforts into developing vaccines. It is, however, hoped that vaccines against leishmaniosis and malaria will be developed in the course of the century.

In terms of arboviruses, only three vaccines are currently (commercially) available: tick-borne encephalitis, yellow fever, and Japanese encephalitis. A vaccine against dengue fever is highly desirable and is expected in the near future (Martin and Hermida 2016, Vannice et al. 2016). Vaccines against other arboviruses (e.g. chikungunya, West Nile, zika) are of

considerable, even urgent, importance (Schwameis et al. 2015, Brandler and Tangy 2013, Fan and Moon 2016, Nandy et al. 2016).

6.3 Strategies against Vectors

In addition to the traditional methods of vector control, i.e. (careful and responsible!) alterations to ecosystems, application of insecticides and acaricides, genetic methods will becoming increasingly significant. These include the introduction of large numbers of genetically altered individuals into the populations. Genetic engineering may consist of lethal genes or alterations to the genome leading to reduced vector capacity. At any rate monitoring in the field (Kampen and Schaffner 2008, Schaffner et al. 2013) is and will be an important precondition for specific vector control. This will require extensive field work – an area of research which merits more attention. In addition, passive monitoring has also proven to be very effective (Kampen et al. 2015).

7. Arboviruses in *statu nascendi*?

In recent years it has become obvious that many arthropods harbour many arthropod-specific viruses (Calzorali et al. 2016). Many of these belong to virus families which contain arboviruses: Flaviviridae (mosquito-only Flaviviridae = MOF; Junglen et al. 2009, Junglen and Drosten 2013, Calzorali et al. 2012, 2016), Togaviridae (Junglen and Drosten 2013), Bunyaviridae (Marklewitz et al. 2011, 2013, 2015), Reoviridae (Hermanns et al. 2014), Rhabdoviridae (Quan et al. 2009).

This is not surprising as it is generally hypothesised that arboviruses originated in arthropods (Aspöck and Dobler 2010) and later achieved the ability to multiply in vertebrates after transmission by a blood-sucking arthropod or possibly by ingestion of the infected arthropod by a vertebrate (Junglen and Drosten 2013).

So far there is no indication of a risk for humans, we should, however, remain aware of the existence of a large variety of insect-specific viruses related to arboviruses. Possibly, or even probably, these viruses belong to an evolutionary line which is restricted to arthropods. These viruses cannot infect vertebrate cells thus being harmless for humans. Our knowledge on the biology of insect-specific viruses is, however, so poor that we cannot exclude that occasionally the ability to replicate in vertebrate cells might be achieved. Perhaps such events may be in a very far future of the evolution of these viruses. At any rate the question must be allowed: Are among the insect-specific viruses arboviruses in *statu nascendi*? Other questions concern possible interaction of insect-specific viruses with arboviruses and also potential use as biological control agents (Bolling et al. 2015).

8. Arthropods and Arthropod-borne Pathogens as Biological Weapons in the 21st Century?

One of the most famous cases of an arthropod-borne pathogen used as a biological weapon dates back to the year 1346, when the tartars gave up their siege of Kaffa, a town on the Crimean Peninsula (today: Feodosia), due to a plague epidemic. Before they left, they cata-

pulted corpses across the walls into the town infecting its inhabitants. These infected inhabitants travelled across Europe causing horrible plague epidemics on the continent. It is estimated that there were more than 30 million deaths, about one third of the population of Europe.

In the following centuries and particularly during the two World Wars of the 20th century, biological weapons – and among these also arthropods and arthropod-borne pathogens – played a significant role (Lockwood 2009). Since 1972, the convention on biological weapons forbids the development, production and use of biological weapons. Almost all countries have signed this convention. Even if biological weapons are not used in wars, they remain an aspect of bioterrorism.

There are about 200 pathogens which could be used as biological weapons, about 20 of these are arthropod-borne (Dar et al. 2013) (Tab. 7).

