# Susceptibility of *Culex* species native to Germany for West Nile virus and the role of *Wolbachia* in virus-vector interaction

**Dissertation**

with the aim of achieving the doctoral degree
doctor rerum naturalium
(Dr. rer. nat.)

at the Faculty of Mathematics, Informatics
and Natural Sciences

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Hamburg, 2016

Day of submission: 21.06.2016

Day of oral defense: 12.09.2016

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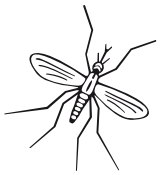

**’’Nothing in life is to be feared.**

**It is only to be understood.**

**Now is the time to understand more,**

**so that we may fear less.’’**

Marie Sklodowska Curie

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# Abbreviations

| | |
|---|---|
| # | quantity |
| % | percent |
| °C | degree Celsius |
| µg | microgram |
| µl | microliter |
| µm | micrometre |
| A | absorbance |
| *Ae.* | *Aedes* |
| ANK | ankyrin repeats |
| approx. | approximately |
| Arbovirus | arthropod-borne virus |
| as | antisense |
| BNITM | Bernhard Nocht Institute for Tropical Medicine |
| bp | basepair |
| C | capsid protein |
| CDC | Centers for Disease Control and Prevention |
| CI | cytoplasmic incompatibility |
| $cm^2$ | square centimetre |
| CPE | cytopathic effect |
| CT | cycle threshold |
| *Cx.* | *Culex* |
| *Cx. mol* | *Culex pipiens* biotype *molestus* |
| *Cx. pip* | *Culex pipiens* biotype *pipiens* |
| *Cx. qui* | *Culex quinquefasciatus* |
| *Cx. tor* | *Culex torrentium* |
| *D.* | *Drosophila* |
| DAPI | 4',6-diamidino-2-phenylindole |
| dd | double-distilled |
| DENV | Dengue virus |
| DIC | differential interference contrast |

| | |
|---|---|
| DMEM | Dulbecco's Modified Eagle Medium |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| dpi | days post infection |
| E | East |
| E | envelope protein |
| EDTA | ethylendiamin tetraacetic acid |
| EIP | extrinsic incubation temperature |
| ER | endoplasmatic reticulum |
| F | filial generation |
| FBS/FCS | fetal bovine serum/fetal calf serum |
| Fig. | figure |
| FITC | fluorescein isothiocyanate |
| GFP | green fluorescent protein |
| Gp | gene product |
| h | hour |
| ID | identification |
| kb | kilobase |
| LL | Langenlehsten |
| min | minute |
| ml | millilitre |
| MLST | multilocus sequence typing loci |
| mm | millimetre |
| MOI | multiplicity of infection |
| N | North |
| n | number |
| n/a | not applicable |
| NCBI | National Center for Biotechnology Information |
| NEAA | non-essential amino acids |
| NEG | negative |
| ng | nanogram |
| nm | nanometre |

| | |
|---|---|
| ns | non-significant |
| NS | non-structural |
| Ø | diameter |
| ORF | open reading frame |
| *p* | *p*-value |
| PBS | phosphate buffered saline |
| PBT | phosphate buffered saline with Triton-X100 |
| PCR | polymerase chain reaction |
| PFU | plaque forming units |
| pH | potential of hydrogen |
| pmol | picomol |
| preM | premembrane protein |
| qRT-PCR /qPCR | quantitative real-time polymerase chain reaction |
| $R^2$ | coefficient of determination |
| RNA | ribonucleic acid |
| rpm | rounds per minute |
| rRNA | ribosomal ribonucleic acid |
| s | second or sense |
| S | South |
| ssRNA | single-stranded ribonucleic acid |
| TAE | TRIS-acetate-EDTA buffer |
| $TCID_{50}$ | 50 % tissue culture infective dose |
| $T_m$ | melting temperature |
| TRIS | tris(hydroxymethyl)aminomethane |
| U | unit |
| UK | United Kingdom |
| USA | United States of America |
| UTR | untranslated region |
| UV | ultraviolet |
| V | volt |
| VC | vectorial capacity |
| W | Wendland |

| | |
|---|---|
| WHO | World Health Organisation |
| WNV | West Nile Virus |
| wsp | *Wolbachia* surface protein |

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# 1 Introduction

In December 2015, Angola's capital Luanda witnessed the start of the worst Yellow fever outbreak of the country in 30 years. Up to now (15.05.2016), 736 confirmed cases were reported and 93 have succumbed to the disease. Around the same time, on the 11th November 2015, Brazil declared a national public health emergency in response to a Zika virus outbreak suspected to be the cause of microcephaly in new-born children (WHO Incident management team Angola 2016; World Health Organisation 2016b).

Within the 2015 transmission season, 301 cases of West Nile virus (WNV) infection were diagnosed in patients from European nations. Simultaneously, worldwide, 2.4 million cases of Dengue virus (DENV) infections are reported to the WHO, although the disease burden is estimated to be much higher (European Centre for Disease Prevention and Control 2015; World Health Organisation 2016a).

These disease outbreaks caused by infections with arboviruses are an ongoing public health concern worldwide. Current severe outbreaks underline the need for continuous surveillance and research targeting the biology of this particular group of viruses and the corresponding transmitting vectors.

## 1.1 Arboviral Infection

An arbovirus (arthropod-borne virus) is defined as a virus that is transmitted from an arthropod to a vertebrate host. In general, the infected vertebrate acts as an amplifying host, reaching sufficiently high viremia so that a hematophagous arthropod can become infected upon a blood meal. After undergoing further replication in the arthropod, the virus has to reach the salivary glands to be transmitted to another vertebrate upon a subsequent blood meal (Calisher 1994). To maintain itself in the arthropod population, some viruses are also able to be transmitted transstadially or transovarially to other life stages and progeny (Calisher 1994). In hosts, which develop high viremia, some viruses could alternatively be transmitted from host to host for example via fecal-oral transmission (van der Meulen et al. 2005).

Hematophagous arthropods transmitting viruses from a vertebrate host via a blood meal are termed vectors. There are several vectors characterised that are also relevant for human public health including mosquitoes, ticks and sandflies among many others (Liang et al. 2015). Depending on the vector, many arboviruses are also zoonotic viruses, which can be transmitted from animal hosts to humans. In these cases, humans are mostly dead-end hosts, which are not the main target for viral amplification. However, some arboviruses, such as the DENV, have evolved further and adapted to humans as amplification hosts increasing the disease burden in urban settings (Weaver 2005).

The continuously growing Centers for Disease Control and Prevention's international catalogue of arthropod-borne viruses currently encompasses 537 arbovirus species (Karabatsos 1978). Out of these, more than 100 species have been identified to cause human, animal or zoonotic disease (Liang et al. 2015). The majority of these viruses

cluster in the virus families *Togaviridae*, *Flaviviridae*, *Bunyaviridae* and *Reoviridae* and include species such as DENV, Chikungunya virus, Rift Valley virus and WNV (Liang et al. 2015).

Arboviruses are a cause of economical loss and a severe public health burden. Extreme examples are the livestock losses sustained during a Rift Valley fever outbreak (Rich & Wanyoike 2010) as well as the disease complications and continuous spread of flaviviruses such as WNV (Lindsey et al. 2010).

This work is focused on WNV and its interactions with putative vector populations in Germany.

## 1.2 WNV: virology and pathology

### 1.2.1 General virology

WNV was first isolated in the West Nile district of Uganda in 1937 from a 37 year old female patient originally suspected to be suffering from sleeping sickness (Smithburn et al. 1940). Since then, the virus has been taxonomically integrated into the virus family *Flaviviridae* and the genus *Flavivirus* (Karabatsos 1978). In addition, it is a member of the Japanese encephalitis serocomplex, including also other viruses of medical importance such as the Japanese encephalitis virus and the St. Louis encephalitis virus (May et al. 2011).

The mature virions of WNV are approximately 50 nm in diameter, spherical and are surrounded by an envelope (Brinton 2014). Their genome consists of a single-stranded, positive-sense RNA ((+) ssRNA) molecule (~ 11kb), encoding for a single open reading frame flanked by untranslated regions (UTR) at both 5' and 3' and a type 1 cap structure at the 5' end (Rossi et al. 2010; Brinton 2014). The RNA molecule is directly translated into a polyprotein (~ 3000 amino acids), which is further processed by cellular and viral proteases and cleaved into ten viral proteins (Nowak et al. 1989). The capsid protein (C), premembrane protein (preM) and envelope protein (E) are termed structural proteins and are required for virion formation and assembly into viral particles. The remaining seven proteins are non-structural (NS) proteins, necessary for genome replication and immune evasion (Fig. 1.1) (Rossi et al. 2010; Brinton 2014).

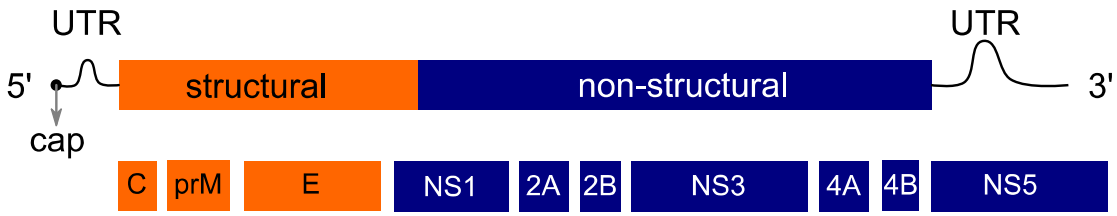



**Fig. 1.1: West Nile virus genome structure and viral protein products.**
Adapted from Rossi, Ross and Evans 2010 and Brinton 2014.

As is the case with all viruses, they require a cellular host and its transcription and translation machinery in order to propagate and get transmitted.

### 1.2.2 Cellular life cycle

WNV entry into the target cell is initiated via the interaction of the viral envelope protein with a cellular receptor. The cellular receptor(s) engaged in WNV entry have not yet been clearly identified. However, some candidate molecules have already been investigated, including various glycosamindoglycans, cellular $\alpha_v\beta_3$ integrins and lamin-binding proteins as well as the G-coupled receptor kinase (GRK) protein family (reviewed in Brinton 2014 and Brinton 2002).

Upon receptor binding, the virus enters the cell via endocytosis into clathrin-coated pits. This is followed by low pH-triggered fusion of the endosomal membrane with the viral envelope, resulting in the subsequent release of the viral nucleocapsid into the cytoplasm. Upon uncoating, the (+) ssRNA is directly translated into a polyprotein at the rough endoplasmic reticulum (ER). The polyprotein is then cleaved into the 10 structural and non-structural viral proteins using viral and cellular proteases. Non-structural proteins, including the viral RNA-dependent RNA polymerase (NS5), synthesise full-length (-) ssRNA intermediates, which serve as templates for the production of full-length (+) ssRNA. Assembly of the viral capsid and encapsidation of (+) ssRNA transcripts occurs at the rough ER membrane and is followed by the transport through the host's secretory pathway. Here, the viral envelope protein is glycosylated and the premembrane protein cleaved into membrane protein to generate mature virus particles, which are released from the cell via exocytosis (Fig. 1.2) (reviewed by Brinton 2002).

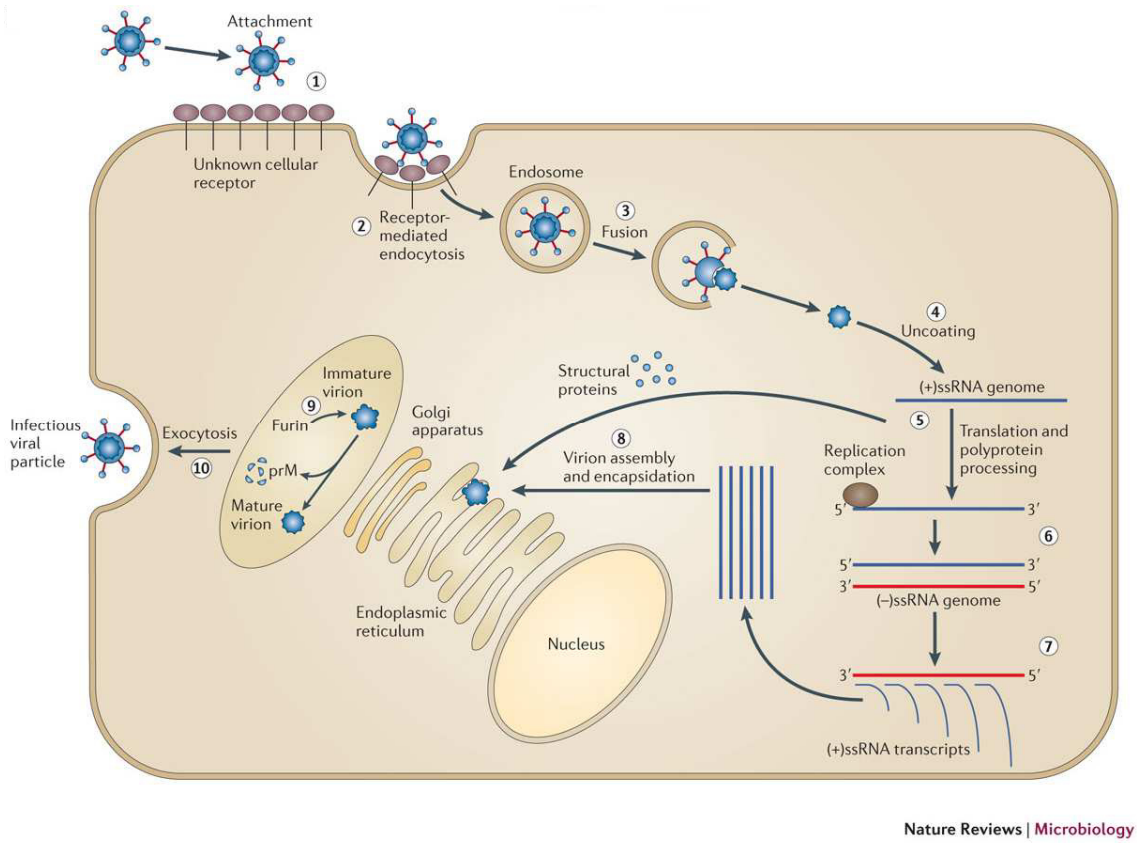



**Fig. 1.2: Schematic representation of the cellular life cycle of WNV.**
Image by Suthar, Diamond, and Gale 2013
1 = attachment of virion to unknown cellular receptor; 2 = endocytosis into clathrin-coated pits; 3 = low pH-induced fusion of viral envelope and endosomal membrane; 4 = uncoating of the capsid and release of viral genome; 5 = direct translation and polyprotein processing; 6 and 7 = synthesis of negative-sense ssRNA and subsequent production of positive-sense ssRNA transcripts; 8 = assembly of viral capsid proteins and encapsidation of viral genome transcripts; 9 = glycosylation of viral envelope proteins and cleavage of preM into M (membrane) proteins to form mature virus particles; 10 = exocytosis of mature virus particles

It is possible to perform WNV *in vitro* culture with primary and immortalised cell lines originating from a variety of different organisms, including avian, mammalian, amphibian and insect species (Brinton 2014). *In vivo*, WNV tropism is limited to keratinocytes, Langerhans cells, dendritic cells, macrophages, neutrophils, endothelial cells as well as neurons (reviewed in Petersen et al. 2013). Especially the infection of neurons can be detrimental for the progression of WNV disease in humans.

### 1.2.3 West Nile disease

WNV disease, which includes West Nile fever and West Nile neuroinvasive disease, is the clinical phenotype caused by infections with WNV. Clinical human cases are reported from all continents, except Antarctica (Chancey et al. 2015). The global magnitude of the disease is not known as case numbers are not reported by the World Health Organisation (WHO). However, data published by the Centers of Disease Control and Prevention (CDC) describe the situation seen for endemic WNV occurrence in the United States of

America (USA). In 2014, a total of 2205 human disease cases and 97 deaths were reported to the CDC (Centers for Disease Control and Prevention 2015b). WNV does not only pose a significant public health burden, but also induces economic loss for affected regions. For instance, the economic impact of a WNV disease outbreak in Sacramento County (California, USA) in 2005 with only 163 reported human cases was calculated at $2.98 million (Barber LM, Schleier JJ III 2005).

Notably, the majority of human infections (~ 80 %) remain subclinical. Clinical cases usually develop after an incubation period of 2-14 days. The majority present as flu-like and are self-limiting (Petersen et al. 2013). The symptoms of West Nile fever may include headache, malaise, fever, myalgia, chills, vomiting, rash, fatigue and eye pain (Zou et al. 2010).

In about 1 out of 150 symptomatic patients, the virus is able to cross the blood-brain barrier and cause West Nile neuroinvasive disease (Suthar et al. 2013). The neurological symptoms of West Nile neuroinvasive disease can range from a mild, self-limiting confusional state to severe encephalopathy, coma and death (Petersen et al. 2013). The illness can prolong for several weeks or months and may be associated with long-term functional and cognitive loss (Klee et al. 2004).

The current treatment against WNV disease remains supportive and, presently, there is no registered, approved vaccine available for human use. However, several licensed equine vaccines have been developed. In addition, studies from pre-clinical and clinical trials of human vaccine candidates show promising results (Petersen et al. 2013; Amanna & Slifka 2014).

Thus, so long as an effective vaccine is not available, the main method for prevention of WNV disease outbreaks remains strict vector control.

## 1.3 WNV: transmission and expansion

### 1.3.1 Transmission and vector competence

In humans, WNV can utilise alternative transmission routes, which include blood transfusion, organ transplantation and infection of infants via transplacental transmission and possibly also breast milk (Hinckley et al. 2007; Iwamoto et al. 2003; Pealer et al. 2003; Centers for Disease Control and Prevention 2002b; Centers for Disease Control and Prevention 2002a). In addition, a current case study has reported a possible sexual transmission of WNV (Kelley Md et al. 2016). The main route for natural transmission, however, is via blood meals of mosquito vectors.

A range of mosquito species are susceptible to WNV infection, with 65 species from 10 genera tested positive between 1999 and 2012 in the USA (Centers for Disease Control and Prevention 2015a). Mosquitoes maintain the primary enzootic transmission cycle, which evolves around birds as the main reservoir host (especially birds that are members

of the genus *Passeriformes*) (May et al. 2011). For some mosquito vector species, vertical transmission from WNV-infected female to offspring has been shown in nature and laboratory settings, indicating a virus reservoir function of mosquitoes as well (Baqar et al. 1993; Nelms et al. 2013).

Notably, it is necessary to investigate the feeding pattern of a potential mosquito vector to determine its relevance for human infection risk (Turell et al. 2005). In general, one can differentiate between ornithophilic and anthropophilic/mammophilic feeding behaviour.

Ornithophilic mosquitoes feed mainly on birds and thus play a role in maintaining the primary enzootic transmission cycle, which evolves around birds as the reservoir host (Fig. 1.3). But these are unlikely to transmit the virus to dead-end hosts such as humans or horses. Similarly, anthropophilic/mammophilic mosquito species feeding mainly on humans/mammals are not likely to play an important role in a WNV disease outbreak, since incidental dead-end host such as humans do not develop sufficient viremia to infect blood-feeding mosquitoes (reviewed in Chancey et al. (2015); van der Meulen et al. (2005)).

Mosquito species with a mixed ornithophilic and anthropophilic/mammophilic feeding pattern are termed bridge vectors, as they are able to cause spillover events from the infected reservoir (birds) to mammalian hosts (Kilpatrick et al. 2005). Such a spillover can initiate a West Nile disease epidemic and/or epizootic (Fig. 1.3).

Notably, previous observations and recent molecular data characterising the blood meals of mosquito species suggest that this differentiation into feeding preferences is not as strict as suggested, increasing the number of potential bridge vectors significantly (Kilpatrick et al. 2005; Börstler et al. 2016).

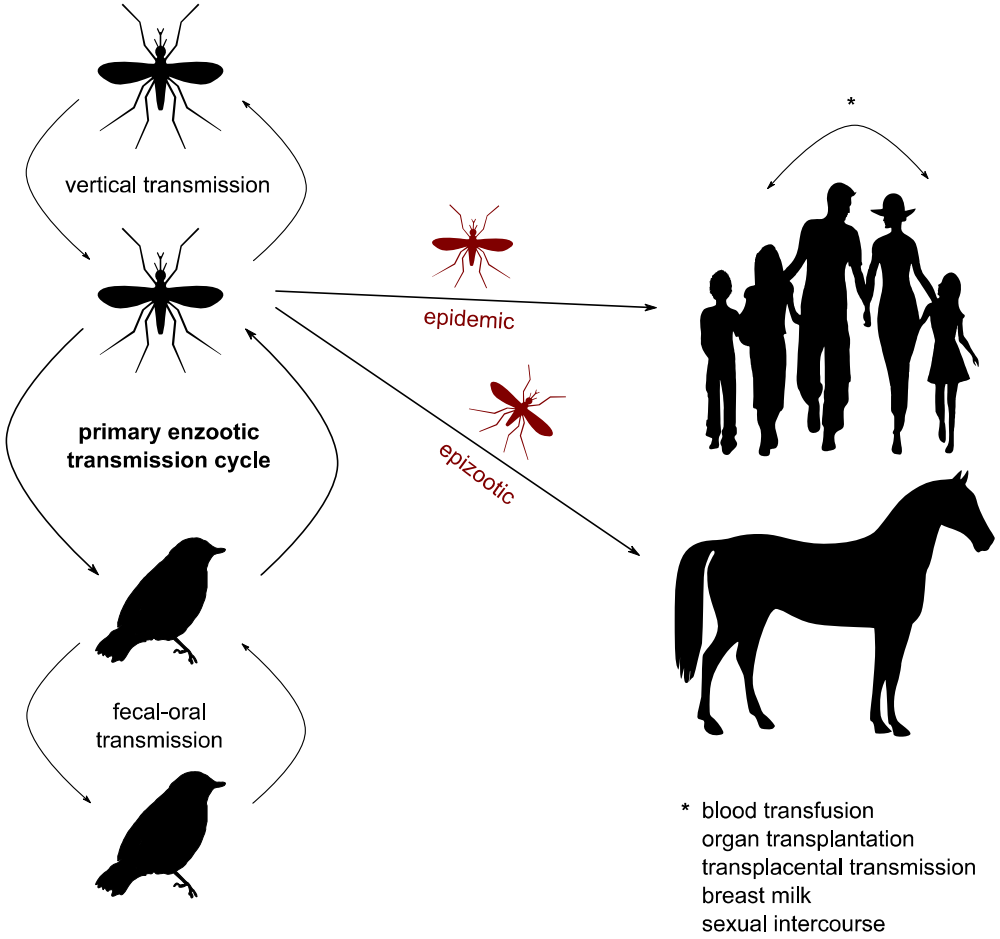



**Fig. 1.3: WNV transmission cycles**
The primary enzootic transmission cycle evolves around mosquito vectors and birds as reservoir hosts. This primary transmission cycle is supplemented by vertical transmission within the mosquito population and fecal-oral transmission from bird to bird. Transmission into the human population via a bridge vector (red) can be the cause of WNV disease epidemics. The virus can further circulate from human to human via blood transfusion, organ transplantation, and transplacental transmission or possibly also via breast milk or sexual intercourse. Transmission of the virus to non-human mammals (such as horses) can initiate epizootic outbreaks.

Important vectors for WNV include members of the *Culex pipiens* complex, such as *Culex tarsalis* and *Culex quinquefasciatus*, as well as mosquitoes from the genus *Aedes* (Chancey et al. 2015).

Feeding patterns alone, are not sufficient to characterise a WNV vector. In addition, the mosquito needs to be classified as a ''competent vector'' for the virus.

A competent vector must be able to become infected with the virus, maintain the infection and allow for systemic dissemination and finally sustain long enough for the colonisation of the salivary glands and transmission to the next host (Hardy et al. 1983; Franz et al. 2015). To fulfil these criteria, the virus is required to overcome four tissue barriers (Fig. 1.4):

Upon uptake via a blood meal, the virus particles enter the gut. Subsequently, they have to cross the first tissue barrier, the midgut infection barrier, establish an infection in the

midgut epithelial cells and replicate and spread within the epithelium. The second tissue barrier is the midgut escape barrier, after which the virus disseminates from the midgut via the haemolymph to other tissues and establishes a systemic infection. To ensure transmission to the next host, the virus then needs to overcome both the salivary gland infection and escape barriers in order to enter the salivary glands and exit the mosquito with the saliva excretions (Hardy et al. 1983; Franz et al. 2015).

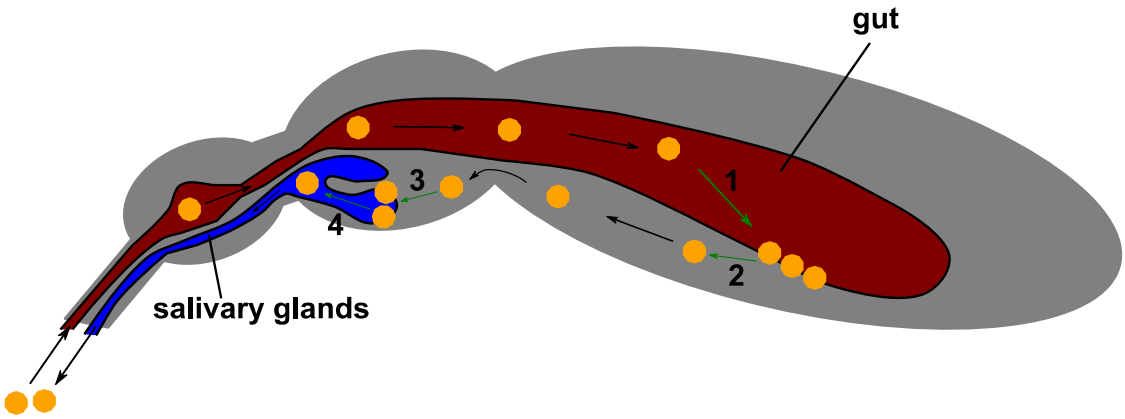



**Fig. 1.4: Schematic illustration of arbovirus tropism in a mosquito vector**
Adapted from Franz et al. 2015
The viral particles (yellow) are taken up via a blood meal on an infected host and travel into the gut area (red). Here, the virus needs to infect the gut epithelial cells, overcoming the midgut infection barrier (1), and replicate. The virus then escapes the gut epithelial cells, crossing the midgut escape barrier (2), and moves into the haemocoel, where transport via the haemolymph mediates a disseminating systemic infection. Once reaching the salivary glands (blue), the virus needs to overcome the salivary gland infection barrier (3) as well as the salivary gland escape barrier (4) to manifest itself in the salivary glands. Here, the virus replicates further and is transmitted via the saliva during the next blood meal.

WNV and corresponding competent vectors have spread to almost every continent, making WNV the most important causative agent of viral encephalitis in the world (Chancey et al. 2015).

### 1.3.2 WNV phylogeny and geographic distribution

Phylogenetic analyses of published isolates have shown that WNV is not only a geographically but also genetically diverse virus. Five distinct genetic lineages have been identified so far, which show genetic variation of 20-25 % among each other and correlate with their geographical origin (Chancey et al. 2015). Only lineage 1, 2 and 5 have so far been associated with West Nile disease outbreaks in humans (Chancey et al. 2015).

Lineage 1 is the most widely distributed lineage and is further subdivided into subclades 1a and 1b (Petersen & Roehrig 2001). It is believed that the origin of subclade 1a has been sub-Saharan Africa (Zehender et al. 2011). From these regions, the virus spread via migratory bird routes to Asia, Europe, and the Middle east and, later, also the USA. Lineage 1b is also known as the Australian Kunjin virus (Chancey et al. 2015).

Lineage 2 remains endemic in sub-Saharan Africa and Madagascar and was associated with zoonotic outbreaks in South Africa and Southern and Eastern Europe (Burt et al. 2002; Erdélyi et al. 2007).

Lineage 3 originated from mosquito isolates collected in 1997 and 1999. This WNV lineage from the Czech Republic border region has been shown to infect mosquitoes and mosquito cell lines only, excluding itself from the pool of potential harmful WNV variants (Bakonyi et al. 2005; Aliota et al. 2012). Similarly, WNV lineage 4 isolates, circulating in Russia, have so far not been found to be associated with any WNV disease outbreak (Lvov et al. 2004; Chancey et al. 2015).

WNV lineage 5, one of the 3 lineages associated with WNV disease outbreaks, originated from and circulates in India. This lineage was formerly considered to be a third subclade of WNV lineage 1 (lineage 1c), but has since been regrouped into its own lineage following extensive phylogenetic review (Bondre et al. 2007).

The presence of WNV lineage 1 and 2 in Europe might indicate that WNV may become a public health threat for Germany.

### 1.3.3 Public health relevance in Europe

The speed by which WNV could spread across a country as has been imposingly demonstrated by WNV outbreaks in the USA. WNV was first introduced to the USA (New York Metropolitan Area) in 1999 via infected migratory birds (Nash et al. 2001; Debiasi & Tyler 2006; Ciota & Kramer 2013). This was followed by an epidemic, which resulted in 59 human cases of WNV disease and 7 subsequent deaths due to encephalitis (Nash et al. 2001). In the following years, it spread rapidly reaching the West coast in 2002 (Fig. 1.5). Today, WNV is considered to be the most common cause of epidemic encephalitis in the USA (Debiasi & Tyler 2006).

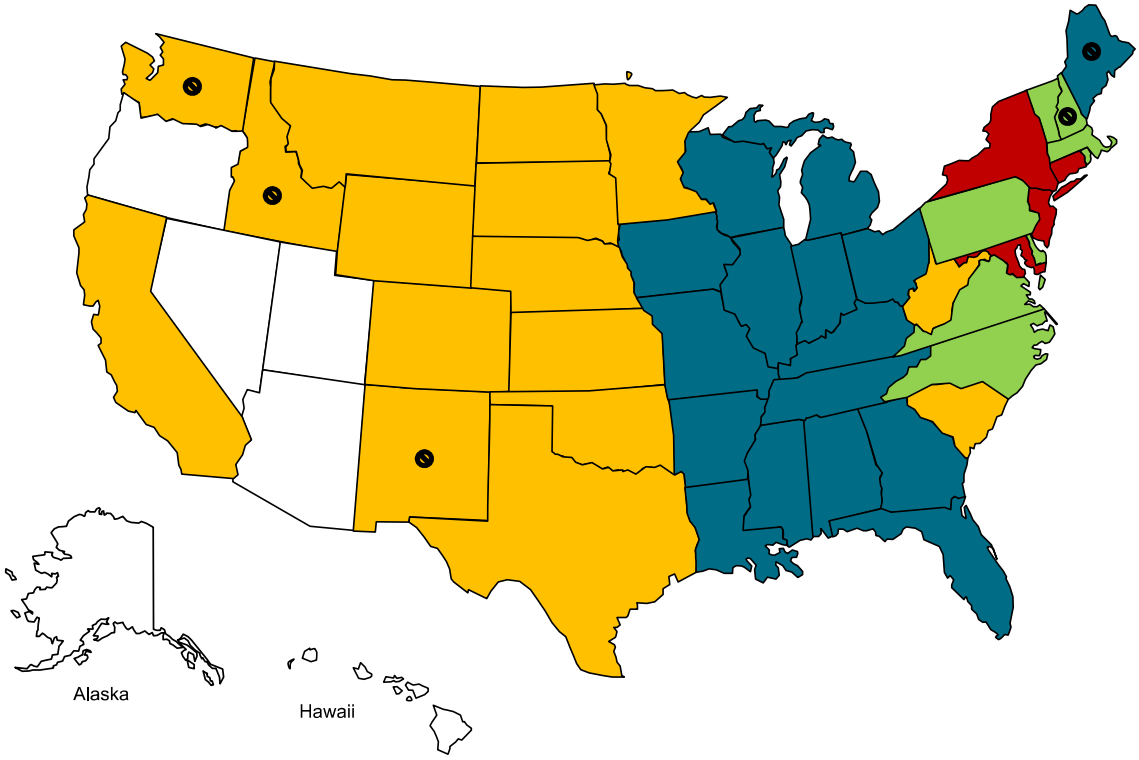



**Fig. 1.5: Geographical distribution of WNV in the USA (1999-2002)**
Adapted from Debiasi & Tyler 2006
Map template produced by www.presentationmagazine.com (last accessed: 25.03.2016)
WNV spread from the east to the west coast of the USA within only four years (1999 (red), 2000 (green), 2001 (blue), 2002 (yellow)). States marked with ⊘ have not reported human cases between 1999 and 2002. White areas have not reported WNV between 1999 and 2002.

In 1958, WNV neutralising antibodies were detected in human sera in Albania, giving the first indication of the presence of WNV in Europe (Bardos et al. 1959; Hubálek & Halouzka 1999). The first documented WNV disease followed in 1962-1963 (France) involving a number of human and equine cases (Murgue et al. 2006; Chancey et al. 2015).

After a long period without reported clinical WNV cases in Europe, human WNV disease started to re-emerge in western Ukraine (1985), Romania (1996) and Russia (1999) (Platonov et al. 2001; Tsai et al. 1998; Zeller & Schuffenecker 2004). The outbreaks in Romania and Russia were substantial with 343 and 183 patients, respectively, tested positive for WNV with neurological symptoms (Tsai et al. 1998; Platonov et al. 2001).

Between 2000 and 2015, several notable WNV disease outbreaks occurred throughout Europe, including France (2000-2003), Italy (2008-2009) and Hungary (2008) (reviewed in Chancey et al. (2015)). Other European countries have reported further human WNV disease cases throughout the years. These include Austria, Bosnia and Herzegovina,

Croatia, Greece, Kosovo, the Former Yugoslav Republic of Macedonia, Montenegro, Serbia, Spain, Czech Republic and Portugal (reviewed in Chancey et al. (2015)).

With the increase in frequency of WNV disease outbreaks in Europe, several surveillance programmes were initiated in a number of European countries, including the UK, Germany, Switzerland and Italy (Linke et al. 2007; Morgan 2006; Läubli et al. 2006; Engler et al. 2013). The Italian surveillance programme has already proven to be very effective, as it allowed for a swift identification of WNV cases during the country's largest recorded outbreak in 2012, involving 28 autochthonous human WNV disease cases (Napoli et al. 2013).

Whether or not enzootic WNV transmission cycles will become established also in Germany is dependent on a number of factors crucial for an effective arboviral life cycle.

## 1.4 Arboviral life cycle

Whether a mosquito-borne transmission cycle can be set up and maintained depends on an array of factors, which influence virus, vector and host biology and corresponding interactions. These factors can be categorised as intrinsic and extrinsic factors. Intrinsic factors include elements such as viral evolution, host and vector immunity, vector competence and other vector life-history traits (Hardy et al. 1983; Chamberlain & Sudia 1961). Extrinsic factors focus on elements influencing the interaction between host and vector and include temperature and density of both populations (Ciota & Kramer 2013). Intrinsic and extrinsic factors can have an effect among as well as between each other (Fig. 1.6).

The importance of each of these factors needs to be evaluated in the context of vectorial capacity (VC). VC is a measure of a mosquito population's capability to transmit a virus to a susceptible host population and can be calculated via the following basic equation (Ciota & Kramer 2013; Macdonald 1961):

$$VC = \frac{m \cdot a^2 \cdot b \cdot p^n}{-\log_e p}$$

This basic equation incorporates the number of female mosquitoes per host (m), the daily blood feeding rate (a), the transmission rate among exposed mosquitoes (b), the probability of daily survival (p) and the extrinsic incubation period (n) (Fig. 1.6).

Based on this basic estimate of VC, a selection of intrinsic and extrinsic factors have been chosen to be discussed in the following.

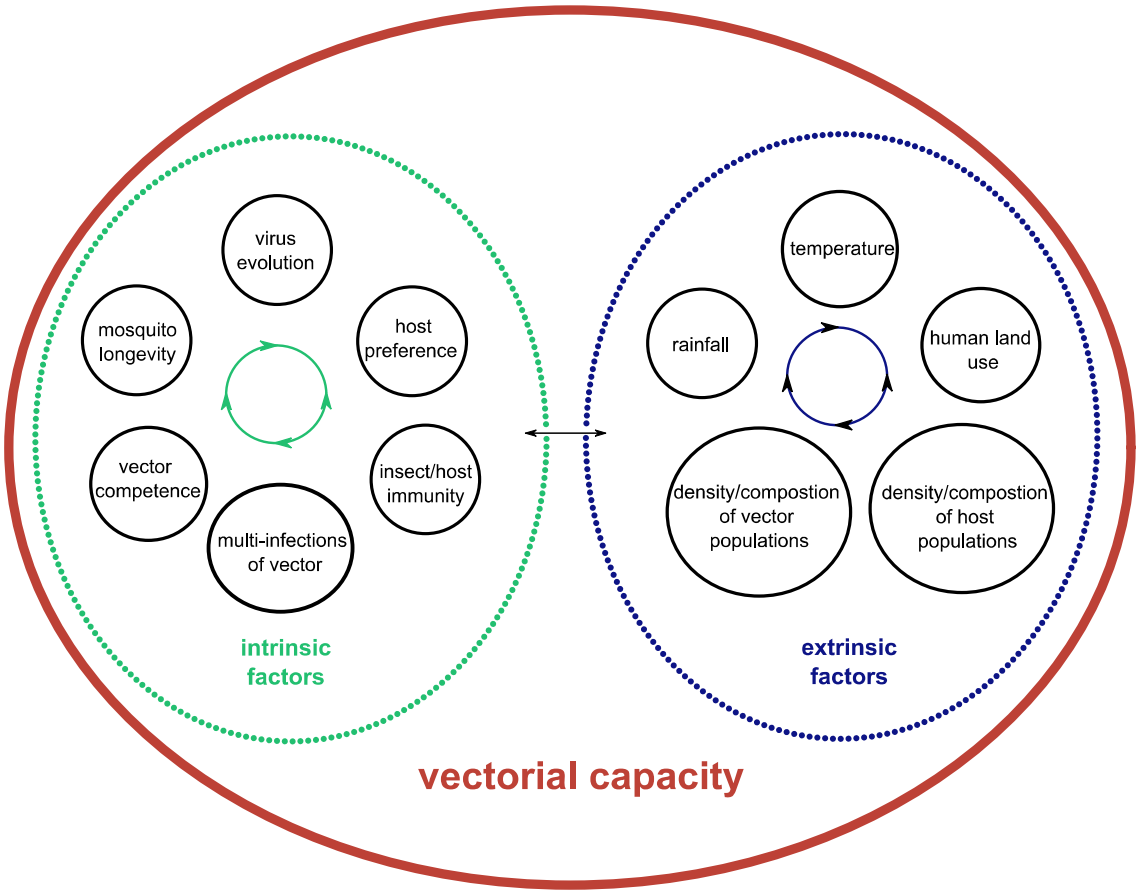



**Fig. 1.6: Schematic illustration of the intrinsic and extrinsic factors influencing vectorial capacity and thus the setup and maintenance of an arboviral life cycle**

## 1.4.1 Extrinsic factors

### 1.4.1.1 Temperature

The importance of the two intrinsic factors, host preference and vector competence, has been discussed previously (refer to 1.3.1). Especially vector competence is highly variable and usually influenced by temperature. To be more specific, temperature can influence the extrinsic incubation period (EIP), which is the number of days between ingestion and possible transmission of the virus (Dohm et al. 2002; Kilpatrick et al. 2008; Ciota & Kramer 2013). Work with *Culex pipiens* and WNV has demonstrated, that increasing temperature has an accelerating effect on EIP (Kilpatrick et al. 2008).

Temperature has been shown to further influence mosquito life history traits crucial for vectorial capacity, such as mosquito survival, developmental rates and, eventually, population density (Delatte et al. 2009; Rueda et al. 1990; Ciota & Kramer 2013). Generally, increasing temperature accelerates mosquito development, although this effect has been shown to be less pronounced at temperatures over 24 °C (Rueda et al. 1990; Ciota et al. 2014). This, however, indicates that increase of temperature as a result of global warming will play a significant role in the spread of arboviral diseases. This is in particular

relevant in areas of temperate climates with lower average temperatures (Ciota et al. 2014), which is the case in Germany.

Notably, increasing temperatures have also been associated with species-specific decrease in longevity of adults and immature mosquito stages. This suggests that the effect of temperature cannot be generalised and must be evaluated in association with other factors including vector and host population dynamics (Ciota et al. 2014).

#### 1.4.1.2 Vector population dynamics

As mentioned above, members of the *Culex pipiens* complex are known vectors for WNV (refer to 1.3.1). The complex includes species such as *Cx. quinquefasciatus*, *Cx. pipiens* and *Cx. torrentium* that have a worldwide distribution, although the latter two are found in temperate climate, whereas *Cx. quinquefasciatus* thrives in tropical and subtropical temperatures (Becker et al. 2012; Simonato et al. 2016). Nonetheless, these species are evolutionary closely related, which makes them morphologically difficult to differentiate, encouraging the development of molecular methods (Becker et al. 2012; Rudolf et al. 2013). Notably, *Culex pipiens* can be further subdivided into two biotypes, *Culex pipiens* biotype *pipiens* and biotype *molestus*, which differ in a number of phenotypic markers, including autogeny, stenogamy, ability to diapause and feeding preference (Farajollahi et al. 2011).

The feeding preference in particular is an important characteristic for vector competence (refer to 1.3.1). Members of the *Culex pipiens* complex are primarily ornithophilic, but occasionally also anthropophilic, which qualifies them as bridge vectors for WNV (Ciota & Kramer 2013). Recent data focusing on mosquito blood meal has indicated that mixed feeding preferences with frequent host switching occurs more often than previously suggested (Börstler et al. 2016).

Members of the *Culex pipiens* complex are able to interbreed and form hybrids such as *Cx. pipiens* biotype *pipiens/molestus* and *Cx. quinquefasciatus*/*pipiens* biotype *pipiens*. It has been shown that this hybridisation can have a significant effect on the vectorial capacity for WNV (Ciota & Kramer 2013). One particular study focusing on these hybrid species concluded, that the transmission rate of the hybrid population by day 13/14 was always significantly higher than in the corresponding parental populations (Ciota et al. 2013).

Finally, it is important to consider, that the vectorial capacity assigned to a mosquito population of one species within a geographical region cannot be automatically assigned to another mosquito population, even of the same species (Kilpatrick et al. 2010). Vector competence can vary on the basis of genetic variation within a population and may also vary seasonally (Hayes et al. 1984; Vaidyanathan & Scott 2006). Thus, when evaluating the effect of vector population dynamics on vectorial capacity, it is important to perform these analyses in a fixed seasonal and geographical context.

#### 1.4.1.3 Host population dynamics

The presence of WNV has been detected in wide variety of vertebrate host species. As described previously, mammalian hosts, such as humans and horses, are considered dead-end hosts, which are not able to produce viremia required for transmission (reviewed in Chancey et al. 2015; van der Meulen et al. 2005). However, several laboratory studies have revealed exceptions, including the eastern gray squirrels (*Sciurus carolinensis*), eastern chipmunks (*Tamias striatus*) and eastern cottontail rabbit (*Sylvilagus floridanus*). These mammals have been shown to develop significant viremia, which might allow WNV transmission (Gómez et al. 2008; Platt et al. 2007; Tiawsirisup et al. 2005).

However, the main amplifying reservoir are birds. In particular, members of the genus *Passeriformes* (May et al. 2011). Although, as primary hosts, these birds usually do not show any clinical signs, WNV-related morbidity in some bird species has been recorded (Colpitts et al. 2012). For example, American crows and birds of prey, such as falcons, are among the bird species that are highly susceptible to the virus and frequently succumb to infection with WNV (Yaremych et al. 2004; Ziegler et al. 2013).

Notably, migratory birds are the primary source of WNV infection, as seen in the USA and globally (Ciota & Kramer 2013). Accordingly, WNV surveillance in local and migratory bird populations is required for WNV risk assessment in Europe (Engler et al. 2013).

