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Phenotypical aspects of *Culex pipiens* biotype *pipiens* during diapause: Lipid utilization, body size, insemination, and parity

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ABSTRACT

In temperate regions, female *Culex pipiens* biotype *pipiens* mosquitoes undergo diapause in winter. Diapausing biotype *pipiens* mosquitoes are potentially important winter reservoirs for mosquito-borne viruses, such as West Nile virus (WNV), Usutu virus (USUV), and Sindbis virus (SINV). Mosquitoes in diapause have not taken a bloodmeal prior to winter. Therefore, they do not become infected by taking an infectious bloodmeal and as a consequence, vertical transmission is considered the primary mechanism of mosquito-borne virus overwintering. Prior to winter, biotype *pipiens* mosquitoes build up fat reserves, which they utilize throughout winter. Furthermore, earlier studies have indicated that larger body size is correlated with increased survival during winter. However, not much is known about lipid utilization and body size of wild biotype *pipiens* mosquitoes in diapause. Therefore, we performed monthly collections of diapausing biotype *pipiens* mosquitoes in two consecutive winters (2020/2021 and 2021/2022) in bunkers of the New Hollandic Waterline in the Netherlands. Every month, we checked the proportion of inseminated and parous females via microscopy. In addition, we measured wing length as proxy for body size, and assessed total lipid content. Furthermore, we monitored indoor temperature in the overwintering locations. We show that the overwintering sites in our study provide relatively stable environments, in which temperatures rarely drop below 0 °C. The vast majority of biotype *pipiens* females were inseminated (84.1 %) and nulliparous (97.5 %). We detected differences in body size between but not within the two years of sampling. Additionally, we detected a difference in lipid content between the sampling years. We confirm that the vast majority of diapausing biotype *pipiens* females are inseminated and nulliparous. This indicates that they did not blood feed prior to winter, which underscores the likeliness of vertical transmission being the primary mechanism behind virus overwintering. The detected difference in body size between years can most likely be attributed to differences in summer conditions the mosquitoes were exposed to as larvae, although this needs confirmation. The difference in lipid depletion could not be explained by differences in climatic conditions. To shed more light on the links between climatic conditions, body size, lipid depletion and the consequences for mosquito population dynamics and arbovirus transmission, future experimental work, for example by arbovirus exposure followed by artificially induced diapause, is desired.

1. Introduction

Usutu virus (USUV) and West Nile virus (WNV) have recently expanded in geographical range in Europe (Bakonyi & Haussig, 2020; Nikolay, 2015; Vilibic-Cavlek et al., 2020). Infections with USUV can cause severe disease symptoms in birds, and outbreaks of USUV are characterized by mass mortality of blackbirds (*Turdus merula*) (DWHC, 2022; Giglia et al., 2021; Rijks et al., 2016). With regards to public health, USUV is considered a limited threat, as clinical cases mainly occur in immunocompromised patients (Vázquez et al., 2011). Infection with WNV, on the other hand, may lead to the development of disease symptoms in horses and humans, although the majority of infections is subclinical. In rare cases, infections with WNV may lead to severe symptoms, such as encephalitis (Colpitts et al., 2012). Both viruses circulate between *Culex* mosquitoes and birds, and *Culex pipiens* s.s. (*sensu stricto*) is recognized as the primary vector (Brugman et al., 2018; Vogels et al., 2017). *Culex pipiens* s.s. can be distinguished into two biotypes (*Culex pipiens* biotype *pipiens* and *Culex pipiens* biotype

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molestus), which are sometimes also called ecotypes or forms. Both biotypes are presumed to exhibit strong behavioural differences in host preference, mating strategy, and overwintering. Hybridization may occur in places where both biotypes occur in sympatry, and hybrids are presumed to exhibit intermediate behaviour (Aardema et al., 2022; Brugman et al., 2018; Haba & McBride, 2022; Kilpatrick et al., 2005).