Tab. 7 Arthropod-borne pathogens considered as possible biological weapons

Viruses
Mosquito-borne Flaviviridae
– Yellow Fever (YF) Virus
– Dengue Viruses
– Japanese Encephalitis (JE) Virus
– Murray Valley Encephalitis (MVE) Virus
– West Nile (WN) Virus
– St. Louis Encephalitis (SLE) Virus
Mosquito-borne Togaviridae
– Chikungunya Virus
– Eastern Equine Encephalitis (EEE) Virus
– Western Equine Encephalitis (WEE) Virus
– Venezuelan Encephalitis (VE) Virus
– Ross River Virus
Tick-borne Flaviviridae
– Tick-borne Encephalitis Virus (TBEV)
– Kyasanur Forest Disease Virus (KFDV)
– Omsk Hemorrhagic Fever Virus (OHFV)
Tick-borne Bunyaviridae
– Crimean Congo Hemorrhagic Fever (CCHF) Virus
Mosquito-borne Bunyaviridae
– Rift Valley Fever (RVF) Virus
South American Hemorrhagic Fever Viruses (Junin, Machupo, ...)
Bacteria
– *Rickettsia prowazekii* (louse-borne)
– *Yersinia pestis* (mainly flea-borne)
– *Francisella tularensis* (partially tick-borne)
– *Coxiella burnetii* (partially tick-borne)

Out of all the pathogens, a list of 12 (groups of) pathogens has been compiled by the Centers for Disease Control and Prevention (CDC) – the so-called "dirty dozen" – which are particularly suited as biological weapons. Five of these are arthropod-borne: *Yersinia pestis*, *Francisella tularensis*, *Coxiella burnetii*, arthropod-borne encephalitis viruses, and arthropod-borne hemorrhagic fever viruses.

It is unlikely that these biological weapons will be a significant problem in the 21st century since terrorists have so many other gruesome possibilities. Nevertheless, it is important to be aware of this aspect of medical entomology in our century.

9. Outlook

The great new challenges in medical entomology in the 21st century almost exclusively concern vectors and vector-borne pathogens, and in particular arboviruses.

Monitoring and controlling the spread, particularly of introduced (neobiotic) vectors, will be a major issue. This will require extensive fieldwork and mass collections of vectors. As a consequence, we will need more entomologists and acarologists – simply put, people who are reliably (!) able to identify ticks, mosquitoes, sandflies, gnats, blackflies and horseflies. In addition, improved methods of rapid identification of arthropods using methods in molecular biology will be necessary.

It will be necessary to improve methods to specifically control vectors instead of waging unspecific battles against unspecific arthropods, sometimes against the whole ecosystem. Genetic engineering seems most promising, but research in this field is still in its infancy.

The development of new drugs against vector-borne pathogens will be another imperative issue in our century, particularly in light of emerging resistance to presently effective drugs. Unfortunately, there is no justified basis for an optimistic prognosis – this may, however, rapidly change.

Our century will hopefully bring new vaccines against some important vector-borne diseases: Lyme borreliosis, leishmaniosis, malaria, dengue, perhaps also against other viruses (West Nile? Zika? Chikungunya?). One must, however, be realistic: The development of vaccines is extremely expensive and – with a few exceptions – the huge sums of money will never be recouped.

One fascinating field of research pertains to viruses in arthropods which are not transmitted to vertebrates, but can be attributed to families which contain arboviruses. The question arises as to whether such viruses in arthropods are candidates for transmission to vertebrates thus becoming arboviruses. How long does evolution need to produce an arbovirus?

The 21st century is the century of molecular biology and its application in almost all disciplines of biology and medicine. Methods in molecular biology are and will increasingly become involved in all fields of medical entomology.

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Plenary Lecture

Nova Acta Leopoldina NF Nr. *411*, 261–268 (2016)

Willkommensgruß zur Abendveranstaltung im Tieranatomischen Theater der Humboldt-Universität Berlin

Theodor Hiepe ML (Berlin)

Meine sehr verehrten Damen und Herren,
liebe Teilnehmer unseres Symposiums,

herzlich willkommen, zugleich im Namen des Senators der Sektion Veterinärmedizin der Leopoldina, Herrn Prof. Hartwig Bostedt, im ältesten und wohl auch schönsten akademischen Lehr- und Forschungsgebäude unserer leidgeprüften Stadt Berlin.