### 1.4.2 Intrinsic factors

Important intrinsic factors are mosquito longevity and vector competence, which have been discussed already (refer to 1.4.1). One other intrinsic factor that may have a considerable impact on vectorial capacity is vector co-infections with multiple microorganisms.

One of the possible co-infections with WNV so far identified include a number of insect-specific flaviviruses. Although noted, the extent to which the presence of these co-infections influences vector competence for WNV is not yet fully characterised (Ciota & Kramer 2013). Interestingly, it is has already been shown that the phenotype of these co-infections seems to be species specific. For example, co-infection of WNV with Nhumirim virus suppressed WNV infection in *Cx. quinquefasciatus*, whereas the same co-infection revealed no significant effects in *Cx. pipiens* (Goenaga et al. 2015).

In addition to insect-specific flaviviruses, microbial agents have also been shown to directly impact vector competence of mosquitoes for arboviruses (Weiss & Aksoy 2011). One of these co-infections, involving the proteobacterium of the genus *Wolbachia*, will be discussed in more detail in the following.

## 1.5 *Wolbachia pipientis*

### 1.5.1 General biology

These intracellular bacteria were discovered by Hertig and Wolbach in 1924 and named *Wolbachia pipientis* due to their location in the reproductive tissues of the mosquito *Cx. pipiens* (Hertig & Wolbach 1924; Hertig 1936; Werren 1997).

*Wolbachia* are gram-negative α-proteobacteria that are members of the order Rickettsiales, which includes other symbiotic bacteria genera, namely *Anaplasma*, *Ehrlichia* and *Rickettsia* (Werren et al. 2008; Micieli & Glaser 2014). These other genera mainly, but not exclusively, infect both invertebrate vectors and vertebrate hosts throughout their lifecycles, whereas *Wolbachia* has only been detected in invertebrates (Werren et al. 1994; Werren et al. 2008). So far, *Wolbachia* is estimated to infect about 40% of terrestrial arthropod species as well as numerous members of the nematoda genus (Zug & Hammerstein 2012; Werren et al. 2008).

*Wolbachia* are mainly transmitted via the egg cytoplasm of infected females and is able to manipulate host reproduction to promote this transmission route (see 1.5.2) (Johnson 2015). In the case of arthropod hosts, it is important to note that there is also strong indication of inter-species lateral transmission (reviewed in Werren et al. 2008). Concerning targets of infection, *Wolbachia* are not only concentrated on the reproductive organs, but are broadly distributed in the somatic tissue of the host (Fig. 1.7) (Micieli & Glaser 2014; Min & Benzer 1997). This is especially interesting considering *Wolbachia*-induced antiviral protection (Micieli & Glaser 2014; Min & Benzer 1997; Dobson et al. 1999; Zouache et al. 2009) (see 1.5.3.2).

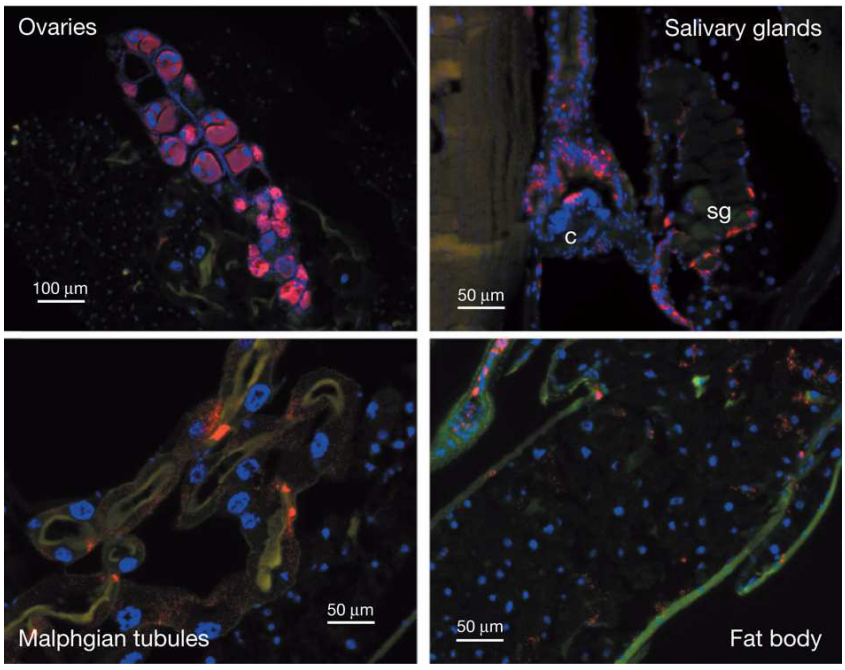



**Fig. 1.7: Tissue distribution of *Wolbachia* in mosquito organs**
Image by Walker et al. 2011
red = *Wolbachia pipientis* 16S rRNA, blue = DAPI DNA staining
green = GFP filter to enhance contrast
sg = salivary gland, c = cardia (midgut)

### 1.5.1.1 Genetics and classification

The species *Wolbachia pipientis* is further classified based on molecular data. The loci usually used for strain discrimination are the 16 rRNA gene, five multilocus sequence typing loci (MLST) as well as the *Wolbachia* surface protein gene (wsp) (Baldo, Dunning Hotopp, et al. 2006; Zhou et al. 1998; Werren et al. 2008). Based on these molecular data, strains can be, phylogenetically classified into evolutionary lineages called supergroups (Zhou et al. 1998). So far, 16 supergroups, named A-Q (G is considered an artifact and is not included), have been identified with this methodology (Comandatore et al. 2015).

It is an ongoing debate in the field, whether or not all bacteria in the *Wolbachia* clade should be designated as the species *Wolbachia pipientis* (Werren et al. 2008). However, since this issue is not yet resolved, the species designation '*Wolbachia pipientis*' is still valid, as well as subdivisions into supergroups and strains. The naming of the strains is dependent on the host species as illustrated in Fig. 1.8. In addition, referring to the bacteria as '*Wolbachia*' and not as '*Wolbachia pipientis*' is a habit in the research community (Werren et al. 2008) and will thus also be applied in this work.

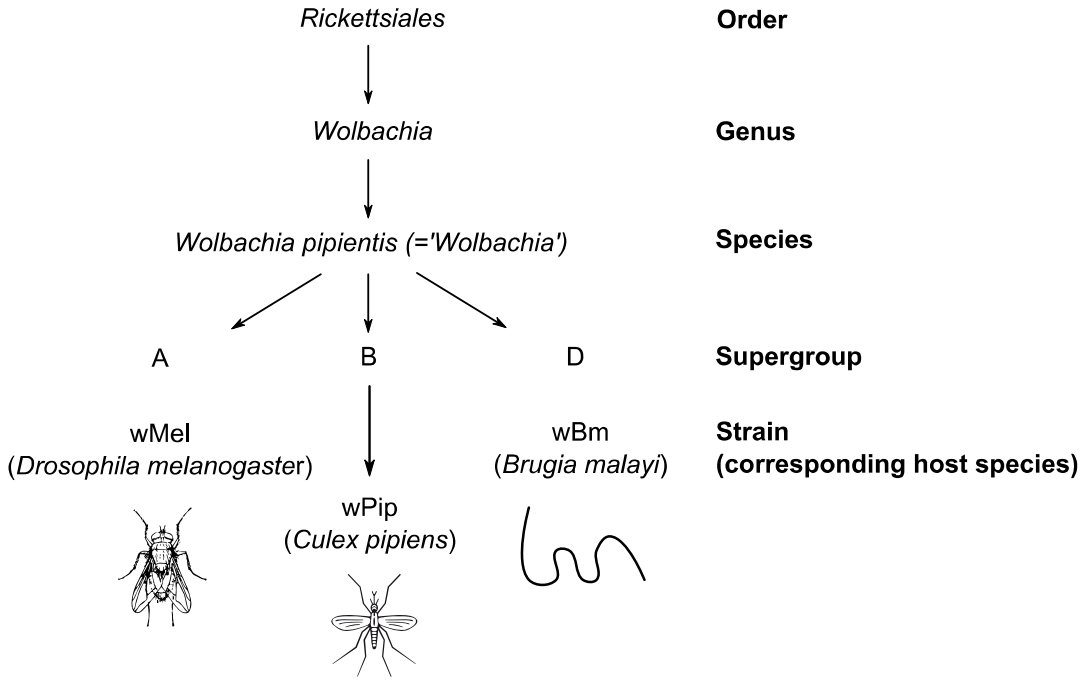



**Fig. 1.8: Taxonomic structure of *Wolbachia***

Sequencing and annotating *Wolbachia* genomes is ongoing. So far, full genome sequences are available for the wMel strain from *D. melanogaster*, the wBm strain from the filarial nematode *Brugia malayi* as well as the wPip strain from *Cx. pipiens* (Wu et al. 2004; Foster et al. 2005; Klasson et al. 2008). The sizes of the Wolbachia genomes comprise between 1.08 and 1.7 Mb and contain a high amount of mobile and repetitive elements. For example, 14 % of the wMel genome consists of repeats (Klasson et al. 2008).

Ankyrin (ANK) repeats mediating host-pathogen interactions in bacteria, duplications of short open reading frames of unknown function as well as variations of the surface proteins make up considerable amounts of the repetitive elements (Wu et al. 2004).

In addition, *Wolbachia* isolated from arthropods harbour various virus-like elements in their genome, including the lambda bacteriophage WO. Interestingly, some phage elements are transcribed and expressed via a lytic cycle, putatively co-expressing ANK proteins which can be integrated in these prophage segments (Masui et al. 2000; Klasson et al. 2008).

It has been shown that *Wolbachia* also undergo recombination, which, among others, affects the primary structure of various molecules such as surface proteins, housekeeping genes, ANK and prophage regions (Baldo et al. 2005; Baldo, Bordenstein, et al. 2006). These recombination are said to be linked to variation in the reproductive phenotype of *Wolbachia* (Klasson et al. 2008). This suggests that in addition to direct involvement in infection phenotype, ANK and prophage genes might also qualify as screening candidates for detection of further *Wolbachia* strain variants (Bordenstein et al. 2006; Klasson et al. 2008). The role of the WO prophage will be discussed in more detail in the following.

#### 1.5.1.2 Lambda bacteriophage WO

Masui et al. first identified phage-related gene clusters in the *Wolbachia* genome (Masui et al. 2000). The first inducible phage particles were then isolated from the Mediterranean flour moth, *Ephestia kuehniella*, giving further indication that the prophage genes are mobile and active (Fujii et al. 2004). Since then, WO phage particles have been isolated from several *Wolbachia*-infected arthropods and have been characterized via transmission electron microscopy as icosahedral particles with a head structure (20-40 nm diameter) and, in some reports, a small tail structure (Kent & Bordenstein 2010; Sanogo & Dobson 2006; Bordenstein et al. 2006).

The WO double-stranded DNA genome is a dynamic element that is present in multiple copies, dispersed throughout the *Wolbachia* genome and, therefore, also responsible for the genetic diversity of *Wolbachia* (Kent & Bordenstein 2010; Duron et al. 2006). Interchange of WO genetic elements can occur via horizontal gene transfer among co-infecting *Wolbachia* or via *Wolbachia*-infected sperm. Other possibilities include intragenic recombination between phage haplotypes (Klasson et al. 2008).

There is evidence that the WO bacteriophage can influence the interaction of *Wolbachia* with host cells. For instance, the WO homolog of a *Rickettsia* gene coding for a patatin-like phospholipase is associated with *Wolbachia* entry into the host cell (Kent & Bordenstein 2010; Tanaka et al. 2009). This might be particularly important for target cell preferences of *Wolbachia* strains.

Duron et al. have utilized the WO-generated genetic diversity of *Wolbachia* to identify more *Wolbachia* variants. They have identified and analysed 10-15 WO gene products (Gp), encoded for in the wPip genome, for sequence variability. Any variability in the

primer regions of the sequences, affecting primer binding, were picked up by absence/presence of the Gp. Additional sequence variability in other regions was found via sequencing. Using this methodology, 49 different wPip variants (mosquito sample size n=103) were found in Southern Europe alone (Duron et al. 2006).

This methodology of *Wolbachia* strain typing was adapted and used in the work presented here as well. The PCR was implemented for 9 Gp, which were previously described for wPip variants isolated from mosquito populations from countries close to Germany. Based on the different sizes of the Gp, a pattern of PCR products was obtained via agarose gel electrophoresis and absence/presence analysis was performed to identify variants (Fig. 1.9).

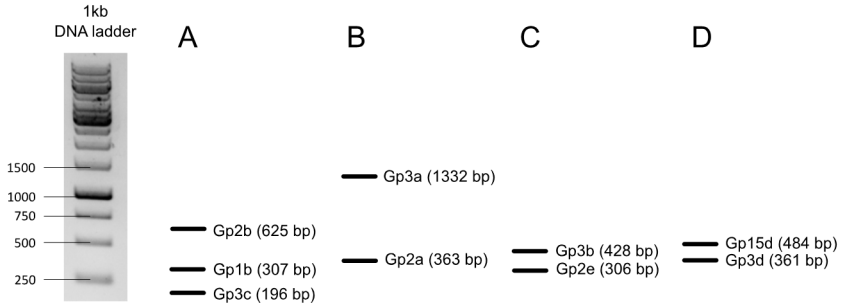



**Fig. 1.9: *Wolbachia* strain typing – PCR product pattern**
The 9 Gp are sorted into to 4 groups (A-D) to obtain a PCR-product pattern when separated via agarose electrophoresis.

### 1.5.2 *Wolbachia* infection

Originally, the term symbiosis was defined as the living together of dissimilar organisms (de Bary 1879). Contrary to popular belief, this definition incorporates a range of interactions, including mutualism (beneficial), commensalism (neutral) and parasitism (harmful), which can occur individually or as a variable mixture (Hentschel et al. 2000; Werren et al. 2008).

*Wolbachia* is able to manipulate cellular and reproductive processes in invertebrates, which may result in distinct phenotypes ranging from mutualisms to parasitism (Werren et al. 2008).

#### 1.5.2.1 *Wolbachia*-induced phenotypes

Feminisation and parthenogenesis are two reproductive phenotypes that both result in the production of more female offspring, which are able to transmit *Wolbachia* to subsequent progeny (Werren et al. 2008). In the case of parthenogenesis, unfertilized, haploid eggs do not develop into male but into female offspring (Arakaki et al. 2001; Stouthamer et al. 1990; Weeks & Breeuwer 2001). During feminization, however, *Wolbachia* somehow interferes with the sex-determination pathway causing genetic males to develop into females (Vandekerckhove et al. 2003; Hiroki et al. 2002; Narita et al. 2007; Negri et al.

2006). Notably, neither of these two reproductive phenotypes has been observed in *Diptera*, which makes them negligible in the context of *Wolbachia* infections in mosquitoes (Werren et al. 2008).

In contrast, the phenotype of *Wolbachia*-induced male-killing has been observed in the order *Diptera*, although not yet in mosquito populations (Dyer & Jaenike 2004; Werren et al. 2008). Here, genetic males do not survive embryogenesis resulting in all-female progeny (Fig. 1.10). Experiments with *Ostrinia scapulalis* indicates, that the cause of death might be lethal feminisation (Kageyama et al. 2002; Kageyama & Traut 2004). It is, however, unclear, if and how this translates to other host species (Werren et al. 2008).

The most common *Wolbachia*-induced reproductive phenotype is cytoplasmic incompatibility (CI), which has also been described in mosquitoes (Jost 1970; Trpis et al. 1981; Werren 1997). The basic principle is that sperm from *Wolbachia* infected males cannot produce viable offspring with females, whose eggs contain no *Wolbachia* or *Wolbachia* of a different type. This is due to the fact, that *Wolbachia* causes modification of infected sperm that must be rescued by the corresponding *Wolbachia* in the female egg, otherwise no viable embryos can develop (Werren 1997). There are two types of CI, uni-directional incompatibility and bi-directional incompatibility (Fig. 1.10).

During uni-directional incompatibility, *Wolbachia*-free mosquito females can only mate successfully with non-infected males, giving an advantage to *Wolbachia* infected females, which can mate with both. Bi-directional incompatibility occurs, when both male and female are infected with differing *Wolbachia* strains, which cannot rescue the sperm modification caused by the respective other strain (Zabalou et al. 2008; Werren 1997).

The intracellular mechanism of CI is still unclear, however a few interesting theories were formulated in this context (reviewed in Poinsot et al. 2003). Notably, the non-rescued phenotype seems to be the result of asynchronous development of male and female pronuclei, which is mainly accounted for by a delay in mitosis in the male pronucleus (Tram & Sullivan 2002). Furthermore, there is evidence of so-called host-induced ‘suicide infections’, during which the genotype of the host can influence CI and potentially hinder *Wolbachia* strains to rescue their own sperm modifications (Zabalou et al. 2008).

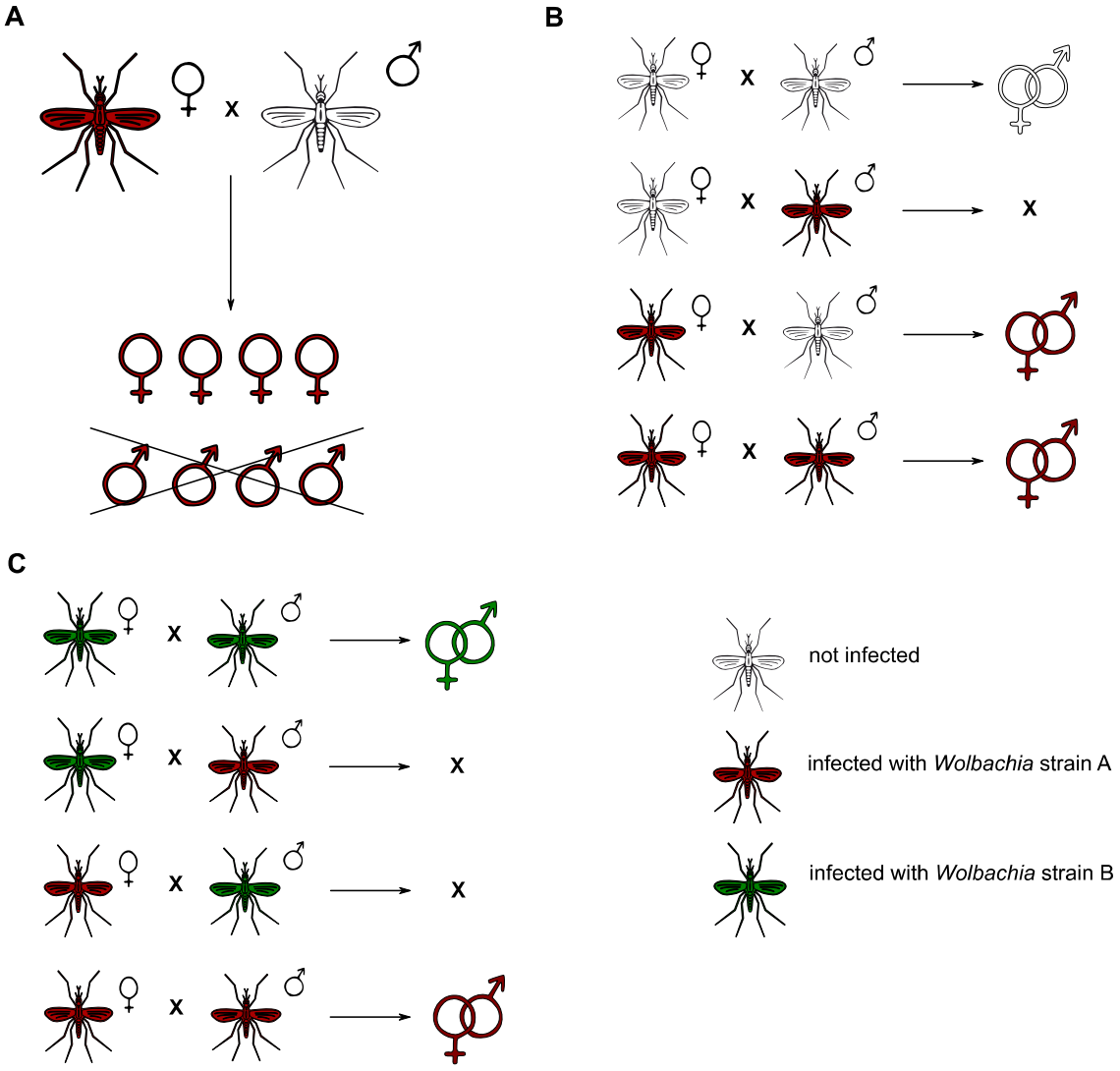



**Fig. 1.10: *Wolbachia*-induced reproductive phenotypes**
Adapted from Werren et al. 2008
Feminisation causes the death of genetic males resulting in all-female progeny (A). Uni-directional CI results in embryonic death when *Wolbachia*-uninfected females mate with infected males (B). Bi-directional incompatibility can occur, when both male and female are infected with differing *Wolbachia* strains, which are not able to rescue the sperm modifications caused by the respective other strain (C).

In addition to reproductive phenotypes, other implications of *Wolbachia* infections have been described.

*Wolbachia* and nematodes seem to have developed a mutualistic relationship. This manifests itself in *Wolbachia* being crucial for moulting of microfilaria as well as reproduction of the mature worm (Taylor & Hoerauf 1999). Furthermore, there is indication that *Wolbachia* might interfere with the vertebrate immune response against nematode infections (Taylor & Hoerauf 1999).

An interesting phenotype termed ‘genetic addiction’ has been observed in the parasitic wasp *Asobara tabida*. Briefly, it has been observed that proper development of the ovaries in this wasp depends on *Wolbachia* infection. This dependence is not observed in

related wasps of the same genus, suggesting that this relationship is the result of individual host-*Wolbachia* co-evolution (Dedeine et al. 2005).

Further possible phenotypes include *Wolbachia*-associated alterations in mating preference (Koukou et al. 2006), changes in responses to olfactory cues (Peng et al. 2008) as well as protection against virus infections (Teixeira et al. 2008; Moreira et al. 2009). The latter as well as implications associated with this phenotype will be discussed in more detail in the following.

### 1.5.3 *Wolbachia* and vector competence for arboviruses

As mentioned previously, *Wolbachia* infections are also present in *D. melanogaster*, both in nature and laboratory stocks (Clark et al. 2005; Hoffmann et al. 1996). Interestingly, only infection with the *Wolbachia* variant wMelPop, originating from a laboratory stock, has so far been observed to cause a striking, albeit destructive phenotype involving tissue degeneration and a shortened lifespan (Min & Benzer 1997). Most naturally occurring *Wolbachia* variants of *D. melanogaster* are only associated with a weak CI phenotype, even though stronger phenotypes have been observed in the closely related *D. simulans* (Yamada et al. 2007; Hoffmann et al. 1986). However, since also the weak *Wolbachia* strains are maintained in nature, albeit a vertical transmission rate of below 100%, it seems likely that other fitness benefits further promote *Wolbachia* infections (Teixeira et al. 2008; Hoffmann et al. 1998). Several studies have focused on these putative fitness benefits. Conclusions range from no effect at all to variable positive and/or negative influences on life span and fecundity (Harcombe & Hoffmann 2004; Fry et al. 2004; Montenegro et al. 2006; Fry & Rand 2002). Fitness benefits due to *Wolbachia* infections have also been observed in other insect species. For instance, Dobson et al. reported an increased life span as well as improved fecundity and hatching rates in infected *Ae. albopictus* females (Dobson et al. 2002).

*Wolbachia*-induced resistance against viral or parasitic infections would also constitute a fitness benefit and is presently a target of research.

#### 1.5.3.1 Antiviral protection

The link between antiviral protection and *Wolbachia* infection was first discovered in *D. melanogaster*. It was shown that, once cured of their *Wolbachia* infection, the pathogenicity associated with the RNA viruses Drosophila C virus (DCV), Cricket paralysis virus, Nora virus and Flock House virus was greatly reduced in the flies (Hedges et al. 2008; Teixeira et al. 2008). Increased survival is also sometimes accompanied by a marked reduction of virus particle accumulation, suggesting that *Wolbachia* have a dual impact on virus infections: reduced/delayed virus accumulation and reduced/delayed host mortality (Hedges et al. 2008; Teixeira et al. 2008; Johnson 2015).

Possible antiviral effects of *Wolbachia* infections are of interest in mosquitoes, which are potential vectors for arboviruses. Numerous studies have reported *Wolbachia*-mediated antiviral effects for a broad range of RNA virus families, including the *Flaviviridae*. These

effects, however, may induce a broad range of phenotypes, including reduced virus proliferation or transmission, reduced infection but also enhanced virus infection rates (reviewed in Johnson 2015).

This is also the case with WNV and *Culex* mosquitoes. Glaser and Meola reported that *Wolbachia* causes WNV resistance in naturally infected *Cx. quinquefasciatus*. They noted that, although there was no effect on the infection rate, lower virus titers and a 2 to 3-fold reduced transmission rate was observed for *Wolbachia*-infected mosquitoes (Glaser & Meola 2010). Interestingly, infection of *Cx. tarsalis*, which usually lack *Wolbachia*, with the *Wolbachia* strain wAlbB enhanced WNV infection rather than causing inhibition (Dodson et al. 2014). To add to this bivalent picture, another study found that WNV resistance in *Cx. quinquefasciatus* as well as in *Cx. pipiens* varies depending on *Wolbachia* density (Micieli & Glaser 2014).

The mechanisms involved in *Wolbachia*-induced antiviral protection are still unknown, although some ideas have been speculated. These include competition for resources and remodelling of the host cell environment, immune stimulation and the expression of microRNAs to mediate antiviral effects (reviewed in Rainey et al. 2014).

Further research into *Wolbachia*-virus-host interaction is necessary to explain and potentially predict the phenotype a *Wolbachia*-virus co-infection would trigger. This can be especially beneficial for the development of arbovirus control strategies.

#### 1.5.3.2 *Wolbachia* and arbovirus control

*Wolbachia*-induced antiviral protection constitutes a promising new tool for arbovirus transmission control, as recently shown with transinfected *Ae. aegypti* (not naturally infected with *Wolbachia*), which become resistant to DENV once infected with *Wolbachia* (Xi et al. 2005; Walker et al. 2011; Rainey et al. 2014). Since March 2011, the Eliminate Dengue program has released *Wolbachia*-infected *Ae. aegypti* in Australia, Vietnam, Colombia, Indonesia and Brazil (Eliminate Dengue Program n.d.). Published results for the Australian trial indicate a successful invasion into the local *Ae. aegypti* population and reduction of DENV transmission (Walker et al. 2011; Hoffmann et al. 2011).

*Wolbachia*-infected mosquitoes seem to be a low risk method to reduce arbovirus transmission. So far, no antibody production against *Wolbachia* could be detected in human volunteers upon a mosquito bite and no transfer to mosquito predators or the environment (Popovici et al. 2010). Nevertheless, long-term risks associated with artificial *Wolbachia* infection in the wild are difficult to predict. Especially putative decrease in *Wolbachia* density in response to mosquito/*Wolbachia* co-adaptation might endanger antiviral protection (Rainey et al. 2014).

## 1.6 Aim

WNV is the causative agent of the debilitating West Nile disease. It has caused severe outbreaks worldwide, including the USA and Europe, and has the potential to spread to Germany (Chancey et al. 2015).

The *Culex* complex has a worldwide distribution and the members *Cx. pipiens* biotype *pipiens*, *Cx. pipiens* biotype *molestus* as well as *Cx. torrentium* are also present in Germany (Rudolf et al. 2013; Krüger et al. 2014). However, due to population-linked diversity of vector competence, their susceptibility for WNV cannot be extrapolated from other populations of the same species in endemic regions (Kilpatrick et al. 2010). Possible population-, temporal- and temperature-linked variations for WNV susceptibility of German *Culex* mosquitoes are also unknown.

In addition, *Wolbachia* has been shown to have the capacity to mediate resistance of arthropods to infection with RNA-viruses (reviewed in Johnson 2015). Thus, *Wolbachia* might also influence the susceptibility of German *Culex* mosquitoes for WNV. However, their *Wolbachia* status and strain variability has not been subject of scientific studies so far.

To assess the rate and variation of susceptibility of German *Culex* mosquitoes for WNV and the influence of a *Wolbachia* infection, the following focus points were formulated:

1. Analyse the distribution of putative WNV vectors in Germany, focusing on *Cx. torrentium, Cx. pipiens* biotype *pipiens* as well as *Cx. pipiens* biotype *molestus* and their hybrids *Cx. pipiens* biotype *pipiens*/*molestus*.
2. Determine the susceptibility of WNV for field-caught *Cx. pipiens* biotype *pipiens* and *Cx. torrentium*. Use lab strains of *Cx. quinquefasciatus* and *Cx. pipiens* biotype *molestus* as positive controls. Determine also how the susceptibility varies according to
    a. temperature (low vs high: 18 °C vs 25 °C)
    b. sampling location of mosquito population (Northern Germany vs Southern Germany)
    c. year of sampling (2012 vs 2013)
3. Assess the infection status and *Wolbachia* strain variability in field-caught *Culex* mosquitoes
4. Assess the effect of a *Wolbachia* infection on the susceptibility for WNV using the lab strain *Cx. pipiens* biotype *molestu*s.

Answering these research questions will be of aid to assess the potential role of *Culex* mosquitoes in establishing an enzootic WNV transmission cycle in Germany.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Equipment

**Table 2.1: Utilised equipment**

| | Technical equipment | Manufacturer/Supplier |
|---|---|---|
| DNA/RNA extraction | Heat block MBT 250 | ETG GmbH (Ilmenau, Germany) |
| | NanoPhotometer® P 300 | Implen (Munich, Germany) |
| | Pistilles | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| | Vortex Genie 1 and 2 | Scientific Industries Inc. (Bohemia, NY, USA) |
| | Vortex mixer | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| | Water bath | Memmert GmbH & Co. KG (Schwabach, Germany) |
| Agarose gel electrophoresis | Agarose gel chamber | Bio-Rad Laboratories GmbH (Munich, Germany) |
| | Magnetic stirrer<br>Stir bars | Hanna Instruments Deutschland GmbH (Vöhringen, Germany)<br>neoLab Migge Laborbedarf-Vertriebs GmbH (Heidelberg, Germany) |
| | Microwave | Severin Elektrogeräte GmbH (Sundern, Germany) |
| | PowerPac Universal | Bio-Rad Laboratories GmbH (Munich, Germany) |
| | Scout® Pro Electronic balance | Ohaus Cooperation (Parsippany, NJ, USA) |
| | UV-transducers | Bio-Rad Laboratories GmbH (Munich, Germany) |
| PCR and qRT-PCR | Flex Cycler | Analytic Jena AG (Eisfeld, Germany) |
| | LightCycler 2.0<br>Cool rack | Roche Diagnostics International AG (Risch-Rotkreuz, Switzerland) |
| | LightCycler 480 | Roche Diagnostics International AG (Risch-Rotkreuz, Switzerland) |
| | RotorGene 6000 | Corbett Research, Qiagen (Hilden, Germany) |
| $TCID_{50}$ | Mini see-saw rocker SSM4 | Bibby Scientific Limited (Staffordshire, UK) |
| | Digital cameras<br>Image printer | Bio-Rad Laboratories GmbH (Munich, Germany)<br>IBI Kodak (Rochester, NY, USA)<br>Mitsubishi Electric Corporation (Ratingen, Germany) |
| | Immunfluorescence microscope, Axio Imager 2 | Zeiss (Oberkochen, Germany) |

| | | |
|---|---|---|
| Mosquito rearing | Sleeper | Inject+matic (Geneva, Switzerland) |
| | Bugdorm | MegaView Science Co., Ltd. (Taichung, Taiwan) |
| | Cold light MLC-150C | Motic Deutschland GmbH (Wetzlar, Germany) |
| | Forceps neoLab-Dumont | neoLab Migge Laborbedarf-Vertriebs GmbH (Heidelberg, Germany) |
| | Gravid traps (System Box M) | KIS (Roncadelle di Ormelle, Italy) |
| | Plastic trays | Bürkle (Bad Bellingen, Germany) |
| | Pump | Vacuubrand GmbH & CO. KG (Wertheim, Germany) |
| | Sieve | various vendors |
| | Stereomicroscope SMZ 168 | Motic Deutschland GmbH (Wetzlar, Germany) |
| Clean benches and fume cupboard | Clean bench HERAsafe | Heraeus Instruments (Hanau, Germany) |
| | Laminar Flow BSB4 (cell culture, S3) | Gelaire (Seven Hills NSW, Australia) |
| | FlowSafe® B-[MaxPro]$^3$-130 (insectary, S3) | BERNER INTERNATIONAL GmbH (Elmshorn, Germany) |
| | Flow Laboratory BSB 6A | Gelaire (Seven Hills NSW, Australia) |
| | Fume cupboard | Werner Hassa Laborbedarf GmbH (Lübeck, Germany) |
| Fridges, freezers and incubators | New Brunswick Ultra-Low Temperature freezer C660 HEF™ (-80 °C) | Eppendorf AG (Hamburg, Germany) |
| | Kryotec Sanyo (-80 °C, cell culture, S3) | Kryotec-Kryosafe GmbH (Hamburg, Germany) Ewald Innovationstechnik GmbH (Bad Nenndorf, Germany) |
| | GFL 6481 (-80 °C, insectary, S3) | GFL Gesellschaft für Labortechnik mbH (Burgwedel, Germany) |
| | Fridges (-20 °C; + 4 °C); Premium and Comfort | Liebherr (Bulle, Switzerland) |
| | Incubator (37 °C) | Memmert GmbH & Co. KG (Schwabach, Germany) |
| | Constant Climate Chamber KBF-240 (insectary, S3) | BINDER GmbH (Tuttlingen, Germany) |
| | Heraus BBD6220 | ThermoScientific (Waltham, MA, USA) |
| | Centrifuge (Himac CT 15 RE) | VWR International GmbH (Darmstadt, Germany) |
| | Centrifuge (MiniStar silverline) | VWR International GmbH (Darmstadt, Germany) |
| | Centrifuge (Sigma 1-15) | Sigma Laborzentrifugen GmbH (Osterode am Harz, Germany) |
| | Centrifuge (Labofuge 400R; insectary, S3) | ThermoScientific (Waltham, MA, USA) |
| | Cool rack | BioCision (San Rafael, CA, USA) |
| | Light microscope AE2000 | Motic Deutschland GmbH (Wetzlar, Germany) |

| | | |
|---|---|---|
| Others | Multichannel pipette (Eppendorf Research® Plus; 50-300 µl) | Eppendorf AG (Hamburg, Germany) |
| | Multichannel pipette (Eppendorf xplorer; 0.5-10 µl) | Eppendorf AG (Hamburg, Germany) |
| | pH meter (SCHOTT instruments) | SI Analytics GmbH (Mainz, Germany) |
| | Pipet aid pipetus® | Hirschmann Laborgeräte GmbH & Co. KG (Eberstadt, Germany) |
| | Pipettes (Eppendorf Research® Plus) | Eppendorf AG (Hamburg, Germany) |
| | Pipettes (Gilson PIPENTMAN® ) | Gilson, Inc. (Middleton, WI, USA) |
| | Thoma cell counting chamber | Paul Marienfeld GmbH & Co. KG (Lauda-Königshofen, Germany) |

### 2.1.2 Consumables

**Table 2.2: Utilised consumables**

| Material | Manufacturer/Supplier |
|---|---|
| Human blood (blood group 0) | Bloodbank University Hospital Hamburg Eppendorf (Hamburg, Germany) |
| Conical tubes (50 ml, 15 ml) | Sarstedt AG & Co. (Nümbrecht, Germany) |
| Cotton balls/pads | various vendors |
| Cover slip | R. Langenbrinck Labor-und Medizintechnik (Emmendingen, Germany) |
| Fish food (Tropical Tablets) | ASTRA Aquaristic GmbH (Hameln, Germany) |
| LightCycler® Capillaries (20 µl) | Roche Diagnostics International AG (Risch-Rotkreuz, Switzerland) |
| Microtubes (1.5 ml; safe-seal and standard) | Sarstedt AG & Co. (Nümbrecht, Germany) |
| Multiply® - µStrip Pro 8-strip | Sarstedt AG & Co. (Nümbrecht, Germany) |
| Omnifix-F Tuberculin syringes (single use; 1 ml) | B. Braun Melsungen AG (Melsungen, Germany) |
| Pasteur pipette (disposable) | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| Petri dishes (92x16 mm) | Sarstedt AG & Co. (Nümbrecht, Germany) |
| Pipette (serological; 5/10/25 ml) | Sarstedt AG & Co. (Nümbrecht, Germany) |
| Pipette tips (with filter and standard; 1000/200/10 µl) | Sarstedt AG & Co. (Nümbrecht, Germany) |
| Plastic vials (175 ml) | Greiner Bio-One GmbH (Frickenhausen, Germany) |
| Plugs for plastic vials (Ø 52 mm) | K-TK e. K. (Retzstadt, Germany) |
| Q tips | various vendors |
| qPCR plate (384 well) | Sarstedt AG & Co. (Nümbrecht, Germany) |
| qPCR seal | 4titude (Surrey, UK) |
| Rotilabo® syringe filters (0.2 µm) | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| Specimen slide (Menzel-Gläser Superfrost® Plus) | Gerhard Menzel GmbH (Braunschweig, Germany) |
| StericapPlus Bottle-Top Vaccum Filtration Device | Merck Millipore (Billerica, MA, USA) |

| | |
|---|---|
| STRIP Tubes (0.1 ml) | LTF-Labortechnik GmbH & Co. KG (Wasserburg, Germany) |
| TC Flask T25/T75, standard, vent. cap | Sarstedt AG & Co. (Nümbrecht, Germany) |
| TC Plate 96 well, standard, round/flat bottom | Sarstedt AG & Co. (Nümbrecht, Germany) |
| Urine cups | neoLab Migge Laborbedarf-Vertriebs GmbH (Heidelberg, Germany) |
| Weighing tray | neoLab Migge Laborbedarf-Vertriebs GmbH (Heidelberg, Germany) |
| Wooden spatula | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |

### 2.1.3 Chemicals

**Table 2.3: Utilised chemicals**

| Chemicals | Manufacturer |
|---|---|
| 2-Propanol | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| 4-Aminobenzoic acid | Sigma-Aldrich (Steinheim am Albuch, Germany) |
| Agarose Standard (Roti®garose) | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| 6X DNA Loading Dye | ThermoScientific (Waltham, MA, USA) |
| D(-)-Fructose | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| Disodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| DMEM | PAN-Biotech GmbH (Aidenbach, Germany) |
| Ethanol (≥ 99.5 %) | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| Ethidium bromide solution (0.5 %) | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| Ethylendiamin tetraacetic acid (EDTA) | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| FBS/FCS (fetal bovine/calf serum) normal and Gold | PAN-Biotech GmbH (Aidenbach, Germany)<br>PAA Laboratories GmbH (Pasching, Austria) |
| Formaldehyde (37 %) | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| Glacial acetic acid | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| Hydrochloric acid (HCl) | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| L-glutamine (200 mM) | PAN-Biotech GmbH (Aidenbach, Germany) |
| Non-essential amino acids (100X; MEM NEAA) | PAN-Biotech GmbH (Aidenbach, Germany) |
| Penicillin/Streptomycin | Gibco® Thermo Fisher (Carlsbad, CA, USA) |

| | |
|---|---|
| Potassium chloride (KCl) | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| Schneider's Drosophila Medium | PAN-Biotech GmbH (Aidenbach, Germany) |
| Sodium chloride (NaCl) | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| Sodium pyruvate (100 mM) | PAN-Biotech GmbH (Aidenbach, Germany) |
| Tetracyclin | Sigma-Aldrich (Steinheim am Albuch, Germany) |
| Tris(hydroxylmethyl)aminomethane (TRIS) | Carl Roth GmbH & Co. KG (Karlsruhe, Germany) |
| Triton® X-100 | MP Biomedicals, LLC. (Santa Ana, CA, USA) |
| Trypsin/EDTA solution (0.05/0.02 % in PBS) | PAN-Biotech GmbH (Aidenbach, Germany) |

### 2.1.4 Recipes: buffers, media and solutions

**Table 2.4: Utilised buffers (A), media (B) and solutions (C)**

A

| Buffer | Recipe |
|---|---|
| PBT buffer (1X) | 1X PBS<br>0.5 % Triton-X100 |
| Phosphate buffered saline (PBS) (10X) | for 1 litre (in $ddH_2O$):<br>80 g NaCl<br>2 g KCl<br>26.8 g $Na_2HPO_4 \cdot 7H_2O$<br>2.4 g $KH_2PO_4$<br>→ pH 7.4 |
| $TCID_{50}$ blocking buffer (1X) | 1X PBT<br>10 % fetal bovine serum (FCS) |
| TRIS-acetate-EDTA (TAE) buffer (50X) | for 1litre (in $ddH_2O$):<br>242 g TRIS<br>57.1 ml glacial acetic acid<br>100 ml 50 mM EDTA |

B

| Medium | Recipe |
|---|---|
| supplemented Dulbecco's Modified Eagle Medium (DMEM) | DMEM<br>10 % FCS (normal cell culture)<br>2 % FCS (virus probagation and infection assays)<br>100 units/ml penicillin<br>100 µg/ml streptomycin<br>1X non-essential amino acids (NEAA)<br>1 mM sodium pyruvate<br>2 mM L-glutamine |
| supplemented Schneider's Drosophila Medium | Schneider's Drosophila Medium<br>10 % FCS<br>100 units/ml penicillin<br>100 µg/ml streptomycin<br>1X non-essential amino acids (NEAA)<br>1 mM sodium pyruvate<br>2 mM L-glutamine |

C

| Solution | Recipe |
|---|---|
| Blood meal (blood feed infection assay) | Human blood (blood group 0)<br>10 % FCS<br>50 % fructose solution (8 %)<br>virus stock (1-1.6x$10^7$ PFU) |
| Blood meal (normal culture) | Human blood (blood group 0)<br>50 % FCS<br>0.5 % fructose solution (8 %) |
| Fixation solution | 1X PBS<br>4 % formaldehyde |
| Fructose solution (8 %) | for 1 litre (in $H_2O$):<br>80 g fructose<br>0.2 g 4-aminobenzoic acid |
| Tetracycline solution | 8 % fructose solution<br>0.5 mg/ml tetracycline |

### 2.1.5 Kits and markers

**Table 2.5: Utilised kits and markers**

| Name | Supplier |
|---|---|
| Dneasy Blood & Tissue Kit | QIAGEN (Hilden, Germany) |
| QIAamp Viral RNA Mini Kit | QIAGEN (Hilden, Germany) |
| QuantiTect Probe PCR Kit | QIAGEN (Hilden, Germany) |
| HotStarTaq Master Mix Kit | QIAGEN (Hilden, Germany) |
| GeneRuler 1 kb DNA Ladder | ThermoScientific (Waltham, MA, USA) |
| GeneRuler 100 bp DNA Ladder | ThermoScientific (Waltham, MA, USA) |

### 2.1.6 Enzymes and antibodies

The enzyme listed in the following table was purchased individually. All other enzymes utilised were purchased as part of a kit (Table 2.5).