The relative abundance of the two biotypes and hybrids differs between habitat types and over a latitudinal gradient, with relatively more biotype *pipiens* occurring in the north of Europe compared to biotype *molestus*, and vice versa towards the south (Haba & McBride, 2022; Vogels et al., 2016). The relative abundance of *Cx. pipiens* s.s. biotypes across this latitudinal gradient may be attributed to differences in tolerance towards seasonal dynamics (Aardema et al., 2022; Haba & McBride, 2022). Biotype *pipiens* is better adapted to climatic conditions in temperate areas, compared to biotype *molestus*. As all other insects, mosquitoes are ectotherms, meaning that body temperature is regulated externally. At high latitudes, ectotherms need adequate survival strategies to endure the low temperatures during winter. Similar to other insect species occurring in temperate regions, biotype *pipiens* mosquitoes enter diapause. Diapause is a physiological state, which is characterized by the cessation of ovariole development and the termination of blood-feeding. In autumn, photoperiod and temperature trigger a hormonal pathway in larvae that induces diapause in the adult stage (Robich et al., 2007; Robich & Denlinger, 2005; Sanburg & Larsen, 1973; Sim & Denlinger, 2008). After larval development, pupation, and adult eclosion, newly emerged adults mate. This is followed by mass mortality of males. Simultaneously, females survive and feed on plant sugars, which are subsequently used to build up fat reserves to prepare for adverse winter conditions. After this, they seek shelter in anthropogenic or natural overwintering sites, such as cellars, bunkers, wells, caves, and animal burrows, where they remain until spring (Bergman et al., 2020; Blom et al., 2023; Dörge et al., 2020; Koenraadt et al., 2019; Onyeka & Boreham, 1987; Sauer et al., 2022; Sauer et al., 2023). Biotype *molestus*, on the other hand, does not enter diapause. As a consequence, this biotype relies on warmer habitats, such as houses, to overwinter. Here they remain actively blood-feeding throughout winter (Vogels et al., 2015).

At higher latitudes, the transmission cycles of mosquito-borne viruses are usually interrupted in winter. Diapausing mosquitoes are, however, considered an important reservoir of viruses in winter. In several European countries, mosquito-borne viruses (USUV, WNV, Sindbis virus (SINV) and Tahyna virus (TAHV)) have been detected in diapausing mosquitoes in winter (Bergman et al., 2020; Kampen et al., 2021; Rudolf et al., 2017, 2020; Sauer et al., 2023). Interestingly, in the US, earlier studies have shown that live virus can be isolated from *Cx. pipiens* s.s. mosquitoes in diapause (Nasci et al., 2001). This indicates that mosquitoes in diapause can serve as an overwintering pathway of mosquito-borne viruses. Vertical transmission of virus (i.e. transmission from parent to offspring) has been suggested as the primary mechanism behind infections in diapausing mosquitoes. It is widely accepted that the biotype *pipiens* generation in which diapause has been induced does not blood-feed before winter. This can be derived by examining mosquitoes for parity status of their ovaries. Overall, the proportion of nulliparous females (i.e. those not having laid eggs and thus not blood-fed before) in winter ranges between 98.4 % and 100 % (Bergman et al., 2020; Jaenson, 1987). Whenever infections are detected in diapausing mosquitoes, blood feeding is generally excluded as a possible infection route. However, rare cases are known in which mosquitoes take a bloodmeal without utilizing it for the production of eggs (gonotrophic dissociation) (Mitchell, 1983; Washino, 1977). Although laboratory experiments have demonstrated that vertical transmission of mosquito-borne viruses is possible for various mosquito-arbovirus combinations, it only occurs at low rates (up to 6.9/1000 mosquitoes getting infected) (Anderson et al., 2008, 2012; Dohm et al., 2002; Goddard et al., 2003).