Anstelle einer wortreichen mündlichen Darstellung habe ich mir erlaubt, Ihnen diesen wissenschaftshistorischen Standort (Abb. 1), Geburtsstätte der Vergleichenden Medizin, und dieses Gebäude (Abb. 2*A*), dem die Studenten der Veterinärmedizin den Kosenamen „Trichinentempel“ verliehen haben, über zwei an jedem Platz ausgelegte Schriftstücke vorzustellen (siehe Anhänge). Im Anhang 1 wird der vom Autor beschriebene Campus erläutert; Anhang 2 beschreibt den „Trichinentempel“/Langhans-Bau.

Gibt es eine Beziehung der Leopoldina zu diesem Haus? Antwort: Ja!

Christian Andreas von Cothenius (1708–1789, Abb. 3*A*), Leopoldina-Mitglied (seit 1743) und Leibarzt von Friedrich dem Grossen (1712–1786), hat in dessen Auftrag seine Vorstellungen über ein Studium der Tiermedizin in dieses Projekt einfließen lassen. Er war der Meinung, dass die Eleven aktiv in den Lehr- und Forschungsprozess einbezogen werden sollten, Präparierübungen ausführen dürfen und überhaupt günstige Bedingungen zum Studieren haben müssen.

Zum anderen wurde 1908 Emil Abderhalden (1877–1950; Abb. 3*B*), Schüler von Nobelpreisträger Emil Fischer, auf den Lehrstuhl für Physiologie der damaligen Königlichen Tierärztlichen Hochschule in Berlin berufen, der in diesem Langhansbau seinen Standort hatte. Abderhalden erhielt 1911 einen Ruf an die Universität Halle; er wirkte von 1932 bis 1950 als Leopoldina-Präsident. Bereits 1932, zu Beginn seiner Präsidentschaft wurde die Fachgruppe für Tierheilkunde (jetzt Sektion 22, Veterinärmedizin) in die Leopoldina und somit in das Akademieensemble der Naturwissenschaften und Medizin eingegliedert.

Abb. 1 Lageplan der Königlichen Thierarzneischule von 1790 (Ausschnitt) (Quelle: Humboldt-Universität-Archiv)

Abb. 2 (*A*) Dieser frühklassizistische Bau wurde 1790, anlässlich der Gründung der Königlichen-Tierarznei-Schule, *École Vétérinaire*, von (*B*) Carl Gotthard Langhans (1732–1808), zur gleichen Zeit mit dem Brandenburger Tor, errichtet (Quelle: www.bz-berlin.de).

Abb. 3 (*A*) Christian Andreas von Cothenius (1708–1789) [Quelle: de.wikipedia.org] und (*B*) Emil Abderhalden (1877–1950) [Quelle: Humboldt-Universität – Archiv]

Anhang 1

Kultur- und wissenschaftshistorischer Campus Nord (ehemalige Veterinärmedizinische Fakultät) der Humboldt-Universität

Im Mittelalter Feldmark des Dorfes Wedding; danach Eigentum der Stadt Berlin. Nach 1540 im Besitz des Oberhofpredigers Johannes Agricola (1494–1566; Schüler und Freund Martin Luthers [1483–1546]); dann mehrmaliger Besitzerwechsel. Seit 1655 im Besitz des preußischen Fürstenhofes – vorübergehend „kurfürstliches" Jagdrevier. Um 1750 Parzellierung, Verkauf und Anlegung von Gärten (Reußscher und Bertramscher Garten).