**Table 2.6: Utilised enzyme (A) and antibodies (B)**

A

| Name | Supplier |
|---|---|
| DreamTaq DNA Polymerase | ThermoScientific (Waltham, MA, USA) |

B

| Name | $TCID_{50}$ dilution | Species | Supplier |
|---|---|---|---|
| anti-WNV envelope protein IgG1 | 1:100 | mouse | antikörper-online GmbH (Aachen, Germany) |
| goat anti-mouse FITC-conjugated IgG (H+L) | 1:200 | goat | Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA) |

### 2.1.7 Oligonucleotides

**Table 2.7: Utilised oligonucleotides**

| ID | Sequence (5' → 3') | Target gene and product size [bp] | Utilisation | Origin |
|---|---|---|---|---|
| OSM_69 | TGGTCCAATAAGTGATGAAGAAAC | *Wolbachia* surface protein; 590-632* | *Wolbachia* detection PCR, sense | Braig et al. 1998; Zhou et al. 1998 |
| OSM_70 | AAAAAATTAAACGCTACTCCA | *Wolbachia* surface protein; 590-632* | *Wolbachia* detection PCR, antisense | Braig et al. 1998; Zhou et al. 1998 |
| 1725 | GCGGCCAAATATTGAGACTT | Microsatellite locus CQ11; 189 | *Wolbachia* detection PCR and molecular taxonomic identification, sense | Rudolf et al. 2013 |
| 1726 | CGTCCTCAAACATCCAGACA | Microsatellite locus CQ11; 189 | *Wolbachia* detection PCR and molecular taxonomic identification, antisense | Rudolf et al. 2013 |
| OSM_78 | GACACAGGACGACAGAAA | ace2 gene (*Cx. torrentium*); 116 | *Wolbachia* detection PCR and molecular taxonomic identification, sense | Rudolf et al. 2013 |

| | | | | |
|---|---|---|---|---|
| OSM_79 | GCCTACGCAACTACTAAA | ace2 gene (*Cx. torrentium*); 116 | *Wolbachia* detection PCR and molecular taxonomic identification, antisense | Rudolf et al. 2013 |
| 1727 | AACATGTTGAGCTTCGGK 5'-Cy55, 3'-BBQ-1 | Microsatellite locus CQ11; 189 | molecular taxonomic identification; *Cx. pipiens* all-probe | Rudolf et al. 2013 |
| 1728 | GCTTCGGTGAAGGTTTGTGT 5'-JOE, 3'-BHQ1 | Microsatellite locus CQ11; 189 | molecular taxonomic identification; *Cx. pipiens* biotype *pipiens*-probe | Rudolf et al. 2013 |
| 1729 | TGAACCCTCCAGTAAGGTATCAAC-TAC 5'-Rox, 3'-BHQ2 | Microsatellite locus CQ11; 189 | molecular taxonomic identification; *Cx. pipiens* biotype *molestus*-probe | Rudolf et al. 2013 |
| OSM_82 | CGATGATGCCTGTGCTACCA 5'-FAM, 3'-BHQ1 | ace2 gene (*Cx. torrentium*); 116 | molecular taxonomic identification; *Cx. torrentium*-probe | Rudolf et al. 2013 |
| OSM_213 | AAGTGGCTGGAAAATGTATAAC | WO prophage Gp1b; 307 | *Wolbachia* strain typing; sense | Duron et al. 2006 |
| OSM_214 | TGAGTTTGCTATTTACTGCTAG | WO prophage Gp1b; 307 | *Wolbachia* strain typing; antisense | Duron et al. 2006 |
| OSM_215 | GCAAATATTTTAGGTGAGGCGC | WO prophage Gp2a; 363 | *Wolbachia* strain typing; sense | Duron et al. 2006 |
| OSM_216 | ACGGAGTTCTCCACAAAGTACT | WO prophage Gp2a/b; 363/642 | *Wolbachia* strain typing; antisense | Duron et al. 2006 |
| OSM_217 | CGTAGTGGCATTGAATTTAACC | WO prophage Gp2b; 642 | *Wolbachia* strain typing; sense | Duron et al. 2006 |
| OSM_218 | TTCTACAACAGATGATCAAACG | WO prophage Gp2e; 306 | *Wolbachia* strain typing; sense | Duron et al. 2006 |
| OSM_219 | CATCATCGGCCTACATAGCCA | WO prophage Gp2e; 306 | *Wolbachia* strain typing; antisense | Duron et al. 2006 |
| OSM_220 | AAGTGGGTTTGATGAAAAATGT | WO prophage Gp3a/d; 1339/361 | *Wolbachia* strain typing; sense | Duron et al. 2006 |
| OSM_221 | TACATCATCATGCGGAATGTGC | WO prophage Gp3a; 1339 | *Wolbachia* strain typing; antisense | Duron et al. 2006 |
| OSM_222 | CAGAGGTCTTTCAATTGAAAAG | WO prophage Gp3b/c; 428/196 | *Wolbachia* strain typing; sense | Duron et al. 2006 |
| OSM_223 | GCGGTTATAAAATTTAAATGCA | WO prophage Gp3b; 428 | *Wolbachia* strain typing; antisense | Duron et al. 2006 |

| | | | | |
|---|---|---|---|---|
| OSM_224 | AAGAACTTCAGTACGATACTTG | WO prophage Gp3c/d; 196/361 | *Wolbachia* strain typing; antisense | Duron et al. 2006 |
| OSM_225 | GTAGAAGCAAAAGAGTTTGTTG | WO prophage Gp15b; 484 | *Wolbachia* strain typing; sense | Duron et al. 2006 |
| OSM_226 | CTTCCTCACAGTATTCGAGTTT | WO prophage Gp15b; 484 | Wolbachia strain typing; antisense | Duron et al. 2006 |
| OSM_323 | TAGCGATTGAAGATATGC | *Wolbachia* surface protein; 82 | *Wolbachia* detection qPCR; sense | Virology, BNITM, Hamburg |
| OSM_324 | CTAGCTTCTGAAGGATTG | *Wolbachia* surface protein; 82 | *Wolbachia* detection qPCR; antisense | Virology, BNITM. Hamburg |
| OSM_325 | CACCAACACCAACACCAACG 5'-FAM, 3'-BHQ1 | *Wolbachia* surface protein; 82 | *Wolbachia* detection qPCR; probe | Virology, BNITM, Hamburg |
| OSM_145 | GGCAATGGAGTCATAATG | WNV lineage 1 polyprotein; 100 | West Nile virus detection qPCR, sense | Virology, BNITM, Hamburg |
| OSM_146 | GCATCTCAGGTTCGAATC | WNV lineage 1 polyprotein; 100 | West Nile virus detection qPCR, antisense | Virology, BNITM, Hamburg |
| OSM_147 | CCAACGGCTCATACATAAGCG 5'-FAM, 3'-BHQ1 | WNV lineage 1 polyprotein; 100 | West Nile virus detection qPCR, probe | Virology, BNITM, Hamburg |

*Based on the newest published sequence of wPip, the wsp-product in *Culex* mosquitoes was determined to have a size of 603 bp (using Primer BLAST, refer to Table 2.9) (Klasson et al. 2008).

### 2.1.8 Infectious agents, cells and organisms

**Table 2.8: Utilised infectious agent (A), cell line (B) and organisms (C)**

A

| Infectious agent | Strain | BSL level | Origin |
|---|---|---|---|
| WNV | NY99 | 3 | BNITM (Hamburg); originally from NCPV Porton Down, (USA; 2008) |

B

| Name | Species | Origin |
|---|---|---|
| Vero | African green monkey (*Chlorocebus sp.*); kidney | BNITM (Hamburg) |

C

| Name | Strain | Origin |
|---|---|---|
| *Culex quinquefasciatus* Say 1823 | Malaysia | Bayer (Leverkusen, Germany) |
| *Culex torrentium* Martini 1925 | n/a | field-caught |
| *Culex pipiens* biotype *pipiens* Linnaeus 1758 | n/a | field-caught |
| *Culex pipiens* biotype *molestus* Forskal 1775 | S | field-collected in Heidelberg, Germany |
| | W | field-collected in Wendland area, Germany |
| | LL | field-collected in Langenlehsten, Germany |

### 2.1.9 Databases and programmes

**Table 2.9: Utilised databases and programmes**

| Database/Programme | Utilisation |
|---|---|
| NCBI http://www.ncbi.nlm.nih.gov/ | literature database and (Primer-) BLAST |
| GraphPad Prism 6 | statistical analysis and image design |
| Inkscape 0.91 | editing of vector graphics |
| Rotor Gene real time analysis 6.0 | qRT-PCR analysis |
| LightCycler Software Release 1.5.0 SP3 | qRT-PCR analysis |
| LightCycler Software 4.05 | qRT-PCR analysis |

## 2.2 Methods

Unless otherwise stated, all experimental methods explained in the following were performed under S2 conditions.

### 2.2.1 Virus production and titration

#### 2.2.1.1 Mammalian tissue culture

Vero cells were cultured at 37 °C as monolayers in supplemented DMEM with 10 % FCS (Table 2.4B). When used for titration experiments or WNV stock production, the concentration of FCS supplement was reduced to 2 %.

### 2.2.1.2 Virus propagation

To cultivate new WNV stock, Vero cells were seeded in a T75 cell culture flask (75 $cm^2$ culture area) in supplemented DMEM medium (incl. 2 % FCS; Table 2.4 B) at a density of $2.5x10^6$ cells/flask and incubated at 37 °C overnight to reach an even monolayer. The cells were then moved to S3 conditions, inoculated with existing WNV stock at an MOI of 0.01 and incubated at 37 °C until CPE was visible (approx. 7 days).

Once CPE was detected, the cell medium was transferred into 50 ml falcons and centrifuged at 1000 rpm for 10 min. The supernatant was then aliquoted into sterile 1.5 ml tubes and stored at -80 °C until titer determination via $TCID_{50}$ (2.2.1.3).

### 2.2.1.3 $TCID_{50}$

All procedures of the following $TCID_{50}$ methodology involving living inoculated cells (i.e. prior to fixation) are performed under S3 conditions. The entire essay is carried out at room temperature.

$3.84x10^6$ cells/96-well plate (corresponds to $4x10^4$ cells/well) were seeded in supplemented DMEM medium (incl. 10 % FCS; Table 2.4 B) and incubated at 37 °C overnight.

To analyse virus titers in the mosquito, organ homogenate was filtered using 0.20 µm filters and diluted in supplemented DMEM medium (incl. 2 % FCS; Table 2.4 B) to make 10-fold serial dilutions. When determining the titer of virus stock solution, no filtering of the medium containing the virus was necessary. The serial dilution of virus stock ranged from $10^{-1}$ to $10^{-11}$, whereas mosquito/mosquito organ homogenate was diluted $10^0$ to $10^{-4}$.

The Vero cells were then inoculated with 50 µl of virus dilution and incubated for 3 days at 37 °C. Inoculation with just DMEM medium (incl. 2 % FCS; Table 2.4 B) served as negative control. Due to the limited availability of filtered homogenate, a $10^0$ ''dilution'' was performed by incubating respective wells with 25 µl of undiluted filtered homogenate for 1 h and then adding medium up to 50 µl.

After the incubation period, inoculated Vero cells were fixed in 4 % formaldehyde for 30 min and, after incubation with PBT for a few minutes, blocked with 100 µl blocking solution/well for 30 min. Following 2 washing steps, the cells were then immunostained using WNV recombinant E protein mouse monoclonal antibody diluted 1:100 in PBT for 1-2 h (50 µl/well). After removal of the primary antibody via washing twice with PBT, the cells were incubated with fluorescein (FITC)-conjugated AffiniPure goat anti-mouse IgG diluted 1:200 in PBT for 1 h (50 µl/well). Once washed again using PBT, infected wells were counted under a fluorescent microscope and viral titers were calculated using the Spearman and Kärber algorithm described by Hierholzer and Killington (Hierholzer & Killington 1996).

### 2.2.2 Mosquito culture

#### 2.2.2.1 Collection and rearing of field-caught mosquitoes

Field-caught mosquitoes were obtained from egg raft collection carried out in Hamburg area in the North (Altes Land 53°35′N 9°32′E, Langenlehsten 53°30′N 10°44′E and Hamburg city 53°32′N, 9°57′E) and at Lake Constance (Radolfzell-Böhringen 47°44′N, 8°58′E and Mettnau 47°43′N, 8°59′E) in the south of Germany.

Gravid traps filled with a hay infusion were placed in proximity to natural breeding sites of the *Culex* mosquitoes to attract gravid females and encourage egg deposition. Traps were checked daily and deposited egg rafts were retrieved using a wooded spatula and placed into individual plastic cups for transportation to the laboratory.

The hatched larvae were reared in a climate chamber and under the same conditions as the mosquito lab strains (2.2.2.2). 4-5 larvae were removed to be used for DNA extraction (2.2.4.1) and subsequent molecular taxonomic identification (2.2.5.2.3).

Once identified, larvae of the same species were pooled and reared until adult status to be used for blood feed infection assays (2.2.3)

#### 2.2.2.2 Mosquito lab strains

The mosquito lab strains were reared in a climate chamber at 23 +/- 2 °C, a relative humidity of 80 % and 16 h : 8 h light:dark cycle. The adults were fed on cotton pads soaked with an 8 % fructose solution (Table 2.4 C). Weekly blood meals (Table 2.4 C), also presented on cotton pads, enabled egg production.

Egg rafts were transferred into plastic trays containing dechlorinated water. Hatched larvae were fed on tablet fish food and transferred into the cages once they had pupated.

#### 2.2.2.3 Tetracycline treatment

In order to cure the lab strain *Cx. pipiens* biotype *molestus* of their natural *Wolbachia* infection, the antibiotic tetracycline was applied. In contrast to previously published methodology focusing on larvae, adult mosquitoes were the treatment target (Suenaga 1993; Dobson & Rattanadechakul 2001).

The mosquito adults were fed with a fructose solution containing 8 % fructose and 0.5 mg/ml tetracycline (Table 2.4 C) for a duration of at least 5 days. After this time, rearing continued normally as explained above (2.2.2.2). Offspring pupae were transferred into a plastic cup containing dechlorinated water and were allowed to hatch in a different cage so as not to be mixed with their parent population. Again, adult mosquitoes were fed with the fructose-antibiotic solution.

This treatment was performed for 3 consecutive generations (F0, F1, F2), after which the *Wolbachia* status was checked using a gel PCR (2.2.5.1.1). Once found to be *Wolbachia*-free, the treatment seized and all subsequent offspring were pooled to form

a *Wolbachia*-free *Cx. pipiens* biotype *molestus* population large enough to be used for blood feed infection assays.

### 2.2.3 Blood feed infection assay

The blood feed infection assay was adapted on the basis of previously published protocols (Huber et al. 2014; Kilpatrick et al. 2010).

4 to 14 day old females were numbed using a $CO_2$-sleeper and sorted into plastic tubes at 10 females/tube. The females were then starved overnight at normal culture conditions. The following day, they were transferred into S3 conditions and received an overnight blood meal.

The blood meal contained 1-1.6x$10^7$ PFU WNV NY99 in addition to 10 % FCS and 50 % of an 8 % fructose solution and was offered on cotton Q-tips (Table 2.4 C) at 2 Q-tips/tube.

The next day, the females were again numbed using a $CO_2$-sleeper and all non-engorged females were discarded. 10 mosquitoes from each study group were then selected at random and frozen at -80 °C as 0-dpi-samples. The remaining individuals were kept in incubators at 18 °C or 25 °C and 80 % humidity and were sacrificed at 14, 21 or 35 dpi via freezing at -80 °C. As many individuals per study group per time point as possible were sacrificed, ideally at least 10.

### 2.2.4 DNA/RNA extraction, quantification and visualisation

#### 2.2.4.1 DNA extraction

Individual adult mosquitoes or pools of larvae were placed in sterile 1.5 ml tubes in preparation of DNA extraction. At this point, samples could be stored at -80 °C prior to performing the procedure.

The DNA extraction procedure was executed using the DNeasy Blood and Tissue kit (Protocol: Purification of Total DNA from Animal Tissues (Spin-Column Protocol)) according to manufacturer's instructions.

The concentration of the obtained DNA solution was determined via a photometric approach (2.2.4.3).

#### 2.2.4.2 Viral RNA extraction

Infected mosquitoes were individually sorted into sterile 1.5 ml tubes and were stored at -80 °C until viral RNA extraction.

If applicable, target organs (head and remaining body) were separated under a stereomicroscope into individual sterile tubes. The organs or individual whole mosquitoes were then triturated by a tissue homogeniser in 500 µl cell culture medium (DMEM or Schneider's Drosophila medium (supplemented or unsupplemented); Table 2.4 B).

The homogenate was then used for RNA extraction using the QIAamp Viral RNA Mini kit according to manufacturer's instructions. Prior to viral deactivation using viral lysis buffer, all procedures were performed under S3 conditions.

#### 2.2.4.3 Photometric determination of nucleic acid concentration

In order to determine the concentration and purity of DNA or RNA solutions, the absorbance at distinct wavelengths was measured using a nanophotometer. To do this, 1-2 µl of the solution was deployed and the light absorbance at 260 and 280 nm was measured. This method was originally developed by Warburg & Christian (1942).

The relationship between absorbance at 260 nm ($A_{260}$) and nucleic acid concentration is based on the following relationship, allowing the determination of DNA and RNA yield:

$A_{260}$ of 1 = 50 µg/ml dsDNA

$A_{260}$ of 1 = 40 µg/ml RNA

The purity of the nucleic acid solution is determined by calculating the ratio of absorbance at 260 and 280 nm.

$A_{260}$ / $A_{280}$ = purity value for nucleic acid solution

Pure DNA solution is expected to have an $A_{260}/A_{280}$ ratio of between 1.7 and 2.0, whereas pure RNA solution has a ratio of around 2.0.

#### 2.2.4.4 Agarose gel electrophoresis

Agarose gel electrophoresis is the common method applied for the separation of nucleic acid fragments according to size. The underlying theory is based on the fact that the negatively charged phosphate backbone of nucleic acids lead to movement of the DNA/RNA fragment through a porous agarose gel towards a positively charged anode within an electric field.

Due to the uniform mass-charge ratio of the nucleic acid fragments, the distance travelled within the agarose gel is inversely proportional to their molecular weight (Lee et al. 2012).

To separate PCR products, a 1 % agarose gel was prepared with TAE Buffer (Table 2.4 A) and supplemented with approximately 0.01 % ethidium bromide.

10 µl of PCR product was mixed with 2 µl 6X DNA loading buffer containing the colour markers bromophenol blue and xylene cyanol, which are used to visualize the ongoing separation of the DNA. In a 1 % agarose gel, bromophenol blue and xylene cyanol will run on the approximately same level as a 500 and 5000 bp DNA fragment respectively. Loading buffer additionally contains glycerol, which ensures that the samples and markers remain in their respective wells, and EDTA, which inhibits nucleases.

To label the sizes of separated PCR products, GeneRuler 100 bp and 1 kb DNA Ladders were applied. The agarose gel electrophoreses was then run for 1 h at 120 V.

Due to the DNA-intercalating ethidium bromide, the fragments could be visualized under UV light and documented using a digital camera.

### 2.2.5 PCR/qPCR

The polymerase chain reaction (PCR) has been developed to allow *in vitro* amplification of DNA using a thermostable DNA polymerase for diagnostic and research purposes (Saiki et al. 1988).

In general, a PCR-cycle is subdivided into 3 basic phases (Fig. 2.1). During the denaturing phase (1), the hydrogen bonds between the double-stranded DNA are broken due to high temperature. This gives small complementary oligonucleotides (primers) access to the individual sense and antisense strand of the template DNA and allows annealing to the respective 3' end (2). To mediate primer binding, the temperature is decreased to the so-called annealing temperature, which is dependent on primer design and is typically set 3-5 °C below the melting temperatures ($T_m$) of the primers. Subsequent increase of the temperature then allows 5' → 3' elongation of the primers along the DNA template by a DNA polymerase (3). This cycle is then continued until sufficient DNA copies have been made.

During the exponential amplification phase, the amount of PCR product doubles after every cycle (assuming 100 % efficiency). The PCR reaction then moves into an intermediate state, where the reaction slows down as PCR reagents such as dNTPs are consumed. Having reached the plateau phase, all limiting reagents have been consumed and, thus, no new product is made.

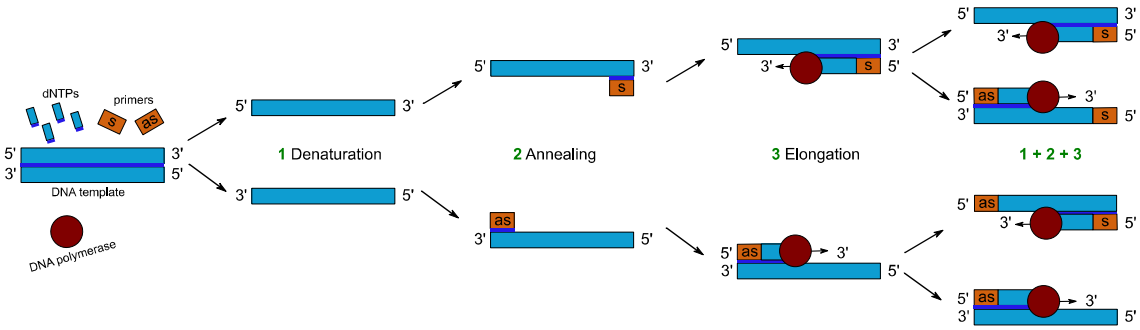



**Fig. 2.1: Basic concept of a polymerase chain reaction**
Primer are labelled as s (sense) and as (antisense).

Primers were ordered from the company Eurofins MWG Operon (Ebersberg, Germany), who also supplied the $T_m$ of the oligonucleotides. When necessary, a gradient PCR was performed to optimise the annealing temperature.

All utilised primers are summarised in Table 2.7.

#### 2.2.5.1 PCR

When using standard gel PCRs, the reaction is performed until end-point (i.e. until the plateau phase) and the resulting product is separated and visualised via agarose gel electrophoresis (2.2.4.4).

#### 2.2.5.1.1 *Wolbachia* detection

The protocol of the Wolbachia detection PCR was developed by Braig et al. 1998 and Zhou et al. 1998. To perform a *Wolbachia* detection PCR, two reaction set-ups were prepared for each sample. One reaction set-up included primers for the actual *Wolbachia* detection and the other functioned as a DNA control (Table 2.9 A).

**Table 2.10: *Wolbachia* detection PCR – pipetting scheme (A) and PCR programme (B)**
MM2 and MM3 include the template DNA as well as primers for *Wolbachia* detection (OSM_69 and OSM_70) and mosquito DNA (1725 and 1726 for *Cx. pipiens*; OSM_78 and OSM_79 for *Cx. torrentium*) respectively. MM1 includes all other reagents necessary and is added to MM2 and MM3 directly before PCR start. The full list of gene products and corresponding primer pairs are summarised in Table 2.7. All primers were utilised at a concentration of 10 pmol/µl.

A

| Master Mix | Reagent | Volume/sample [µl] |
|---|---|---|
| MM1 | 10X DreamTaq Green Buffer | 4 |
| | dNTPs (10 mM) | 1.6 |
| | $MgCl_2$ (25 mM) | 2 |
| | DreamTaq DNA Polymerase (5 U/µl) | 0.2 |
| | $ddH_2O$ | 27 |
| MM2 | MM1 | 17.4 |
| | Primer sense (OSM_69) | 0.8 |
| | Primer antisense (OSM_70) | 0.8 |
| | Template DNA (10 ng) | 1 |
| MM3 | MM1 | 17.4 |
| | Primer sense (1725 or OSM_78) | 0.8 |
| | Primer antisense (1726 or OSM_79) | 0.8 |
| | Template DNA (10 ng) | 1 |

B

| | Temperature [°C] | Time | # Cycles |
|---|---|---|---|
| **Initial denaturation** | 95 | 2 min | 1 |
| **Denaturation** | 95 | 30 s | 30 |
| **Annealing** | 50 | 1 min | |
| **Elongation** | 72 | 1 min 10 s | |
| **Final elongation** | 72 | 10 min | 1 |
| **Cooling** | 4 | ∞ | n/a |

### 2.2.5.1.2 *Wolbachia* strain typing

The Wolbachia strain typing PCR was developed by Duron et al. 2006. To perform a *Wolbachia* strain typing PCR, four reaction set-ups were prepared for each sample. Each of the four reaction set-ups includes two or three primer pairs, generating a different gene product of the *Wolbachia* WO prophage (Table 2.10 A). The full list of gene products and corresponding primer pairs are summarised in Table 2.7.

**Table 2.11: *Wolbachia* strain typing PCR – pipetting scheme (A) and PCR programme (B)**
MM2-5 include primers for the detection of different gene products of the *Wolbachia* WO prophage as well as the template DNA and $ddH_2O$. MM1 includes all other reagents necessary and is added to MM2-5 directly before PCR start. The full list of gene products and corresponding primer pairs are summarised in Table 2.7. All primers were utilised at a concentration of 10 pmol/µl.

A

| Master Mix | Reagent | Volume/sample [µl] |
|---|---|---|
| MM1 | 10X DreamTaq Green Buffer | 8 |
| | dNTPs (10 mM) | 3.2 |
| | $MgCl_2$ (25 mM) | 4 |
| | DreamTaq DNA Polymerase (5 U/µl) | 0.4 |
| MM2 | MM1 | 3.9 |
| | Primer sense (OSM_213) | 0.8 |
| | Primer antisense (OSM_214) | 0.8 |
| | Primer antisense (OSM_216) | 0.8 |
| | Primer sense (OSM_217) | 0.8 |
| | Primer sense (OSM_222) | 0.8 |
| | Primer antisense (OSM_224) | 0.8 |
| | Template DNA (100 ng) | 1 |
| | $ddH_2O$ | 10.3 |
| MM3 | MM1 | 3.9 |
| | Primer sense (OSM_215) | 0.8 |
| | Primer antisense (OSM_216) | 0.8 |
| | Primer sense (OSM_220) | 0.8 |
| | Primer antisense (OSM_221) | 0.8 |
| | Template DNA (100 ng) | 1 |
| | $ddH_2O$ | 11.9 |
| MM4 | Mastermix 1 | 3.9 |
| | Primer sense (OSM_218) | 0.8 |
| | Primer antisense (OSM_219) | 0.8 |
| | Primer sense (OSM_222) | 0.8 |
| | Primer antisense (OSM_223) | 0.8 |
| | Template DNA (100 ng) | 1 |
| | $ddH_2O$ | 11.9 |
| MM5 | Mastermix 1 | 3,9 |
| | Primer sense (OSM_225) | 0,8 |
| | Primer antisense (OSM_226) | 0,8 |
| | Primer sense (OSM_220) | 0,8 |

| | | |
|---|---|---|
| | Primer antisense (OSM_224) | 0,8 |
| | Template DNA (100 ng) | 1 |
| | $ddH_2O$ | 11,9 |

B

| | Temperature [°C] | Time | # Cycles |
|---|---|---|---|
| **Initial denaturation** | 94 | 2 min | 1 |
| **Denaturation** | 94 | 30 s | 30 |
| **Annealing** | 52 | 30 s | |
| **Elongation** | 72 | 1 min | |
| **Final elongation** | 72 | 10 min | 1 |
| **Cooling** | 4 | ∞ | n/a |

### 2.2.5.2 qRT-PCR

Quantitative real-time PCR (qRT-PCR) is a further development of the standard PCR that allows the detection of PCR-product as it is generated in real-time.

In this work, a TaqMan® probe carrying a fluorescent dye and a quencher has been utilised (originally developed by Heid et al. 1996). In its intact form, the probe ensures that the fluorescent signal of the dye is suppressed by the quencher.

The probe is further designed to bind to the target sequences. Upon elongation, the probe is cleaved via the 5' nuclease activity of the Taq DNA polymerase. This leads to special separation of the fluorescent dye and the quencher and to subsequent generation of a fluorescent signal (Fig. 2.2).

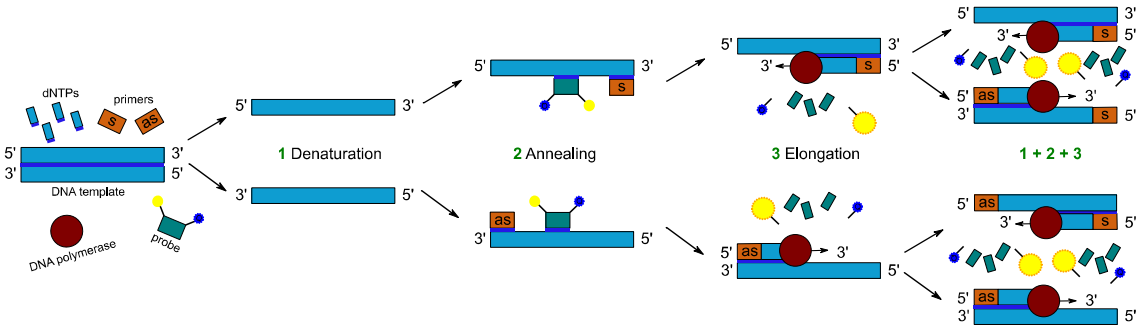



**Fig. 2.2: Basic concept of a qRT-PCR**
Primers are labelled as s (sense) and as (antisense). The quencher and fluorescent dye are depicted as a blue and yellow sphere on the probe respectively.

The read-out of the qRT-PCR is the so-called cycle threshold (CT), which is defined as the amount of PCR cycles necessary for the fluorescent signal to surmount background noise and grow exponentially. Notably, qRT-PCR focuses on the exponential phase of a PCR reaction, which is in contrast to the standard gel PCR.

The CT-value is especially useful to quantify the presence of an unknown amount of template. In this work, the standard curve methodology has been utilised, whereby a

serial dilution of known concentration of target template is analysed. When plotting the concentration of these standard samples to their respective CT-values, linear regression should generate a line, also known as a standard curve. The more efficient the PCR reaction, the closer $R^2$ is to the ideal value of 1.

Using the standard curve as well as their respective CT-values, the concentration of unknown samples can be interpolated.

#### 2.2.5.2.1 WNV detection

The WNV detection qRT-PCR is differentiated from the other qRT-PCRs utilised in this work in that it also involves a reverse-transcription step (Table 2.11B). As the PCR template is viral RNA, a reverse transcriptase present in the QuantiTect RT enzyme mix (kit utilised: QuantiTect Probe PCR kit (Table 2.5)) is required to transcribe the RNA into cDNA. This then acts as a template for the DNA polymerase and all subsequent PCR cycles.

This PCR protocol as well as primers and probes were developed by Prof. Dr. med. Dr. med. habil. Jonas Schmidt-Chanasit (Virology, BNITM, Hamburg).

**Table 2.12: WNV detection PCR – pipetting scheme (A) and PCR programme (B)**
The QuantiTect Probe PCR kit was utilised for this PCR setup. Information about this gene product and the corresponding primer pair is summarised in Table 2.7. All primers and probe were utilised at a concentration of 10 pmol/µl.

A

| Reagent | Volume/sample [µl] |
|---|---|
| dd$H_2O$ | 2.5 |
| 2X QuantiTect Mix | 5 |
| Primer sense (OSM_145) | 0.6 |
| Primer antisense (OSM_146) | 0.6 |
| Probe (OSM_147) | 0.2 |
| QuantiTect RT Mix | 0.1 |
| RNA template | 2 |

B

| | Temperature [°C] | Time | # Cycles |
|---|---|---|---|
| **Reverse transcription** | 50 | 20 min | 1 |
| **Initial denaturation** | 95 | 15 min | 1 |
| **Denaturation** | 95 | 5 s | 45 |
| **Annealing and elongation** | 60 | 60 s | |

#### 2.2.5.2.2 *Wolbachia* detection

The final qRT-PCR protocol for the detection of *Wolbachia* was developed as part of this work. Refer to section 3.2.1 for a detailed description.

The primers and probes were designed by Prof. Dr. med. Dr. med. habil. Jonas Schmidt-Chanasit (Virology, BNITM, Hamburg).

**Table 2.13: *Wolbachia* detection qRT-PCR –pipetting scheme (A) and PCR programme (B)** The HotStarTaq Master Mix kit was utilised for this PCR setup. Information about this gene product and the corresponding primer pair is summarised in Table 2.7. All primers and probe were utilised at a concentration of 10 pmol/µl.

A

| Reagent | Volume/sample [µl] |
|---|---|
| $MgCl_2$ (25 mM) | 1.8 |
| $ddH_2O$ | 0.8 |
| 2X HotStar Master Mix | 5 |
| Primer sense (OSM_323) | 0.6 |
| Primer antisense (OSM_324) | 0.6 |
| Probe (OSM_325) | 0.2 |
| DNA template | 1 |

B

| | Temperature [°C] | Time | # Cycles |
|---|---|---|---|
| **Initial denaturation** | 95 | 15 min | 1 |
| **Denaturation** | 95 | 5 s | 45 |
| **Annealing** | 55 | 60 s | |
| **Elongation** | 72 | 30 s | |

### 2.2.5.2.3 Molecular taxonomic identification

The qRT-PCR for molecular taxonomic identification of members of the *Culex* complex was designed by Rudolf et al. 2013. As more than one primer pair and probe are utilized in this PCR setup, it is also considered a multiplex qRT-PCR. In addition, the PCR programme also involves a touchdown step (Table 2.14 B). As the annealing temperature is gradually reduced until reaching its final value, the specificity of the PCR, especially when involving multiple primer pairs, is increased.

The full list of gene products and corresponding primer pairs are summarised in Table 2.7. The primer concentrations utilised are indicated in the PCR pipetting scheme (Table 2.14 A).

**Table 2.14: qRT-PCR for molecular taxonomic identification of members of the *Culex* complex – pipetting scheme (A) and PCR programme (B)**
The HotStarTaq Master Mix kit was utilised for this PCR setup. The full list of gene products and corresponding primer pairs are summarised in Table 2.6.
Within the PCR programme (B), the touchdown and standard cycling are marked in red and green respectively.

A

| Reagent | Stock concentration | Volume/sample [µl] |
|---|---|---|
| 2X HotStarTaq Master Mix | 2X | 10 |
| $MgCl_2$ | 25 mM | 2 |
| Primer sense (1725) | 30 pmol/µl | 0.2 |
| Primer antisense (1726) | 30 pmol/µl | 0.2 |
| Probe (1727) | 20 pmol/µl | 0.2 |
| Probe (1728) | 20 pmol/µl | 0.2 |
| Probe (1729) | 20 pmol/µl | 0.2 |
| Primer sense (OSM_78) | 10 pmol/µl | 0.9 |
| Primer antisense (OSM_79) | 10 pmol/µl | 1 |
| Probe (OSM_82) | 10 pmol/µl | 0.4 |
| $ddH_2O$ | n/a | 2.7 |
| DNA template | n/a | 2 |

B

| | Temperature [°C] | Time | # Cycles |
|---|---|---|---|
| **Initial denaturation** | 95 | 15 min | 1 |
| **Denaturation** | 95 | 15 s | 10 |
| **Annealing** | 65-60.5 | 20 s | |
| **Elongation** | 72 | 30 s | |
| **Denaturation** | 95 | 15 s | 40 |
| **Annealing** | 60 | 20 s | |
| **Elongation** | 72 | 30 s | |
| **Final elongation** | 40 | 30 s | 1 |

# 3 Results

## 3.1 The susceptibility of *Culex* mosquitoes native to Germany for WNV

WNV is a highly pathogenic arbovirus, which is primarily transmitted by *Culex ssp.* mosquitoes. Increasing incidences of West Nile virus disease outbreaks in various parts of Europe illustrate the growing concern of WNV infections for the continent (Chancey et al. 2015).

It is thus of interest to investigate the distribution of the *Culex* species, which are considered important vectors for WNV, in Germany. These are primarily *Cx. pipiens* biotype *pipiens* and *Cx. torrentium* (Chancey et al. 2015; Rudolf et al. 2013; Krüger et al. 2014).

### 3.1.1 The distribution of putative WNV vectors is subject to temporal and spatial variation

In order to investigate the distribution of putative WNV vectors in Germany, egg rafts were sampled at selected locations in both Northern and Southern Germany throughout the mosquito season from May to September. The field-collected egg rafts were floated separately in dechlorinated water and 4-5 larvae hatched from each egg raft were used for molecular taxonomic identification by qRT-PCR.

In 2012, egg rafts were sampled only in the North of Germany and a total of 344 rafts were analysed. *Cx. pipiens* biotype *pipiens* was the most prevalent species collected throughout all months with a maximum prevalence of 80 % (n=8) in September. Similarly to *Cx. pipiens* biotype *pipiens*, *Cx. torrentium* egg rafts were found in every sampling-month in 2012. However, its maximum prevalence, in July, stands at only 39 % (n=62). *Cx. pipiens* biotype *molestus* was sampled only in August and September with prevalences of 2 % (n=2) and 10% (n=1) respectively (Fig. 3.1).

In order to pick up on putative temporal changes in species distribution, egg raft collections in the North were repeated in 2013 with an added collection site (total egg rafts identified=428). In contrast to 2012, the majority of egg rafts identified in May, June and July were *Cx. torrentium* with a maximum species prevalence of 83 % in June (n=24). Considering *Cx. pipiens* biotype *pipiens*, the prevalence was 29 % (May, n=17; July, n=51) and 17 % (June, n=5) in the first three months. The prevalence increased, however, to 72 % (n=101) and 89 % (n=16) in August and September respectively. Notably, no *Cx. pipiens* biotype *molestus* egg rafts were sampled in 2013. Instead, one *Cx. pipiens* biotype *pipiens/molestus* Hybrid egg raft was identified in Northern Germany in August (Fig. 3.2).

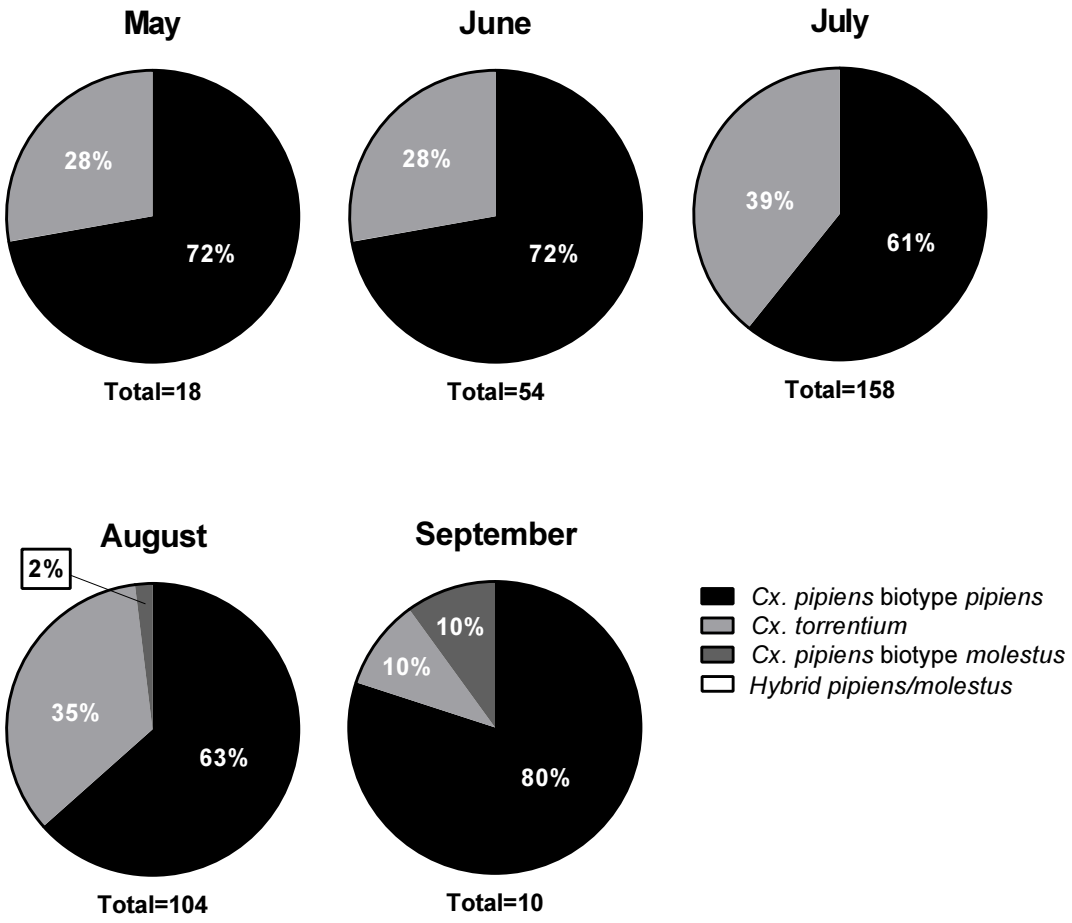



**Fig. 3.1: Distribution of putative WNV vectors in the North of Germany in 2012**
Using gravid traps to promote egg deposition, egg rafts were collected at two locations in Northern Germany (Langenlehsten 53°30′N 10°44′E and Hamburg city 53°32′N, 9°57′E). Each individual egg raft was floated separately in dechlorinated water. Once the larvae had hatched, 4-5 larvae were used for molecular taxonomic identification. The total number of egg rafts successfully identified per month is depicted below the respective pie chart.
In 2012, samples were collected and identified by my colleagues in the Molecular Entomology lab (refer to acknowledgements).

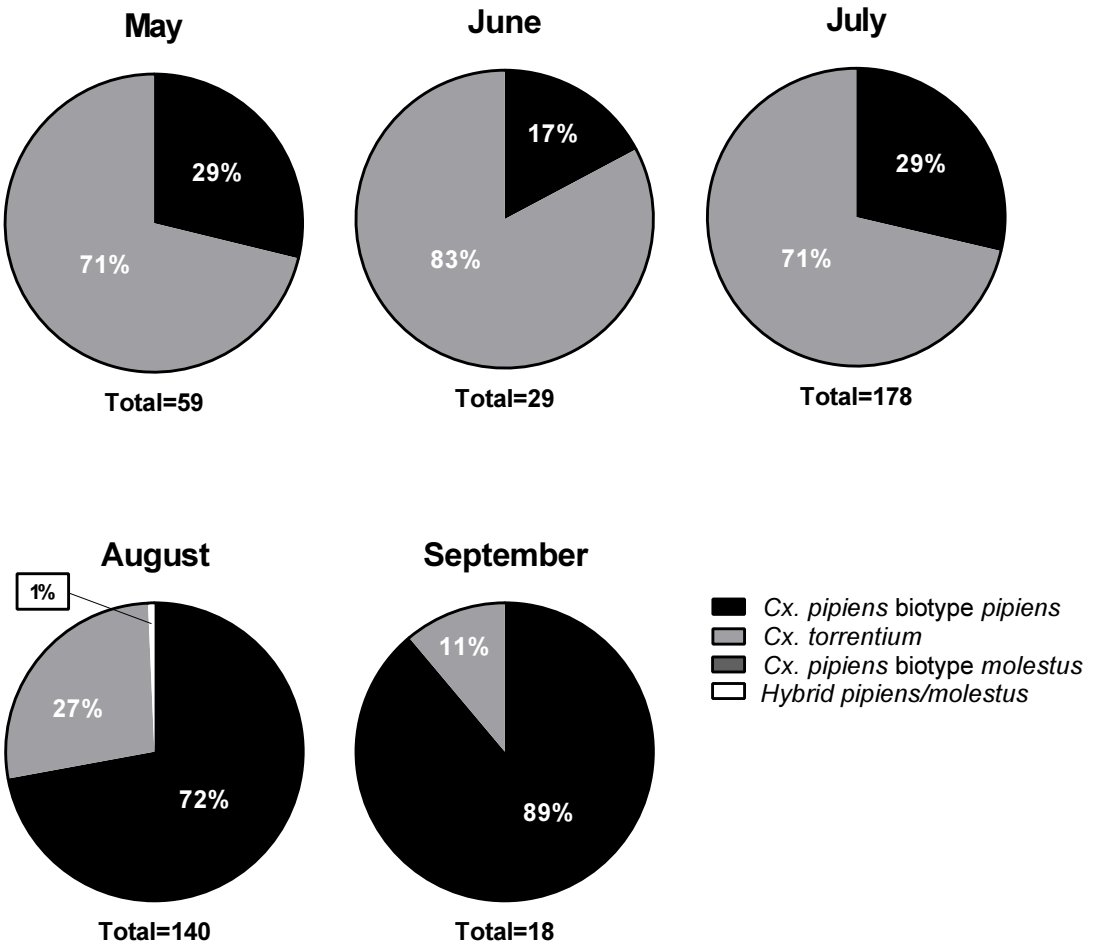



**Fig. 3.2: Distribution of putative WNV vectors in the North of Germany in 2013**
Using gravid traps to promote egg deposition, egg rafts were collected at three locations in Northern Germany (Altes Land 53°35′N 9°32′E, Langenlehsten 53°30′N 10°44′E and Hamburg city 53°32′N, 9°57′E). Each individual egg raft was floated separately in dechlorinated water. Once the larvae had hatched, 4-5 larvae were used for molecular taxonomic identification. The total number of egg rafts successfully identified per month is depicted below the respective pie chart.