Even though biotype *pipiens* mosquitoes in diapause play a pivotal role in virus overwintering, much remains unknown about their biology in winter. In the Netherlands, up to 70 % of the biotype *pipiens* population in diapause may survive throughout winter (Koenraadt et al., 2019). However, the factors that underlie their overwintering survival are poorly understood. The lipid reserves accumulated by biotype *pipiens* mosquitoes are considered to be a prime determinant of their winter survival (Hahn & Denlinger, 2007; Robich et al., 2007; Rozsypal et al., 2021; Sauer et al., 2023). Studies on lipid depletion in wild populations of diapausing biotype *pipiens* mosquitoes are scarce, however. As a consequence, it remains unclear how the depletion of lipid reserves occurs throughout winter. Not only lipid utilization plays an important role in survival of diapausing mosquitoes, body size may do so as well (Sauer et al. (2022). Body size of biotype *pipiens* mosquitoes has been positively correlated with survival, an association that was not found for *Anopheles messae* (Sauer et al. 2022). On the one hand, larger mosquitoes can hold more lipid reserves in an absolute sense than smaller mosquitoes. On the other hand, maintaining homeostasis may cost relatively more reserves in larger specimens. It is unknown whether the observations by Sauer et al. (2022) are also clear at the population level. If mosquitoes of larger body size have a higher survival throughout winter than mosquitoes of smaller body size, it is expected that the distribution of body size of the diapausing population will shift towards larger individuals over time. In general, body size of mosquitoes is positively correlated with fitness (i.e. fecundity, mating success), which is a pattern that is observed across various mosquito taxa (Briegel, 1990; Lima et al., 2003; Lyimo & Takken, 1993). Therefore, body size of the overwintering population can be an important determinant of the size of the emerging population in spring and its potential for further pathogen transmission later in the season.

In the Netherlands, several *Culex*-borne viruses (USUV, WNV and SINV) have been repeatedly detected in wild birds, animals, humans, and/or mosquitoes (Oude Munnink et al., 2020; Rijks et al., 2016; Sikkema et al., 2020; Streng et al., in prep. b; Vlaskamp et al., 2020). Therefore, there is a possibility that these viruses will overwinter in diapausing mosquitoes. This was already confirmed for USUV (Koenraadt et al., in prep.) and highlights the importance of understanding the biology of *Cx. pipiens* biotype *pipiens* diapause, as it will help us understand the seasonal dynamics of mosquito-borne viruses. We collected wild diapausing biotype *pipiens* mosquitoes at monthly intervals during the winters of two consecutive years. We ran a series of analyses on these mosquitoes, in order to provide fundamental insights in biotype *pipiens* diapause. We scored the proportion of inseminated and parous female mosquitoes. Mosquitoes were identified morphologically, followed by molecular identification to determine the biotype. Lastly, on the molecularly identified mosquitoes, we conducted monthly measurements of body size and lipid reserves, in order to study lipid depletion and distribution of body size at population level.

2. Methods

2.1. Mosquito collection

Monthly mosquito collections were carried out during the winters of 2020/2021 (sampling year 1) and 2021/2022 (sampling year 2) in six bunkers of the New Hollandic Waterline in the municipality of West-Betuwe, the Netherlands. The area is characterized by a mosaic of river floodplains, pastures, fruit orchards, and tiny patches of forest, with only a few human settlements. The bunkers are located primarily next to dykes, and host a range of arthropods during winter, including diapausing biotype *pipiens, Anopheles maculipennis s.l., Culiseta annulata,* and *Culex territans* mosquitoes (Blom et al., 2023; Ibáñez-Justicia et al., 2022; Koenraadt et al., 2019). Fungus gnats of the family Mycetophilidae, moths and butterflies, and various arachnid species can also be observed in the bunkers. Furthermore, the bunkers can serve as an overwintering location for bats. Prior to every collection, bunkers were checked for the presence of overwintering bats. Whenever bats were present, mosquito collections were not carried out in that particular bunker in order to avoid disturbance of the bats. Mosquitoes were collected from the walls and ceilings of the bunkers via automatic and/or manual aspiration as described by Blom et al. (2023). In sampling year 1, mosquito collections were carried out from December 2020 until April 2021. In sampling year 2, collections were carried out from September 2021 until March 2022. Diapausing mosquitoes could no longer be detected in May and April of year 1 and 2, respectively. Consequently, field collections were terminated after those months. Live mosquitoes were transported to the Laboratory of Entomology, Wageningen University & Research, the Netherlands, where they were placed in incubators at 6 °C. After assessment of parity and insemination rates, remaining mosquitoes were stored at −20 °C. At the start of each sampling year, dataloggers (TinyTag plus2, Gemini Data Loggers UK Ltd, Chichester, UK) were placed in three out of six bunkers to measure relative humidity and temperature at an hourly interval. We considered the combination of three bunkers to be representative for all other overwintering locations included in our study. In the first year of sampling, one datalogger could no longer be found halfway through the sampling period.