Von 1790 bis 1993 Standort der Veterinärmedizin als Bildungs- und Forschungsstätte

Bereits 1767 Auftrag des Preußenkönigs Friedrich II. an seinen Leibarzt Christian Andreas Cothenius eine für die Gesundheit und Heilung der Tiere geeignete Schule („École Veterinaire") zu projektieren. Von 1790 bis 1887 Königliche Thierarzneischule; danach Königlich-Tierärztliche Hochschule (1887–1934). In dieser Periode entstand durch enges Zusammenwirken mit der benachbarten Charité der Gründungsstandort „Vergleichende Medizin". 1934 Eingliederung in die Friedrich-Wilhelms-Universität (ab 1946 Humboldt-Universität zu Berlin), zunächst als Landwirtschaftlich-Tierärztliche Fakultät (1934–1937), anschließend als Veterinärmedizinische Fakultät (1937–1968) – 1951 und 1956 wechselt ein Großteil des Lehrkörpers und der Studenten aus politischen Gründen an die Freie Universität –, von 1968 bis 1990 als Sektion Tierproduktion und Veterinärmedizin – schließlich von 1990 bis 1993 erneut Veterinärmedizinische Fakultät der Humboldt-Universität.

Nach dem Mauerfall und der Wiedervereinigung Deutschlands erfolgte auf Empfehlung des Wissenschaftsrates 1992 die Fusion der beiden veterinärmedizinischen Bildungsstätten Berlins, zugeordnet der Humboldt-Universität. Das Abgeordnetenhaus beschloss jedoch später die Eingliederung in die Freie Universität mit dem Hauptstandort Düppel.

Auf diesem denkmalgeschützten Terrain hier in der Innenstadt, durchzogen von dem Flüsschen Panke, sind u. a. drei Baustile vertreten:

- Frühklassizismus: Tieranatomisches Theater, Architekt: C. G. Langhans (1790), Haus 3;
- Spätklassizismus: Hauptgebäude der Königlichen Tierarzneischule (1840), Architekt: Ludwig Ferdinand Hesse (1795–1876), Haus 1 (mit den 12 Aposteln der Veterinärmedizin);
- Moderne: Schmiede- und Apothekengebäude (1932), Architekt: Walter Wolff, Haus 10.

Seit 2014 wird das ehemalige veterinärmedizinische Gelände auf dem Campus Nord durch die Lebenswissenschaftliche Fakultät der Humboldt-Universität genutzt.

Anhang 2

Der Trichinentempel – ältestes akademisches Lehr- und Forschungsgebäude der Stadt Berlin – ein Symbol für Frühklassizismus[1]

Dieses frühklassizistische Haus, der „Trichinentempel", ist das älteste akademische Lehr- und Forschungsgebäude der Stadt Berlin; es wurde von Carl Gotthard Langhans (1732–1808) erbaut und anlässlich der Gründung der Königlichen Thierarzneischule (*École Veterinaire* Berlin) 1790 eingeweiht. Es trägt verschiedene Namen: Zootomie, *Theatrum anatomicum animale*, Tieranatomisches Theater, Langhansbau und (frühestens seit 1920) Trichinentempel.

Anlass zur Einrichtung einer tierärztlichen Bildungsstätte waren die seinerzeit verheerend verlaufenden Tierseuchen, die vor allem durch das Massensterben von Rindern infolge der sogenannten Rinderpest zu Hungersnot führten, auch die Errichtung von Tierarzneischulen in Lyon (1754), Alfort und Wien (1767) und kurz danach in Dresden (1780) sowie – preußenspezifisch – das Auftreten der Sarkoptesräude der Pferde und Reiter während des Siebenjährigen Krieges. Friedrich II., damaliger König, beauftragte deshalb seinen Leibarzt Christian Andreas Cothenius (1708–1789), ein Konzept für Struktur und Funktion dieser veterinärmedizinischen Lehr- und Forschungsanstalt vorzubereiten; dies erfolgte umgehend und tiefgründig. Als Standort wurde der über 9 ha große Reußsche Park, ein lieblich-kleinhügeliges Gelände gegenüber der Charité, vor den nordwestlichen Toren der Stadt Berlin gelegen, ausgewählt. Der Baubeginn konnte aber aus ökonomischen Gründen (Kostenvoranschlag = 12 844 Taler) zunächst nicht realisiert werden. Der auf einem „sanften Hügel" errichtete Bau erfolgte erst unter seinem Nachfolger Friedrich Wilhelm II. (1744–1797). Die Leitung der Tierarzneischule wurde Karl Heinrich August Graf von Lindenau (1755–1842) übertragen.