It has been shown previously, that mosquito vector competence is subject to spatial and temporal variation (Hayes et al. 1984; Kilpatrick et al. 2010; Vaidyanathan & Scott 2006). It is thus important put mosquito populations from different sampling areas as well as from different sampling years under consideration when analysing vector competence. To account for this variation, egg raft sampling was repeated in 2013, with additional sampling sites also in the South of Germany (Lake Constance).

The prevalence of *Cx. pipiens* biotype *pipiens* was highest among all species sampled in both the North and South of Germany with 72 % (n=101) and 87.7 % (n=342) respectively. Notable, the occurrence of *Cx. torrentium* egg rafts varied with a prevalence of 27 % (n=38) in Northern and 7.4 % (n=29) in Southern Germany. One *Cx. pipiens* biotype *molestus* egg raft was found in the South, whereas none were found at Northern German sampling sites in 2013. This, however, correlates well with the higher prevalence

of *Cx. pipiens* biotype *pipiens/molestus* Hybrid egg rafts identified in the South (4.6 %; n=18) as opposed to the North of Germany (1 %; n=1) (Fig. 3.3).

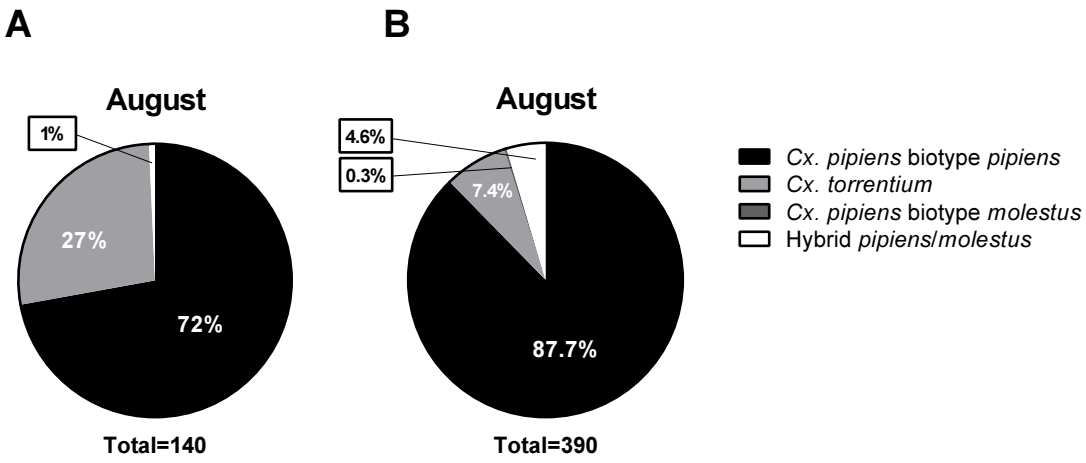



**Fig. 3.3: Distribution of putative WNV vectors in the North vs the South of Germany in August 2013**
Using gravid traps to promote egg deposition, egg rafts were collected at three locations in (**A**) Northern Germany (Altes Land 53°35′N 9°32′E, Langenlehsten 53°30′N 10°44′E and Hamburg city 53°32′N, 9°57′E) and one location in (**B**) Southern Germany (Lake Constance: Radolfzell-Böhringen 47°44′N, 8°58′E and Mettnau 47°43′N, 8°59′E). Each individual egg raft was floated separately in dechlorinated water. Once the larvae had hatched, 4-5 larvae were used for molecular taxonomic identification. The total number of egg rafts successfully identified at each location is depicted below the respective pie chart.

### 3.1.2 *Cx. pipiens* biotype *pipiens* and *Cx. torrentium* are susceptible to WNV infection

After having established the presence and varying distribution of putative WNV vectors through egg raft sampling, the next step was to investigate, whether or not these field-caught mosquitoes are susceptible to WNV infection.

To do this, a blood feed infection essay was established and calibrated using lab strains of the two WNV-vectors *Cx. quinquefasciatus* (Andreadis 2012; Richards et al. 2012; Fall et al. 2014) and *Cx. pipiens* biotype *molestus* (Thaori et al. 1955) as positive controls. The protocol is based on previously published protocols (Huber et al. 2014; Kilpatrick et al. 2010).

Briefly, 4-14 days old adult females were sorted into plastic containers and were fed with human blood containing 1.0 to 1.6x$10^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C or 18 °C and 80 % humidity in a climate chamber for 14 to 35 days. WNV-positive individuals were determined via WNV-specific qRT-PCR. In addition, selected mosquito homogenates were screened for infectious virus particles as explained in the following chapter.

#### 3.1.2.1 Calibration of the blood feed infection assay using lab strains of *Cx. quinquefasciatus* and *Cx. pipiens* biotype *molestus* as positive control

To verify the use of virus RNA detection via qRT-PCR as a proxy for WNV infection and to define the cut-off CT for qPCR detection, the mosquito homogenate identified as WNV RNA-positive had to be screened for viable virus particles.

To do this, a $TCID_{50}$ assay applying fluorescence-tagged antibody staining was employed. Briefly, Vero cells were infected with serial dilutions of mosquito homogenate previously filtered through 0.2 µm pore filters and incubated at 37 °C for 3 days. Cells treated only with medium functioned as negative controls and accounted for background signals visible as diffuse green colouration (Fig. 3.4). The cells were then fixed using 4 % formaldehyde, antibody- tagged and stained and subsequently viewed under an inverse fluorescence microscope (Fig. 3.4 and 3.5). Viral titers were calculated using the Spearman and Kärber algorithm described by Hierholzer and Killington (Hierholzer & Killington 1996).

Upon detection of virus RNA via qRT-PCR, mosquito samples from day 0 post infection of both *Cx. quinquefasciatus* and *Cx. pipiens* biotype *molestus* were selected at random from at least 3 independent blood feed assays and analysed for viable, infectious virus particles. The results indicated that all samples that were identified as positive for WNV RNA by qRT-PCR were also positive for viable virus particles. Virus titers on day 0 post infection for *Cx. quinquefasciatus* ranged from $1.38x10^1$ PFU/ml as the lowest to $7.76x10^3$ PFU/ml as the highest titer measured respectively, whereas the lowest titer measured for *Cx. pipiens* biotype *molestus* at day 0 post infection was $7.76x10^0$ PFU/ml and the highest titer $7.76x10^4$ PFU/ml (Table 6.7; supplementary materials). Subsequently, the same procedure was performed with day 0-samples from *Cx. torrentium* and *Cx. pipiens* biotype *pipiens*. In both cases, all qRT-PCR positive samples were also positive for infectious viral particles. The lowest and highest viral titers in *Cx. torrentium* were measured at $7.76x10^0$ and $7.76x10^2$ PFU/ml respectively (Table 6.7; supplementary materials). In the case of *Cx. pipiens* biotype *pipiens*, the lowest viral titer was $1.38x10^1$ PFU/ml and the highest $2.45x10^2$ PFU/ml (Table 6.7; supplementary materials).

To further verify the correlation between viral RNA and infectious viral particles at different time points after infection, selected samples from at least three different blood feed assays at day 14 and 21 post infection were tested. The results indicated that, all samples with a CT < 35 (n=81) were found to contain infectious virus particles (Table 6.7; supplementary materials). In contrast, 23 out of 34 samples from day 14 and 21 with a CT ≥ 35 were found to contain viable virus (Table 6.7; supplementary materials).

Based on these results, the CT cut-off was placed at 35 and was used in all blood feed assays. It is important to note, that only experiments where all samples from day 0 post infection were found positive were included into the analysis, in order to make sure that all mosquitoes have been successfully infected with WNV and loss of infection on subsequent days are not due to a lack of initial infection.

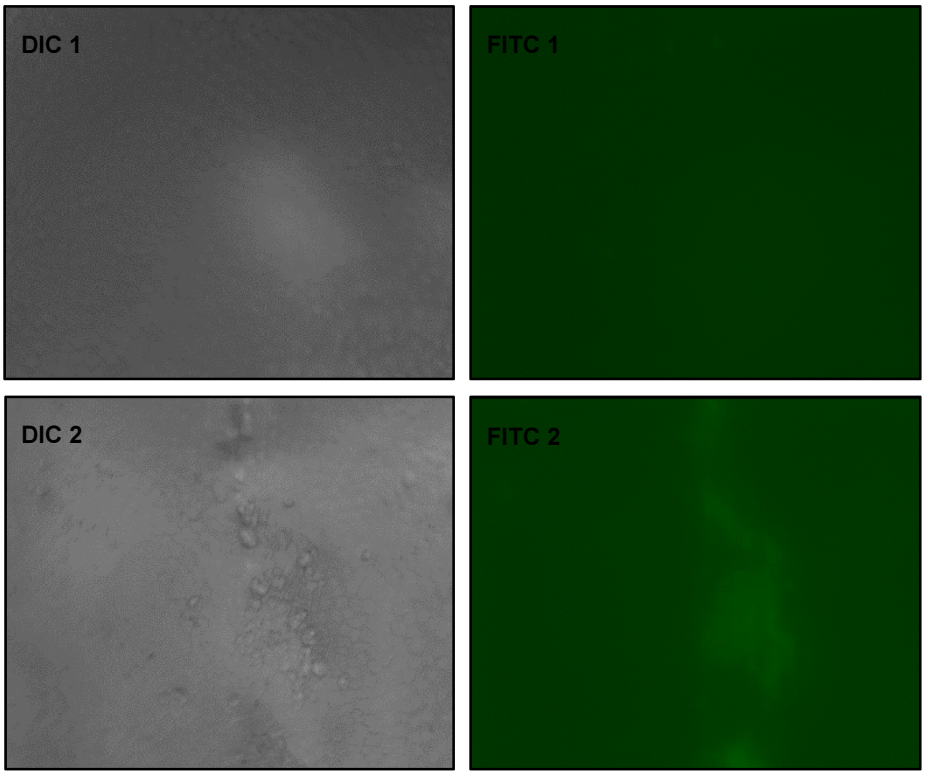



**Fig. 3.4: Vero cells ''infected'' with mosquito homogenate free of viable WNV particles illustrating the background signal of $TCID_{50}$ staining.**
Vero cells were infected with serial dilutions of mosquito homogenate previously filtered through a 0.2 µm pore filter and incubated at 37 °C for 3 days. The cells were then fixed using 4 % formaldehyde and tagged with a WNV recombinant E protein mouse monoclonal antibody. Staining was performed using a fluorescein (FITC)-conjugated AffiniPure goat anti-mouse IgG secondary antibody. The samples were then viewed under an inverse fluorescence microscope. DIC=differential interference contrast

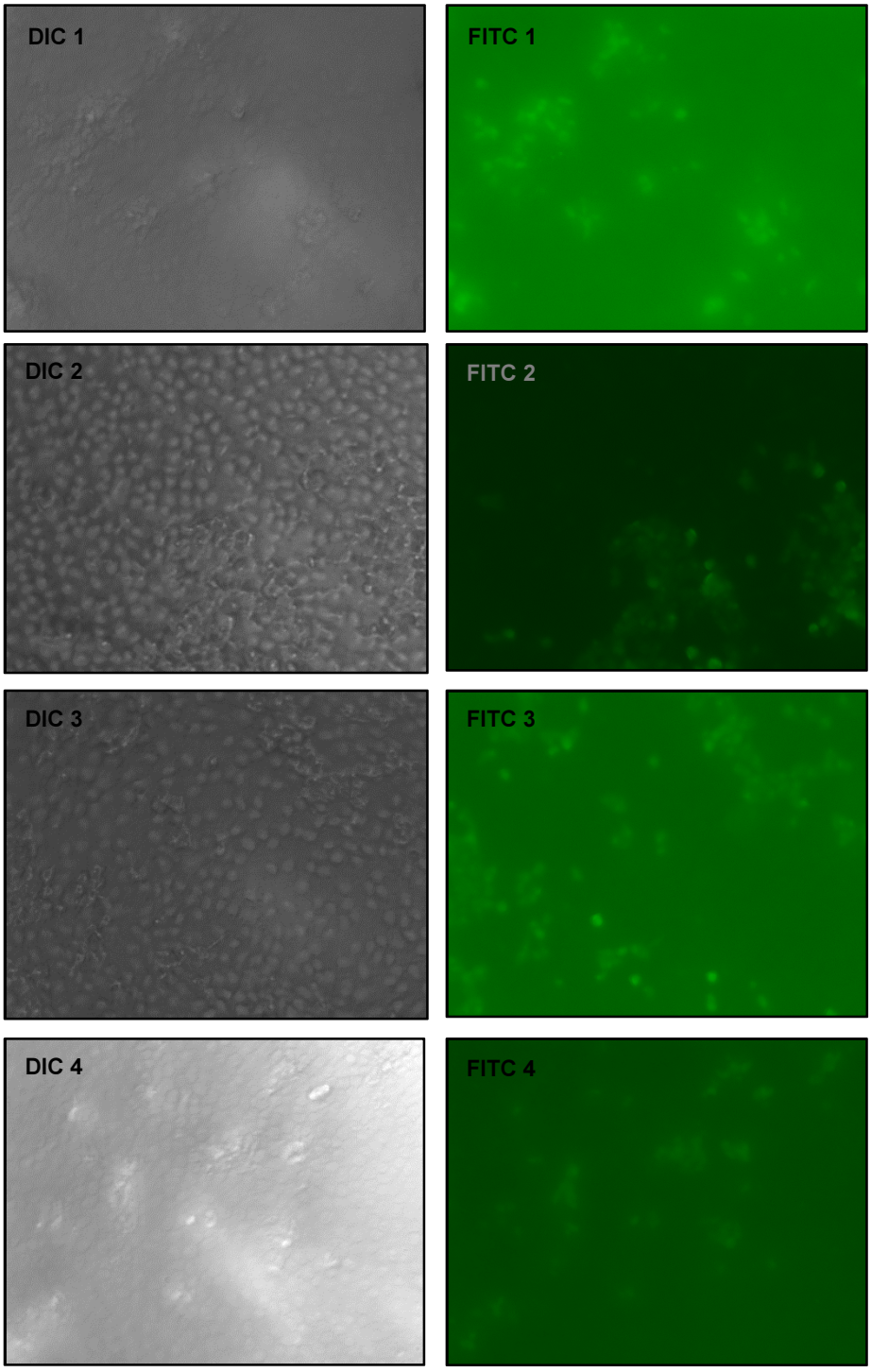



**Fig. 3.5: Vero cells infected with mosquito homogenate containing viable WNV particles illustrating the positive signal of $TCID_{50}$ staining.**
Vero cells were infected with serial dilutions of mosquito homogenate previously filtered through a 0.2 µm pore filter and incubated at 37 °C for 3 days. The cells were then fixed using 4 % formaldehyde and tagged with a WNV recombinant E protein mouse monoclonal antibody. Staining was performed using a fluorescein (FITC)-conjugated AffiniPure goat anti-mouse IgG secondary antibody. The samples were then viewed under an inverse fluorescence microscope.
DIC=differential interference contrast

The infection rates of *Cx. quinquefasciatus* and *Cx. pipiens* biotype *molestus* were investigated using the established qRT-PCR. The infection rate was calculated by determining the percentage of WNV-positive females with respect to the total number of blood-fed females.

In the case of *Cx. quinquefasciatus*, the maximum infection rate at both temperatures was reached at day 28 post infection (25 °C: 78 %, n=41; 18 °C: 82 %, n=38). There were no statistically significant differences between the infection rates at the two temperatures throughout the entire sampling period (Fig 3.6, **A**).

Looking at the data obtained for *Cx. pipiens* biotype *molestus*, the maximum infection rate was obtained at an incubation temperature of 18 °C on day 14 post infection (67 %, n=15). Significantly different infection rates between the two temperatures 25 °C and 18 °C were found only at day 35 post infection with infection rates of 63 % (n=90) and 42 % (n=67) respectively (Fig. 3.6, **B**).

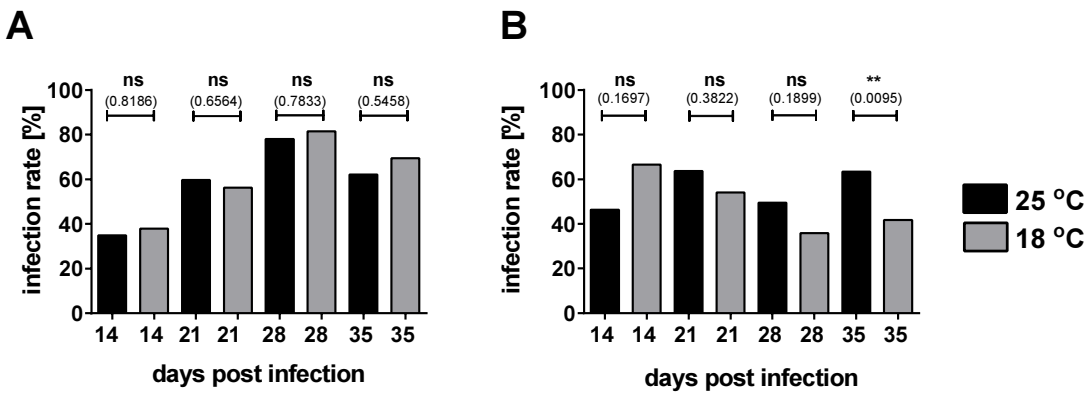



**Fig. 3.6: WNV infection rate of lab strains *Cx. quinquefasciatus* and *Cx. pipiens* biotype *molestus***

Adult females were sorted into plastic containers 4-14 days after emergence and received human blood containing 1–1.6x$10^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C or 18 °C and 80 % humidity in a climate chamber for 14 to 35 days.

The graph depicts the WNV infection rate of *Cx. quinquefasciatus* (**A**) and *Cx. pipiens* biotype *molestus* (**B**) at 25 °C (black) and 18 °C (grey) as determined by a WNV-specific qRT-PCR (CT cut-off = 35 cycles). Detailed number of individuals and number of independent blood feed assays are depicted in Table 6.1 and Table 6.2 (supplementary material). The data shown here are pooled data sets from the Northern and Southern populations. Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

### 3.1.2.2 Temperature dependency of the WNV infection rate differs between mosquito species

Having calibrated and confirmed the usability of the blood feed infection assay to investigate susceptibility to WNV, it was then possible to perform the assay using the field-caught mosquito samples. Detailed number of individuals and the number of independent blood feed assays are depicted in Table 6.1, 6.2 and 6.3 (supplementary material).

#### 3.1.2.2.1 *Cx. torrentium*

*Cx. torrentium* displayed a very high infection rate throughout the assay timeline with a maximum of 96 % (n=46) at 25 °C on day 21 post infection. Notably, significant differences between 18 and 25 °C were observed on day 14, 21 and 35 days post infection. Incubation at 25 °C for 14 and 21 days revealed significantly higher infection rates than incubation at 18 °C (Fig. 3.7).

The dynamics of viral infection seemed temperature-dependent. At 25 °C, infection rates of 83 % (n=12) were detected as early as 14 days post infection, whereas only 17 % (n=12) of females were tested positive at 18 °C incubation temperature on the same time point. However, the infection rates in *Cx. torrentium* at 18 °C increased over time and were found to be significantly higher (46 percentage points) compared to the infection rates at 25 °C at day 35 post infection (Fig. 3.7).

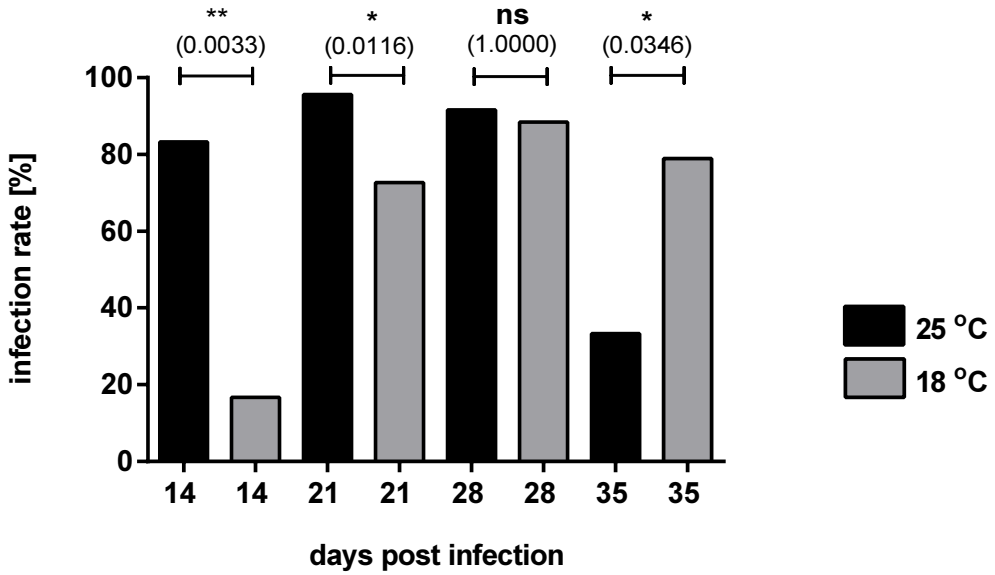



**Fig. 3.7: WNV infection rate of field-caught *Cx. torrentium* in 2013**
Adult females were sorted into plastic containers 4-14 days after emergence and received human blood containing 1–1.6x$10^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C or 18 °C and 80 % humidity in a climate chamber for 14 to 35 days. The graph depicts the WNV infection rate of *Cx. torrentium* at 25 °C (black) and 18 °C (grey) as determined by a WNV-specific qRT-PCR (CT cut-off = 35 cycles). Detailed number of individuals and number of independent blood feed assays are depicted in Table 6.1 and Table 6.2 (supplementary material). The data shown here are pooled data sets from the Northern and Southern populations. Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

### 3.1.2.2.2 *Cx. pipiens* biotype *pipiens*

WNV infection rates of *Cx. pipiens* biotype *pipiens* were slightly lower than those of *Cx. torrentium* with a maximum infection rate of 75 % (n=67) at 18 °C on day 21 post infection. The percentage of WNV RNA-positive females increased over time at both temperatures with maximum infection rates at 21 days post infection in both cases. Notably, there were no statistically significant differences between the infection rate at 25 °C and 18 °C (Fig. 3.8).

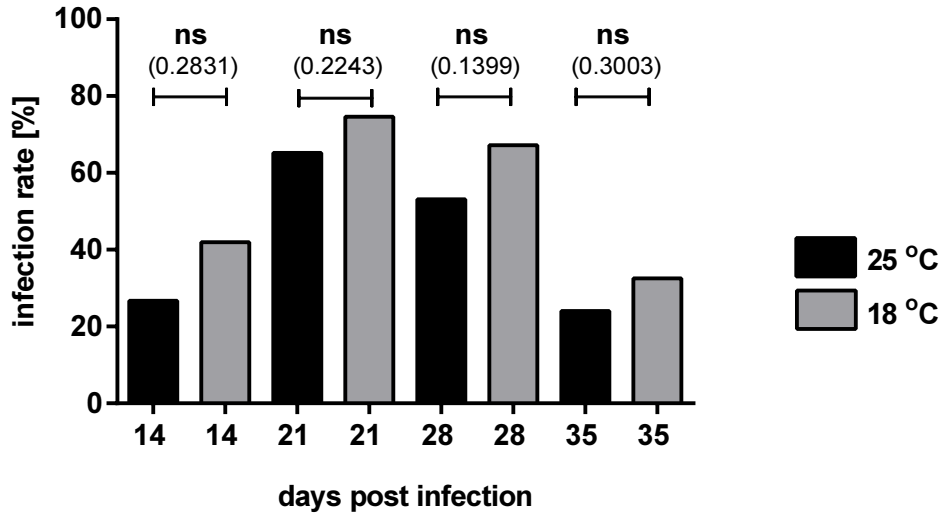



**Fig. 3.8: WNV infection rate of field-caught *Cx. pipiens* biotype *pipiens* in 2013**
Adult females were sorted into plastic containers 4-14 days after emergence and received human blood containing 1–1.6x10$^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C or 18 °C and 80 % humidity in a climate chamber for 14 to 35 days.
The graph depicts the WNV infection rate of *Cx. pipiens* biotype *pipiens* at 25 °C (black) and 18 °C (grey) as determined by a WNV-specific qRT-PCR (CT cut-off = 35 cycles). Detailed number of individuals and number of independent blood feed assays are depicted in Table 6.1 and Table 6.2 (supplementary material). The data shown here are pooled data sets from the Northern and Southern populations. Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

### 3.1.2.3 Spatial variation of the WNV infection rate is species dependent

It is highly likely that WNV would be introduced into Germany via infected hosts, i.e. migratory birds from the South of Europe (Ciota & Kramer 2013; Engler et al. 2013). Thus, it has to establish an endemic infection cycle in Southern Germany before being able to spread to other areas of the country.

Thus, in addition to analyses of field-caught mosquitoes from the North of Germany, it was of interest to investigate putative spatial separation-induced variation of WNV susceptibility in *Cx. torrentium* and *Cx. pipiens* biotype *pipiens* mosquitoes from the South of Germany.

To do so, the data obtained from field-caught mosquitoes in 2013 were stratified according to sample origin – Northern Germany (Altes Land 53°35′N 9°32′E, Langenlehsten

53°30′N 10°44′E and Hamburg city 53°32′N, 9°57′E) and Southern Germany (Lake Constance: Radolfzell-Böhringen 47°44′N, 8°58′E and Mettnau 47°43′N, 8°59′E). In order to be able to analyse as many samples as possible from the same incubation period, only data from day 21 post infection were included.

#### 3.1.2.3.1 *Cx. torrentium*

In the case of *Cx. torrentium*, the infection at 18 °C differed by 44 percentage points between the Northern (87 %, n=15) and Southern (43 %, n=7) populations. However, this difference was not statistically significant according to analysis with the Fisher's exact test. Looking at the data obtained at 25 °C incubation temperature, similar infection rates were observed for *Cx. torrentium* from the North (94 %, n=34) and South (100 %, n=12) of Germany (Fig. 3.9).

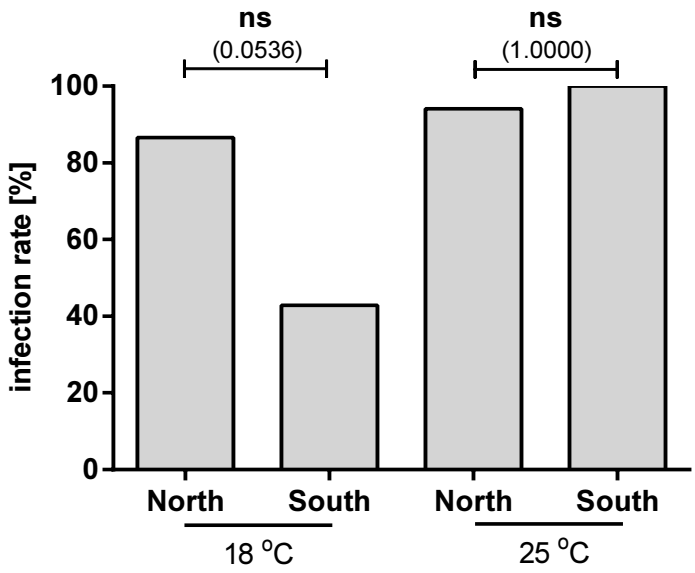



**Fig. 3.9: WNV infection rate of two field-caught *Cx. torrentium* populations from Northern and Southern Germany in 2013**
Adult females were sorted into plastic containers 4-14 days after emergence and received human blood containing 1–1.6x10$^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C or 18 °C and 80 % humidity in a climate chamber for 14 to 35 days.
The graph depicts the WNV infection rate of *Cx. torrentium* population from Northern (Altes Land 53°35′N 9°32′E, Langenlehsten 53°30′N 10°44′E and Hamburg city 53°32′N, 9°57′E) and Southern Germany (Lake Constance: Radolfzell-Böhringen 47°44′N, 8°58′E and Mettnau 47°43′N, 8°59′E) at 25 °C and 18 °C, respectively, and 21 days post infection. The infection rate is determined by a WNV-specific qRT-PCR (CT cut-off = 35 cycles). Detailed number of individuals and number of independent blood feed assays are depicted in Table 6.1 and Table 6.2 (supplementary material). Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

#### 3.1.2.3.2 *Cx. pipiens* biotype *pipiens*

*Cx. pipiens* biotype *pipiens* revealed significant differences between the infection rates of the Southern and Northern populations at both temperatures. At 18 °C, the infection rate of the South (88 %, n=26) was 22 percentage points higher than the rate of the North (66 %, n=41). When incubated at 25 °C, mosquitoes from the South show an infection

rate 37 percentage points higher (92 %, n=24) than the mosquito population from the North (55 %, n=65) (Fig. 3.10).

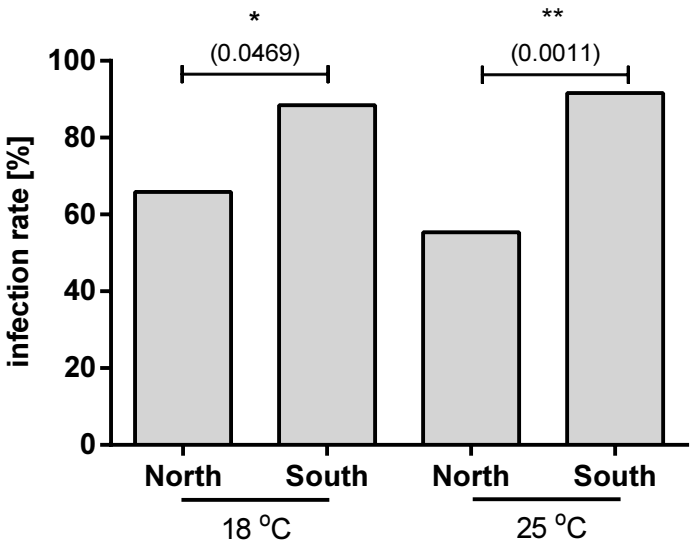



**Fig. 3.10: WNV infection rate of two field-caught *Cx. pipiens* biotype *pipiens* populations from Northern and Southern Germany in 2013**
Adult females were sorted into plastic containers 4-14 days after emergence and received human blood containing 1–1.6x$10^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C or 18 °C and 80 % humidity in a climate chamber for 14 to 35 days.
The graph depicts the WNV infection rate of *Cx. pipiens* biotype *pipiens* population from Northern (Altes Land 53°35′N 9°32′E, Langenlehsten 53°30′N 10°44′E and Hamburg city 53°32′N, 9°57′E) and Southern Germany (Lake Constance: Radolfzell-Böhringen 47°44′N, 8°58′E and Mettnau 47°43′N, 8°59′E) at 25 ºC and 18 ºC, respectively, and 21 days post infection. The infection rate is determined by a WNV-specific qRT-PCR (CT cut-off = 35 cycles). Detailed number of individuals and number of independent blood feed assays are depicted in Table 6.1 and Table 6.2 (supplementary material). Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

### 3.1.2.4 WNV infection rate seems to underlie temporal variation

In addition to spatial variation, susceptibility for WNV infection of a particular mosquito population may also be subject to temporal variation (Kilpatrick et al. 2010; Vaidyanathan & Scott 2006; Hayes et al. 1984). To investigate this possibility, the data obtained from populations in Northern Germany in 2013 were compared to data obtained by my colleagues (refer to acknowledgements) using the same methodology in 2012.

Comparing the infection rates of *Cx. pipiens* biotype *pipiens*, a significantly higher infection rate was measured in 2013 (23 %, n=22) than in 2012 (5 %, n=94) at 14 days post infection. On the other hand, there were no significant differences in infection rates between the two years when the mosquitoes were incubated for 21 days.
In the case of *Cx. torrentium*, the infection rates were generally higher than with *Cx. pipiens* biotype *pipiens* in both subsequent years. Notably, however, there were no significant difference in infection rate at 14 days (2012: 55 %, n= 130; 2013: 83 %, n=12), but at 21 days post infection (2012: 69 %, n=138; 2013: 94 %, n=34) (Fig. 3.11).

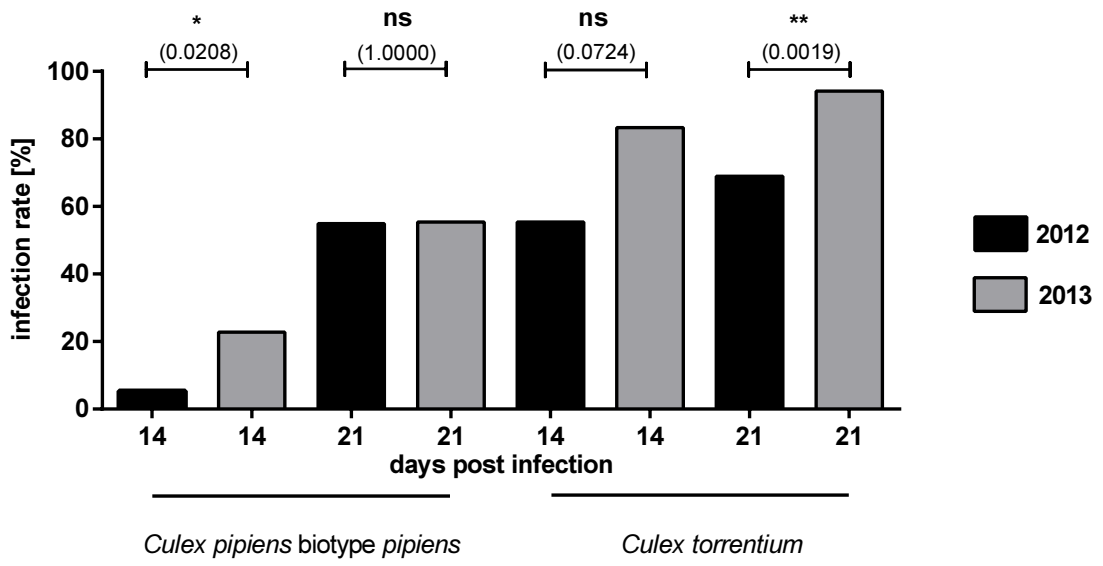



**Fig. 3.11: WNV infection rate of field-caught *Cx. pipiens* biotype *pipiens* and *Cx. torrentium* from Northern Germany at 25 °C in 2012 and 2013**
Adult females were sorted into plastic containers 4-14 days after emergence and received human blood containing 1–1.6x$10^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C or 18 °C and 80 % humidity in a climate chamber for 14 to 35 days.
The graph depicts the WNV infection rate of *Cx. pipiens* biotype *pipiens* and *Cx. torrentium* population from Northern Germany (Altes Land 53°35′N 9°32′E, Langenlehsten 53°30′N 10°44′E and Hamburg city 53°32′N, 9°57′E) at 25 °C. The infection rate is determined by a WNV-specific qRT-PCR (CT cut-off = 35 cycles). For this graph, infection rates from 2013 are matched with data from 2012. Detailed number of individuals and number of independent blood feed assays are depicted in Table 6.1 and Table 6.2 (supplementary material). Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.
In 2012, data was generated by my colleagues in the Molecular Entomology lab (refer to acknowledgements).

### 3.1.2.5 WNV dissemination rates in *Cx. pipiens* biotype *pipiens* and *Cx. torrentium* do not seem to be species-dependent

WNV enters its vertebrate host through saliva expelled by infected mosquitoes during blood-feeds. It is therefore essential for the virus to reach the salivary glands of the mosquito in order to get transmitted.

To be able to assess the vector competence of a mosquito species for WNV, it is necessary to analyse not only infection rates but also dissemination rates. The latter is an indicator of movement of viral particles in the mosquito.

In this work, the dissemination rate is determined by separate testing for virus RNA in head and body (= everything excluding the head) via qRT-PCR (CT cut-off =35) and calculating the number of WNV-positive heads with respect to the amount of WNV-positive mosquito females.

### 3.1.2.5.1 *Cx. torrentium*

In the case of *Cx. torrentium*, the maximum dissemination rate at 25 °C (100 %, n=11) and 18 °C (91 %, n=23) incubation temperature was reached 28 days post infection (Fig. 3.12).

Notably, the dissemination rate was uniformly high (>60 %) at 21, 28 and 35 days post infection, with no significant differences between the two temperatures analysed (Fig. 3.12).

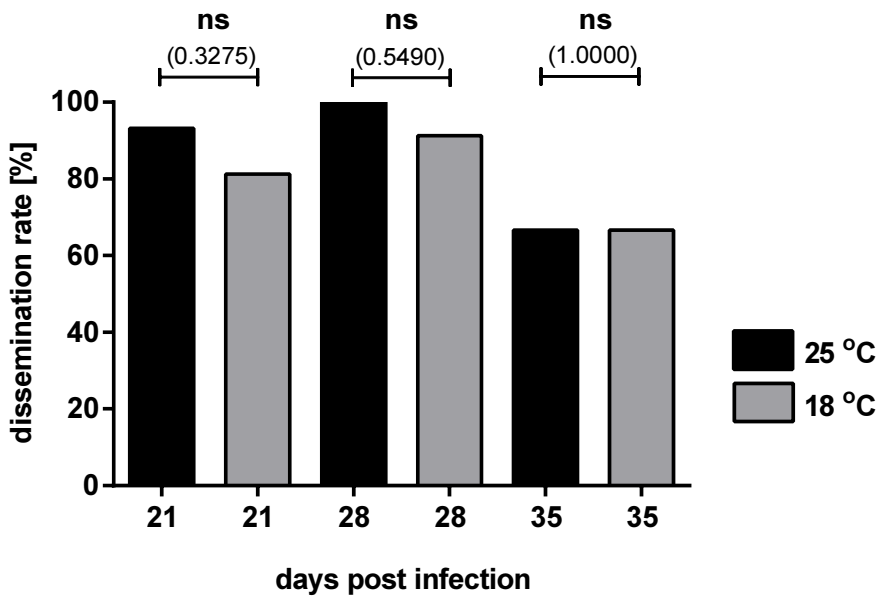



**Fig. 3.12: WNV dissemination rate of field-caught *Cx. torrentium* in 2013**
Adult females were sorted into plastic containers 4-14 days after emergence and received human blood containing 1–1.6x$10^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C or 18 °C and 80 % humidity in a climate chamber for 14 to 35 days.
The graph depicts the WNV dissemination rate of *Cx. torrentium* at 25 °C (black) and 18 °C (grey) as determined by a WNV-specific qRT-PCR (CT cut-off = 35 cycles). Detailed number of individuals and number of independent blood feed assays are depicted in Table 6.1 and Table 6.3 (supplementary material). The data shown here are pooled data sets from the Northern and Southern populations. Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

### 3.1.2.5.2 *Cx. pipiens* biotype *pipiens*

In the case of *Cx. pipiens* biotype *pipiens*, the maximum dissemination rate at 25 °C was reached on day 28 post infection (94 %, n=34). When incubated at 18 °C, the maximum dissemination rate was reached at 21 days post infection already (88 %, n=50) (Fig. 3.13).

Comparable to the situation with *Cx. torrentium* (Fig. 3.12), there were no significant differences between the WNV dissemination rates in *Cx. pipiens* biotype *pipiens* at 18 °C and 25 °C throughout the entire sampling period (Fig. 3.13).

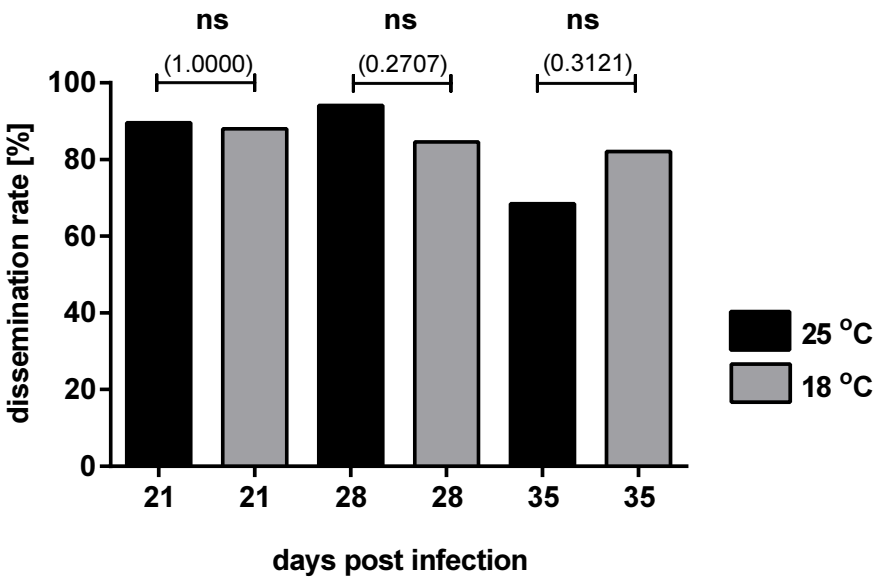



**Fig. 3.13: WNV dissemination rate of field-caught *Cx. pipiens* biotype *pipiens* in 2013**
Adult females were sorted into plastic containers 4-14 days after emergence and received human blood containing 1–1.6x$10^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C or 18 °C and 80 % humidity in a climate chamber for 14 to 35 days.
The graph depicts the WNV dissemination rate of *Cx. pipiens* biotype *pipiens* at 25 °C (black) and 18 °C (grey) as determined by a WNV-specific qRT-PCR (CT cut-off = 35 cycles). Detailed number of individuals and number of independent blood feed assays are depicted in Table 6.1 and Table 6.3 (supplementary material). The data shown here are pooled data sets from the Northern and Southern populations. Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

## 3.2 Influence of *Wolbachia* infection on the susceptibility of *Culex* mosquitoes native to Germany for WNV

The results presented here indicate that the various German *Culex* species focused on in this work are susceptible to WNV infection. In addition, temperature, an abiotic and extrinsic factor, was found to influence susceptibility in a species-dependent manner.

Next, it is of interest, whether *Wolbachia* infections may influence susceptibility of German *Culex* mosquitoes to WNV infection as has been previously shown in other species (Hussain et al. 2013; Glaser & Meola 2010).

As a first step, it is necessary to determine the *Wolbachia* infection status of the local mosquito populations.

### 3.2.1 Establishment of qRT-PCR specific for *Wolbachia* for high-throughput screening

For the determination of *Wolbachia* infection, a qRT-PCR was designed and optimised as a tool for high-throughput screening with minimal contamination risk.

Primers were designed on the basis of sequences available for the wsp-gene of the *Wolbachia* known to infect *Culex* mosquitoes. The primer pair (and probe) generate a 82 bp product and fit within the PCR product generated by the *Wolbachia* detection PCR published previously (Braig et al. 1998; Zhou et al. 1998).

As a working basis, the first protocol for the *Wolbachia* qRT-PCR was based on the WNV detection qRT-PCR used to screen for WNV (using the QuantiTect Probe RT-PCR kit from Qiagen). Using this combination of new primers and old protocol led to a PCR with low efficiency and non-sigmoid amplification curves (Fig. 3.14 A and B).

To improve PCR reaction conditions, $MgCl_2$ titrations were performed as well as a gradient PCR to determine the ideal annealing temperature. The PCR was then repeated under optimised conditions (6 mM $MgCl_2$ and 55 °C annealing temperature). The results indicated a much better reaction efficiency, albeit still below 90 % (Fig. 3.14 C and D).