2.2. Sample selection

Collected mosquitoes were identified morphologically to the species group (C*ulex pipiens/torrentium*) level following the identification key of Becker et al. (2020). Per timepoint and location of sampling, a subset of 5–10 *Cx. pipiens/torrentium* mosquitoes was selected for molecular identification and lipid analysis. Subsequently, *Cx. pipiens/torrentium* mosquitoes were further identified to the species/biotype level following a DNA extraction and real-time PCR assay slightly adjusted from the protocol of Vogels et al. (2015) targeting the CQ11 microsatellite region. DNA extractions were performed on mosquito legs collected from morphologically identified *Cx. pipiens/torrentium* mosquitoes via lysis in ammonium hydroxide. Materials (e.g. forceps) were cleaned in 96 % ethanol in between handling of different samples to reduce potential contamination. Subsequently, this was followed by real-time PCR, with adjustments described by Blom et al. (2024). All real-time PCR reactions were performed on a CFX OPUS (Bio-Rad) qPCR machine. Up to five mosquitoes identified as biotype *pipiens* per sampling timepoint were selected for further lipid analysis and wing length measurements.

2.3. Insemination and parity

From every timepoint and location of sampling, up to 10 live mosquitoes were dissected and scored for parity and insemination. In short, live mosquitoes were briefly anesthetized with CO_2. Subsequently, up to 5 individuals were placed on a microscope slide with the abdomens placed in phosphate buffered saline (PBS) solution. Parity was determined by visually examining ovarian tracheation. Two incisions were made between tarsal segments six and seven. Subsequently, the apical end of the abdomen was removed in order to expose ovaria. Ovaria were checked visually on coiling of the tracheoles using a Olympus SZ51 stereo microscope (Olympus Life Sciences, Tokyo, Japan). Nulliparous females have short, coiled tracheoles, whereas the tracheoles of the ovaria of parous females are uncoiled and form a web-, or net-like structure (Detinova, 1962; Tyndale-Biscoe, 1984). Subsequently, from every female the spermathecae were isolated. Spermathecae were covered with a cover glass and gently crushed to expose the spermatozoa within. Spermathecae were visually checked for the presence of spermatozoa using a Euromex iScope trinocular microscope (Euromex, Arnhem, the Netherlands).

2.4. Correlation between wing length and body size

We used mosquito wing length as a proxy for body size. To validate the suitability of wing length as a proxy for body size, wing and thorax lengths of 42 laboratory-reared biotype *pipiens* mosquitoes were measured. Biotype *pipiens* mosquitoes, originating from a summer population collected in Wageningen, the Netherlands, were reared under the following conditions: 22–23 °C, 16:8 l:D photoperiod and 60 % relative humidity. As described by Yeap et al. (2013), thorax length of mosquitoes was measured from the scutellum to the most anterior point of the thorax from a lateral angle (Fig. 1). Per trapping location and timepoint of sampling, up to five field collected mosquitoes (previously identified as biotype *pipiens*) were used for body size analysis and subsequent lipid analysis. For both the lab-reared as well as field-collected mosquitoes, wings were removed from mosquitoes with forceps and mounted on glass microscope slides using double sided tape and covered with a slip glass. Mosquito wing and thorax photographs were taken using a Dino-Lite AM7915MZT EDGE 5 Megapixel (AnMo Electronics, Hsinchu City, Taiwan) digital microscope. Wing size was determined by measuring the mosquito wing from the alula to the most apical point, excluding the fringe scales (Mohammed & Chadee, 2011) (Fig. 1). All measurements were performed using DinoCapture 2.0 (Version 1.5.47C, 2016).