Um die architektonische Gestaltung dieses Baues zu verstehen, sei erwähnt, dass Langhans als einer der frühesten Vertreter des Klassizismus gilt. Er erfasst und überträgt die Antike nicht nur in der äußeren Form, sondern vor allem in ihrem Wesen. Johann Gottfried Schadow (1764–1850) sagt: „Langhans war daran gelegen, einen reineren Stil einzuführen."[2] Wieweit er auf Reisen nach Italien, England, Frankreich gewonnene Eindrücke mit eigenem Können glücklich vereinen kann, lässt sich am Bau der Zootomie besonders gut nachweisen. Schadow bemerkt hierzu mit leichter Ironie: „Auf seinen Reisen hatte er seine Mappen gefüllt und eine Wiederholung anerkannter Meisterwerke dünkte ihm sicherer als neue Originale von unsereinem."[3]

Dem *Theatrum anatomicum* legt Langhans die Form des griechischen Kreuzes zugrunde, das aus einem quadratischen Kern mit vier gleichen Vorsprüngen gebildet wird. Den Mittelbau überragt eine Flachkuppel, die in der damaligen Zeit auf Grund ihrer Konstruktion eine kleine Sensation darstellte. Sie ist als Bohlendach ausgeführt und, wie Friedrich Nicolai (1733–1811) in seiner *Reise durch Deutschland und die Schweiz* (1796) überrascht und bewundernd feststellte, „ein Kuppeldach ganz ohne Dachstuhl und überhaupt ohne Balken und Sparren".[4]

1 Kurzfassung eines Vortrages von Th. Hiepe.
2 Hoffmann und Farchhin 1965.
3 Ebenda.
4 Nicolai 1796.

Im Mittelpunkt der Beschreibung des Gebäudes, das parallel zum Brandenburger Tor innerhalb von zwei Jahren entstand, steht dessen architektonische und funktionelle Betrachtung. Sowohl Cothenius als auch Langhans waren der Grundauffassung, optimale Bedingungen für diese tiermedizinische Bildungsstätte anzustreben. Zweifellos stand der 140–150 Plätze umfassende Hör- und Demonstrationssaal (Abb. 4), der im Stile eines Amphitheaters gestaltet wurde, im Zentrum dieses zweigeschossigen Baus. Außer dem Hörsaal wurden Räume für wissenschaftliche Sammlungen, eine großangelegte Bibliothek sowie zur Lagerung und Obduktion von Tierleichen sowie Präparierstuben und eine Wohnung eingerichtet. Als technische Attraktion galt der sogenannte Hubtisch, auf welchen die Tierleichen bis zu Rinder- und Pferdegröße vom Untergeschoss in den Hör- und Demonstrationssaal transportiert werden konnten. Die im Winkel von 60° angelegten Sitzreihen ermöglichten außergewöhnliche Sicht aus der Nähe. Die architektonische Gestaltung erfolgte in Form eines freitragenden Kuppelbaus. Die malerische Ausgestaltung dieser Zootomie-Kuppel besorgte Bernhard Rode (1725–1797), der seit 1783 als Direktor der Akademie der Künste vorstand. Die Freskenmalerei erfolgte in der Grau-in-Grau-Maltechnik, welche in der Periode der wiedererwachenden Antike seinerzeit beliebt war.