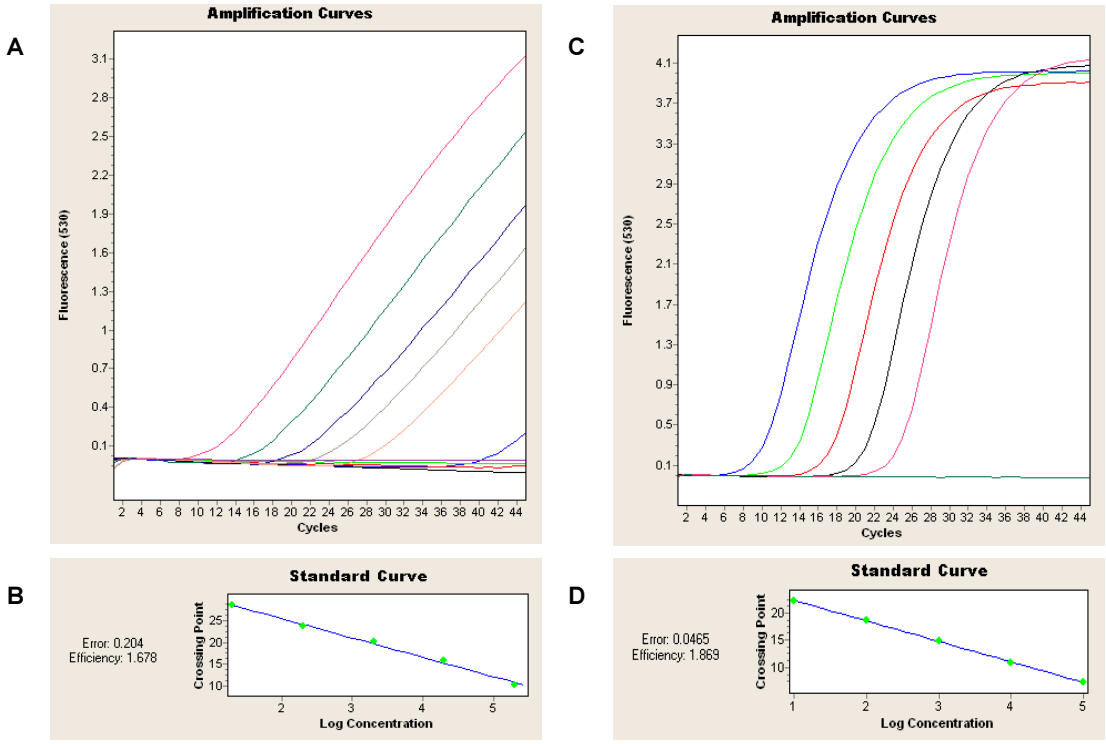



**Fig. 3.14: Amplification and standard curves of *Wolbachia* qRT-PCR obtained with QuantiTect Probe RT-PCR kit (Qiagen) and the LightCycler 2.0 (Roche)**
**A** and **B**: Amplification and standard curves obtained with newly designed primers targeting the wsp-gene and the PCR protocol used for WNV diagnostics
**C** and **D**: Amplification and standard curves obtained with optimised PCR conditions (6mM $MgCl_2$ and 55 °C annealing temperature)

To further improve the PCR reaction efficiency, an alternative PCR kit (HotStarTaq Master Mix kit from Qiagen) was tested leading to a reaction efficiency of 91% (Fig. 3.15 A and B). Once optimised thus far, all screenings were performed on the RotorGene 6000 (Corbett Research, Qiagen).

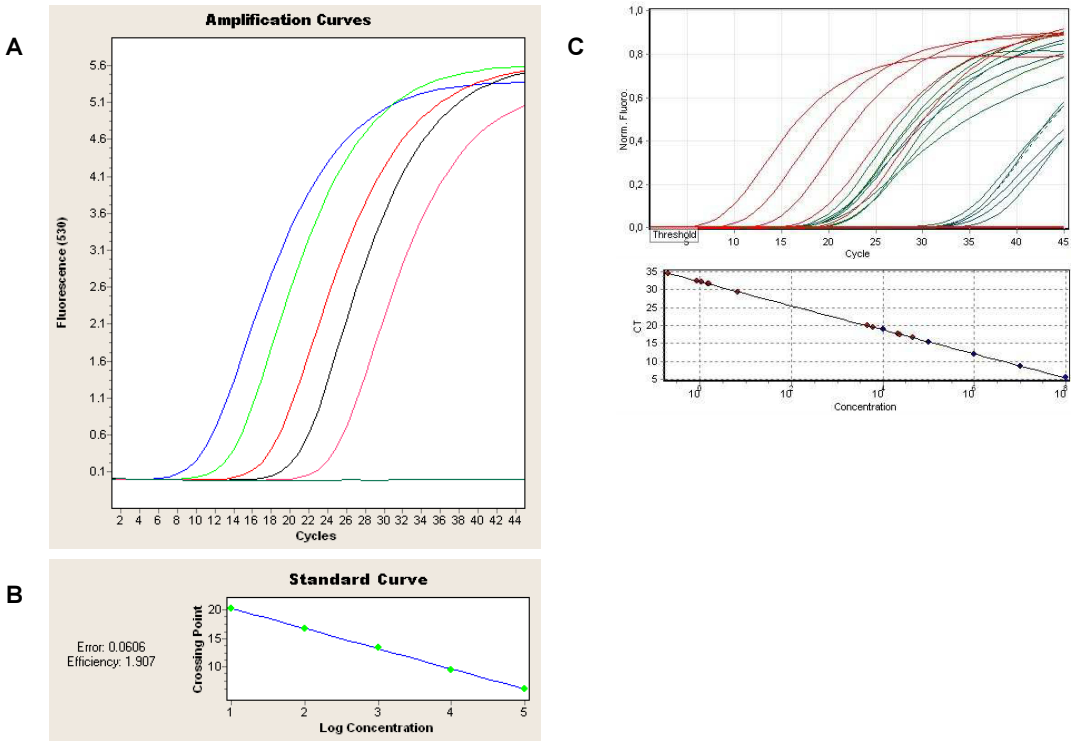



**Fig. 3.15: Amplification and standard curves using HotStarTaq Master Mix kit (Qiagen)**
**A** and **B**: Amplification and standard curve obtained with optimised PCR conditions (6 mM $MgCl_2$ and 55 °C annealing temperature) using the LightCycler 2.0 (Roche)
**C**: Amplification and standard curve obtained with optimised PCR conditions (6 mM $MgCl_2$ and 55 °C annealing temperature) using the RotorGene 6000 (Corbett Research, Qiagen)

The essential hallmarks of an optimised qPCR assay are high amplification efficiency, linearity of the standard curve and consistency across replicate reactions (reproducibility).

Efficient amplification ensures reliable and accurate results. Ideally, the amplification efficiency should be 100 %, which indicates a doubling of the amount of PCR product after each cycle (Bustin et al. 2009). Generally, efficiencies of 80-110 % are considered to produce reliable results. The amplification efficiencies reached for this essay lie between 92 and 100 %, with one experiment having run at an efficiency of 85 % (Fig. 3.16 A).

The linearity of the standard curve ensures, that there is a direct correlation between the CT-values and quantification of gene products. The linearity of a standard curve is generally assessed by calculating the coefficient of determination ($R^2$), which should have a value of >0.980, ideally of 1.0 (Bustin et al. 2009). The $R^2$ values calculated for the essay presented here ranged between 0.9900 and 0.9999 (Fig. 3.16 B).

Consistency across replicate reactions is important to allow comparisons between data obtained in independent experiments. Fig. 3.16 C shows the concentrations of wsp standard dilutions calculated based on their CT for 7 independent reactions. To assess the degree of range of concentrations that were be measured at each standard dilution step, a linear curve was fitted to the data points. The nearer the $R^2$ value is to the value 1.0, the better it is fitted to the data points, indicating a small range of CT values. The $R^2$ value obtained here is 0.9805.

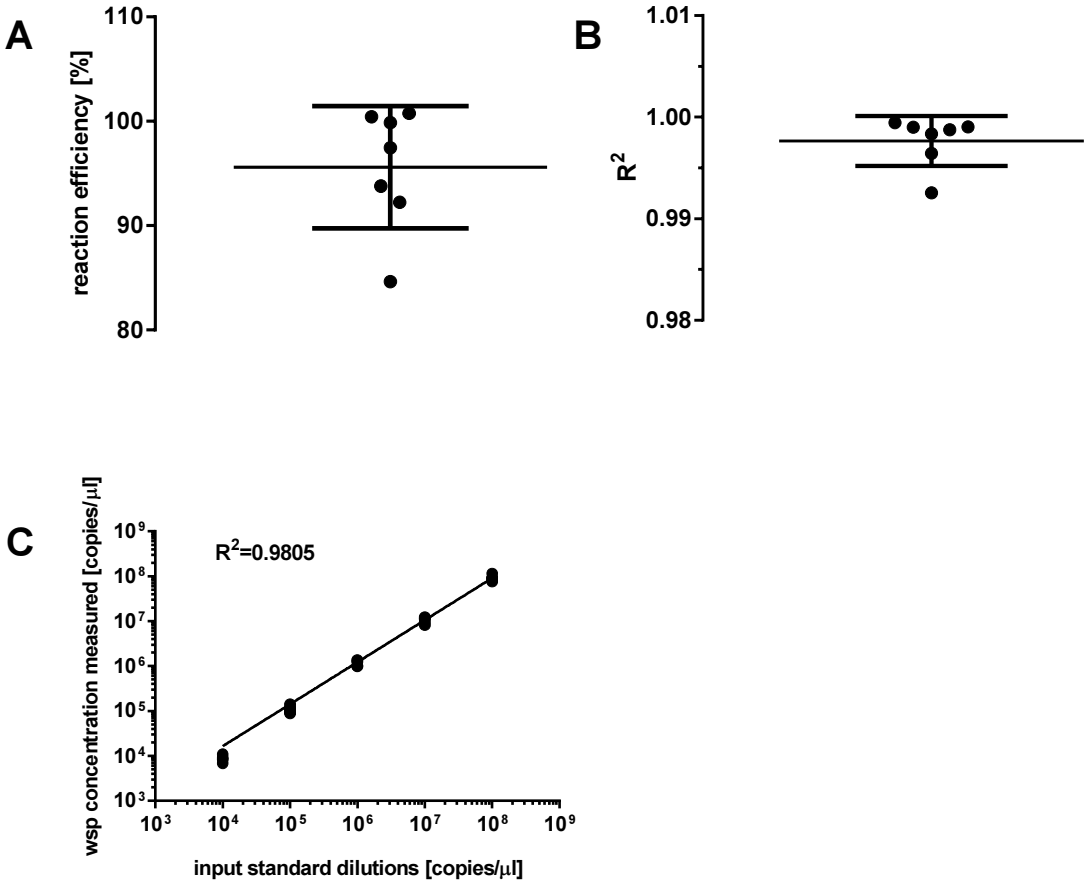



**Fig. 3.16: Analysis of the reaction efficiency, linearity of the standard curves ($R^2$) and reproducibility of the optimised qRT-PCR for *Wolbachia* screening**
All values are obtained from the standard dilutions of 7 independent qRT-PCR reactions. The reaction efficiency (**A**) and the $R^2$-values (**B**) are individually plotted with the mean and standard deviation indicated in the graph. The reproducibility (**C**) is plotted as the calculated input standard dilutions against the actual measured concentration based on the CT-value. The $R^2$ of the regression line is noted in the graph. Statistical analysis was performed using GraphPad Prism software.

The hallmarks of the established qRT-PCR lie within generally acceptable ranges. However, it was noticeable, that, among clear positive samples, some samples generate a very low CT (that is correlated with a wsp copy number of $1x10^3$ copies/µl) (Fig. 3.15, C and Table 6.9). To verify the *Wolbachia* status of these samples, they were again tested using the *Wolbachia* detection PCR published previously (Braig et al. 1998; Zhou et al. 1998).

The newly established *Wolbachia* qRT-PCR was then used to analyse larvae samples reared from field-collected egg rafts. The taxonomic status of these larvae had already been established previously by molecular methods (see above).

In total, 515 individual samples were screened for the presence of *Wolbachia* (Table 6.9). Out of the *Cx. pipiens* biotype *pipiens* from the North, 87 % (n=151) were found to be *Wolbachia* positive, whereas in the South the prevalence increased to 98% (n=166) (Table 6.9). None of the samples classified as *Cx. torrentium* were found to contain *Wolbachia* DNA, irrespective of the origin of the samples (North or South) (Table 6.9).

Thus, there is a significant difference in *Wolbachia* infestation between *Cx. pipiens* biotype *pipiens* and *Culex torrentium*, irrespectively of whether the egg rafts were collected in the North or the South of Germany (Fig. 3.17). Comparing the two *Cx. pipiens* biotype *pipiens* populations, the prevalence of *Wolbachia*-positive egg rafts is higher in the South (Fig. 3.17).

Furthermore, high-throughput screening included eight *Cx. pipiens* biotype *pipiens*/*molestus* Hybrids and one *Cx. pipiens* biotype *molestus*, which were all found to be positive for *Wolbachia* (Table 6.9). Interestingly, there was one egg raft out of which two DNA samples (from two independent sets of larvae) were prepared. Since originating from the same egg raft, both samples were identified as *Cx. pipiens* biotype *pipiens*, but one sample was *Wolbachia* positive and the other negative (Table 6.9).

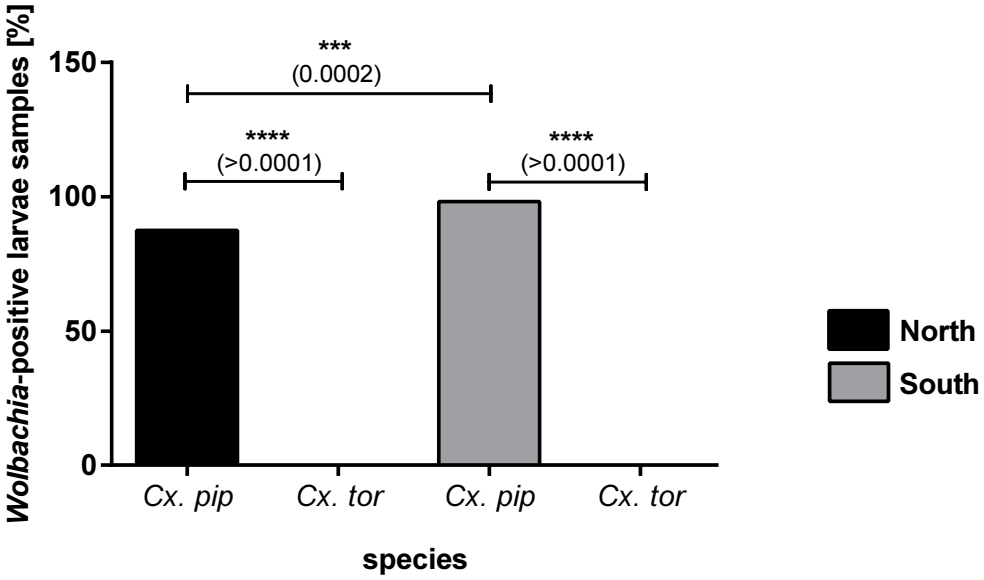



**Fig. 3.17: Percentage of *Wolbachia*-positive field-collected larvae samples**
Field-collected larvae samples previously identified as *Cx. pipiens* biotype *pipiens* (*Cx. pip*) or *Cx. torrentium* (*Cx. tor*) are screened for the presence of *Wolbachia* using a combination of high-throughput qRT-PCR screening and verification via gel PCR. Individuals of the same species collected in the North of Germany (Altes Land 53°35′N 9°32′E, Langenlehsten 53°30′N 10°44′E and Hamburg city 53°32′N, 9°57′E) are compared to the Southern German population (Lake Constance: Radolfzell-Böhringen 47°44′N, 8°58′E and Mettnau 47°43′N, 8°59′E) as well as each the species from each individual sample among each other origin (e.g. *Cx. pip* from the North vs. *Cx. tor* from the North).
Detailed number of individuals, qRT-PCR and PCR-results are depicted in Table 6.9 (supplementary material). Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

The mere presence of *Wolbachia* infection is not the only factor potentially influencing vector competence. The specific variations of *Wolbachia* might also be of importance. It has been shown previously that different wMel variants, *Wolbachia* infecting *D. melanogaster*, confer different degrees of protection against Drosophila C virus infection (Chrostek et al. 2013).

Thus, the *Wolbachia* infection of *Cx. pipiens* biotype *molestus* mosquito was analysed for the presence of different strain variations.

### 3.2.2 *Cx. pipiens* biotype *molestus* lab strain is infected with several different variants of *Wolbachia*

One of the key phenotypes of a *Wolbachia* infection is the induction of cytoplasmic incompatibility (CI), which leads to embryonic mortality when infected males mate with uninfected females or females that are infected with an incompatible *Wolbachia* strain (Yen & Barr 1973). Due to incompatibilities of crosses between mosquitoes of the *Culex* complex from different origins, it has been hypothesised that these CI patterns are due to the presence of different *Wolbachia* variants (Duron et al. 2006). Duron et al. developed a method to identify polymorphisms that are characteristic for different *Wolbachia* strains by targeting the WO prophage sequences within the genome of infected *Cx. pipiens* (Duron et al. 2006).

Based on information on variants around Germany as described by Duron et al., gene products that were most likely present in local mosquito populations were chosen, separated into 4 convenient subgroups (A, B, C and D) according to size and primer compatibility.

Subsequently, the different *Wolbachia* variants are characterised by combining the sizes of the resulting gene products. Fig. 3.18 displays different *Wolbachia* variants found in the lab strain *Cx. pipiens* biotype *molestus* LL.

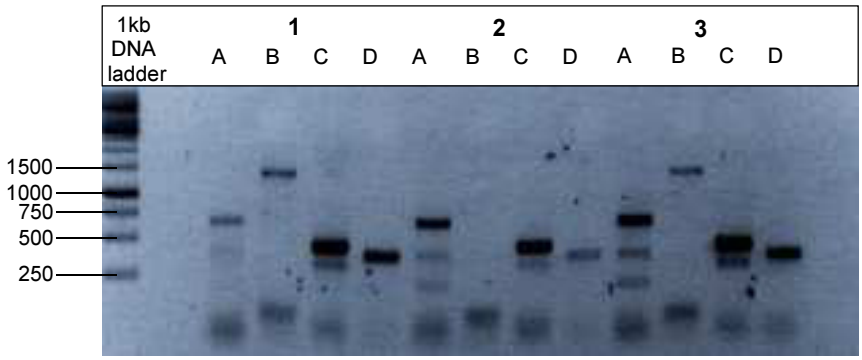



**Fig. 3.18: *Wolbachia* variants in *Cx. pipiens* biotype *molestus* LL as determined via the *Wolbachia* variant gel PCR**
100 ng DNA of three *Cx. pipiens* biotype *molestus* LL mosquitoes were used as template for the *Wolbachia* variant gel PCR. The different gene products (Gp) are sorted into four different primergroups A (Gp 3c, 1b, 2b), B (Gp 2a and 3a), C (Gp 2e, 3b) and D (Gp 3d and 15b). Sizes of the specific Gp are listed in tables 3.1 and 3.2.
The PCR product was run on a 1 % Agarose gel for 1 h at 120 V. DNA ladder used was the GeneRuler 1 kb DNA Ladder from ThermoScientific.

Analysis of 10 individuals of each *Cx. pipiens* biotype *molestus* lab strain (S, W and LL) indicated that the different mosquito populations are not monoclonal but contain various *Wolbachia* variants. In total, 4 different variants per lab strain could be detected using this method (Table 3.1).

**Table 3.1: Selection of different *Wolbachia* variants found in lab strains *Cx. pipiens* biotype *molestus* S, W and LL**
These lab strains are naturally infected with *Wolbachia* and originated from the Wendland area and Langenlehsten in the North of Germany (W and LL respectively) and from Heidelberg in the South of Germany (S).
10 individuals from each lab strain were randomly selected and screened for their *Wolbachia* variant via the *Wolbachia* variant gel PCR. The different gene products (Gp) are sorted into four different primer-groups A (Gp 3c, 1b, 2b), B (Gp 2a and 3a), C (Gp 2e, 3b) and D (Gp 3d and 15b). Sizes of the specific Gp are listed below the respective Gp name.
1 = Gp is present; 0 = Gp is not present

| | | A | | | B | | C | | D | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **Gp 3c** | **Gp 1b** | **Gp 2b** | **Gp 2a** | **Gp3a** | **Gp2e** | **Gp3b** | **Gp3d** | **Gp 15b** |
| **Spe-cies** | **Ori-gin** | **196 bp** | **307 bp** | **642 bp** | **363 bp** | **1339 bp** | **306 bp** | **428 bp** | **361 bp** | **484 bp** |
| *Culex pipiens* biotype *mo-lestus* | S | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | W | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| | | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 |
| | | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 |
| | | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| | LL | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 |
| | | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 |
| | | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |

The variety of different *Wolbachia* strains present in German *Cx. pipiens* biotype *molestus* raised the question, whether it would be possible to generate a population of mosquitoes infected with only one *Wolbachia* variant (= monoclonal).

To do this, one egg raft was raised to adult status and analysed for the *Wolbachia* variant (F0). Egg rafts from the F0-population were then pooled and individuals from this next generation (F1) also screened for their *Wolbachia* variant. If applicable, the same was performed with consecutive generations.

In the case of *Cx. pipiens* biotype *molestus* W, one clone was picked out. However, the variant changed in the F1 generation and was discarded. A similar situation was observed with the LL-lab strain. Although the variant expressed remained stable for generation F1, it changed in the F2 generation. In the case of *Cx. pipiens* biotype *molestus* S, two clones were generated. One clone changed the *Wolbachia* variant in generation F1. Another remained stable. However, due to a small number of individuals, it was not possible to rear a F2 generation (Table 3.2).

**Table 3.2: Selection of *Wolbachia* monoclonal *Cx. pipiens* biotype *molestus* S, W and LL lab strains**
These lab strains are naturally infected with *Wolbachia* and originated from the Wendland area and Langenlehsten in the North of Germany (W and LL respectively) and from Heidelberg in the South of Germany (S).
1 egg raft was raised to adult status and checked for their *Wolbachia* variant (F0) via the *Wolbachia* gel PCR. Egg rafts from the F0-generation were pooled and individuals from this next generation (F1) screened for their *Wolbachia* variant. If applicable, the same was performed with the consecutive generations.
The different gene products (Gp) are sorted into four different primer-groups A (Gp 3c, 1b, 2b), B (Gp 2a and 3a), C (Gp 2e, 3b) and D (Gp 3d and 15b). Sizes of the specific Gp are listed below the respective Gp name.
1 = Gp is present; 0 = Gp is not present

| | | | | A | | | B | | C | | D | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Gp 3c | Gp 1b | Gp 2b | Gp 2a | Gp3a | Gp 2e | Gp 3b | Gp 3d | Gp 15b |
| **Species** | **Origin** | **Clone** | **Generation** | **196 bp** | **307 bp** | **642 bp** | **363 bp** | **1339 bp** | **306 bp** | **428 bp** | **361 bp** | **484 bp** |
| *Cx. pipiens* biotype *molestus* | S | 1 | F0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | | | F1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | | 2 | F0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | F1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | W | 1 | F0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 |
| | | | F1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 |
| | LL | 1 | F0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 |
| | | | F1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 |
| | | | F2 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 |

Accordingly, we failed to establish a monoclonal mosquito population in order to investigate individual *Wolbachia* variants in the natural host. Thus, the following experiments are designed to analyse the susceptibility of *Cx. pipiens* biotype *molestus* for WNV in the presence of a variety of *Wolbachia* variants – as would be the situation in the field.

### 3.2.3 Influence of *Wolbachia* infection on the susceptibility of *Cx. pipiens* biotype *molestus* for WNV seems to be population-dependent

Having established the presence of *Wolbachia* in some *Cx.* mosquito species native to Germany, it is interesting to next establish, whether the presence of said *Wolbachia* has any effect on susceptibility for WNV infection.

Due to the difficulty in establishing a self-sustaining lab strain of the species *Cx. pipiens* biotype *pipiens* and *Cx. torrentium*, these experiments were performed with the lab strain *Cx. pipiens* biotype *molestus*. This species is naturally infected with *Wolbachia* and has previously been identified as a vector for WNV (Thaori et al. 1955).

To investigate the influence of *Wolbachia* on WNV susceptibility, it is necessary to establish and characterise a *Wolbachia*-free control colony of our target species population.

#### 3.2.3.1 Establishment and characterisation of *Wolbachia*-negative *Cx. pipiens* biotype *molestus* populations

In order to cure a *Wolbachia* infection, the antibiotic tetracycline was employed. Contrary to previously published methodology focusing on larvae, adult mosquitoes were the treatment target (Suenaga 1993; Dobson & Rattanadechakul 2001).

Adult mosquitoes were fed on fructose solution containing 8 % fructose and 0.5 mg/ml tetracycline for a duration of at least five days. The lab strains received treatment for three consecutive generations (F0, F1, F2). Analyses of the *Wolbachia* status by PCR revealed that offspring of the treated F2 adults (=F3) were free of *Wolbachia* in both *Cx. pipiens* biotype *molestus* S and *Cx. pipiens* biotype *molestus* W.

Having established a negative *Wolbachia* status, the lab strains were kept in culture until generation F10 (to ensure a stable Wolbachia-negative infection status), after which they were subjected to infection experiments. In addition, prior to infection experiments, 10 mosquitoes were selected at random to control the *Wolbachia* status again. Fig. 3.19 exemplifies the picture obtained for *Wolbachia*-free and infected lab strains.

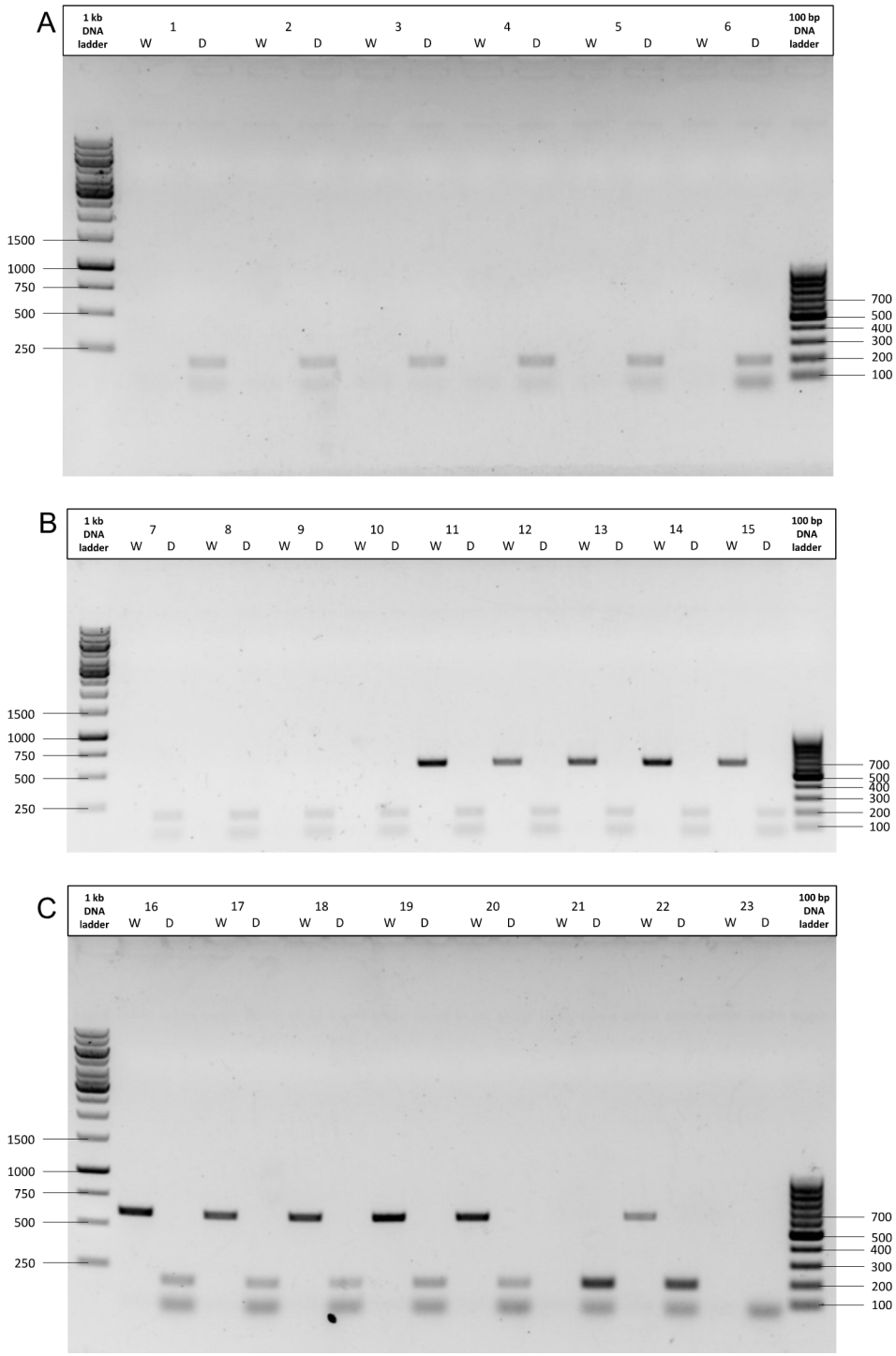



**Fig. 3.19: Standard gel PCR screening for presence of *Wolbachia* in *Culex* mosquitoes**
10 females were randomly selected from the *Cx. pipiens* biotype *molestus* W lab strain treated with tetracycline (1-10; **A** and **B**) and wildtype (11-20; **B** and **C**). All individuals belonged to generations > F10. 100 ng DNA was used in standard gel PCR targeting the wsp gene for *Wolbachia* screening (W; size: 603 bp) and, separately, the microsatellite locus CQ11 as DNA control (D; size: 189 bp). Samples 21 and 22 are *Cx. pipiens* biotype *molestus* previously tested negative and positive for *Wolbachia* respectively and which were employed to control for PCR functionality. Sample 23 is the non-template control.
The PCR product was run on a 1 % Agarose gel for 1 h at 120 V. DNA ladders used were the GeneRuler 100 bp DNA Ladder and the GeneRuler 1kb DNA Ladder from ThermoScientific.

Having established *Wolbachia*-free homologs for the *Cx. pipiens* biotype *molestus* S and W lab strains, blood feed infection assays were performed as described above.

Interestingly, the assay indicated changes in blood feed behaviour among the different lab strains. While there were no significant differences in the percentage of blood engorged females in the case of *Cx. pipiens* biotype *molestus* S free (n=1063, 74 %) and *Cx. pipiens* biotype *molestus* infected (n=881, 76 %), a significant difference was observed in the case of *Cx. pipiens* biotype *molestus* W. Here, only 47 % of *Wolbachia*-free females took up a blood meal (n=495), whereas 70 % of the corresponding *Wolbachia*-infected females did so (n=472) (Fig. 3.20).

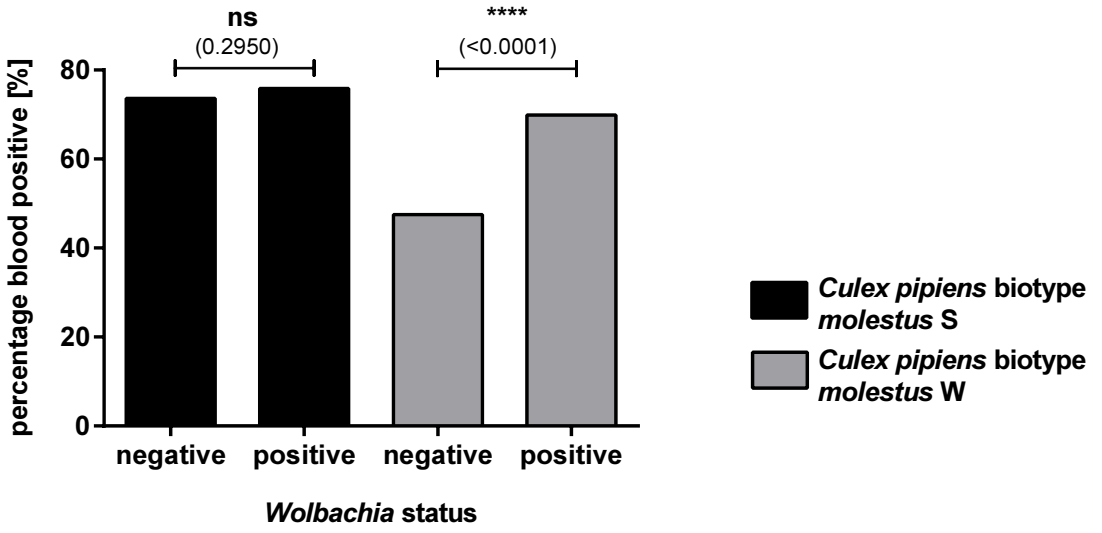



**Fig. 3.20: Blood feeding rate of *Cx. pipiens* biotype *molestus* S und W**
Adult females were sorted into plastic containers 4-14 days after emergence and received human blood overnight. These lab strain is naturally infected with *Wolbachia* and originated from the Wendland area in the North of Germany (W) and from Heidelberg in the South of Germany (S). *Wolbachia*-free *Cx. pipiens* biotype *molestus* W and S have been generated through treatment with tetracycline throughout three consecutive generations (F0, F1 and F2).
The graph depicts the percentage of engorged females after having received the blood meal overnight. Detailed number of individuals are listed in Table 6.10. Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

### 3.2.3.2 WNV infection and dissemination rates appear to be greater in *Wolbachia*-negative *Cx. pipiens* biotype *molestus* S

Looking at the infection rate of *Wolbachia*-negative and *Wolbachia*-positive *Cx. pipiens* biotype *molestus* S, a definite trend was observed:

At 14 days post infection, the infection rate in *Wolbachia*-negative mosquitoes (26 %, n=43) was found to be 8 percentage points higher than the infection rate seen with corresponding *Wolbachia*-positive mosquitoes (18 %, n=39) (Fig. 3.21).

Similarly, at 21 days post infection, the infection rate in *Wolbachia*-negative *Cx. pipiens* biotype *molestus* (45 %, n=58) was 20 percentage points greater than in *Wolbachia*-positive mosquitoes (25 %, n=52) (Fig. 3.21).

*Wolbachia*-negative mosquitoes display higher infection rates at both 14 and 21 days post infection. However, this difference was significant, using the Fisher's exact test, in the case of 21 days post infection only (Fig 3.21).

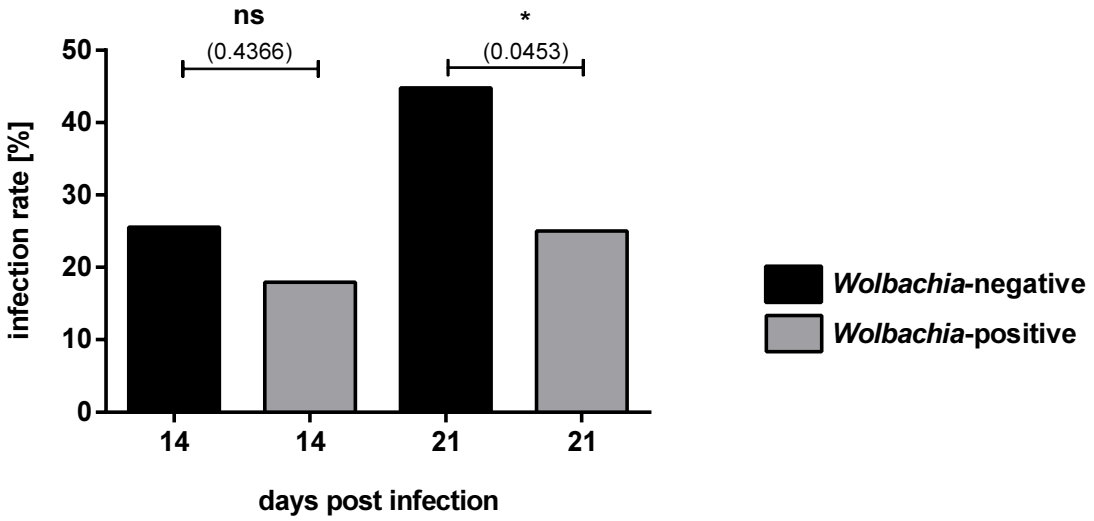



**Fig. 3.21: WNV infection rate of *Wolbachia*-negative and -positive *Cx. pipiens* biotype *molestus* S**
Adult females were sorted into plastic containers 4-14 days after emergence and received human blood containing $1.4x10^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C and 80 % humidity in a climate chamber for 14 to 21 days. The graph depicts the WNV infection rate of *Cx. pipiens* biotype *molestus* S positive (grey) or negative (black) for *Wolbachia* infection. This lab strain is naturally infected with *Wolbachia* and originated from Heidelberg in the South of Germany. *Wolbachia*-free *Cx. pipiens* biotype *molestus* S have been generated through treatment with tetracycline throughout three consecutive generations (F0, F1 and F2).
The WNV infection rate is determined by a WNV-specific qRT-PCR (CT cut-off = 35 cycles). Detailed number of individuals and number of independent blood feed assays are depicted in Table 6.4 and 6.5 (supplementary material). Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

Similar to the infection rate, there were differences in dissemination rates between *Wolbachia*-infected and *Wolbachia*-free *Cx. pipiens* biotype *molestus*.

At day 14 post infection, the dissemination rate of *Wolbachia*-negative mosquitoes (73 %, n=11) was found to be 30 percentile points higher than the one measured in *Wolbachia*-positive mosquitoes (43 %, n=7). Similarly, at day 21 post infection, the dissemination rate of *Wolbachia*-negative mosquitoes was 73 % (n=26) and 69 % (n=13) in *Wolbachia*-positive *Cx. pipiens* biotype *molestus* S (Fig. 3.22).

These observed differences were, however, not found to be statistically significant using the Fisher's exact test (Fig. 3.22).

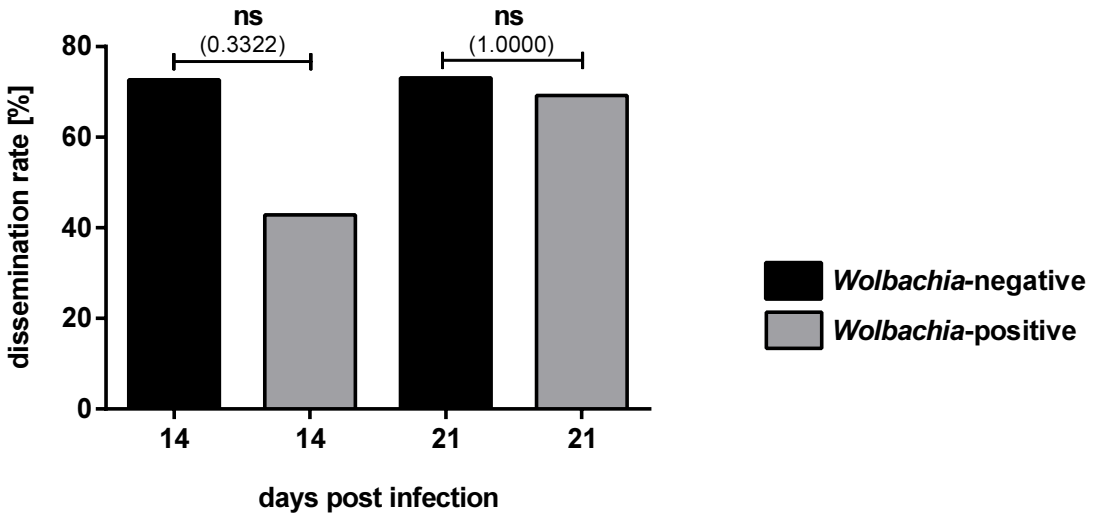



**Fig. 3.22: WNV dissemination rate of *Wolbachia*-negative and -positive *Cx. pipiens* biotype *molestus* S**
Adult females were sorted into plastic containers 4-14 days after emergence and received human blood containing 1.4x$10^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C and 80 % humidity in a climate chamber for 14 to 21 days. The graph depicts the WNV dissemination rate of *Cx. pipiens* biotype *molestus* S positive (grey) or negative (black) for *Wolbachia* infection. This lab strain is naturally infected with *Wolbachia* and originated from Heidelberg in the South of Germany. *Wolbachia*-free *Cx. pipiens* biotype *molestus* S have been generated through treatment with tetracycline throughout three consecutive generations (F0, F1 and F2).
The WNV dissemination rate is determined by a WNV-specific qRT-PCR (CT cut-off = 35 cycles). Detailed number of individuals and number of independent blood feed assays are depicted in Table 6.4 and 6.6 (supplementary material). Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

### 3.2.3.3 WNV infection and dissemination rates do not differ between *Wolbachia*-negative and *Wolbachia*-positive *Cx. pipiens* biotype *molestus* W

In the case of *Cx. pipiens* biotype *molestus* W, both infection and dissemination rates in *Wolbachia*-negative and *Wolbachia*-positive populations did not follow a clear trend.

Considering the infection rates at 14 days post infection, *Wolbachia*-positive mosquitoes seemed to be more susceptible (*Wolbachia*-positive: 14 %, n=50; *Wolbachia*-negative: 8 %, n=40). However, this relationship is turned at day 21 post infection, where *Wolbachia*-negative mosquitoes displayed a slightly higher infection rate (*Wolbachia*-positive: 15 %, n=65; *Wolbachia*-negative: 17 %, n=36). Albeit clearly visible, especially at day 14 post infection, none of these differences were found to be statistically significant (Fig. 3.23).

The dissemination rates at day 14 post infection are found to be higher with *Wolbachia*-negative mosquitoes (67 %, n=3) when compared to *Wolbachia*-positive mosquitoes (29 %, n=7). However, as seen with the infection rates, this relationship again turned at day 21 post infection with *Wolbachia*-negative mosquitoes (17 %, n=6) displaying a 3 percentage points lower dissemination rate than *Wolbachia*-positive mosquitoes (20 %, n=10) (Fig. 3.24).

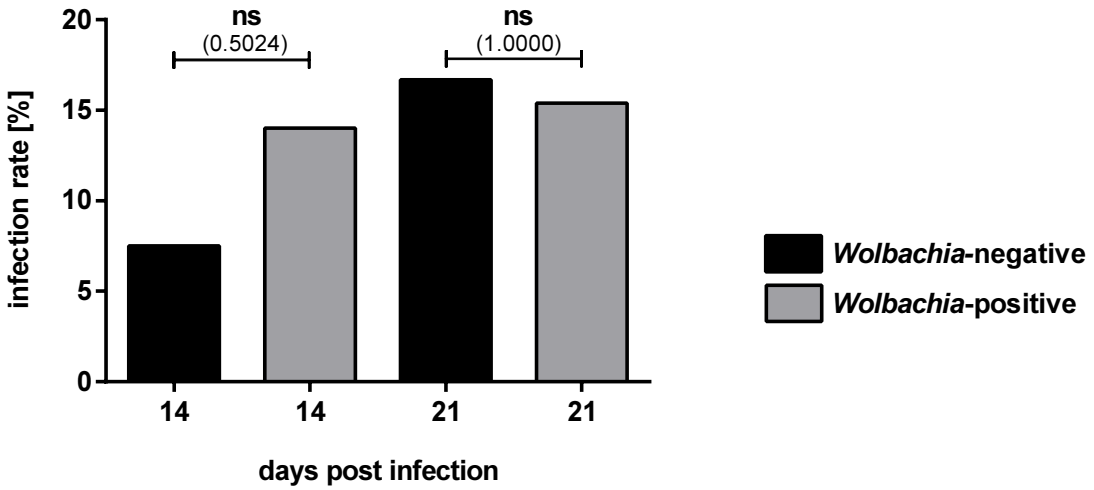



**Fig. 3.23: WNV infection rate of *Wolbachia*-negative and -positive *Cx. pipiens* biotype *molestus* W**

Adult females were sorted into plastic containers 4-14 days after emergence and received human blood containing $1.4x10^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C and 80 % humidity in a climate chamber for 14 to 21 days. The graph depicts the WNV infection rate of *Cx. pipiens* biotype *molestus* W positive (grey) or negative (black) for *Wolbachia* infection. This lab strain is naturally infected with *Wolbachia* and originated from the Wendland area in the North of Germany. *Wolbachia*-free *Cx. pipiens* biotype *molestus* W have been generated through treatment with tetracycline throughout three consecutive generations (F0, F1 and F2).