2.5. Lipid analysis

Total lipid content per individual mosquito was measured following the phosphoric acid-vanillin assay described by Van Handel, with slight adjustments (Van Handel, 1985). In short, mosquitoes were placed in borosilicate tubes (FisherScientific, Hampton, NH, US), after which they were crushed using glass rods. Subsequently, 200 μl of sodium sulphate was added, followed by further homogenization. To each sample, 1.5 ml 1:1 chloroform:methanol was added. Samples were centrifuged at 20,000 rpm for 1 min. The supernatant was transferred to a new borosilicate tube, and these samples were evaporated at 80 °C. After evaporation, 200 μl of sulphuric acid was added, and samples were heated at 95 °C for ten minutes. Per sample, 3 ml of phosphoric acid-vanillin reagent was added. Absorbance at 525 nm was measured with a Multiskan Sky (ThermoScientific, Waltham, MA, US) plate reader, using Cellstar 96 well plates (Greiner Bio-One, Kremsmünster, Austria). Sunflower oil (Jumbo, Veghel, the Netherlands) was used to set up a standard curve,

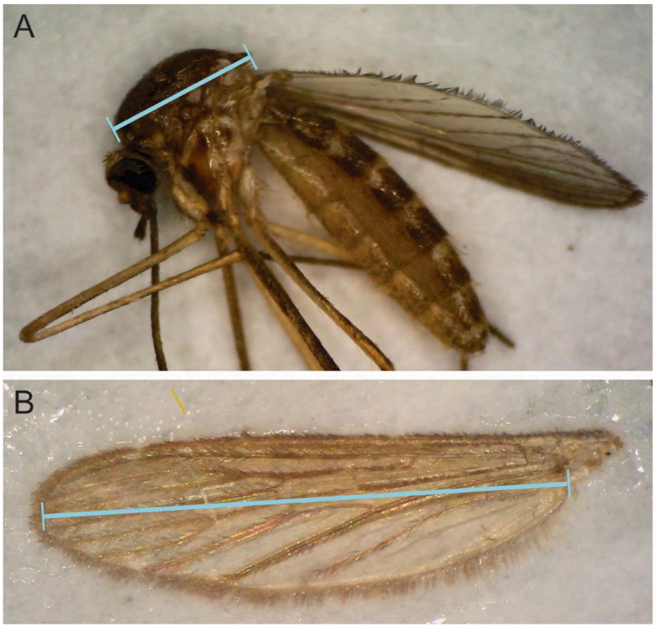

Fig. 1. Depiction of body size measurements of laboratory reared *Cx. pipiens* biotype *pipiens* mosquitoes. A) Thorax lengths were measured from the scutellum to the most anterior point of the thorax. B) Wing lengths were measured from the alula to the most apical point of the wing, excluding the fringe scales.

with which the total lipid content of each sample was determined. In April 2022, an insufficient number of diapausing biotype *pipiens* was collected from the bunkers. Therefore, this month was excluded from further lipid and body size analyses.

2.6. Statistical analyses

The correlation between outdoor temperatures and indoor temperatures was evaluated. Daily outdoor temperature data (minimum, average and maximum), measured at the closest nearby weather station (Herwijnen, municipality of West-Betuwe) were acquired from the Dutch meteorological institute (KNMI) (KNMI, 2021a). Indoor temperatures were averaged per day for each individual sampling site. Only data collected between January 5th and April 18th of both sampling years were included, as this was the timeframe covered in both sampling years. From the 17th up until the 20th of December 2021, no temperature was logged at the KNMI weather station. Those timepoints were omitted from the correlation analysis. For each separate bunker, the linear relationship between the mean daily indoor and outdoor temperature (minimum, average, and maximum) was calculated using a Spearman correlation test, as the data have a non-parametrical distribution.

We tested whether wing size is an appropriate proxy for mosquito body length. We calculated the linear correlation between the body size and wing length of laboratory-reared biotype *pipiens* mosquitoes. For field collected mosquitoes, wing length was analysed by running a linear mixed effect model (LMEM), with wing length as response variable, collection month and sampling year as explanatory variables and bunker as random effect variable. For the statistical analysis on lipid content, we corrected lipid content for mosquito wing length, as larger individuals were expected to contain a higher absolute total lipid weight compared to smaller mosquitoes. In order to identify differences in corrected lipid content between months, we ran an LMEM, with timepoint of sampling and year as fixed variables and bunker as a random effect variable. In this model, timepoint of sampling was included as a nominal factor. In addition, in order to calculate the slope of lipid depletion (i.e. lipid decrease per time interval), we ran a similar LMEM, but this time with month as a numerical factor. This model was run for the months of December until March. For all statistical tests, the relevant assumptions were visually checked. All statistical analyses were performed with RStudio 2023.06.0 (RStudio Team, 2023).