Abb. 4 Hör- und Demonstrationssaal im *Theatrum anatomicum*. Die Freskenmalerei besorgte Christian Bernhard Rode (1725–1797), seinerzeit Direktor der einheimischen Akademie der Künste. (Aufnahme: Adam Al Halbouni)

Im Vortrag werden detailliert die acht Gruppendarstellungen und deren figürliche und architektonische Motive erläutert: eine Interpretation mit Zitatcharakter der tiefgründigen Beschreibung durch die Tierärztin und Feuilletonistin Dr. Renate Hoffmann. In jeder Gruppe stehen die Mensch-Tier (Ziege, Schaf, Schwein, Hund, Rind und Pferd) -Beziehungen im Mittelpunkt.

Die Motive sind aufgeteilt in figürliche und architektonische. Erstere liegen in acht Gruppendarstellungen zwischen den Bogenfenstern, und letztere füllen die Kuppel aus. Das Figurenmotiv ist jeweils aus Menschen und Tieren zusammengestellt, wobei die Mitte von einer Tiergruppe eingenommen wird und die Flächen nach den Fenstern zu durch Männergestalten abgeschlossen sind. Eine Figur ist regelmäßig dem Beschauer zugewandt, während die zweite in abgewandter Stellung erscheint. Dies bringt in die ohnehin lebendigen Bilder starke Bewegung. Bemerkenswert sind die fast jeder Gruppe beigefügten Attribute, die meist auf die Nutzung der Tiere hindeuten.

- Da ist die ruhende Ziegenherde, deren rechter Hirte auf dem Arm ein Zicklein trägt, nach dem das Muttertier besorgt aufschaut. Auf dem Boden liegen Hirtenstab und Panflöte.
- Die nächste Szene wird von der Schafherde bestimmt. In der Hand der rechten Figur erkennt man eine Schere zur Schafschur.
- Daneben werden die Schweine dargestellt. Die rechte Gestalt ist in sitzender, entspannter Haltung entworfen und stützt den Kopf ruhend in die Hand.
- Dann folgt das neckisch-heitere Spiel mit den Hunden. Diesmal ist es die linke Figur, die mehr in Aktion tritt; ihr leckt ein Hund zutraulich übers Gesicht, während sie mit der linken Hand versucht, ein zweites freundlich-zudringliches Tier zurückzuhalten. Es ist sicherlich die Jagdmeute, Jagdspieß und Jagdhorn am Boden verraten es, außerdem trägt die zweite Figur einen Köcher.
- Zwei Motive mit Rindern erscheinen ruhig und ausgewogen. Auf dem einen Bild ist ein Pflug dargestellt.
- Außerordentlich bewegt hingegen sind die Rossebändiger gestaltet. Auf beiden Abbildungen scheinen Tiere und Menschen über den Rahmen hinauszudrängen, eine Wirkung, die Rode durch leichtes Hineinmalen in die korrekte Fensterumrandung noch unterstützt. Die rechte Gestalt hält die Lanze, und zu Füßen liegt ihr ein Helm. In der Gesamtheit und Wucht der Ausführung erinnern gerade diese beiden Motive an antike Vorbilder.

Um die einzelnen Szenen miteinander zu verbinden, lässt Rode oberhalb im vollen Rund eine Girlande verlaufen, die von den Hirten mit einer Hand getragen wird und sich über den Fensterbögen zu einem Tierkopf aufschwingt. Aus diesen acht Gruppen erwächst das architektonische Deckenfresko. Die stützenden Pfeiler dieses gemalten Linienspiels erheben sich fast unmerklich hinter den Figuren, so dass es frei zu schweben scheint. Es ist aus Gurten im Rhombenmuster zusammengesetzt, die sich verjüngend bis zum Oberlicht spannen.

Diese künstlerische Szenerie ist nach Schadow als ein außergewöhnliches meisterhaftes Kunstwerk einzuordnen.

Nebenbei bemerkt: Die Rodeschen Fresken weisen einen anatomischen Fehler auf: Ein „Rind mit Schneidezähnen im Oberkiefer“ gibt es gar nicht (dafür existiert dort eine derbe Hornplatte, die sogenannte „Gaumenplatte“).