The WNV infection rate is determined by a WNV-specific qRT-PCR (CT cut-off = 35 cycles). Detailed number of individuals and number of independent blood feed assays are depicted in Table 6.4 and 6.5 (supplementary material). Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

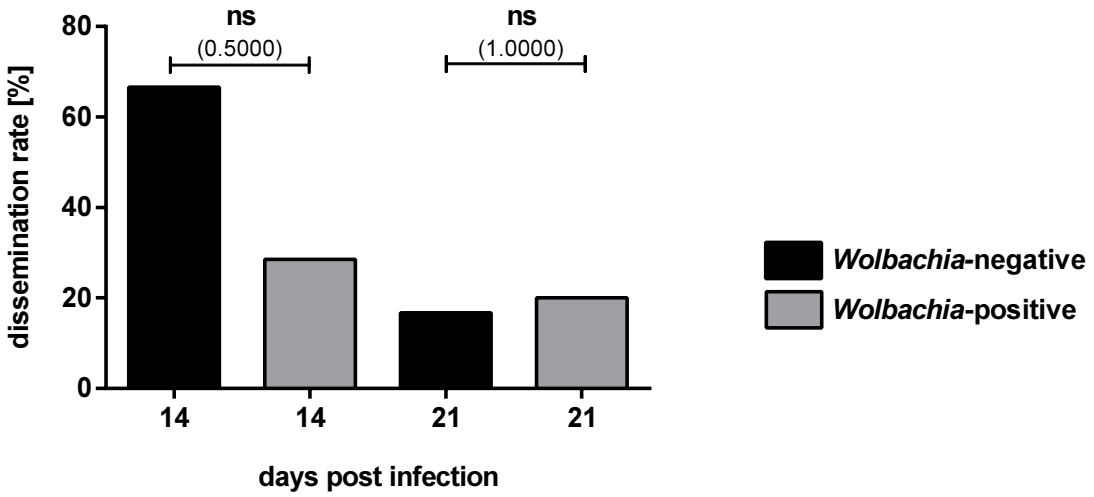



**Fig. 3.24: WNV dissemination rate of *Wolbachia*-negative and -positive *Cx. pipiens* biotype *molestus* W**

Adult females were sorted into plastic containers 4-14 days after emergence and received human blood containing $1.4x10^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C and 80 % humidity in a climate chamber for 14 to 21 days. The graph depicts the WNV dissemination rate of *Cx. pipiens* biotype *molestus* W positive (grey) or negative (black) for *Wolbachia* infection. This lab strain is naturally infected with *Wolbachia* and originated from the Wendland area in the North of Germany. *Wolbachia*-free *Cx. pipiens* biotype *molestus* W have been generated through treatment with tetracycline throughout three consecutive generations (F0, F1 and F2).

The WNV dissemination rate is determined by a WNV-specific qRT-PCR (CT cut-off = 35 cycles). Detailed number of individuals and number of independent blood feed assays are depicted in Table 6.4 and 6.6 (supplementary material). Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

# 4 Discussion

In the year 2015, 108 human cases of West Nile disease were reported by member states of the European Union and an additional 193 human cases from neighbouring countries, illustrating the danger of the virus and the disease to spread to Germany as well (European Centre for Disease Prevention and Control 2015). Vectors for the transmission of West Nile virus (WNV) are species of the *Culex* complex, the most common being *Cx. pipiens* biotype *pipiens*, *Cx. pipiens* biotype *molestus* and *Cx. torrentium*, which are native to Germany (Rudolf et al. 2013; Krüger et al. 2014). Both *Cx. pipiens* biotype *pipiens* and biotype *molestus* have been previously attributed to West Nile disease outbreaks (Chancey et al. 2015; Farajollahi et al. 2011; Fonseca et al. 2004). However, vector capacity of populations native to Germany have not yet been assessed and susceptibility of the species *Cx. torrentium* for WNV is entirely unknown.

As these mosquito species are most abundant in Germany, their role in setting up and maintaining an enzootic and epidemic WNV infection cycle, is pivotal in assessing the potential risk of WNV outbreaks in Germany.

The focus of this work was to investigate several crucial extrinsic and intrinsic factors influencing the vectorial capacity of German *Culex* mosquitoes and to evaluate whether these factors substantiate the risk of WNV outbreaks in Germany.

## 4.1 Assessment of the vectorial capacity of German *Culex* mosquitoes for WNV

The vectorial capacity of a mosquito vector population describes its ability to transmit a virus to a susceptible host population (Ciota & Kramer 2013; Macdonald 1961). This characteristic is influenced by an array of intrinsic and extrinsic factors, which include viral evolution, host and vector immunity, vector competence and other life-history traits (intrinsic factors) (Chamberlain & Sudia 1961; Hardy et al. 1983) as well as climate and density of vector and host population (extrinsic factors) (Ciota & Kramer 2013).

The extrinsic factors focused on in this work are the distribution of putative vector populations and temperature. The intrinsic factors investigated are confined to vector competence and the influence of *Wolbachia*, an intracellular proteobacterium, on vector biology and susceptibility to virus infections.

It is to note, that vector competence or, more specific, the susceptibility to WNV infection, is assessed in conjunction with temperature and vector population dynamics in this work. This is why this actual intrinsic factor will be discussed under the heading of the extrinsic factor ‘temperature’.

### 4.1.1 Distribution of putative vector populations

*Cx. torrentium*, *Cx. pipiens* biotype *pipiens*, *Cx. pipiens* biotype *molestus* and the hybrid species *Cx. pipiens* biotype *pipiens*/*molestus* are potential candidates for WNV transmission events in Germany. Their presence and distribution in Germany has been recently investigated (Rudolf et al. 2013; Krüger et al. 2014). However, temporal as well as spatial variation of species distribution are important information for WNV risk assessments.

To assess potential variation in species distribution and, simultaneously, gather field-caught individuals for experimental infections, mosquito egg rafts were collected at locations in the North (Altes Land, Langenlehsten and Hamburg city) and South of Germany (Lake Constance: Radolfzell-Böhringen and Mettnau) (Fig. 4.1).

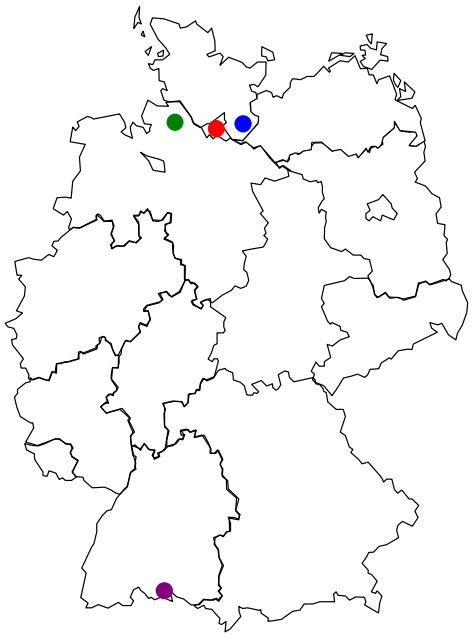


**Fig. 4.1: Mosquito egg raft sampling locations**
Map template produced by www.presentationmagazine.com (last accessed: 25.03.2016)
The approximate location of the egg raft sampling points are indicated as coloured dots. Green = Altes Land; Red = Hamburg city; Blue = Langenlehsten; Purple = Lake Constance (Radolfzell-Böhringen and Mettnau)

To assess temporal variation in species distribution, egg collections in the North was conducted in two successive years (2012 and 2013). One collection area, namely Altes Land, was added in 2013 but the majority of samples were obtained from Langenlehsten in both years. Notably, *Cx. pipiens* biotype *pipiens* was most prevalent in May, June and July 2012. However, in 2013, the species composition changed in these three months with *Cx. torrentium* being the most abundant species. Nevertheless, the species composition for August and September 2012 and 2013 were not significantly different, with *Cx. pipiens* biotype *pipiens* being the more prevalent species (refer to Fig. 3.1 and Fig. 3.2). Annual as well as seasonal (i.e. monthly) variation has been described before in the context of Malaria vector control (Anopheline mosquitoes) (Fontenille et al. 1997;

Munhenga et al. 2014). Investigations on the dynamics of mosquito populations in Istanbul over a 5 year-period included also *Culex* mosquitoes. Although *Cx. pipiens* was the most dominant species throughout the observation periods, seasonal and annual variations were detected here as well (Sengil et al. 2011). However, since both *Cx. pipiens* biotype *pipiens* and *Cx. torrentium* have been found to take blood meals from both mammals and birds (Börstler et al. 2016), it seems that the relationship between the two species at any time point is not relevant for the occurrence of spill over events, potentially causing WNV outbreaks in humans. Nevertheless, it might be possible that one or the other mosquito species is more susceptible to WNV infection under the climatic conditions usually present in Germany, possibly making 'successful' spill over events more likely in times of high prevalence of the more susceptible species. This will be discussed in more detail in section 4.1.2.

Considering spatial variation of species composition, egg rafts from sampling locations in the North and South of Germany in August 2013 were collected and compared species prevalence (refer to Fig. 3.3). Interestingly, the prevalence of *Cx. pipiens* biotype *pipiens* does not vary significantly among the locations investigated (North: 72 %; South: 87.7 %). However, the prevalence of *Cx. torrentium* is markedly higher in the North (27 %; South: 7.4 %). This North-South-variation has been shown recently by Hesson et al in 2014. They conducted a species prevalence study, ranging from Scandinavia to the Mediterranean coast, focusing on *Cx. pipiens* and *Cx. torrentium* and found that *Cx. torrentium* dominated in Northern and *Cx. pipiens* in Southern Europe (South of the Alps) respectively. Germany is situated in Central Europe, an area where the two species have almost equal prevalence (Hesson et al. 2014). However, the observations made in Germany reflect the trend made for the whole of Europe with a clear North-South variation.

Interestingly, very few *Cx. pipiens* biotype *molestus* and biotype *molestus*/*pipiens* egg rafts were identified, which make any assumptions as to the natural prevalence variation difficult. Although, looking at spatial variance, more hybrid egg rafts were found in the South, which might correlate with the higher prevalence of *Cx. pipiens* populations in the South as described above (Hesson et al. 2014). The low *Cx. pipiens* biotype *molestus* collection number might originate from the method of sampling. Egg rafts were collected out of large water-filled plastic containers placed near natural breeding sites and areas, where mosquitoes generally occurred to lure gravid females. However, *Cx. pipiens* biotype *molestus* prefers underground breeding sites and might thus not be attracted by the openly accessible containers (Weitzel et al. 2011).

### 4.1.2 Temperature-dependency of susceptibility to WNV infection

The temperature-dependency of the WNV infection susceptibility was assessed by performing a blood infection assay at 25 °C and 18 °C ambient temperature. Temperatures at or around 25 °C are often used in vector competence studies and represent the maximum monthly average temperature limit for Germany. However, an ambient temperature of 18 °C is a more accurate reflection of the temperature situation. Climate data from 2015 shows, that there were two months in the North of Germany (Hamburg) with a

mean air temperature of or around 18 °C (July: 17.9 °C; August: 18.8 °C), with 18.8 °C being the highest monthly mean air temperature. In the South of Germany (Constance), there was one month with a mean temperature of close to 25 °C (July: 22.9 °C), however 3 months depicted a mean air temperature of clearly over 18 °C (June: 18.9 °C; July: 22.9 °C; August: 21.3 °C) (Fig. 4.2) (Deutscher Wetterdienst 2016). Thus, to fulfil both the need for comparability and biological significance, these two incubation temperature conditions were used in this study.

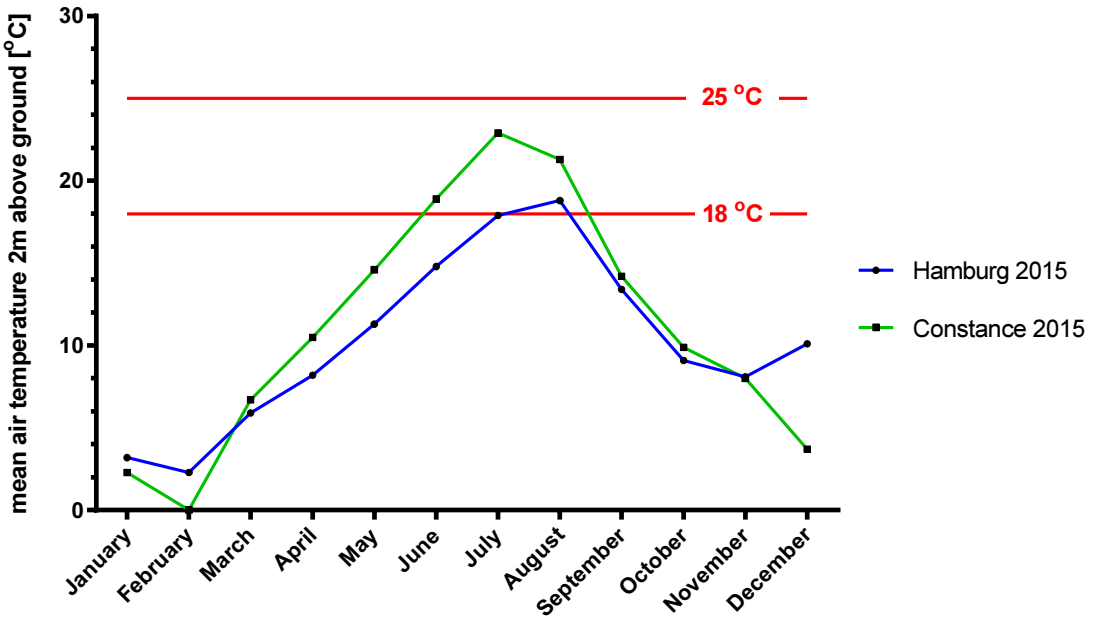



**Fig. 4.2: Mean air temperature 2 m above ground in °C for the year 2015 in Hamburg and Constance**
Data from the Deutscher Wetterdienst 2016
18 °C and 25 °C temperature limits are indicated as red lines within the graph.

Having defined the blood feed assay conditions, the positive control species *Cx. quinquefasciatus* and *Cx. pipiens* biotype *molestus* were used to calibrate the subsequent qRT-PCR and $TCID_{50}$ analysis for the detection of WNV RNA and viable particles. Briefly, all samples that were tested positive for viral RNA on day 0 post infection also contained viable virus particles in the $TCID_{50}$ assay. Subsequent analysis of randomly chosen samples from day 14 and 21 post infection via $TCID_{50}$ allowed to set the qRT-PCR cut-off CT to 35.

Both positive controls have been found to be susceptible to WNV via the blood feed infection assay as evidenced by subsequent qRT-PCR. This further confirms the suitability of the assay to be used for vector competence studies. There were no significant differences in infection rates between the two temperatures, except for *Cx. pipiens* biotype *molestus,* which revealed a 21 percentage point higher infection rate at 25 °C at 35 days post infection (dpi).

The infection rate obtained for *Cx. quinquefasciatus* at 25 °C correspond well with results previously reported by Richard et al., who performed a study with similar conditions. They reported an infection rate after 13 days of 30 %, matching an infection rate of 35 % at

14 dpi in this study (Richards et al. 2007). Also the infection rates obtained for *Cx. pipiens* biotype *molestus* confirm their susceptibility for WNV infection, which has again been shown by recent studies of American and European populations (Ciota et al. 2013; Fortuna et al. 2015). The susceptibility of these two species for WNV at 18 °C has not been shown previously. However, a recent publication from the Netherlands has shown, that Dutch *Cx. pipiens* lab cultures are susceptible to WNV lineage 2 at 18 °C (Fros et al. 2015). This observation substantiates the possibility that especially *Cx. pipiens* biotype *molestus* populations, that are native to Germany, could also be potential vectors for WNV.

#### 4.1.2.1 *Cx. pipiens* biotype *pipiens*

Data focusing on mosquito populations in the United States have shown that the infection rate of *Cx. pipiens* mosquitoes for WNV can be very high but generally also very variable among field-caught mosquitoes (Kilpatrick et al. 2010; Ciota et al. 2013). Considering this, the infection rates measured within this work fit into the spectrum obtained in the USA. Interestingly, a very recent study working with Italian field-caught *Cx. pipiens* populations found slightly higher infection rates in later stages of infections. However, this study was conducted under Italian temperature conditions (28 ± 1 °C) and utilising a lineage 1 WNV strain originating from Europe (Sardinia) as opposed to a lineage 1 strain from the USA, which was used in this work (Fortuna et al. 2015). It remains to be determined, whether infection rates differ in German *Culex* mosquitoes when virus strains adapted to European mosquito populations are used.

Furthermore, no significant difference was observed in the infection rate at 25 °C and 18 °C in the case of *Cx. pipiens* biotype *pipiens*. Interestingly, a recent study from the Netherlands, focusing on Dutch *Cx. pipiens* lab cultures, has reported a different relationship (Fros et al. 2015). Amongst others, they investigated the infection rate of these lab cultures upon infection with a WNV lineage 2 strain at three different temperatures (18 °C, 23 °C and 28 °C). They reported significant increases in infection rate with increasing temperature (Fros et al. 2015). It is, however, difficult to make any assumptions to whether these differences originate from the origin of the mosquito population (field-caught vs laboratory colony; German vs Dutch) or from the different WNV lineages used (lineage 1 vs lineage 2). It has been previously shown, that both genetic variability of mosquito populations and the WNV lineage may cause differential susceptibility to arboviral infection (Fros et al. 2015; Hardy et al. 1976; Vazeille et al. 2001; Vazeille et al. 2003). For example, Fros et al. have demonstrated that although European mosquitoes are competent for both WNV lineage 1 and 2, the competence of North American populations from WNV lineage 2 is significantly decreased (Fros et al. 2015). Thus, further infection experiments with field-collected mosquitoes involving WNV of both lineages are necessary to gain a clearer picture concerning the infection rate of *Cx. pipiens* biotype *pipiens* for WNV.

Nonetheless, the dissemination rate is a more crucial factor for vector competence, since a successful escape of the midgut barrier and a systemic infection is the prerequisite of

transmission (Franz et al. 2015). And seeing that the dissemination rate remains equally high, irrespective of temperature, further supports the possibility that German *Cx. pipiens* biotype *pipiens* populations have the potential to transmit WNV in Germany.

#### 4.1.2.2 *Cx. torrentium*

The high prevalence of *Cx. torrentium* in Europe has been discovered only recently through analyses of the *Culex* complex via molecular methods (Rudolf et al. 2013; Hesson et al. 2014). In addition, *Cx. torrentium* has not yet been associated with WNV transmission. It is thus of particular interest that the highest overall infection rate measured in this work has been obtained for *Cx. torrentium*. Notably, *Cx. torrentium* distribution seems to be restricted to North and Central Europe, which does not allow much overlap with most endemic regions for WNV in the South-Eastern regions (Hesson et al. 2014; European Centre for Disease Prevention and Control 2015). Nonetheless, *Cx. torrentium* has previously been identified as a vector for other arboviruses, such as the Sindbis virus, both in a laboratory setting and via isolation of the virus from field-caught mosquitoes (Lundström, Niklasson, et al. 1990; Lundström, Turell, et al. 1990; Hesson et al. 2015). The fact that Sindbis virus-infected *Cx. torrentium* are present in Germany (Jost et al. 2010) further supports the necessity of incorporating this species into WNV risk assessments for Germany.

Although high in both cases (up to 80-90 %), the infection at 18 °C is delayed by 7 days when compared to 25 °C, resulting in significantly higher infection rates at 25 °C at 14 and 21 dpi. Together with the finding of similar dissemination rates at the two temperatures, this observation points towards a delayed virus replication at lower temperature. In other words, although the proportion of positive mosquitoes at 18 °C exceeds the amount at 25 °C, virus replication rate at 18 °C has not yet allowed midgut barrier escape to the same extend that would also cause significant differences in dissemination rates. Studies on the immune system of insects, namely the RNAi pathway, aimed at explaining the phenomena of higher susceptibility at lower temperatures as observed here. Briefly, WNV is targeted by the RNAi pathway and cooler temperatures destabilizes RNAi (Brackney et al. 2009; Adelman et al. 2013). Adelmann et al. further hypothesised a link between impaired RNAi pathway and increased susceptibility of *Ae. aegypti* to Chikungunya virus infection. Temperature-sensitive RNAi pathway could be a genetic trait facilitating susceptibility to virus infection (Adelman et al. 2013).

However, the moderate sample sizes for *Cx. torrentium* make it difficult to draw a definite conclusion. In particular, previous results indicating high infection rates of *Cx. torrentium* for other arboviruses independent of temperature encourage future investigations (Lundström, Turell, et al. 1990). Nevertheless, the high infection and dissemination rates at both temperatures qualify *Cx. torrentium* to be incorporated into WNV risk assessment studies.

#### 4.1.2.3 Population-dependency of susceptibility to WNV infection

The susceptibility of a mosquito species for West Nile virus infection can vary on the basis of changes that occur with time (seasonal variation) or through spatial separation into independent populations (spatial variation) (Hayes et al. 1984; Kilpatrick et al. 2010; Vaidyanathan & Scott 2006).

Temporal variation was observed for both *Cx. torrentium* and *Cx. pipiens* biotype *pipiens* in this study, with higher infection rates in 2013 when compared to data from 2012. This observation is in line with data obtained from field-collected *Cx. pipiens* from the USA, which hypothesise a link between temporal variation and genetic as well as environmental factors (Kilpatrick et al. 2010).

Spatial variation was determined by focusing on the WNV susceptibility measured in 2013 only for populations of the same species, which are spatially isolated from each other in Northern and Southern Germany, respectively.

Interestingly, no significant differences between the Northern and South populations of *Cx. torrentium* were observed, although there is a visible, albeit not significant, trend favouring the Northern population at lower temperature of 18 °C. However, it has to be taken into consideration that only moderate sample sizes of *Cx. torrentium*, especially from the South of Germany, were investigated. For the case that the observed trend is verified in the future, *Cx. torrentium* would be a relevant candidate vector for potential WNV transmission in the North and at lower temperatures. This hypothesis is supported by previous studies on Sindbis virus transmission in Sweden, for which *Cx. torrentium* is a confirmed vector (Lundström, Niklasson, et al. 1990; Lundström, Turell, et al. 1990; Hesson et al. 2015).

In the case of *Cx. pipiens* biotype *pipiens*, the infection rate was significantly higher for the South population irrespective of the extrinsic incubation temperature. This indicates that intrinsic factors, such as genetic variability, are responsible for this differential susceptibility. This hypothesis is supported by work on American *Cx. pipiens* populations, which displayed spatial variation concerning WNV vector competence (Kilpatrick et al. 2010). Furthermore, studies performed on other mosquito-arbovirus-relationships, such as *Cx. tarsalis* and Western equine encephalomyelitis or *Ae. albopictus* and DENV, underline the importance of population analysis as well as the use of both, field-collected as well as laboratory strains to investigate the vector competence of native mosquito species (Hardy et al. 1976; Vazeille et al. 2001; Vazeille et al. 2003).

Notably, a recent study from Italy seems to break ranks concerning these observations. Fortuna et al. reported significant differences between lab strains and field-caught populations as well as among two independent field-caught populations of *Cx. pipiens* when looking at infection and dissemination rates. However, this did not translate to the transmission rates, which were similar throughout all study populations (Fortuna et al. 2015). There are several possible scenarios that could explain this observation. Firstly, the lab colonies were cultivated for approximately 1 year (F7 and F11), which suggests that, possibly, the genetic aberration with respect to the field-caught populations are not large

enough to cause variation in transmission-phenotype (Lainhart et al. 2015). Secondly, there is the possibility that the *Cx. pipiens* populations in Italy are naturally more uniform. However, a very recent study focusing on the genetic diversity of *Cx. pipiens* in Italy, although not on the exact same populations, indicate high genetic diversity (Simonato et al. 2016) making this hypothesis more unlikely. A third possibility could be that German and Italian mosquito populations harbor distinct *Wolbachia* populations that, in turn, influence their differing susceptibility to arboviral infections and, subsequently, vector competence (Johnson 2015).

### 4.1.3 *Wolbachia*-mediated effects on vectorial capacity

#### 4.1.3.1 *Wolbachia* infection status of German *Culex* mosquitoes

Before assessing any potential effects of *Wolbachia* infections on WNV susceptibility, it is important to know the natural *Wolbachia* infection status of the German *Culex* population.

*Wolbachia* were first discovered in the reproductive organs of *Cx. pipiens* mosquitoes (Hertig & Wolbach 1924; Hertig 1936). They have also been identified in German populations via CI crossing experiments (Laven 1951). It is thus highly likely, but not confirmed, that the *Cx. pipiens* biotype *pipiens*, biotype *molestus* and hybrid populations investigated in this work are also positive for *Wolbachia*. In contrast, the infection status for *Cx. torrentium* populations in Germany is entirely unknown.

Various larvae samples from the North and South of Germany were screened for *Wolbachia* infection. Most, but not all, *Cx. pipiens* biotype *pipiens*/biotype *molestus* and hybrid samples were found to be *Wolbachia* positive. In contrast, none of the *Cx. torrentium* samples, were tested positive for *Wolbachia* (refer to Fig. 3.17).

Previous studies have investigated the infection status of *Cx. pipiens* mosquitoes from various countries, including Russia, Kazakhstan, Kyrgyzstan, Belarus, Portugal, Spain, France, Italy, Switzerland, Belgium, Great Britain, the Netherlands, Greece, Turkey, Cyprus, Tunisia and Australia. They all confirmed a *Wolbachia* infection status of up to 100% (Duron et al. 2006; Khrabrova et al. 2009; Raharimalala et al. 2016; Vinogradova et al. 2007; Shaikevich & Zakharov 2010). In contrast, none of the studies, including a study performed 2016 in Belgium, reported presence of Wolbachia in *Cx. torrentium* mosquitoes (Vinogradova et al. 2007; Khrabrova et al. 2009; Shaikevich & Zakharov 2010; Raharimalala et al. 2016). However, a single study from Italy, which included two *Cx. torrentium* individuals, reported both of them to be positive for arthropod-specific *Wolbachia* (Ricci et al. 2002).

In any case, none of the German *Cx. torrentium* populations investigated in this study were found to contain *Wolbachia*, which is in contrast to the high prevalence of *Wolbachia* that was found in *Cx. pipiens* biotype *pipiens*, biotype *molestus* and hybrid populations. This finding might explain other interesting phenomena such as the difference in genetic variability between *Cx. torrentium* and *Cx. pipiens* reported by others

(Werblow et al. 2014). Werblow et al. hypothesised that the decreased mitochondria diversity in *Cx. pipiens*, with respect to *Cx. torrentium* populations, is the result of CI and subsequent mitochondrial sweep. A *Wolbachia*-free *Cx. torrentium* population in Germany substantiates this statement.

About 80 to 100% of the *Cx. pipiens* biotype *pipiens* samples were *Wolbachia*-positive, which indicates that a small percentage was free of *Wolbachia*. In addition, one egg raft was tested both positive and negative for *Wolbachia*. These two findings might indicate that vertical transmission of *Wolbachia* is imperfect as previously suggested by Turelli (Turelli 1994).

Interestingly, there was a significant difference in *Wolbachia* prevalence between *Cx. pipiens* biotype *pipiens* populations in the North and South of Germany. This finding is in line with previous observations of differing *Wolbachia* infection rates among spatially isolated mosquito populations of the same species (Vinogradova et al. 2007; Khrabrova et al. 2009; Shaikevich & Zakharov 2010).

It would be interesting to analyse the *Wolbachia* prevalence within and among mosquito populations over time in order to evaluate, whether there are any changes, which could possibly be connected to arbovirus susceptibility. Fortunately, the newly developed *Wolbachia* qRT-PCR enables the handling of large amounts of samples via high-throughput screening, which is indispensable for such studies.

#### 4.1.3.2 Variation assessment of wPip infection

Intrigued by the complex CI patterns observed in mosquito populations, previous studies have established that *Wolbachia* show high genetic variability, which allows further subdivision of strains (such as wPip) into variants (Duron et al. 2006; Klasson et al. 2008). These are generally characterised by screening of repetitive and mobile elements, including ANK repeat domains and WO prophage genes (Klasson et al. 2008).

Using the method developed by Duron et al., focusing on WO prophage genes, three *Cx. pipiens* biotype *molestus* lab strains were screened for wPip variants. In total, 12 different wPip variants (four per lab strain) could be identified via screening of only 10 individuals each. This reflects the high amount of different wPip variants discovered in Southern European *Cx. pipiens*, in which up to 10 different variants were detected in a single population (Duron et al. 2006).

However, different wPip variants might not only ensure evolutionary flexibility of the *Wolbachia* species but are also relevant for other effects on mosquito biology, including vectorial capacity. A recent study revealed that different wMel variants, *Wolbachia* that infect *Drosophila melanogaster*, confer different degrees of protection against Drosophila C virus infection (Chrostek et al. 2013). In addition, Riegler et al. have reported evidence that, in the case of wMel and *D. melanogaster*, one single wMel variant has replaced all others circulating in nature within the last 80 years, whereas a selection of variants continue to circle in lab strains (Riegler et al. 2005).

It would thus be of interest to see, whether different wPip variants also confer different levels of protection against WNV. To do this, it is necessary to make 'monoclonal populations' infected with only one single wPip variant as was reported for wMel in nature (Riegler et al. 2005). This was attempted in this study (refer to Table 3.2). However, it was not possible to establish a stable monoclonal *Cx. pipiens* biotype *pipiens* population thus far.

During this work, it was not possible to successfully generate a mosquito population with a monoclonal infection of wPip, even though establishment is possible with wMel strains in nature. One could argue that colony establishment in the lab was started with one single egg raft, leading to relatively few individuals and an extreme genetic bottleneck. In the case of *D. melanogaster* in nature, the population was not simply reduced to one wMel strain, but the seemingly monoclonal wMel infection established itself over several decades due to fitness benefits, that are not generally decisive in a lab setting (Miller 2013; Teixeira et al. 2008). Texeira et al. studied this replacement process and found that flies carrying the previous ancestral strain wMelCS infection are more protected against viral infections but pay a cost in life expectancy. wMel strains grow to lower titers, thus reducing fitness costs, but are still protecting against viral infections. This promoted the replacement of wMelCS by wMel over time (Miller 2013; Teixeira et al. 2008).

In addition, Atyame et al. proposed that the lower genetic diversity seen in other arthropods, such as *D. melanogaster*, are the result of restricted methodology used and lack of studies on CI. In other words, the variation is simply missed (Atyame et al. 2011). To add to this, recombination and other gene modifications involving mobile genetic elements have been shown to be responsible for wPip variability and are necessary to promote and explain the complex CI patterns seen in mosquitoes (Duron et al. 2006; Atyame et al. 2011). It is therefore likely, although not detectable with the method used, that one mosquito individual is infected with several wPip variants, which prevents establishment of monoclonal populations (Atyame et al. 2011).

Accordingly, it was decided to investigate *Wolbachia*-mediated changes in mosquito biology using mosquitoes with non-monoclonal wPip infections as is the case in nature.

#### 4.1.3.3 *Wolbachia*-mediated changes in mosquito biology

*Wolbachia* have been shown to influence their host's biology in ways that range from classic parasitism to mutualism (Werren et al. 2008). The most common phenotypes, which ensure spread of *Wolbachia* within the mosquito population, comprise manipulation of the reproductive system, cytoplasmic incompatibility and male killing (Werren et al. 2008). In addition, other interesting phenotypes initiated by *Wolbachia* include behavioural changes such as alterations in mating preferences (Koukou et al. 2006) and response to olfactory cues (Peng et al. 2008) as well as resistance against viral infections (Moreira et al. 2009; Teixeira et al. 2008).

These *Wolbachia*-mediated effects, especially resistance against viral infections, can influence mosquito fitness and reproductive capacity in the long run, which are major parameters defining vectorial capacity (Hegde et al. 2015).

#### 4.1.3.3.1 Blood-feeding behaviour

A *Wolbachia*-mediated effect was observed while performing blood feed assays with *Wolbachia*-infected and *Wolbachia*-cured *Cx. pipiens* biotype *molestus* lab strains S and W. Whereas no significant differences were detected in the proportion of blood-engorged females in *Cx. pipiens* biotype *molestus* S (originating from the South of Germany), an interesting variation was observed with the W populations from the North. Here, significantly less of the *Wolbachia*-cured mosquitoes (47%; n=495) took a blood meal in comparison to their *Wolbachia*-infected counterparts (70%; n=472). Thus, it seems that *Wolbachia* might have an effect on the feeding behaviour of *Cx. pipiens* biotype *molestus* W (refer to Fig. 3.20).

An increase in feeding rates, in connection with elevated pathogen infection burden, has been shown previously. However, these manipulations of hosts originate from the fact that these pathogens need to be transmitted to a new host via a blood meal, which is not the case in *Wolbachia* (Turley et al. 2009; Grimstad et al. 1980; Rossignol et al. 1984; Añez & East 1984). Nevertheless, a relatively recent study in bees has shown that infection can lead to an increased feeding rate although pathogen transmission occurs via a different route (Mayack & Naug 2009).

Interestingly, only very few studies have looked on the influence of *Wolbachia* infection on mosquito behaviour. Moreover, all have focused on artificial interactions of *Ae. aegypti* and the life-shortening *Drosophila Wolbachia* strain wMelPop. In one of the studies, Turley et al. have shown that *Wolbachia*-infected individuals obtain fewer blood meals than their *Wolbachia*-uninfected counterparts. Although this effect was shown for older mosquitoes (26 and 35 days) only, it is in contrast to the results obtained with *Cx. pipiens* biotype *molestus* W in this study. However, as previously mentioned, the infection with wMelPop is severe, leading to tissue damage that increases with age and most likely causes reduction in blood feeding success. This is impressively illustrated by the 'bendy proboscis', a behavioural trait that Turley et al. described for infected mosquitoes (Fig. 4.3) (Turley et al. 2009).

It is more likely that increased blood feeding rates of *Wolbachia*-infected *Cx. pipiens* biotype *molestus* W stems from the need of *Wolbachia* for female mosquitoes to produce large amounts of (infected) eggs and to ensure a fitness benefit via increased nutrient uptake. Alternatively, or in addition, the increased nutrient uptake could be a behaviour to compensate for the energy demand of infections, which would be comparable to the situation observed in parasite-infected bees (Mayack & Naug 2009). This hypothesis is also supported by work from Evans et al., who observed increased locomotor activity and metabolic rates in *Ae. aegypti* infected with wMelPop (Evans et al. 2009).

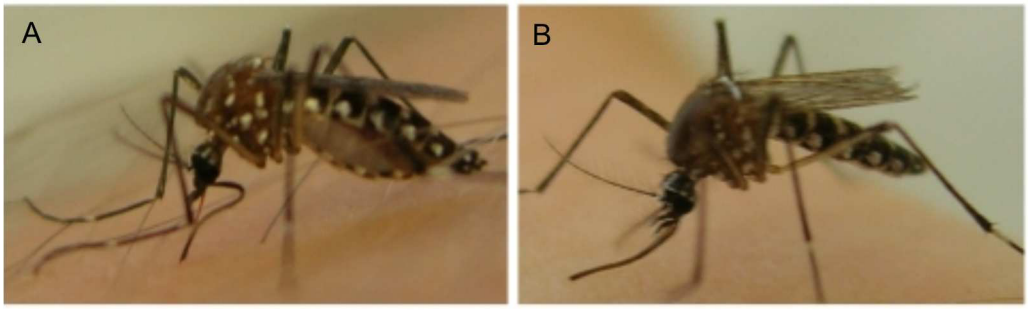



**Fig. 4.3: Behavioural trait 'bendy proboscis' observed in *Ae. aegypti* infected with wMelPop**
Image by Turley et al. 2009
A: Successful bite of a *Wolbachia*-uninfected *Ae. aegypti* female
B: wMelPop infected *Ae. aegypti* female is unable to penetrate the skin due to a flexible proboscis possibly caused by tissue damage

In the case presented here, the effect appears to be population specific. In contrast to the population from the North, *Cx. pipiens* biotype *molestus* from the South did not display a *Wolbachia*-mediated change in the blood feed rate.

Screenings for wPip variants in this study revealed that the *Cx. pipiens* biotype *molestus* lab strains are all infected with a different set of variants (refer to Table 3.1). It could be possible that the diverse variants have different impacts on the host behaviour. This has not been extensively studied in the case of wPip. However, it is known from studies in mosquitoes and fruit flies that *Wolbachia* variants can grow to different levels and may induce lower or higher energetic stress on insect hosts (Berticat et al. 2002; Chrostek et al. 2013). Thus, a possible hypothesis could be that increasing *Wolbachia* density is positively correlated with behavioural changes involving blood meal intake.

Naturally, there is also a realistic possibility that, due to tetracycline treatment, the remaining microbiota in *Cx. pipiens* biotype *molestus* W has changed in such a way that feeding behaviour is affected. The microbiota of mosquitoes is known to be very complex and variable (Minard et al. 2013). Different members of the microbiota are also known to interact and influence each other. For instance, bacteria of the genus *Asaia* were found to inhibit vertical transmission of *Wolbachia* in *Anopheles*. A similar mechanism might be instrumental for the lack of *Wolbachia* in *Cx. torrentium* mosquitoes (Hughes et al. 2014). It is to note, however, that much of the microbiota diversity of the mosquito is acquired through interaction with the environment (e.g. food intake) and this is expected to be stable in a laboratory setting (Minard et al. 2013).

#### 4.1.3.3.2 WNV susceptibility

From a public health point of view, resistance to viral infections is one of the most important *Wolbachia*-mediated effects on mosquito biology. The interaction between mosquito vector, *Wolbachia* and viral infection is already implemented in arbovirus control programmes today. Based on the observation that artificial *Wolbachia*-infections induce resistance against DENV in *Ae. aegypti*, the Eliminate Dengue program was established,

which, until now, reports exclusively positive results (Walker et al. 2011; Ferguson et al. 2015; Eliminate Dengue Program n.d.).

Research aiming for similar interactions in *Culex* mosquitoes revealed a somewhat inexplicit picture. Looking at the natural system, i.e. in mosquitoes naturally infected with *Wolbachia* such as *Cx. quinquefasciatus*, *Wolbachia* mediated reduction in WNV transmission in a density dependent manner (Glaser & Meola 2010; Micieli & Glaser 2014). Furthermore, studies into artificial systems involving naturally *Wolbachia*-free species, do not resemble the situation seen with *Ae. aegypti*. In fact, *Wolbachia*-free *Cx. tarsalis* are even more susceptible to WNV infection upon transient infection with wAlbB from *Ae. albopictus* (Dodson et al. 2014).

These studies all cover different *Culex* species-*Wolbachia* strain-interactions, which likely contribute to the different phenotypes observed. This further underlines the need for thorough investigations before implementing *Wolbachia* as an arbovirus control method.

The results obtained in this work further contribute to this view.

A blood feed assay was performed with two *Cx. pipiens* biotype *molestus* lab strains representing Southern (S) and Northern (W) populations. Susceptibility for WNV was investigated in lab strains representing populations infected with wPip and corresponding cured groups, respectively. The obtained infection and dissemination rates again indicated population-based variations (refer to Fig. 3.21-3.24).

In the case of the S-population, infection rates were generally higher for *Wolbachia*-free mosquitoes. This difference increased over time and was found statistically significant at day 21 post infection. However, no significant differences were observed in the case of *Cx. pipiens* biotype *molestus* W. The dissemination rates obtained for both strains mimic respective infection rates.

It is interesting to connect these results with the blood feeding rates obtained for the respective strains. Although there is no change in feeding rate, WNV susceptibility seems to increase with the loss of *Wolbachia* infection in the case of *Cx. pipiens* biotype *molestus* S. The reverse is seen for the strain W from the North – feeding is reduced with the loss of *Wolbachia.* However, there was no effect on WNV susceptibility. This seems counterintuitive since it might be expected that both feeding rate and resistance against viral infection simultaneously increase with increasing *Wolbachia* titers. A positive correlation has already been shown previously in the case of viral resistance in mosquitoes (Micieli & Glaser 2014).

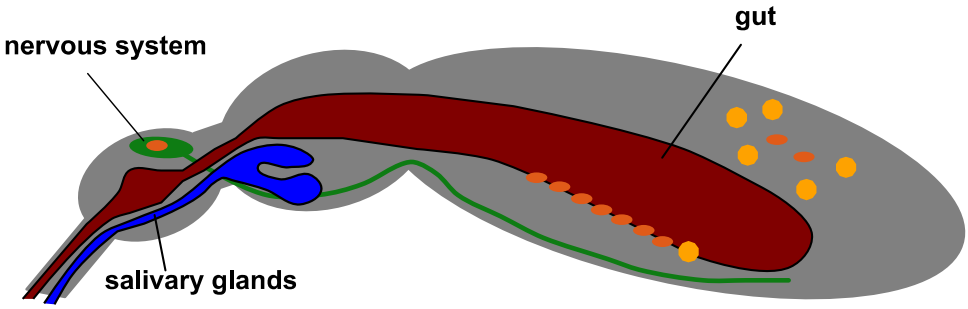
Studies focusing on DENV in *Ae. aegypti* mosquitoes or in respective cell lines might resolve this dilemma. These studies have shown that not only the general or cellular density but also the tissue location of *Wolbachia* in the host are important factors for providing antiviral protection (Frentiu et al. 2010; Moreira et al. 2009). Assuming that cellular density and the location of *Wolbachia* influences resistance to WNV, the following scenario could be proposed, based on discussion from Glaser & Meola 2010 (Fig. 4.4):

In *Cx. pipiens* biotype *molestus* S, the *Wolbachia* titers in the midgut, where the viral infection begins, are high, resulting in a decreased infection rate when compared with *Wolbachia*-free mosquitoes. However, in all subsequent tissues, the *Wolbachia* titers are lower, making dissemination rates less dependent on the presence or absence of *Wolbachia* (Fig. 4.4 A). In the case of *Cx. pipiens* biotype *molestus* W, the *Wolbachia* titers are low in all tissues that are important for infection and dissemination of the virus, resulting in infection and dissemination rates independent of the *Wolbachia* infection (Fig. 4.4 B). Instead, the *Wolbachia* cluster in areas controlling mosquito behaviour, i.e. the nervous system, and affect the blood feeding rate. This causes a decrease in feeding stimuli once the mosquito is cured of *Wolbachia*.

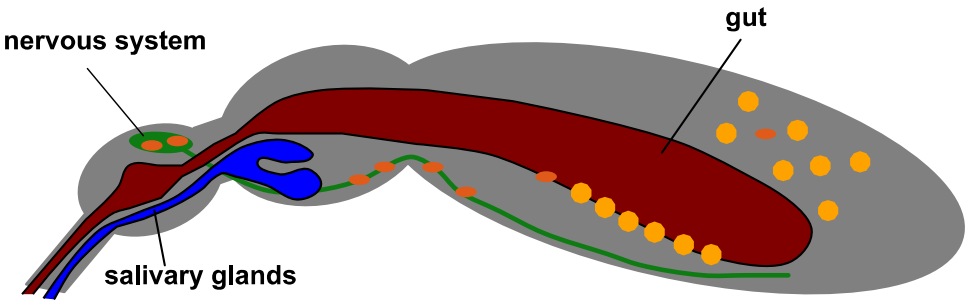
The link between *Wolbachia* and the nervous system with respect to feeding behaviour in *Culex* mosquitoes has not yet been subject of scientific studies. It is only known, that the attraction to human odours is not influenced by *Wolbachia* infections in *Ae. aegypti*, but locomotion is increased (Evans et al. 2009; Turley et al. 2014). Nevertheless, it is known from *D. melanogaster* that *Wolbachia* infection of the nervous system can markedly influence behaviour. This effect might be density dependent (Rohrscheib et al. 2015).