3. Results

3.1. Insemination and parity

In total, parity and insemination proportions were assessed in 478 female *Cx. pipiens/torrentium* mosquitoes. Spermatozoa were detected in the spermathecae of 402 (84.1 %) individual mosquitoes (Table 1). Of 12 mosquitoes (2.5 %), we were unable to assess insemination status. In those cases, presence nor absence of spermatozoa could be visually confirmed, as spermatozoa were not visible. A total number of 466 (97.5 %) mosquitoes were scored as nulliparous, as coiling of the tracheoles of the ovaria was observed. This indicated that these mosquitoes had not produced eggs (and thus not fed blood) prior to diapause. Parity of two individuals (0.4 %) could not be determined, as the coiling of the tracheoles was difficult to visually assess.

Table 1

Overview of parity status and insemination rates of *Cx. pipiens/torrentium* mosquitoes collected from overwintering locations in winter. In some cases, we were unable to visually confirm coiling of the tracheoles or presence of spermatozoa (= NA).

Parity	No.	Percentage
Nulliparous	466	97.5 %
Parous	10	2.1 %
NA	2	0.4 %
Insemination		
Inseminated	402	84.1 %
Not inseminated	64	13.4 %
NA	12	2.5 %

3.2. Temperature

In sampling year 1, the lowest recorded temperature outdoors was −11 °C, on the 9th and 12th of February. In the two bunkers in which we measured, lowest recorded temperatures were −0.7 °C and −3.5 °C, both on the 13th of February. In one of the two bunkers, temperatures reached values below 0 °C for 5 consecutive days, whereas in the other bunker temperatures only reached values below 0 °C for part of a day. In sampling year 2, outdoor temperatures reached below 0 °C several times through the sampling period (Fig. S1, Fig. S2). The lowest recorded temperature was −5.8 °C, which occurred twice, on December 22nd and April 3rd. However, temperatures in bunkers always remained above 0 °C (Fig. S2). Temperature in bunkers was weakly correlated with outdoor minimum temperatures. In contrast, we observed a strong correlation between outdoor average and maximum temperatures and indoor temperature in sampling year 2. In sampling year 1, however, the correlation between outdoor temperatures and indoor temperature was much weaker (Table S1, Fig. S1).

3.3. Body size

For laboratory-reared mosquitoes, we found a strong linear relation between wing length and thorax length ($R^2 = 0.86$, Fig. 2). Therefore, we deemed wing length a suitable proxy for body size for our field-collected specimens. For both years of sampling (2020/2021 and 2021/2022), no statistically significant differences in wing length over the months of a single sampling year could be observed. However, we did detect a statistically significant effect of sampling year on wing length (LMEM, $t = 2.97$, $p < 0.01$). In sampling year 1, wing length of mosquitoes was 4.10 mm on average, whereas in sampling year 2 the wing length was 2.9 % higher (4.23 mm on average).

3.4. Lipid content

From December onwards, lipid content decreased in both years, which was also expressed in statistically significant differences based on our LMEM (Fig. 3, Table S2, Table S3). A statistically significant effect of the sampling year (LMEM, $t = -2$, $n = 282$, $p = 0.05$) and sampling

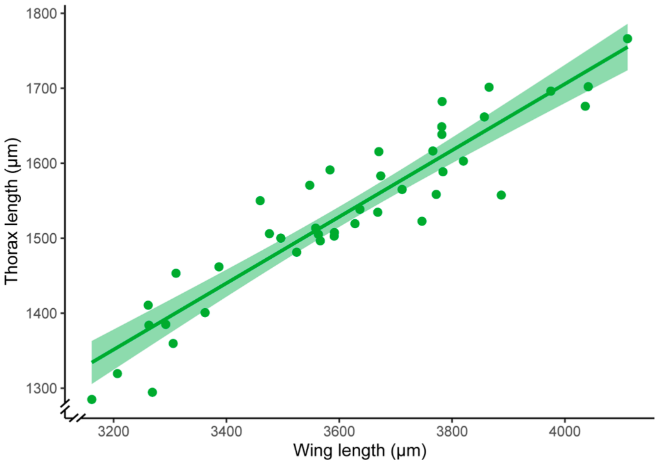

Fig. 2. Linear relation between wing length (μm) and thorax length (μm) of laboratory-reared *Cx. pipiens* biotype *pipiens* mosquitoes ($R^2 = 0.86$).