Im Vortrag wird auf die 200-jährige Nutzung des Gebäudes durch die Veterinärmedizin detailliert eingegangen, und die hervorragenden Gelehrten, die in diesem Haus wirkten, werden vorgestellt sowie die traditionell engen Beziehungen der Veterinärmedizin zur benachbarten Charité betont. Das Gebäude diente nicht nur der Anatomie, sondern auch der Pathologie, der Physiologie und schließlich seit 1920 der Nahrungsmittelkunde/Lebensmittelhygiene der veterinärmedizinischen Bildungsstätte. Dieser Standort darf als weltweiter Gründungsplatz der Vergleichenden Medizin (*Comparative Medicine*) bezeichnet werden.

Die Grundsanierung des Trichinentempels erfolgte von 2005 bis 2012; mit einem Kostenaufwand von 6,87 Mio. Euro. In einer eindrucksvollen Feier am 12. Oktober 2012 wurde

er der Helmholtz-Stiftung der Humboldt-Universität zu Berlin als Repräsentationsgebäude übergeben. – Die beiden Anbauten (Gerlachbau von 1873 und Schlachthalle von 1934) bedürfen noch der Sanierung.

Neben diesem frühklassizistischen Kleinod wird auf zwei weitere Gebäude (der von Schinkel vorprojektierte spätklassizistische Bau, das sogenannte Hauptgebäude, von 1840 mit den 12 Aposteln der Veterinärmedizin und die von der Gropius-Schule im Bauhausstil in den 1920er Jahren gestaltete Apotheke und Lehrschmiede der vormaligen Veterinärmedizinischen Fakultät der Humboldt-Universität) aufmerksam gemacht.

Unter dem Aspekt „Wir, die Gegenwart, sind das Bindeglied von Vergangenheit und Zukunft" wird abschließend auf die Wechselbeziehung von Kunst und Wissenschaft (beide tragen fundamental – globalen Charakter) hingewiesen.

Nachbetrachtung

Dieser Reusssche Park mit seinen historischen Gebäuden kann als eine Oase in Berlins Stadtmitte, in unmittelbarer Nachbarschaft von Natur-, Bio-, Agrarwissenschaften und Charité gelegen, zum Studieren, Lehren und Forschen eingeordnet werden. Der frühklassizistische Trichinentempel, das spätklassizistische Hauptgebäude, die im Bauhausstil gestaltete Schmiede und Apotheke sowie die Denkmale von Christian Gerlach, Wilhelm Schütz und Wilhelm Dieckerhoff sind Zeugen der Vergangenheit des von der Veterinärmedizin aufgegebenen Standortes, welcher der Universität erhalten werden konnte und Spekulanten der Wendeperiode 1989/90 verwehrt worden ist.

Für den nun folgenden Plenarvortrag „Vielfalt der Arthropoden – eine molekularbiologische Sicht" wurde Herr Prof. Bernhard Misof (Bonn) ausgewählt. Nach einer kurzen Vorstellung und Würdigung seiner wissenschaftlichen Leistungen ergriff dieser das Wort.

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Die Vielfalt der Arthropoden – Eine molekularbiologische Sicht

Bernhard Misof (Bonn)

Arthropoden stellen, gegeben ihr geologisches Alter, die bei weitem arten- und formenreichste Gruppe vielzelliger Organismen dar. Sehr wahrscheinlich eroberten Arthropoden vielfach terrestrische Lebensräume und sind für die Entwicklung terrestrischer Ökosysteme im Wechselspiel mit Pflanzen bis heute verantwortlich. Innerhalb der Arthropoden kommt den Insekten, als einer ursprünglich ausschließlich terrestrischen Gruppe, eine besondere Rolle zu. Ihr Artenreichtum, ihre ökologische Vielfalt sucht seinesgleichen. Bis heute haben wir allerdings keine fundierte Hypothese zur Evolutionsgeschichte der Insekten. In meinem Vortrag zeigte ich, in welcher Form umfangreiche genomische und transkriptomische Daten uns helfen werden, etwas zur Evolutionsgeschichte der Insekten abzuleiten. Zusätzlich konnte ich an Hand von Beispielen zeigen, dass mit diesen umfangreichen molekularen Daten wesentlich präzisere Aussagen zur Evolution einzelner Gene, Merkmalskomplexe, bzw. Eigenschaften der Tiere getroffen werden können. Abschließend erörterte ich kurz das Potenzial der Genom-/Transkriptomsequenzierung bezüglich der Identifikation von bakteriellen bzw. viralen Parasiten der Insekten. Zusammenfassend kann man sagen, dass die neuen Techniken der Genomsequenzierung völlig neue spektakuläre Einsichten zur Evolutionsgeschichte ermöglichen werden und uns einem evolutionsbiologischen Verständnis dieser Gruppe näherbringen.