Further investigations are required to validate the proposed scenario. This could be done, for instance, via immunofluorescent tagging of individual organs as it has been described by Moreira et al. 2009. In any case, the results presented here indicate that *Wolbachia* infections may induce complex interactions with the host. These interactions can influence vector competence for pathogens and have to be considered for future investigations into vector control measures involving *Wolbachia*.

**A** *Cx. pipiens* biotype *molestus* S; *Wolbachia*-infected




**B** *Cx. pipiens* biotype *molestus* W; *Wolbachia* -infected




**C** *Cx. pipiens* biotype *molestus* W and S; *Wolbachia*-free

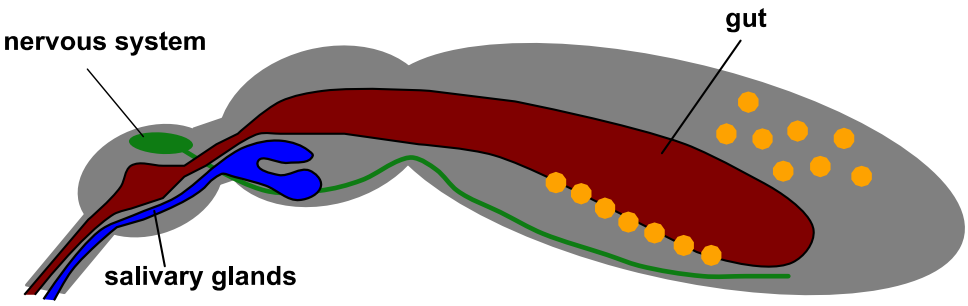



**Fig. 4.4: Schematic illustration of the putative effect of *Wolbachia* density and location on WNV infection and dissemination rate as well as feeding behaviour of *Cx. pipiens* biotype *molestus***

In *Cx. pipiens* biotype *molestus* S (A), the *Wolbachia* (orange spheres) density is high in the gut area, allowing inhibition of viral reproduction (yellow spheres) and a lower infection rate with respect to *Wolbachia*-free mosquitoes (C). However, the dissemination rate is similar due to a lower *Wolbachia* density in subsequent tissues.

In *Cx. pipiens* biotype *molestus* W (B), the *Wolbachia* density is generally low in tissues crucial for viral infection and dissemination, leading to similar infection and dissemination rates as for the *Wolbachia*-free mosquitoes. Due to a high level of *Wolbachia* in the nervous system, feeding behaviour is influenced.

## 4.2 Conclusions and future perspectives

Outbreaks of West Nile disease is a current and serious concern for Europe, illustrated by a total of 301 human cases in the 2015 transmission season (European Centre for Disease Prevention and Control 2015).

Members of the *Culex* complex of mosquitoes act as vectors for WNV in other countries (Chancey et al. 2015). Some of these species are also present in Germany (Rudolf et al. 2013; Krüger et al. 2014). To perform a risk assessment in the case of WNV entry into the country, it is necessary to investigate the distribution of putative vectors. Subsequently, their susceptibility for the virus in the context of influencing factors, including temperature, population origin, time and *Wolbachia* infection must be analysed.

The focus of this work was to perform corresponding investigation for selected *Culex* species native to Germany. The results obtained and future perspectives are summarised and outlined in the following.

Via the collection of egg rafts, mainly *Cx. pipiens* biotype *pipiens* and *Cx. torrentium* were found in both the South and North of Germany. Although there were temporal (i.e. yearly and monthly) variations in the prevalence of each species, these were deemed less defining for risk assessment, since both *Cx. pipiens* biotype *pipiens* and *Cx. torrentium* have been characterised as ornithophilic and mammophilic (Börstler et al. 2016). Investigation into spatial variation indicated an increasing prevalence of *Cx. torrentium* towards the North of Germany.

*Cx. torrentium* displayed the highest overall infection rates and was found to be susceptible to WNV infection at both 18 °C and 25 °C. Notably, the peak of infection at the lower temperature was delayed by 7 days, indicating slower virus replication. Nevertheless, infection and dissemination occurred irrespective of temperature. There is even a trend towards a higher infection rate at 18 °C in the Northern population. When added to the higher prevalence of *Cx. torrentium* in the North, this observation would contribute towards *Cx. torrentium* being a main vector for WNV in Northern Germany. Despite the moderate sample sizes of *Cx. torrentium*, the infection data clearly indicated that *Cx. torrentium* must be incorporated into future WNV risk assessments in Germany.

*Cx. pipiens* biotype *pipiens*, both from Northern and Southern Germany, are susceptible to WNV infection irrespective of the incubation temperature. Interestingly, a significantly higher infection rate was determined for the Southern population. Highly susceptible vector populations in Southern Germany are of particular concern for establishment of viral transmission cycles, as this area represents the most likely point of entry into the country (Ciota & Kramer 2013; Engler et al. 2013).

Susceptibility for WNV revealed temporal variations between sampling years. However, this is not expected to disrupt an established viral transmission cycle as temporal variation is also observed in endemic areas (Kilpatrick et al. 2010).

These results provide helpful information on the vectorial capacity for WNV. However, for complete assessment of vector competence, and by extension vectorial capacity, it

is necessary to determine the transmission rate as well. Furthermore, to obtain the most realistic picture, WNV circulating in Europe rather than isolates from the USA should be used to assess the vector competence of European mosquitoes. To improve risk assessment for Germany, it is thus imperative that future work focuses on infection, dissemination and transmission rates obtained with European WNV strains. It is also of interest to incorporate other potential WNV vectors that are not members of the *Culex* complex into the investigations. These should include other abundant mosquito species native to Germany such as *Ae. vexans* as well as new invasive species such as *Ochlerotatus japonicus japonicus* or *Ae. albopictus*, which have recently entered Germany (Werner et al. 2012; Becker et al. 2011; Börstler et al. 2016) and have been associated with the transmission of arboviruses (Ndiaye et al. 2016; Huber et al. 2014; de Lamballerie et al. 2008). Furthermore, based on very current global events, arboviral research in Germany should not only focus on WNV but also on other arboviruses, such as the Zika virus, that have the potential to be of high public health risk in future (Di Luca et al. 2016).

Returning to the WNV risk assessment for Germany, the effect of *Wolbachia* on the susceptibility for WNV has been investigated as well. It is interesting to see that the *Wolbachia* prevalence of *Cx. pipiens* biotype *pipiens* is higher in the South, and that *Cx. torrentium*, which is more prevalent in the North of Germany, does not harbour any *Wolbachia* at all. Should *Wolbachia* have a debilitating effect on WNV susceptibility for German mosquitoes, this protective characteristic would be beneficial in areas, where WNV entry into the country is most likely (Ciota & Kramer 2013; Engler et al. 2013).

Considering the effect of *Wolbachia*, a diverging phenotypes for the two *Cx. pipiens* biotype *molestus* lab strains W and S for blood-feeding behaviour and susceptibility to WNV was observed. To explain this observation, it was hypothesised that *Wolbachia*-mediated resistance to WNV infection and changes in feeding behaviour could be positively correlated with density and location in the gut and nervous system, respectively. However, further work is required to substantiate this proposition.

As mentioned above, it was found that *Wolbachia*-free *Cx. torrentium* generally have a higher WNV infection rate than the *Wolbachia*-infected *Cx. pipiens* biotype *pipiens* populations. It would be interesting to clarify, whether this difference in infection rate is indeed *Wolbachia*-mediated and whether an artificial infection of *Cx. torrentium*, similar to *Ae. aegypti*, would lead to a resistance phenotype (Walker et al. 2011). If so, this would be an interesting starting point for the development of vector and virus control measures. To do this, it would be necessary to establish a *Cx. torrentium* laboratory colony. This has proven to be difficult in the past, since *Cx. torrentium* adults are considered eurygamous (Vinogradova 2003). Nevertheless, establishment of laboratory colonies has been shown with a Swedish population, increasing the likelihood of success also for Germany (Lundström, Turell, et al. 1990).

The intracellular mechanism of *Wolbachia*-mediated resistance to arboviruses should also be subject of future research. Several possible mechanisms have been proposed, including the competition for resources, remodelling of the host cell environment, immune stimulation and the expression of microRNAs (reviewed in Rainey et al. 2014). In

particular the unravelling of *Wolbachia*-mediated changes in gene regulation that are linked to vector competence could substantiate knowledge about the complex tripartite interactions between *Wolbachia*, mosquito and arbovirus. This information is important for the development of risk assessments and arbovirus control measures.

All in all, the results of the work presented here show that German *Cx. torrentium* and *Cx. pipiens* biotype *pipiens* populations are realistic WNV vector candidates. The *Wolbachia* status of these vector populations provide potential for the development of arbovirus control measures. Most importantly, this work stresses the need for continuous surveillance and foresighted research to minimise the risk of WNV establishment in Germany.

# 5 Literature

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# 6 Supplementary Material

## 6.1 The susceptibility of *Culex* mosquitoes native to Germany for WNV

**Table 6.1: Summary of mosquito samples used for WNV infection assays in 2013 and 2012**
The following lab strains were kept in captivity for 2 month to 3 years prior to the WNV infection assay: *Cx. pipiens* biotype *molestus* Heidelberg (*Cx. mol* S), *Cx. pipiens* biotype *molestus* Wendland (*Cx. mol* W) and *Cx. pipiens* biotype *molestus* Langenlehsten (*Cx. mol* LL). A lab strain of *Cx. quinquefasciatus* (*Cx. qui*; Malaysia strain, Bayer Company) was also used.
The following species were reared from field-collected egg rafts: *Cx. pipiens* biotype *pipiens* from Hamburg/Langenlehsten/Altes Land (*Cx. pip* North), *Cx. pipiens* biotype *pipiens* from Lake Constance (*Cx. pip* South), *Cx. torrentium* from Hamburg/Langenlehsten/Altes Land (*Cx. tor* North) and *Cx. torrentium* from Lake Constance (*Cx. tor* South).
The numbers written in brackets represent the number of individuals, assays and field-collected egg rafts used in 2012. In 2012, samples were collected and identified by my colleagues in the Molecular Entomology lab (refer to acknowledgements). Experiments = blood feed assays

| Species | Origin | Egg rafts | Temperature [°C] | Infection time (days) | Individuals [#] | Experiments [#] |
|---|---|---|---|---|---|---|
| *Cx. qui* | Malaysia | Lab strain | 25 | 14 | 66 | 9 |
| | | | 25 | 21 | 169 | 12 |
| | | | 25 | 28 | 41 | 5 |
| | | | 25 | 35 | 103 | 7 |
| | | | 18 | 14 | 29 | 6 |
| | | | 18 | 21 | 64 | 6 |
| | | | 18 | 28 | 38 | 5 |
| | | | 18 | 35 | 36 | 5 |
| *Cx. pip* | North | 186 (222) | 25 | 14 | 22 (94) | 5 (3) |
| | | | 25 | 21 | 65 (173) | 7 (3) |
| | | | 25 | 28 | 53 | 5 |
| | | | 25 | 35 | 69 | 5 |
| | | | 18 | 14 | 23 | 5 |
| | | | 18 | 21 | 41 | 5 |
| | | | 18 | 28 | 46 | 5 |
| | | | 18 | 35 | 72 | 5 |
| *Cx. pip* | South | 311 | 25 | 14 | 8 | 2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | 25 | 21 | 24 | 4 |
| | | | 25 | 28 | 11 | 3 |
| | | | 25 | 35 | 10 | 3 |
| | | | 18 | 14 | 8 | 2 |
| | | | 18 | 21 | 26 | 4 |
| | | | 18 | 28 | 12 | 3 |
| | | | 18 | 35 | 14 | 3 |
| *Cx. mol* | S,W,LL | Lab strains | 25 | 14 | 80 | 6 |
| | | | 25 | 21 | 273 | 14 |
| | | | 25 | 28 | 109 | 11 |
| | | | 25 | 35 | 90 | 6 |
| | | | 18 | 14 | 15 | 3 |
| | | | 18 | 21 | 24 | 4 |
| | | | 18 | 28 | 39 | 5 |
| | | | 18 | 35 | 67 | 5 |
| *Cx. tor* | North | 225 (119) | 25 | 14 | 12 (130) | 3 (4) |
| | | | 25 | 21 | 34 (138) | 6 (4) |
| | | | 25 | 28 | 9 | 3 |
| | | | 25 | 35 | 9 | 3 |
| | | | 18 | 14 | 12 | 3 |
| | | | 18 | 21 | 15 | 3 |
| | | | 18 | 28 | 16 | 3 |
| | | | 18 | 35 | 19 | 3 |
| *Cx. tor* | South | 29 | 25 | 14 | 0 | 0 |
| | | | 25 | 21 | 12 | 3 |
| | | | 25 | 28 | 3 | 1 |
| | | | 25 | 35 | 0 | 0 |
| | | | 18 | 14 | 0 | 0 |
| | | | 18 | 21 | 7 | 2 |
| | | | 18 | 28 | 10 | 2 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | 18 | 35 | 0 | 0 | |

**Table 6.2: : Infection rates of WNV in field-caught *Cx. torrentium* and *Cx. pipiens* biotype *pipiens (2013 and 2012)* as well as lab strains *Cx. quinquefasciatus* and *Cx. pipiens* biotype *molestus***

The following lab strains were kept in captivity for 2 month to 3 years prior to the WNV infection assay: *Cx. pipiens* biotype *molestus* Heidelberg (*Cx. mol* S), *Cx. pipiens* biotype *molestus* Wendland (*Cx. mol* W) and *Cx. pipiens* biotype *molestus* Langenlehsten (*Cx. mol* LL). A lab strain of *Cx. quinquefasciatus* (*Cx. qui*; Malaysia strain, Bayer Company) was also used.
The following species were reared from field-collected egg rafts: *Cx. pipiens* biotype *pipiens* from Hamburg/Langenlehsten/Altes Land (*Cx. pip* North), *Cx. pipiens* biotype *pipiens* from Lake Constance (*Cx. pip* South), *Cx. torrentium* from Hamburg/Langenlehsten/Altes Land (*Cx. tor* North) and *Cx. torrentium* from Lake Constance (*Cx. tor* South).
The infection rate is calculated by determining the number of WNV-infected females with respect to the number of blood-fed females (n).

| Species | Origin | Sampling year | Temperature [°C] | Infection time (days) | n [#] | infected females [#] | infection rate [%] |
|---|---|---|---|---|---|---|---|
| *Cx. qui* | Lab strain | n/a | 25 | 14 | 66 | 23 | 35 |
| | | | 25 | 21 | 169 | 101 | 60 |
| | | | 25 | 28 | 41 | 32 | 78 |
| | | | 25 | 35 | 103 | 64 | 62 |
| | | | 18 | 14 | 29 | 11 | 38 |
| | | | 18 | 21 | 64 | 36 | 56 |
| | | | 18 | 28 | 38 | 31 | 82 |
| | | | 18 | 35 | 36 | 25 | 69 |
| *Cx. mol* | Lab strain (S,W,LL) | n/a | 25 | 14 | 80 | 37 | 46 |
| | | | 25 | 21 | 273 | 174 | 64 |
| | | | 25 | 28 | 109 | 54 | 50 |
| | | | 25 | 35 | 90 | 57 | 63 |
| | | | 18 | 14 | 15 | 10 | 67 |
| | | | 18 | 21 | 24 | 13 | 54 |
| | | | 18 | 28 | 39 | 14 | 36 |
| | | | 18 | 35 | 67 | 28 | 42 |
| *Cx. pip* | North | 2013 | 25 | 14 | 22 | 5 | 23 |
| | | | 25 | 21 | 65 | 36 | 55 |
| | | | 18 | 21 | 41 | 27 | 66 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Cx. pip* | South | 2013 | 25 | 21 | 24 | 22 | 92 |
| | | | 18 | 21 | 26 | 23 | 88 |
| *Cx. pip* | North + South | 2013 | 25 | 14 | 30 | 8 | 27 |
| | | | 25 | 21 | 89 | 58 | 65 |
| | | | 25 | 28 | 64 | 34 | 53 |
| | | | 25 | 35 | 79 | 19 | 24 |
| | | | 18 | 14 | 31 | 13 | 42 |
| | | | 18 | 21 | 67 | 50 | 75 |
| | | | 18 | 28 | 58 | 39 | 67 |
| | | | 18 | 35 | 86 | 28 | 33 |
| *Cx. pip* | North | 2012 | 25 | 14 | 94 | 5 | 5 |
| | | | 25 | 21 | 173 | 95 | 55 |
| *Cx. tor* | North | 2013 | 25 | 14 | 12 | 10 | 83 |
| | | | 25 | 21 | 34 | 32 | 94 |
| | | | 18 | 21 | 15 | 13 | 87 |
| *Cx. tor* | South | 2013 | 25 | 21 | 12 | 12 | 100 |
| | | | 18 | 21 | 7 | 3 | 43 |
| *Cx. tor* | North + South | 2013 | 25 | 14 | 12 | 10 | 83 |
| | | | 25 | 21 | 46 | 44 | 96 |
| | | | 25 | 28 | 12 | 11 | 92 |
| | | | 25 | 35 | 9 | 3 | 33 |
| | | | 18 | 14 | 12 | 2 | 17 |
| | | | 18 | 21 | 22 | 16 | 73 |
| | | | 18 | 28 | 26 | 23 | 88 |
| | | | 18 | 35 | 19 | 15 | 79 |
| *Cx. tor* | North | 2012 | 25 | 14 | 130 | 72 | 55 |
| | | | 25 | 21 | 138 | 95 | 69 |

**Table 6.3: Dissemination rates of WNV in field-caught *Cx. torrentium* and *Cx. pipiens* biotype *pipiens* (2013)**
The following species were reared from field-collected egg rafts: *Cx. pipiens* biotype *pipiens* from Hamburg/Langenlehsten/Altes Land (*Cx. pip* North), *Cx. pipiens* biotype *pipiens* from Lake Constance (*Cx. pip* South), *Cx. torrentium* from Hamburg/Langenlehsten/Altes Land (*Cx. tor* North) and *Cx. torrentium* from Lake Constance (*Cx. tor* South).
The dissemination rate is calculated by determining the number of WNV-infected heads with respect to the number of WNV-positive females.

| Species | Origin | Sampling year | Temperature [°C] | Infection time (days) | infected females [#] | infected head [#] | dissemination rate [%] |
|---|---|---|---|---|---|---|---|
| *Cx. pip* | North | 2013 | 25 | 21 | 58 | 52 | 90 |
| | + | | 25 | 28 | 34 | 32 | 94 |
| | South | | 25 | 35 | 19 | 13 | 68 |
| | | | 18 | 21 | 50 | 44 | 88 |
| | | | 18 | 28 | 39 | 33 | 85 |
| | | | 18 | 35 | 28 | 23 | 82 |
| *Cx. tor* | North | | 25 | 21 | 44 | 41 | 93 |
| | + | | 25 | 28 | 11 | 11 | 100 |
| | South | | 25 | 35 | 3 | 2 | 67 |
| | | | 18 | 21 | 16 | 13 | 81 |
| | | | 18 | 28 | 23 | 21 | 91 |
| | | | 18 | 35 | 15 | 10 | 67 |

## 6.2 Influence of Wolbachia infection on the susceptibility of *Culex* mosquitoes native to Germany for WNV

**Table 6.4: Summary of mosquito samples used for *Wolbachia*/WNV infection assays**
The *Cx. pipiens* biotype *molestus* (*Cx. mol*) used here is a lab strain. *Cx. pipiens* biotype *molestus* S (''Südland'') originated from Heidelberg in Southern Germany. *Cx. pipiens* biotype *molestus* W (''Wendland'') originated from the Wendland area in Northern Germany. All *Wolbachia*-negative strains have been treated with tetracycline for 3 generation (F0, F1 and F2) and kept for another 8 consecutive generations (F3-F10) until used for assays. Experiments = blood feed assays

| Species | *Wolbachia* status | Temperature [°C] | Infection time (days) | Individuals [#] | Experiments [#] |
|---|---|---|---|---|---|
| *Cx. mol* S | negative | 25 | 14 | 43 | 5 |
| | | 25 | 21 | 58 | 7 |
| *Cx. mol* S | positive | 25 | 14 | 39 | 5 |
| | | 25 | 21 | 52 | 7 |
| *Cx. mol* W | negative | 25 | 14 | 40 | 3 |
| | | 25 | 21 | 36 | 3 |
| *Cx. mol* W | positive | 25 | 14 | 50 | 3 |
| | | 25 | 21 | 65 | 3 |

**Table 6.5: WNV infection rates of *Wolbachia*-negative and *Wolbachia*-positive lab strains of *Cx. pipiens* biotype *molestus* S and W**
The *Cx. pipiens* biotype *molestus* (*Cx. mol*) used here is a lab strain. *Cx. pipiens* biotype *molestus* S (''Südland'') originated from Heidelberg in Southern Germany. *Cx. pipiens* biotype *molestus* W (''Wendland'') originated from the Wendland area in Northern Germany. All *Wolbachia*-negative strains have been treated with tetracycline for 3 generation (F0, F1 and F2) and kept for another 8 consecutive generations (F3-F10) until used for WNV infection assay.
The infection rate is calculated by determining the number of WNV-infected females with respect to the number of blood-fed females (n).

| Species | *Wolbachia* status | Temperature [°C] | Infection time (days) | n [#] | infected females [#] | infection rate [%] |
|---|---|---|---|---|---|---|
| *Cx. mol S* | negative | 25 | 14 | 43 | 11 | 26 |
| | | 25 | 21 | 58 | 26 | 45 |
| *Cx. mol S* | positive | 25 | 14 | 39 | 7 | 18 |
| | | 25 | 21 | 52 | 13 | 25 |
| *Cx. mol W* | negative | 25 | 14 | 40 | 3 | 8 |
| | | 25 | 21 | 36 | 6 | 17 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Cx. mol W* | positive | 25 | 14 | 50 | 7 | 14 |
| | | 25 | 21 | 65 | 10 | 15 |

**Table 6.6: WNV dissemination rates of *Wolbachia*-negative and *Wolbachia*-positive lab strains of *Cx. pipiens* biotype *molestus* S and W**

The *Cx. pipiens* biotype *molestus* (*Cx. mol*) used here is a lab strain. *Cx. pipiens* biotype *molestus* S (''Südland'') originated from Heidelberg in Southern Germany. *Cx. pipiens* biotype *molestus* W (''Wendland'') originated from the Wendland area in Northern Germany. All *Wolbachia*-negative strains have been treated with tetracycline for 3 generation (F0, F1 and F2) and kept for another 8 consecutive generations (F3-F10) until used for WNV infection assay.
The dissemination rate is calculated by determining the number of WNV-infected heads with respect to the number of WNV-positive females.

| Species | *Wolbachia* status | Temperature (°C) | Infection time (days) | infected females [#] | infected head [#] | dissemination rate [%] |
|---|---|---|---|---|---|---|
| *Cx. mol S* | negative | 25 | 14 | 11 | 8 | 73 |
| | | 25 | 21 | 26 | 19 | 73 |
| *Cx. mol S* | positive | 25 | 14 | 7 | 3 | 43 |
| | | 25 | 21 | 13 | 9 | 69 |
| *Cx. mol W* | negative | 25 | 14 | 3 | 2 | 67 |
| | | 25 | 21 | 6 | 1 | 17 |
| *Cx. mol W* | positive | 25 | 14 | 7 | 2 | 29 |
| | | 25 | 21 | 10 | 2 | 20 |

## 6.3 $TCID_{50}$ Screening

**Table 6.7: Summary of $TCID_{50}$ screening of selected mosquito samples for viable virus particles.**
For each species, samples from at least 3 independent experiments were chosen at random and checked for viable virus particles via $TCID_{50}$ on Vero cells using antibody-mediated immunofluorescence targeted at the WNV E-protein. The final virus titers were calculated using a $TCID_{50}$ calculator developed by Marco Binder (Dept. Infectious Diseases, Molecular Virology, Heidelberg University), which is employing the Spearman and Kärber algorithm described by Hierholzer and Killington (1996).
Titers in the graph are marked according to the following colour scheme for ease of use:
Green: CT ≤ 35 and virus titer ≠ 0; Red: CT ≤35 and virus titer = 0; White: CT > 35 or CT = 0 and virus titer = 0 or ≠ 0
Note that the Spearman and Kärber algorithm does not account for a titer of 0 PFU/ml. When no viable virus particles are visible via immunfluoresence, the final titer is calculated as 4.36E+00.
*Cx qui* = *Cx. quinquefasciatus*; Cx. *pip* = *Cx. pipiens* biotype *pipiens*; *Cx. mol* = *Cx. pipiens* biotype *molestus*; *Cx. tor* = *Cx. torrentium*

| **Sample name** | **Species** | ***Wolbachia* status** | **dpi** | **Material** T = total mosquito B = only body | **CT PrB** | **final TCID50 titer [PFU/ml]** |
|---|---|---|---|---|---|---|
| Q26/27 0-1 | *Cx. qui* | n/a | 0 | T | 27 | 4.36E+01 |
| Q26/27 0-3 | | | 0 | T | 29 | 4.36E+01 |
| Q33/34 0-1 | | | 0 | T | 25 | 2.45E+01 |
| Q33/34 0-2 | | | 0 | T | 28 | 1.38E+01 |
| Q36/35 0-1 | | | 0 | T | 0 | 2.45E+01 |
| Q35/36 0-5 | | | 0 | T | 25 | 7.76E+03 |
| Q27 14-1 | | | 14 | T | 31 | 1.38E+01 |
| Q27 14-2 | | | 14 | T | 32 | 1.38E+02 |
| Q27 14-4 | | | 14 | T | 32 | 2.45E+01 |
| Q34 14-2B | | | 14 | B | 34 | 1.38E+01 |
| Q43 14-3B | | | 14 | B | 39 | 2.45E+01 |
| Q43 14-5B | | | 14 | B | 37 | 2.45E+01 |
| Q36 14-1B | | | 14 | B | 0 | 7.76E+01 |
| Q36 14-3B | | | 14 | B | 0 | 2.45E+01 |
| Q36 14-4B | | | 14 | B | 39 | 7.76E+01 |
| Q27 21-1B | | | 21 | B | 36 | 1.38E+02 |
| Q27 21-2B | | | 21 | B | 34 | 2.45E+01 |
| Q27 21-8B | | | 21 | B | 34 | 7.76E+02 |
| Q34 21-5B | | | 21 | B | 35 | 2.45E+01 |
| Q34 21-3B | | | 21 | B | 30 | 2.45E+01 |
| Q34 21-10B | | | 21 | B | 33 | 2.45E+01 |
| Q36 21-2B | | | 21 | B | 37 | 1.38E+01 |
| Q36 21-3B | | | 21 | B | 33 | 1.38E+01 |
| Q36 21-5B | | | 21 | B | 35 | 7.76E+00 |
| P13/14 0-5 | *Cx. pip* | n/a | 0 | T | 27 | 1.38E+01 |
| P13/14 0-1 | | | 0 | T | 29 | 2.45E+01 |
| P19/18 0-2 | | | 0 | T | 26 | 4.36E+01 |
| P19/18 0-4 | | | 0 | T | 30 | 1.38E+01 |
| P20/21 0-1 | | | 0 | T | 27 | 2.45E+02 |
| P20/21 0-2 | | | 0 | T | 28 | 2.45E+01 |

| | | | | | | |
|---|---|---|---|---|---|---|
| P14 14-1 | | | 14 | T | 37 | 4.36E+04 |
| P14 14-2 | | | 14 | T | 36 | 2.45E+01 |
| P14 14-4 | | | 14 | T | 34 | 1.38E+06 |
| P19 14-2B | | | 14 | B | 0 | 4.36E+00 |
| P19 14-4B | | | 14 | B | 37 | 7.76E+00 |
| P19 14-3B | | | 14 | B | 42 | 7.76E+00 |
| P21 14-5B | | | 14 | B | 36 | 6.90E+00 |
| P21 14-8B | | | 14 | B | 39 | 1.38E+01 |
| P21 14-10B | | | 14 | B | 29 | 7.76E+00 |
| P14 21-4B | | | 21 | B | 28 | 2.45E+01 |
| P14 21-1B | | | 21 | B | 15 | 2.45E+02 |
| P14 21-2B | | | 21 | B | 30 | 7.76E+00 |
| P19 21-4B | | | 21 | B | 29 | 2.45E+01 |
| P19 21-5B | | | 21 | B | 30 | 2.45E+01 |
| P19 21-7B | | | 21 | B | 37 | 1.38E+02 |
| P21 21-5B | | | 21 | B | 32 | 2.45E+01 |
| P21 21-10B | | | 21 | B | 28 | 2.45E+01 |
| P21 21-13B | | | 21 | B | 16 | 1.38E+02 |
| T19/20 0-1 | *Cx. tor* | n/a | 0 | T | 25 | 2.45E+01 |
| T19/20 0-2 | | | 0 | T | 29 | 7.76E+00 |
| T23/24 0-2 | | | 0 | T | 26 | 4.36E+02 |
| T23/24 0-5 | | | 0 | T | 28 | 7.76E+02 |
| T25/26 0-1 | | | 0 | T | 25 | 2.45E+01 |
| T25/26 0-2 | | | 0 | T | 27 | 2.45E+01 |
| T20 14-1 | | | 14 | T | 32 | 2.45E+01 |
| T20 14-2 | | | 14 | T | 19 | 1.38E+04 |
| T20 14-3 | | | 14 | T | 34 | 1.38E+01 |
| T24 14-1B | | | 14 | B | 19 | 4.36E+02 |
| T24 14-3B | | | 14 | B | 18 | 2.18E+03 |
| T24 14-4B | | | 14 | B | 37 | 2.45E+01 |
| T26 14-1B | | | 14 | B | 35 | 1.38E+05 |
| T26 14-2B | | | 14 | B | 16 | 2.18E+01 |
| T26 14-3B | | | 14 | B | 26 | 4.36E+03 |
| T20 21-2B | | | 21 | B | 26 | 1.38E+02 |
| T20 21-1B | | | 21 | B | 18 | 1.38E+03 |
| T20 21-4B | | | 21 | B | 15 | 1.38E+03 |
| T24 21-1B | | | 21 | B | 19 | 1.38E+01 |
| T24 21-3B | | | 21 | B | 18 | 1.38E+02 |
| T24 21-4B | | | 21 | B | 37 | 1.38E+02 |
| T26 21-2B | | | 21 | B | 29 | 2.45E+01 |
| T26 21-1B | | | 21 | B | 20 | 1.38E+02 |
| T26 21-3B | | | 21 | B | 29 | 7.76E+00 |
| S7K 0-2 | *Cx. mol S* | positive | 0 | T | 29 | 7.76E+01 |
| S7K 0-4 | | | 0 | T | 27 | 2.45E+01 |
| S7K 0-3 | | | 0 | T | 28 | 2.45E+01 |
| mol 14 0-2 | | | 0 | T | 26 | 7.76E+04 |
| mol 14 0-4 | | | 0 | T | 23 | 7.76E+00 |

| | | | | | | |
|---|---|---|---|---|---|---|
| S8K 0-1 | | | 0 | T | 28 | 7.76E+00 |
| S8K 0-2 | | | 0 | T | 30 | 7.76E+00 |
| mol 32 0-4 | | | 0 | T | 27 | 7.76E+00 |
| mol 32 0-9 | | | 0 | T | 26 | 7.76E+00 |
| mol 32 0-6 | | | 0 | T | 25 | 4.36E+01 |
| mol 31 0-3 | | | 0 | T | 28 | 1.38E+01 |
| mol 31 0-4 | | | 0 | T | 27 | 7.76E+00 |
| S7K 14-1B | | | 14 | B | 35 | 7.76E+00 |
| S7K 14-2B | | | 14 | B | 0 | 4.36E+00 |
| S7K 14-3B | | | 14 | B | 0 | 4.36E+00 |
| S7K 14-4B | | | 14 | B | 35 | 4.36E+00 |
| S7K 14-5B | | | 14 | B | 37 | 4.36E+00 |
| mol 14 14-1 | | | 14 | T | 21 | 7.76E+00 |
| mol 14 14-3 | | | 14 | T | 31 | 2.45E+01 |
| mol 14 14-5 | | | 14 | T | 17 | 2.45E+01 |
| S8K 14-11B | | | 14 | B | 36 | 7.76E+00 |
| S8K 14-14B | | | 14 | B | 36 | 4.36E+00 |
| S8K 14-15B | | | 14 | B | 37 | 4.36E+00 |
| S7K 21-1B | | | 21 | B | 32 | 7.76E+00 |
| S7K 21-2B | | | 21 | B | 39 | 4.36E+00 |
| S7K 21-3B | | | 21 | B | 19 | 2.45E+01 |
| S7K 21-4B | | | 21 | B | 0 | 4.36E+00 |
| S7K 21-5B | | | 21 | B | 0 | 4.36E+00 |
| mol 14 21-1 | | | 21 | T | 27 | 7.76E+01 |
| mol 14 21-2 | | | 21 | T | 30 | 1.38E+02 |
| mol 14 21-5 | | | 21 | T | 29 | 2.45E+01 |
| S8K 21-6B | | | 21 | B | 27 | 2.45E+01 |
| S8K 21-7B | | | 21 | B | 31 | 7.76E+00 |
| S8K 21-9B | | | 21 | B | 32 | 7.76E+00 |
| S8tet 21-12B | | negative | 21 | B | 17 | 7.76E+00 |
| S8tet 21-13B | | | 21 | B | 30 | 2.45E+01 |
| S8tet 21-16B | | | 21 | B | 32 | 7.76E+00 |
| W3K 21-29B | *Cx. mol W* | positive | 21 | B | 30 | 1.38E+02 |
| W3K 21-28B | | | 21 | B | 35 | 4.36E+00 |
| W3K 21-12B | | | 21 | B | 34 | 7.76E+00 |
| W3tet 21-2B | | negative | 21 | B | 32 | 7.76E+00 |
| W3tet 21-3B | | | 21 | B | 33 | 4.36E+01 |
| W3tet 21-5B | | | 21 | B | 35 | 7.76E+00 |

## 6.4 Dissemination rate target: leg vs head

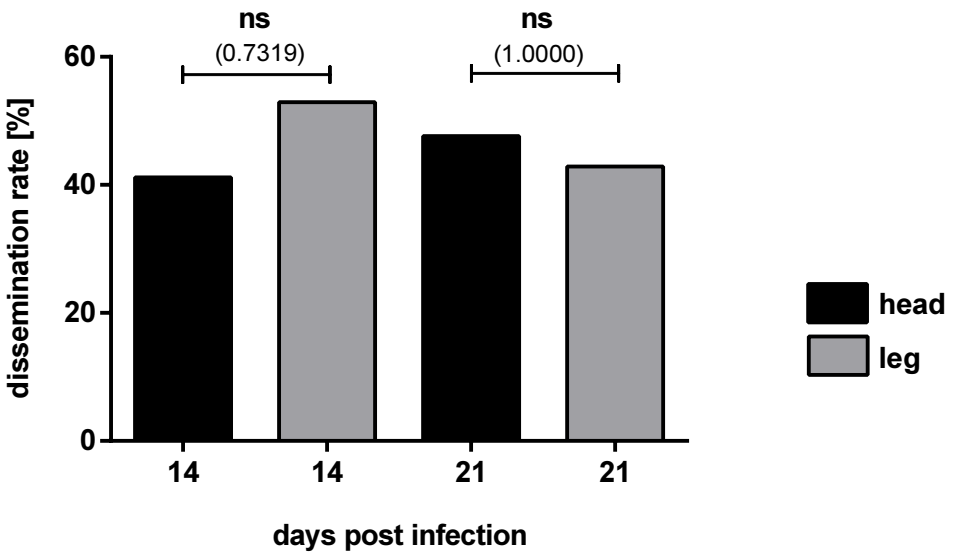


**Fig. 6.1: WNV dissemination rate based on head area vs leg area of laboratory-strain *Cx. pipiens* biotype *molestus***
Adult females were sorted into plastic containers 4-14 days after emergence and received human blood containing $1.4x10^7$ PFU WNV lineage 1 strain NY99 overnight. Fully engorged females were separated and incubated at 25 °C and 80 % humidity in a climate chamber for 14 to 21 days. The graph depicts the WNV dissemination rate of *Cx. pipiens* biotype *molestus* using either the head as requirement for dissemination (black) or the legs (grey) as determined by a WNV-specific qRT-PCR (CT cut-off = 35 cycles). Detailed number of individuals and number of independent experiments are depicted in Table 6.8. The data shown here are pooled data sets from *Cx. pipiens* biotype *molestus* S and W (all *Wolbachia* infected). Statistical analysis was performed using GraphPad Prism software and the Fisher's exact test ($p < 0.05$). P-values are noted in brackets within the graph.