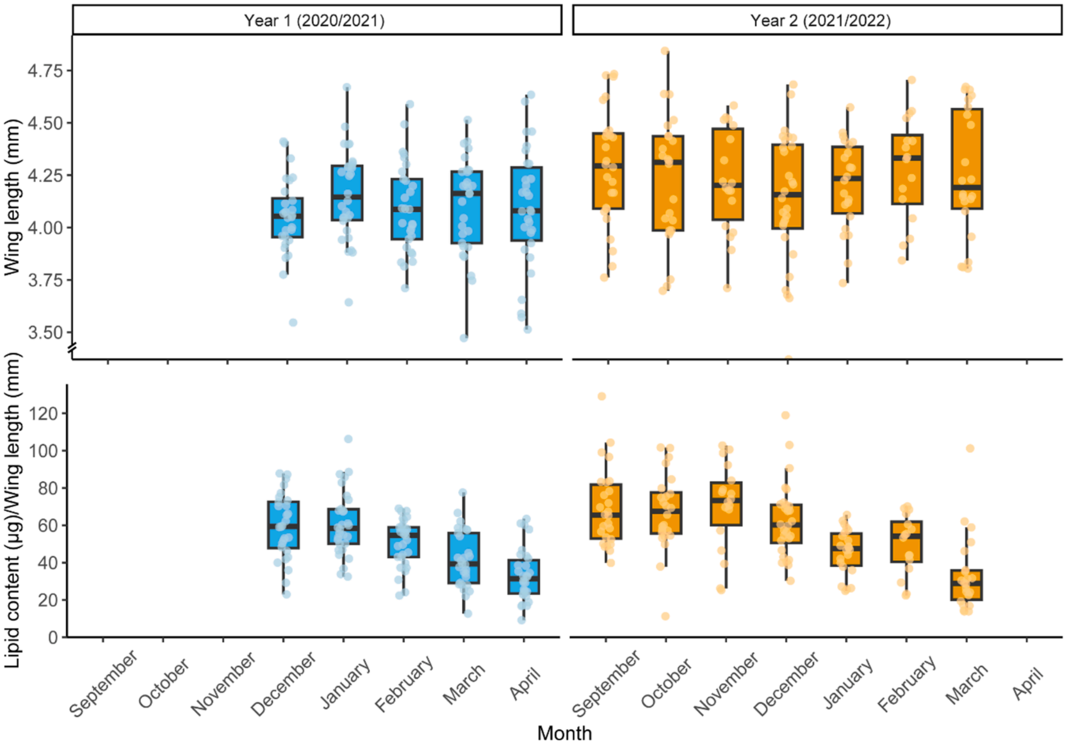

Fig. 3. Top panels: wing length of mosquitoes collected in sampling year 1 (December 2020 to April 2021) and sampling year 2 (September 2021 to March 2022). Statistically significant differences were detected between sampling years (F = 9.84, p < 0.01), but not between the months within sampling years. Bottom panels: lipid content corrected for wing length of mosquitoes collected in sampling year 1 (December 2020 to April 2021) and sampling year 2 (September 2021 to March 2022). Statistically significant differences between the sampling months were observed, with a decrease detected from December to April when both years are pooled (β = -7.76 ± 5.47 SE, n = 222, p < 0.01) (Table S3).

month on corrected lipid content was found (summarized in Table S3). We observed a statistically significant decrease of lipid content for the sampling months (7.76 µg/mm) between December and March (LMEM, β = -7.76 ± 5.47 SE, n = 222, p < 0.01), when both years are pooled. In sampling year 1, corrected lipid content decreased from 59.6 µg/mm in December to 41.9 µg/mm in March (29.6 % decrease). In sampling year 2, corrected lipid content decreased from 62.6 µg/mm in December to 33.4 µg/mm in March (46.7 % decrease).