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Summary

1. Politics and Society

The appearance of epidemic Bluetongue and Schmallenberg virus infections in Central Europe in the last decade increased the awareness of arthropod-transmitted diseases in temperate climatic zones in Europe. Arthropod-borne infectious agents are a threat to human and animal health worldwide, but have mostly been given inadequate attention with the exception of malaria, one of the three major 'killer' infections in humans. With their focus on neglected tropical diseases, the G7 recognized that arthropod-transmitted infections warrant increased attention realizing that they no longer occur in far-away tropical developing countries only. Growing globalization of trade, expanding worldwide travel activity and long term alterations in climate increase the risk to import previously exotic arthropods to Europe and to introduce novel arthropod-transmitted pathogens. Thus, in recent years various invasive mosquito species have established populations in Europe, with a high vector competence for a wide range of pathogens. The demonstration of several new arthropod-borne viruses and exotic nematodes in Central Europe document an increasing entry of these pathogens.

To maintain a high level of awareness even over longer periods it is necessary to explain the difference between the presence of a potential vector and the actual occurrence of a vector-borne disease to policy-makers and the general public. Furthermore, it is imperative to raise the awareness in the medical community of new pathogens transmitted by vectors. This could include a request to submit specimens, to check for vector-borne diseases or to consult experts in unclear medical cases. The creation of a network of specialists focusing on vector-borne infections has been suggested and supported by the attendees of this symposium.

2. Translation of Surveillance

Currently, significant financial resources are invested in expensive surveillance projects. However, it often remains unclear how a maximum benefit can be drawn from these data. It is essential to support the harmonization and practical application of surveillance data by specific programmes aimed at translating research data into the control of vector populations and vector-borne infections. Thus, it is indicated to finance programmes for combating neglected endemic diseases in the countries of origin. This should be done by a combination of treatment of the hosts, control of vector density and clear establishment of clean regulations and responsibilities.

3. Standardization and Quality Assurance

To ensure comparability of results and confidence in dealing with sample material, detailed procedures for the different steps are required. This includes clear guidelines for taxonomical assessment, maintenance and handling of specimens, and management of results. Standard operating procedures, validation of methods, and quality assurance measures including ring trials are fundamental components of such a policy. Taxonomical classification and differentiation should include new OMICS technologies to support and complement morphology-based classical approaches.

4. Clustering of Expertise and Definition of Responsibilities at the International Level

There exists a variety of experts with in-depth knowledge of their specific areas, but this multitude of expertise must be brought together. This symposium offered a first platform. Furthermore, it is necessary to define the responsibilities on federal and EU level, and to identify appropriate competent authorities and institutions. A harmonization of sampling, surveillance and classification procedures at EU level is needed. This should also include the establishment of an EU reference laboratory for mosquito surveillance. Likewise, existing surveillance networks for vectors and pathogens must be developed further and expanded at least EU-wide. This includes the implementation and expansion of citizen science databases.

If necessary, new expert commissions need to be formed – preferably at EU level. The responsibilities of these committees should include the identification of the current funding requirements and giving advice to the appropriate agencies.

For that purpose the German National Academy of Sciences Leopoldina is asked to establish a working group in which national experts in close collaboration with international colleagues will deal with current topics of arthropod-borne infectious diseases and arthropods as pests.

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