**Table 6.8: WNV dissemination rate based on head area vs leg area of laboratory-strain *Cx. pipiens* biotype *molestus***
The lab strains *Cx. pipiens* biotype *molestus* Heidelberg (*Cx. mol* S) and *Cx. pipiens* biotype *molestus* Wendland (*Cx. mol* W) are lab strains. The dissemination rate is calculated by determining the number of respective WNV-infected target organs (head or leg) with respect to the number of WNV-positive females.

| *Species* | *Origin* | *Target organ* | *Temperature (°C)* | *Infection time (days)* | *Infected females [#]* | *infected target organ [#]* | *dissemination rate [%]* |
|---|---|---|---|---|---|---|---|
| *Cx. mol* | S + W | head | 25 | 14 | 17 | 7 | 41 |
| | | | 25 | 21 | 21 | 10 | 48 |
| | | leg | 25 | 14 | 17 | 9 | 53 |
| | | | 25 | 21 | 21 | 9 | 43 |

## 6.5 *Wolbachia* Screening

**Table 6.9: Raw data of *Wolbachia* high-throughput qRT-PCR screening in combination with standard gel PCR results**

Only the *Wolbachia*-status of samples with a wsp concentration of lower than 1x10$^3$ copies/µl were confirmed via standard gel PCR. All *Wolbachia* negative samples are marked in red. Samples encased in a bold edge are 2 probes from the same egg raft (i.e. one sample).
Cx pip = *Cx. pipiens* biotype *pipiens*; Cx mol = *Cx. pipiens* biotype *molestus*; Cx tor = *Cx. torrentium*; Hybrid = *Cx. pipiens* biotype *pipiens/molestus* Hybrid
NEG = negative; POS = positive
Please note that the German numerical notation is used here (e.g. 4,62E+03 corresponds to 4.62E+03 in the English numerical notation system).

| Origin | Sample ID | Taxonomic identification | CT wsp | concentration wsp [copies/µl] | PCR result |
|---|---|---|---|---|---|
| North | LL6 | Cx pip | 20 | 4,62E+03 | |
| | LL24 | Cx pip | 19 | 8,61E+03 | |
| | LL39 | Cx pip | 19 | 8,32E+03 | |
| | LL35 | Cx pip | 18 | 1,97E+04 | |
| | BNI5 | Cx pip | 20 | 1,78E+04 | |
| | LL32 | Cx pip | 21 | 8,98E+03 | |
| | LL31 | Cx pip | 21 | 1,12E+04 | |
| | BNI3 | Cx pip | 19 | 3,90E+04 | |
| | LL14 | Cx pip | NEG | | |
| | LL17 | Cx pip | 19 | 3,83E+04 | |
| | LL23 | Cx pip | 18 | 7,66E+04 | |
| | LL27 | Cx pip | 20 | 1,59E+04 | |
| | LL48 | Cx pip | 19 | 4,05E+04 | |
| | LL46 | Cx pip | 18 | 7,29E+04 | |
| | LL34 | Cx pip | NEG | | |
| | LL41 | Cx pip | 18 | 8,66E+04 | |
| | LL55 | Cx pip | 18 | 4,96E+04 | |
| | LL105 | Cx pip | NEG | | |
| | LL76 | Cx pip | 18 | 2,37E+04 | |
| | HH1 | Cx pip | 17 | 4,51E+04 | |
| | BNI16 | Cx pip | 18 | 2,32E+04 | |
| | LL65 | Cx pip | 20 | 4,49E+03 | |
| | BNI11 | Cx pip | 17 | 4,54E+04 | |
| | LL68 | Cx pip | 18 | 2,30E+04 | |
| | LL63 | Cx pip | 19 | 6,13E+03 | |
| | LL69 | Cx pip | 18 | 2,11E+04 | |
| | BNI47 | Cx pip | 18 | 5,05E+04 | |
| | LL169 | Cx pip | 20 | 1,05E+04 | |
| | LL142 | Cx pip | 18 | 3,86E+04 | |
| | LL132 | Cx pip | 18 | 4,82E+04 | |

| Origin | Sample ID | Taxonomic identification | CT wsp | concentration wsp [copies/µl] | PCR result |
|---|---|---|---|---|---|
| North | LL195 | Cx tor | NEG | | |
| | LL193 | Cx tor | NEG | | |
| | LL192 | Cx tor | 31 | 9,73E+00 | NEG |
| | LL191 | Cx tor | 33 | 3,53E+00 | NEG |
| | LL187 | Cx tor | 31 | 7,64E+00 | NEG |
| | LL185 | Cx tor | NEG | | |
| | LL184 | Cx tor | 32 | 4,71E+00 | NEG |
| | LL181 | Cx tor | 33 | 2,28E+00 | NEG |
| | LL180 | Cx tor | NEG | | |
| | LL179 | Cx tor | NEG | | |
| | LL178 | Cx tor | NEG | | |
| | LL177 | Cx tor | NEG | | |
| | LL176 | Cx tor | NEG | | |
| | LL168 | Cx tor | NEG | | |
| | LL167 | Cx tor | NEG | | |
| | LL166 | Cx tor | NEG | | |
| | LL165 | Cx tor | NEG | | |
| | LL164 | Cx tor | 32 | 6,45E+00 | NEG |
| | LL163 | Cx tor | NEG | | |
| | LL162 | Cx tor | NEG | | |
| | LL161 | Cx tor | NEG | | |
| | LL160 | Cx tor | NEG | | |
| | LL158 | Cx tor | 33 | 2,09E+00 | NEG |
| | LL156 | Cx tor | NEG | | |
| | LL155 | Cx tor | NEG | | |
| | LL134 | Cx tor | NEG | | |
| | BNI45 | Cx tor | NEG | | |
| | LL205 | Cx tor | 32 | 4,84E+00 | NEG |
| | LL203 | Cx tor | NEG | | |
| | BNI30 | Cx tor | NEG | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | LL159 | Cx pip | 19 | 2,71E+04 | | | LL88 | Cx tor | NEG | | |
| | BNI54 | Cx pip | 19 | 1,84E+04 | | | BNI25 | Cx tor | 35 | 6,37E-01 | NEG |
| | BNI52 | Cx pip | NEG | | | | BNI60 | Cx tor | 27 | 1,26E+02 | NEG |
| | HH7 | Cx pip | 18 | 4,43E+04 | | | BNI62 | Cx tor | 28 | 8,33E+01 | NEG |
| | HH6 | Cx pip | 20 | 1,42E+04 | | | LL334 | Cx tor | 31 | 1,02E+01 | NEG |
| | HH5 | Cx pip | 18 | 4,29E+04 | | | LL278 | Cx tor | NEG | | |
| | LL204 | Cx pip | 18 | 4,15E+04 | | | LL281 | Cx tor | 31 | 9,35E+00 | NEG |
| | BNI53 | Cx pip | 18 | 5,01E+04 | | | LL283 | Cx tor | 34 | 1,32E+00 | NEG |
| | BNI29 | Cx pip | 18 | 4,12E+04 | | | LL285 | Cx tor | NEG | | |
| | LL274 | Cx pip | 18 | 5,48E+04 | | | LL286 | Cx tor | 32 | 6,00E+00 | NEG |
| | LL275 | Cx pip | 19 | 3,89E+04 | | | LL295 | Cx tor | NEG | | |
| | LL277 | Cx pip | 20 | 1,97E+04 | | | LL288 | Cx tor | 30 | 2,59E+01 | NEG |
| | LL279 | Cx pip | 20 | 1,52E+04 | | | LL313 | Cx tor | 31 | 1,15E+01 | NEG |
| | LL280 | Cx pip | 20 | 1,83E+04 | | | LL257 | Cx tor | 32 | 4,12E+00 | NEG |
| | LL284 | Cx pip | 17 | 1,45E+05 | | | LL276 | Cx tor | 35 | 7,79E-01 | NEG |
| | LL331 | Cx pip | 20 | 1,74E+04 | | | LL247 | Cx tor | NEG | | |
| | LL329 | Cx pip | 32 | 6,99E+00 | NEG | | LL250 | Cx tor | NEG | | |
| | LL327 | Cx pip | 30 | 2,47E+01 | NEG | | LL251 | Cx tor | NEG | | |
| | LL326A | Cx pip | 20 | 1,74E+04 | POS | | LL252 | Cx tor | NEG | | |
| | LL326B | Cx pip | 30 | 1,45E+01 | NEG | | LL254 | Cx tor | NEG | | |
| | LL324 | Cx pip | 19 | 2,22E+04 | | | LL258 | Cx tor | 29 | 4,78E+01 | NEG |
| | LL335 | Cx pip | 19 | 3,34E+04 | | | LL262 | Cx tor | 30 | 1,96E+01 | NEG |
| | LL299 | Cx pip | 18 | 7,33E+04 | | | LL266 | Cx tor | NEG | | |
| | LL333 | Cx pip | 32 | 7,22E+00 | NEG | | LL236 | Cx tor | 31 | 8,47E+00 | NEG |
| | LL332 | Cx pip | 21 | 6,88E+03 | | | LL282 | Cx tor | 30 | 7,61E+00 | NEG |
| | LL206 1 | Cx pip | 19 | 3,52E+04 | | | LL248 | Cx tor | 32 | 2,61E+00 | NEG |
| | LL206 2 | Cx pip | 21 | 9,21E+03 | | | HH11 | Cx tor | NEG | | |
| | LL323 | Cx pip | 30 | 2,73E+01 | NEG | | LL340 | Cx tor | 31 | 4,27E+00 | NEG |
| | LL322 | Cx pip | 19 | 2,29E+04 | | | LL341 | Cx tor | NEG | | |
| | LL315 | Cx pip | 19 | 2,40E+04 | | | LL345 | Cx tor | 34 | 7,01E-01 | NEG |
| | LL306 | Cx pip | 19 | 3,74E+04 | | | LL346 | Cx tor | 30 | 9,64E+00 | NEG |
| | LL289 | Cx pip | 20 | 1,76E+04 | | | LL354 | Cx tor | 33 | 1,33E+00 | NEG |
| | LL290 | Cx pip | 19 | 3,52E+04 | | | LL355 | Cx tor | 30 | 9,91E+00 | NEG |
| | LL300 | Cx pip | 31 | 7,87E+00 | NEG | | LL359 | Cx tor | 30 | 7,56E+00 | NEG |
| | LL246 | Cx pip | 19 | 2,78E+04 | | | LL363 | Cx tor | 30 | 9,37E+00 | NEG |
| | LL249 | Cx pip | 20 | 1,45E+04 | | | BNI69 | Cx tor | 32 | 2,64E+00 | NEG |
| | LL253 | Cx pip | 19 | 2,55E+04 | | | BNI75 | Cx tor | NEG | | |
| | LL259 | Cx pip | 20 | 1,43E+04 | | | G5 | Cx tor | NEG | | |
| | LL260 | Cx pip | 19 | 3,27E+04 | | | G6 | Cx tor | NEG | | |
| | LL261 | Cx pip | 20 | 1,89E+04 | | | BNI81 | Cx tor | NEG | | |
| | LL263 | Cx pip | 18 | 6,09E+04 | | | LL369 | Cx tor | 30 | 1,19E+01 | NEG |
| | LL264 | Cx pip | 17 | 1,13E+05 | | South | B1-84 | Cx pip | 19 | 4,01E+04 | |
| | LL265 | Cx pip | 19 | 2,48E+04 | | | B1-85 | Cx pip | 19 | 4,07E+04 | |
| | LL267 | Cx pip | 19 | 2,97E+04 | | | B1-86 | Cx pip | 19 | 2,57E+04 | |

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|---|---|---|---|---|---|---|---|---|---|---|
| LL268 | Cx pip | 31 | 1,02E+01 | NEG | | B1-87 | Cx pip | 20 | 1,22E+04 | |
| LL269 | Cx pip | 19 | 2,60E+04 | | | B1-89 | Cx pip | 19 | 3,85E+04 | |
| LL270 | Cx pip | 20 | 1,89E+04 | | | B1-90 | Cx pip | 19 | 3,69E+04 | |
| LL271 | Cx pip | 18 | 4,59E+04 | | | B1-91 | Cx pip | 19 | 2,39E+04 | |
| LL272 | Cx pip | 18 | 5,13E+04 | | | B1-93 | Cx pip | 19 | 3,14E+04 | |
| LL273 | Cx pip | 19 | 2,29E+04 | | | B1-96 | Cx pip | 19 | 2,79E+04 | |
| LL291 | Cx pip | 18 | 3,50E+04 | | | B1-97 | Cx pip | 21 | 6,54E+03 | |
| LL292 | Cx pip | 20 | 1,25E+04 | | | B1-82 | Cx pip | 18 | 4,91E+04 | |
| LL293 | Cx pip | 18 | 2,94E+04 | | | B1-98 | Cx pip | 19 | 2,77E+04 | |
| LL308 | Cx pip | 22 | 3,32E+03 | | | B1-101 | Cx pip | 18 | 4,55E+04 | |
| LL307 | Cx pip | 20 | 9,22E+03 | | | B1-102 | Cx pip | 20 | 2,07E+04 | |
| LL298 | Cx pip | 19 | 2,39E+04 | | | B1-103 | Cx pip | 21 | 9,14E+03 | |
| LL312 | Cx pip | 20 | 7,59E+03 | | | B1-104 | Cx pip | 18 | 5,16E+04 | |
| LL316 | Cx pip | 18 | 4,08E+04 | | | B2-124 | Cx pip | 19 | 2,53E+04 | |
| LL304 | Cx pip | 18 | 4,23E+04 | | | B2-127 | Cx pip | 18 | 4,71E+04 | |
| LL303 | Cx pip | NEG | | | | B2-129 | Cx pip | 19 | 2,36E+04 | |
| LL305 | Cx pip | 19 | 2,15E+04 | | | B2-131 | Cx pip | 19 | 4,27E+04 | |
| LL294 | Cx pip | 18 | 3,53E+04 | | | B2-132 | Cx pip | 20 | 1,79E+04 | |
| LL320 | Cx pip | 33 | 1,42E+00 | NEG | | B2-134 | Cx pip | 19 | 3,16E+04 | |
| LL321 | Cx pip | 20 | 1,32E+04 | | | B2-135 | Cx pip | 20 | 1,71E+04 | |
| LL311 | Cx pip | 30 | 1,31E+01 | NEG | | B2-136 | Cx pip | 19 | 2,68E+04 | |
| LL314 | Cx pip | 18 | 3,25E+04 | | | B2-137 | Cx pip | 19 | 2,97E+04 | |
| LL319 | Cx pip | 28 | 3,90E+01 | NEG | | B2-138 | Cx pip | 19 | 3,64E+04 | |
| LL317 | Cx pip | 17 | 6,46E+04 | | | B2-140 | Cx pip | 20 | 1,35E+04 | |
| LL301 | Cx pip | 20 | 9,95E+03 | | | B3-68 | Cx pip | 22 | 3,87E+03 | |
| LL309 | Cx pip | 20 | 1,34E+04 | | | B3-69 | Cx pip | 19 | 3,30E+04 | |
| LL302 | Cx pip | 18 | 3,36E+04 | | | B3-71 | Cx pip | 17 | 8,82E+04 | |
| LL310 | Cx pip | 20 | 9,68E+03 | | | B3-72 | Cx pip | 18 | 5,12E+04 | |
| LL318 | Cx pip | 19 | 1,50E+04 | | | B3-74 | Cx pip | 19 | 3,56E+04 | |
| HH10 | Cx pip | 21 | 5,91E+03 | | | B3-93 | Cx pip | 20 | 1,57E+04 | |
| LL337 | Cx pip | 20 | 8,42E+03 | | | B3-90 | Cx pip | 18 | 8,12E+04 | |
| LL338 | Cx pip | 20 | 1,16E+04 | | | B1-114 | Cx pip | 18 | 5,85E+04 | |
| LL339 | Cx pip | NEG | | | | B2-159 | Cx pip | 19 | 4,02E+04 | |
| LL342 | Cx pip | 20 | 9,09E+03 | | | B5-36 | Cx pip | 21 | 7,89E+03 | |
| LL343 | Cx pip | 32 | 2,63E+00 | NEG | | x-07 | Cx pip | 20 | 2,58E+04 | |
| LL344 | Cx pip | 22 | 3,03E+03 | | | B3-75 | Cx pip | 19 | 3,19E+04 | |
| LL348 | Cx pip | 18 | 3,94E+04 | | | B3-76 | Cx pip | 19 | 3,63E+04 | |
| LL347 | Cx pip | 17 | 8,53E+04 | | | B3-77 | Cx pip | 18 | 6,84E+04 | |
| LL349 | Cx pip | 20 | 7,86E+03 | | | B3-78 | Cx pip | 18 | 9,96E+04 | |
| LL350 | Cx pip | 19 | 1,61E+04 | | | B3-79 | Cx pip | 18 | 8,01E+04 | |
| LL351 | Cx pip | 19 | 1,76E+04 | | | B5-37 | Cx pip | 18 | 8,69E+04 | |
| LL352 | Cx pip | 20 | 7,48E+03 | | | B5-38 | Cx pip | 18 | 1,00E+05 | |
| LL353 | Cx pip | 20 | 7,69E+03 | | | B5-39 | Cx pip | 25 | 5,09E+02 | NEG |
| LL356 | Cx pip | 19 | 1,87E+04 | | | B5-40 | Cx pip | 19 | 4,02E+04 | |

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|---|---|---|---|---|---|
| LL357 | Cx pip | 18 | 3,27E+04 | | |
| LL358 | Cx pip | 30 | 6,95E+00 | NEG | |
| LL360 | Cx pip | 21 | 5,55E+03 | | |
| LL361 | Cx pip | 32 | 1,83E+00 | NEG | |
| LL362 | Cx pip | 22 | 2,56E+03 | | |
| BNI64 | Cx pip | 21 | 5,59E+03 | | |
| BNI65 | Cx pip | 21 | 6,51E+03 | | |
| BNI66 | Cx pip | 19 | 2,11E+04 | | |
| BNI67 | Cx pip | 20 | 1,14E+04 | | |
| BNI68 | Cx pip | 19 | 1,80E+04 | | |
| BNI70 | Cx pip | 20 | 8,08E+03 | | |
| BNI71 | Cx pip | 22 | 3,39E+03 | | |
| BNI72 | Cx pip | 20 | 1,71E+04 | | |
| BNI73 | Cx pip | 20 | 1,57E+04 | | |
| BNI74 | Cx pip | 20 | 2,02E+04 | | |
| BNI76 | Cx pip | 21 | 1,07E+04 | | |
| BNI77 | Cx pip | 19 | 3,01E+04 | | |
| BNI78 | Cx pip | 23 | 2,60E+03 | | |
| BNI79 | Cx pip | 22 | 5,34E+03 | | |
| G7 | Cx pip | 22 | 4,42E+03 | | |
| BNI80 | Cx pip | 20 | 1,38E+04 | | |
| BNI82 | Cx pip | 19 | 3,66E+04 | | |
| HH12 | Cx pip | 20 | 1,57E+04 | | |
| HH13 | Cx pip | 21 | 1,04E+04 | | |
| LL364 | Cx pip | 18 | 6,63E+04 | | |
| LL365 | Cx pip | 19 | 3,28E+04 | | |
| LL366 | Cx pip | 19 | 2,62E+04 | | |
| LL367 | Cx pip | 19 | 2,55E+04 | | |
| LL368 | Cx pip | 19 | 2,73E+04 | | |
| LL370 | Cx pip | 21 | 1,01E+04 | | |
| LL371 | Cx pip | 19 | 3,87E+04 | | |
| LL372 | Cx pip | 20 | 1,28E+04 | | |
| LL373 | Cx pip | 19 | 3,71E+04 | | |
| LL374 | Cx pip | 20 | 1,91E+04 | | |
| LL375 | Cx pip | 20 | 1,79E+04 | | |
| BNI83 | Cx pip | NEG | | | |
| LL7 | Cx tor | NEG | | | |
| LL8 | Cx tor | NEG | | | |
| LL9 | Cx tor | NEG | | | |
| LL10 | Cx tor | NEG | | | |
| LL1 | Cx tor | NEG | | | |
| LL2 | Cx tor | NEG | | | |
| LL3 | Cx tor | NEG | | | |
| BNI2 | Cx tor | NEG | | | |

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|---|---|---|---|---|---|
| B5-41 | Cx pip | 21 | 1,29E+04 | | |
| B5-42 | Cx pip | 21 | 7,73E+03 | | |
| B1-105 | Cx pip | 20 | 2,18E+04 | | |
| B1-106 | Cx pip | 21 | 1,04E+04 | | |
| B1-107 | Cx pip | 18 | 8,34E+04 | | |
| B1-108 | Cx pip | 18 | 5,43E+04 | | |
| B1-109 | Cx pip | 20 | 2,28E+04 | | |
| B1-110 | Cx pip | 21 | 7,03E+03 | | |
| B2-141 | Cx pip | 20 | 1,99E+04 | | |
| B2-142 | Cx pip | 20 | 1,66E+04 | | |
| B2-143 | Cx pip | 18 | 6,31E+04 | | |
| B2-144 | Cx pip | 18 | 1,02E+05 | | |
| B2-146 | Cx pip | 17 | 1,20E+05 | | |
| B2-147 | Cx pip | 18 | 7,28E+04 | | |
| B2-150 | Cx pip | 18 | 6,89E+04 | | |
| B2-151 | Cx pip | 20 | 1,74E+04 | | |
| B2-152 | Cx pip | 20 | 2,05E+04 | | |
| B2-154 | Cx pip | 19 | 4,28E+04 | | |
| B3-80 | Cx pip | 18 | 5,28E+04 | | |
| B3-81 | Cx pip | 30 | 1,86E+01 | NEG | |
| B3-82 | Cx pip | 18 | 9,49E+04 | | |
| B3-83 | Cx pip | 19 | 4,83E+04 | | |
| B3-84 | Cx pip | 19 | 3,26E+04 | | |
| B3-85 | Cx pip | 22 | 5,10E+03 | | |
| B3-86 | Cx pip | 20 | 2,19E+04 | | |
| B3-88 | Cx pip | 27 | 1,56E+02 | NEG | |
| B3-89 | Cx pip | 20 | 1,89E+04 | | |
| B4-05 | Cx pip | 18 | 8,12E+04 | | |
| B5-43 | Cx pip | 22 | 4,24E+03 | | |
| B5-44 | Cx pip | 19 | 2,98E+04 | | |
| B5-45 | Cx pip | 17 | 1,55E+05 | | |
| B5-46 | Cx pip | 18 | 5,81E+04 | | |
| x-8 | Cx pip | 19 | 4,64E+04 | | |
| x-9 | Cx pip | 22 | 6,39E+03 | | |
| x-10 | Cx pip | 20 | 2,15E+04 | | |
| x-11 | Cx pip | 19 | 3,13E+04 | | |
| x-12 | Cx pip | 19 | 4,61E+04 | | |
| B2-30 | Cx pip | 16 | 2,23E+05 | | |
| B2-32 | Cx pip | 21 | 1,20E+04 | | |
| B2-48 | Cx pip | 20 | 1,38E+04 | | |
| B2-123 | Cx pip | 18 | 6,22E+04 | | |
| B2-126 | Cx pip | 17 | 1,15E+05 | | |
| B2-128 | Cx pip | 21 | 1,02E+04 | | |
| B2-139 | Cx pip | 19 | 8,38E+03 | | |

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|---|---|---|---|---|---|---|---|---|---|---|
| | LL5 | Cx tor | NEG | | | | B2-156 | Cx pip | 17 | 3,36E+04 | |
| | LL11 | Cx tor | NEG | | | | B2-157 | Cx pip | 18 | 1,68E+04 | |
| | LL12 | Cx tor | 33 | 7,76E-01 | NEG | | B2-155 | Cx pip | 17 | 3,17E+04 | |
| | BNI1 | Cx tor | NEG | | | | B2-201 | Cx pip | 14 | 2,01E+05 | |
| | LL15 | Cx tor | 32 | 1,03E+00 | NEG | | B2-204 | Cx pip | 19 | 7,53E+03 | |
| | LL16 | Cx tor | NEG | | | | B2-207 | Cx pip | 21 | 2,90E+03 | |
| | LL18 | Cx tor | NEG | | | | B2-206 | Cx pip | 18 | 1,94E+04 | |
| | LL20 | Cx tor | NEG | | | | B2-210 | Cx pip | 20 | 5,43E+03 | |
| | LL26 | Cx tor | NEG | | | | B2-212 | Cx pip | 15 | 1,24E+05 | |
| | LL38 | Cx tor | NEG | | | | B2-218 | Cx pip | 19 | 1,09E+04 | |
| | LL33 | Cx tor | 32 | 1,03E+01 | NEG | | B2-219 | Cx pip | 16 | 5,33E+04 | |
| | LL30 | Cx tor | NEG | | | | B2-220 | Cx pip | 17 | 3,95E+04 | |
| | BNI6 | Cx tor | NEG | | | | B2-221 | Cx pip | 18 | 1,67E+04 | |
| | LL36 | Cx tor | NEG | | | | B2-28 | Cx pip | 18 | 2,22E+04 | |
| | BNI4 | Cx tor | NEG | | | | B3-70 | Cx pip | 16 | 5,33E+04 | |
| | LL13 | Cx tor | NEG | | | | B3-53 | Cx pip | 27 | 5,95E+01 | POS |
| | LL19 | Cx tor | NEG | | | | B3-95 | Cx pip | 17 | 2,90E+04 | |
| | LL25 | Cx tor | 32 | 1,37E+01 | NEG | | B3-97 | Cx pip | 18 | 1,79E+04 | |
| | LL29 | Cx tor | 32 | 1,20E+01 | NEG | | B5-48 | Cx pip | 17 | 3,55E+04 | |
| | LL22 | Cx tor | NEG | | | | B1-1 Hy | Cx pip | 17 | 3,84E+04 | |
| | LL28 | Cx tor | NEG | | | | B1-22B | Cx pip | 19 | 1,06E+04 | |
| | LL21 | Cx tor | NEG | | | | B1-27 | Cx pip | 18 | 1,91E+04 | |
| | LL49 | Cx tor | 32 | 1,02E+01 | NEG | | B1-83 | Cx pip | 18 | 1,83E+04 | |
| | LL47 | Cx tor | 33 | 8,59E+00 | NEG | | B1-88 | Cx pip | 21 | 2,92E+03 | |
| | LL45 | Cx tor | NEG | | | | B1-95 | Cx pip | 17 | 4,85E+04 | |
| | LL44 | Cx tor | 33 | 6,68E+00 | NEG | | B1-99 | Cx pip | 18 | 2,19E+04 | |
| | LL43 | Cx tor | 32 | 1,30E+01 | NEG | | B1-100 | Cx pip | 17 | 3,06E+04 | |
| | LL40 | Cx tor | NEG | | | | B5-50 | Cx pip | 16 | 7,29E+04 | |
| | LL50 | Cx tor | 32 | 1,05E+01 | NEG | | B2-158 | Cx pip | 17 | 2,77E+04 | |
| | G3 | Cx tor | NEG | | | | B3-92 | Cx pip | 16 | 5,62E+04 | |
| | LL51 | Cx tor | NEG | | | | B5-49 | Cx pip | 17 | 2,77E+04 | |
| | G2 | Cx tor | 32 | 1,48E+01 | NEG | | B2-01 | Cx pip | 20 | 4,24E+03 | |
| | LL53 | Cx tor | NEG | | | | B5-03 | Cx pip | 17 | 2,83E+04 | |
| | LL56 | Cx tor | NEG | | | | B3-15 | Cx pip | 19 | 1,20E+04 | |
| | LL54 | Cx tor | 33 | 7,19E+00 | NEG | | B3-14 | Cx pip | 18 | 1,58E+04 | |
| | LL52 | Cx tor | 32 | 1,24E+01 | NEG | | B3-13 | Cx pip | 17 | 3,23E+04 | |
| | LL75 | Cx tor | NEG | | | | B3-12 | Cx pip | 18 | 2,19E+04 | |
| | BNI9 | Cx tor | NEG | | | | B3-11 | Cx pip | 19 | 9,33E+03 | |
| | BNI20 | Cx tor | NEG | | | | B3-10 | Cx pip | 17 | 2,75E+04 | |
| | LL104 | Cx tor | 32 | 8,60E-01 | NEG | | B3-08 | Cx pip | 20 | 3,81E+03 | |
| | BNI8 | Cx tor | 31 | 1,53E+00 | NEG | | B3-07 | Cx pip | 18 | 1,66E+04 | |
| | LL61 | Cx tor | NEG | | | | B3-06 | Cx pip | 18 | 2,33E+04 | |
| | BNI21 | Cx tor | 32 | 1,07E+00 | NEG | | B3-05 | Cx pip | 19 | 1,07E+04 | |
| | G1 2 | Cx tor | NEG | | | | B3-03 | Cx pip | 17 | 3,62E+04 | |

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|---|---|---|---|---|---|---|---|---|---|---|
| | LL82 | Cx tor | NEG | | | | B3-02 | Cx pip | 18 | 1,61E+04 | |
| | BNI15 | Cx tor | NEG | | | | B3-01 | Cx pip | 18 | 1,96E+04 | |
| | LL67 | Cx tor | NEG | | | | B2-14 | Cx pip | 19 | 1,16E+04 | |
| | LL60 | Cx tor | NEG | | | | B2-13 | Cx pip | 18 | 1,69E+04 | |
| | BNI13 | Cx tor | NEG | | | | B2-11 | Cx pip | 20 | 5,08E+03 | |
| | LL81 | Cx tor | NEG | | | | B2-10 | Cx pip | 17 | 4,11E+04 | |
| | LL83 | Cx tor | NEG | | | | B2-09 | Cx pip | 18 | 1,94E+04 | |
| | BNI10 | Cx tor | NEG | | | | B2-08 | Cx pip | 18 | 2,12E+04 | |
| | BNI14 1 | Cx tor | NEG | | | | B2-07 | Cx pip | 18 | 1,57E+04 | |
| | BNI14 2 | Cx tor | NEG | | | | B2-06 | Cx pip | 16 | 6,18E+04 | |
| | LL58 | Cx tor | NEG | | | | B2-05 | Cx pip | 18 | 2,25E+04 | |
| | BNI19 | Cx tor | 34 | 1,98E-01 | NEG | | B2-04 | Cx pip | 17 | 2,97E+04 | |
| | LL85 | Cx tor | NEG | | | | B2-02 | Cx pip | 17 | 3,47E+04 | |
| | BNI18 | Cx tor | NEG | | | | B1-04 | Cx pip | 18 | 1,64E+04 | |
| | LL106 | Cx tor | NEG | | | | B1-03 | Cx pip | 17 | 3,24E+04 | |
| | BNI17 | Cx tor | 31 | 1,48E+00 | NEG | | B1-02 | Cx pip | 17 | 3,54E+04 | |
| | LL103 | Cx tor | NEG | | | | B5-04 | Cx pip | 18 | 1,47E+04 | |
| | LL66 | Cx tor | NEG | | | | B3-17 | Cx pip | 18 | 2,02E+04 | |
| | LL115 | Cx tor | NEG | | | | B3-16 | Cx pip | 16 | 6,61E+04 | |
| | LL86 | Cx tor | NEG | | | | B2-24 | Cx pip | 18 | 2,05E+04 | |
| | LL80 | Cx tor | NEG | | | | B2-23 | Cx pip | 18 | 2,00E+04 | |
| | BNI12 | Cx tor | 29 | 6,51E+00 | NEG | | B2-21 | Cx pip | 17 | 3,35E+04 | |
| | LL70 | Cx tor | NEG | | | | B2-20 | Cx pip | 17 | 4,42E+04 | |
| | LL111 | Cx tor | NEG | | | | B2-19 | Cx pip | 17 | 4,18E+04 | |
| | LL90 | Cx tor | NEG | | | | B2-18 | Cx pip | 19 | 1,20E+04 | |
| | G4 | Cx tor | NEG | | | | B2-17 | Cx pip | 17 | 2,89E+04 | |
| | LL73 | Cx tor | NEG | | | | B2-15 | Cx pip | 18 | 2,02E+04 | |
| | LL64 | Cx tor | NEG | | | | B1-06 | Cx pip | 19 | 1,22E+04 | |
| | LL84 | Cx tor | NEG | | | | B1-05 | Cx pip | 17 | 3,94E+04 | |
| | LL59 | Cx tor | NEG | | | | B2-22 | Cx pip | 19 | 1,23E+04 | |
| | LL77 | Cx tor | NEG | | | | B5-05 | Cx pip | 16 | 6,44E+04 | |
| | LL79 | Cx tor | NEG | | | | B1-94 | Cx tor | 30 | 1,25E+01 | NEG |
| | LL72 | Cx tor | NEG | | | | B2-133 | Cx tor | 32 | 4,30E+00 | NEG |
| | BNI22 | Cx tor | NEG | | | | B1-111 | Cx tor | 29 | 3,62E+01 | NEG |
| | LL154 | Cx tor | 32 | 6,49E+00 | NEG | | B2-145 | Cx tor | 33 | 3,18E+00 | NEG |
| | LL153 | Cx tor | 33 | 2,12E+00 | NEG | | B2-149 | Cx tor | 28 | 5,79E+01 | NEG |
| | LL152 | Cx tor | NEG | | | | B2-153 | Cx tor | 31 | 9,45E+00 | NEG |
| | LL150 | Cx tor | NEG | | | | B1-112 | Cx tor | NEG | | |
| | LL149 | Cx tor | NEG | | | | B5-02 | Cx tor | 29 | 8,93E+00 | NEG |
| | LL147 | Cx tor | NEG | | | | B5-01 | Cx tor | 32 | 1,96E+00 | NEG |
| | LL146 | Cx tor | NEG | | | | B3-09 | Cx tor | NEG | | |
| | LL143 | Cx tor | NEG | | | | B2-12 | Cx tor | 31 | 3,90E+00 | NEG |
| | LL139 | Cx tor | 31 | 1,09E+01 | NEG | | B2-03 | Cx tor | NEG | | |
| | LL137 | Cx tor | 32 | 4,50E+00 | NEG | | B2-16 | Cx tor | NEG | | |

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|---|---|---|---|---|---|---|---|---|---|---|
| | LL136 | Cx tor | 33 | 2,98E+00 | NEG | North | HH9 | Hybrid | 22 | 3,12E+03 | |
| | LL133 | Cx tor | NEG | | | South | B2-125 | Hybrid | 19 | 2,20E+04 | |
| | LL130 | Cx tor | NEG | | | | B2-148 | Hybrid | 18 | 9,76E+04 | |
| | LL122 | Cx tor | 32 | 4,74E+00 | NEG | | B3-87 | Hybrid | 20 | 1,93E+04 | |
| | LL121 | Cx tor | 31 | 8,16E+00 | NEG | | B3-94 | Hybrid | 16 | 9,28E+04 | |
| | LL120 | Cx tor | NEG | | | | B3-18 | Hybrid | 20 | 4,21E+03 | |
| | LL199 | Cx tor | 32 | 3,80E+00 | NEG | | B3-41 | Hybrid | 15 | 1,01E+05 | |
| | LL198 | Cx tor | 31 | 7,59E+00 | NEG | | B5-47 | Cx mol | 17 | 3,48E+04 | |
| | LL197 | Cx tor | 31 | 1,31E+01 | NEG | | B1-22A | Hybrid | 16 | 5,37E+04 | |

## 6.6 Blood feeding rate

**Table 6.10: Summary of amount of samples and blood feeding rates of *Cx. pipiens* biotype *molestus* S und W**
The *Cx. pipiens* biotype *molestus* (*Cx. mol*) used here is a lab strain. *Cx. pipiens* biotype *molestus* S (''Südland'') originated from Heidelberg in Southern Germany. *Cx. pipiens* biotype *molestus* W (''Wendland'') originated from the Wendland area in Northern Germany. All *Wolbachia*-negative strains have been treated with tetracycline for 3 generation (F0, F1 and F2) and kept for another 8 consecutive generations (F3-F10) until used for WNV infection assay.
The blood feeding rate is determined by calculating the percentage of blood-fed females after an overnight blood meal with respect to the total number of females entered into the experiment (n).

| Species | Origin | *Wolbachia* status | n [#] | blood-fed females [#] | blood feeding rate [%] |
|---|---|---|---|---|---|
| *Cx. pipiens* biotype *molestus* | S (South) | negative | 1063 | 783 | 74 |
| | | positive | 881 | 668 | 76 |
| | W (North) | negative | 495 | 235 | 47 |
| | | positive | 472 | 330 | 70 |

# Abstract

West Nile virus (WNV; genus *Flavivirus*, family *Flaviviridae*) is an arbovirus and the causative agent of West Nile disease. The enzootic transmission cycle of WNV includes mosquitoes and birds. However mammals, such as horses and humans, may act as incidental, dead-end hosts and develop clinical symptoms, ranging from mild febrile illness to encephalitis and death (Petersen et al. 2013). Outbreaks of the disease are a current medical concern for Europe, which is illustrated by ongoing reports of autochthonous human cases in different European countries (European Centre for Disease Prevention and Control 2015).

Some of the mosquito species that act as vectors for WNV are grouped into the genus *Culex*. The most abundant member species native to Germany are *Cx. torrentium* and *Cx. pipiens* biotype *pipiens* in addition to *Cx. pipiens* biotype *molestus* and hybrid forms of the latter two (Rudolf et al. 2013; Krüger et al. 2014). The presence of potential vector species as well as continuing WNV case reports from various European countries indicate infection risk for Germany (European Centre for Disease Prevention and Control 2015). However, the susceptibility to WNV infection of these potential vectors and their subsequent role in establishing an enzootic transmission cycle in Germany is so far unknown. This work strives to change this status quo and improve future risk assessments and safety measures.

To assess the vectorial capacity of mosquito species, it is necessary to investigate some of its main influencing factors (Ciota & Kramer 2013). In this work, the distribution of putative vectors as well as conditions for susceptibility for WNV infection in the context of temperature, population origin, time and co-infection with *Wolbachia*, an α-proteobacterium, were analysed. Emphasis was put on field-collected *Cx. torrentium* and *Cx. pipiens* biotype *pipiens* mosquitoes from sampling areas in the North and South of Germany. *Cx. quinquefasciatus* and *Cx. pipiens* biotype *molestus* were used as positive controls. A previously established blood feed assay was applied to mimic the natural infection route (Huber et al. 2014; Kilpatrick et al. 2010).

Generally, it was found that both *Cx. torrentium* and *Cx. pipiens* biotype *pipiens* are susceptible to WNV at 25 °C as well as under ambient temperature at 18 °C, irrespective of population origin or time. Nevertheless, some minor species-specific variations were detected.

*Cx. torrentium* mosquitoes, which are more prevalent in the North of Germany, displayed the highest overall infection rates. Although the infection rates at lower temperature lagged behind 7 days compared to incubation temperature of 25 °C, indicating delayed viral growth, *Cx. torrentium* from the North even tend towards increased susceptibility for WNV at 18 °C. In contrast, *Cx. pipiens* biotype *pipiens*, which are more prevalent in the South of Germany, tend towards a higher infection rate when originating from the South, irrespective of incubation temperature.

Interestingly, resistance to WNV infection and change in feeding behaviour could be correlated with density and location of *Wolbachia* co-infection in cells of the mosquito gut

area and nervous system, respectively. Notably, this hypothesis is based on results obtained with *Cx. pipiens* biotype *molestus* lab strains and was not confirmed in this work. Therefore, further experiments have to be performed, ideally with a focus on natural *Culex* populations.

All in all, the high susceptibility for WNV at both low and high temperatures indicates that German *Cx. torrentium* and *Cx. pipiens* biotype *pipiens* populations are realistic WNV vector candidates. Insights obtained considering the effect of *Wolbachia* could open doors to further research possibly directed at arbovirus control measures, which would be in line with current programs focusing on arbovirus elimination such as the Eliminate Dengue Program (Eliminate Dengue Program n.d.).

Ultimately, this work underlines the need for further surveillance and early vector control measures to minimise the risk for the establishment and transmission of WNV in Germany.

# Zusammenfassung

Das humanpathogene West Nil Virus (WNV; Genus: *Flavivirus*, Familie: *Flaviviridae*) gehört zu den Arboviren und ist als Auslöser des West Nil Fiebers nahezu weltweit von aktueller medizinischer Relevanz. Auch in der Europäischen Union wurden allein 2015 108 Krankheitsfälle gemeldet. Hinzu kamen weitere 193 Meldungen aus umgebenen Nationen (European Centre for Disease Prevention and Control 2015). Der primäre Übertragungszyklus des Virus findet zwischen Vögeln als Hauptwirt und Mücken als Vektoren statt. Die Infektion von Fehlwirten wie Pferden und Menschen kann zum Ausbruch von West Nil Fieber führen. Das Krankheitsbild umfasst in der Regel mildes Fieber und leichte Erkältungssymptome. In Einzelfällen kommt es jedoch auch zu schweren Enzephalitiden mit tödlichem Ausgang (Petersen et al. 2013).

Viele Vektoren von WNV gehören zur Gattung *Culex*. Die in Deutschland am häufigsten vorkommenden Arten sind *Cx. torrentium*, die beiden *Cx. pipiens* Biotypen *pipiens* und *molestus* sowie deren Hybride (Rudolf et al. 2013; Krüger et al. 2014). Das Auftreten von Krankheitsfällen in Nachbarstaaten begünstigt eine potentielle Einschleppung nach Deutschland (European Centre for Disease Prevention and Control 2015). Zur besseren Risikoabschätzung ist eine genaue Kenntnis über des Vektorpotenzials der Mückenpopulationen notwendig. Dieses Vektorpotenzial und damit die Bedeutung der einheimischen Mückenpopulationen für die Etablierung eines enzootischen Transmissionszyklus sind allerdings bisher unbekannt.

Ziel dieser Arbeit ist es, das Vektorpotenzial einheimischer *Culex* Arten zu untersuchen, um zukünftige Risikoabschätzungen und die Einleitung von Sicherheitsmaßnahmen zu ermöglichen.

Die Fähigkeit von *Culex* Arten, als Überträger des West Nil Virus zu wirken, wird anhand einer Auswahl wichtiger Einflussfaktoren bewertet (Ciota & Kramer 2013). Es wurden sowohl Unterschiede in der Verbreitung potenzieller Vektorspezies in Deutschland untersucht als auch deren Vektorpotenzial in Abhängigkeit von Faktoren wie Umgebungstemperatur, geographische Herkunft der Population, Sammelzeitraum und Infektion mit *Wolbachia*, einem α-Proteobakterium.

Der Schwerpunkt der Arbeit lag auf Wildfängen der Arten *Cx. torrentium* und *Cx. pipiens* Biotyp *pipiens* aus dem Norden und Süden Deutschlands. Laborkulturen von *Cx. quinquefasciatus* und *Cx. pipiens* Biotype *molestus* fungierten als Positivkontrollen und, im Falle von Biotyp *molestus*, als Vektorspezies für die *Wolbachia*-WNV-Infektionsexperimente. Der natürliche Infektionsweg wurde via einer mit Virus versetzten Blutmahlzeit simuliert (Huber et al. 2014; Kilpatrick et al. 2010).

In der vorliegenden Arbeit gezeigt werden, dass sowohl *Cx. torrentium* als auch *Cx. pipiens* Biotyp *pipiens* bei 25 °C als auch schon bei 18 °C anfällig für eine WNV-Infektion sind. Diese Anfälligkeit ist unabhängig von der Herkunft der Mücken (Nord- oder Süddeutschland) und ebenfalls unabhängig vom Sammelzeitraum (Jahr der Sammlung).

Mücken der Art *Cx. torrentium* wiesen eine höhere Prävalenz im Norden Deutschlands auf und zeigten die höchste Infektionsrate für WNV auf. Im Vergleich zu 25 °C wurden bei *Cx. torrentium* die höchsten Infektionsraten bei 18 °C um 7 Tage verzögert gemessen, was auf eine verlangsamte Virusreplikation hindeutet. Überraschenderweise wurde für die *Cx. torrentium* Population aus dem Norden Deutschlands bei 18 °C tendenziell höhere Infektionsraten beobachtet als bei 25 °C. Dieser Unterschied war jedoch nicht signifikant. Im Gegensatz dazu tendieren *Cx. pipiens* Biotyp *pipiens* Mücken aus dem Süden, wo sie auch eine höhere Prävalenz aufweisen, zu einer höheren Infektionsrate für WNV, jedoch unabhängig von der Temperatur.

Die Ergebnisse der *Wolbachia*-WNV-Infektionsversuche legen die Vermutung nahe, dass sowohl Virusreplikation als auch Blutsaugverhalten abhängig sind von der Lokalisation der *Wolbachia* im Darm oder Nervensystem und der dort vorherrschenden jeweiligen Bakteriendichte. Da diese Experimente jedoch mit *Culex* Laborkulturen vorgenommen wurden, sollte diese Beobachtung mit *Cx. torrentium* sowie *Cx. pipiens* Biotype *pipiens* Wildfängen weiter untersucht werden. Zudem sind weitere Experimente im Hinblick auf die genaue Ermittlung der Bakteriendichte in den jeweiligen Organen notwendig.

Alles in Allem zeigt das hohe Vektorpotential der *Cx. torrentium* und *Cx. pipiens* Biotyp *pipiens* Populationen aus Deutschland insbesondere auch bei niedrigen Umgebungstemperaturen, dass diese Arten als realistische WNV-Vektoren in Betracht gezogen werden müssen. Erkenntnisse im Bereich der WNV-*Wolbachia*-Interaktionen könnten zu weiterer Forschung auch in Richtung Vektor- und Viruskontrolle animieren. Solche Arbeiten haben sich bereits im Kontext der Dengue Virus Kontrolle als erfolgreich herausgestellt (Eliminate Dengue Program n.d.).

Abschließend unterstreicht diese Arbeit den Bedarf eines kontinuierlichen WNV-Monitoring und entsprechender Vektorkontrollen, um für Deutschland das Risiko einer WNV-Etablierung und -Transmission zu minimieren.

# Cooperations

The protocol, primers and probes of the WNV qRT-PCR as well as the primers and probe of the *Wolbachia* qRT-PCR were designed by Prof. Dr.med. Dr.med. habil. Jonas Schmidt-Chanasit (Virology, BNITM, Hamburg).

# Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

_____ _____

Ort, Datum (Mayke Leggewie)

# Declaration on oath

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids.

_____ _____

city, date (Mayke Leggewie)

# Publications

**Some results presented in this dissertation were published in form of the following article:**

**Leggewie, M.**, Badusche, M., Rudolf, M., Jansen, S., Jessica, B., Krumkamp, R., Huber, K., Krüger, A., Schmidt-Chanasit, J., Tannich, E., Becker, S. C., 2016. *Culex pipiens* and *Culex torrentium* populations from Central Europe are susceptible to West Nile infection. *One Health*, 2, pp. 88-94.

**Other publications:**

Lühken, R., Steinke, S., **Leggewie, M.**, Tannich, E., Krüger, A., Becker, S. C., Kiel, A. E., 2015. Physio-Chemical Characteristics of *Culex pipiens* sensu lato and *Culex torrentium* (Diptera: Culicidae) Breeding Sites in Germany. *Journal of Medical Entomology*, 52 (5), pp. 932-936.

Huber, K., Jansen, S., **Leggewie, M.**, Badusche, M., Schmidt-Chanasit, J., Becker, N., Tannich, E., Becker, S. C., 2014. *Aedes japonicus japonicus* (Diptera: Culicidae) from Germany have vector competence for Japan encephalitis virus but are refractory to infection with West Nile virus. *Parasitology research*, 113 (9), pp. 3195-3199.

**Leggewie, M.**, Sreenu, V. B., Abdelrahman, T., Leitch, E. C. M., Wilkie, G. S., Klymenko, T., Muir, D., Thursz, M., Main, J., Thomson, E. C., 2013. Natural NS3 resistance polymorphisms occur frequently prior to treatment in HIV-positive patients with acute hepatitis C. *AIDS*, 27 (15), pp. 2485-8.

# Curriculum Vitae

Entfällt aus datenschutzrechtlichen Gründen

# Acknowledgements

It is done. I have analysed, summarized and discussed the scientific insights I gathered during my time as a PhD student. This is my first notable contribution to the scientific community. And, thanks to a lot of people, I was able to do it to the best of my abilities.

But science is not all fun and games. All trials and errors, failed hypotheses and difficult times are not part of this thesis, although these experiences are equally as important, I think. Because, thanks again to a lot of people, I was able to learn from them and grow not only as a scientist but also as a person.

So, without further ado:

Thank you to all senior scientists – my supervisor Prof. Stefanie Becker, my evaluators Prof. Egbert Tannich and Prof. Tim-Wolf Gilberger, my co-supervisor PD Dr. Minka Breloer and Prof. Jonas Schmidt-Chanasit. I am grateful for your extensive advise, guidance and illustrative scientific discussions.

It all starts somewhere. And I am especially indebted to Egbert for looking beyond the naïve enthusiasm of a 21-year-old Biology student and wanting to recruit me as a PhD student even before I had received my Bachelor's degree.

Speaking of enthusiasm – an engaging, helpful, motivational and all in all stimulating environment is a necessary prerequisite for good work. Thank you to Marlies, Martin and all other alumni of the Medical Entomology lab – especially to those, who helped in the collection of the 2012 data! This includes also the BTA students that helped out to tackle an often overwhelming amount of work. Another special thank you must go to Marlies for being such a big help throughout the SAW project!

To the guys from the technical department, who juggled the sensitive moods of our S3 labs and to everyone at the BNITM and other institutes who, in one way or another, helped me on my PhD journey: Thank you!

Many thanks also to the Graduate School IMPact-Vector for offering opportunities to extent my professional and interpersonal skill set. In this context, a special shout-out to the best roommate ever – Lisa!

Steph, Jessica, Heidi and Anna: You are trusty colleagues, brilliant scientists, motivational speakers and wonderful friends. Thank you for all the lovely memories!

A special thank you needs to go out to Anna, Steph and Ralf. Thank you for your input and the constructive discussions during the completion of this dissertation. Your help was truly invaluable!

And thank you Anna McNaughton for being 'my native speaker'. ;-)

To my friends and family: You are the important people behind the scenes without whom none of this would have happened. I thank you with all my heart.

And finally: Janko - thank you for being there for me. Jeg elsker deg!