4. Discussion

We have shown that nearly all mosquitoes that are in diapause are nulliparous, while the vast majority is also inseminated prior to winter. In addition, the sampled bunkers provided a stable environment for diapausing biotype *pipiens* mosquitoes. In our study, bunker temperatures only seldom dropped below 0 °C, whereas outdoor temperatures dropped below freezing point more frequently. It seems that, as long as outdoor temperatures do not remain below 0 °C for extended periods of time, anthropogenic structures such as bunkers provide a stable and relatively warm environment for mosquitoes in diapause. Interestingly, we noticed that bunker temperature responded differently to outdoor temperature conditions in the sampling year of 2020/2021 compared to 2021/2022. This is most likely due to delayed temperature changes in bunkers in response to outdoor temperature dynamics, and the sudden shifts from very cold temperatures to exceptionally high temperatures in February 2021 (KNMI, 2021a). It is important to understand how climate may have an effect on the diapause period of important vector species and persistence of their associated pathogens. In earlier studies, it was shown that survival of mosquitoes in diapause is drastically reduced when they are exposed to sub-zero temperatures (Rozsypal et al., 2021; Sauer et al., 2022). The bunkers in our study may therefore be ideal hibernacula, facilitating the survival of diapausing mosquitoes due to relatively high temperatures. However, it should be noted that our sampling locations contained a high abundance of potential predators, which may contribute to mosquito mortality during winter. These effects are unknown and should be taken into account in future studies on diapausing mosquito population dynamics.

4.1. Wing length differs between years

In contrast to our expectations, no shift in mosquito wing length was detected during winters in both sampling years. Given the higher survival of larger biotype *pipiens* mosquitoes (Sauer et al., 2022), we expected a shift towards larger specimens over the course of winter. We did, however, detect a difference in wing length between the two years. We hypothesize that the differences in wing length can be attributed to a difference in climatic conditions between the summers of the respective years. Overall, the summer of 2020 was extraordinarily warm, especially August and September (KNMI, 2021b). The summer of 2021 was characterized by a warm June, with below average temperatures in July and August. September 2021 was cooler than September 2020, although temperatures were still above average (KNMI, 2022). As adult insect body size is negatively correlated with temperature, the higher temperatures in August prior to sampling year 1 may explain the shorter wing length observed in that year (Atkinson, 1994; Kingsolver & Huey, 2008). Earlier laboratory studies have shown this negative correlation in several mosquito species. For instance, Ciota et al. (2014) showed this for several *Culex* species, including *Cx. pipiens* s.l., *Culex quinquefasciatus,* and *Culex restuans*. Similar temperature-mediated effects were also detected in *Anopheles gambiae* mosquitoes (Barr et al., 2023). However, in their study, not all body parts were affected equally, and wing length was not affected at all. Body size is a primary determinant of insect fitness, and body size of the diapausing population may affect the size of the population in spring, as larger insects generally produce more offspring. However, the link between parameters at the individual level (e.g. body size, lipid depletion) and population level (e.g. survival) of diapausing mosquitoes and the abundance in spring/summer remain unclear.

4.2. Lipid utilization throughout winter

Interestingly, we did not observe a decrease in lipid reserves before December. Firstly, it could be that mosquitoes that were already in diapause continued sugar feeding, a phenomenon earlier described by Sauer et al. (2022). Interestingly, higher temperatures in winter may trigger diapausing mosquitoes to initiate ovarian development and blood-feeding (Fyie et al., 2023; Nelms et al., 2013). However, in our study we did not detect any blood-engorged mosquitoes, even after the extraordinarily warm temperatures in February 2021. Secondly, it could be that part of the population was already in diapause, while the influx of more recently emerged mosquitoes was still ongoing. Diapause is primarily triggered by a combination of temperature and photoperiod, although other factors (e.g. altitude) may also play a role. An earlier study from the United States shows that diapause induction starts in late August and peaks in mid-September (Field et al., 2022). This shows that diapause induction may occur over a relatively long period of time, which then differs between years. For the Netherlands, such data is unavailable, and the time period of diapause induction is unclear. An additional reason for the absence of lipid depletion in the earliest months of our study period might be due to the phenology of flowering plants on which mosquitoes rely for sugar feeding. Prior to diapause, newly emerged mosquitoes derive plant-sugars from flowering plants to build up their lipid reserves. Possibly the phenology of mosquito diapause is related to plant phenology. Climate change will most likely induce shifts in plant phenology, including their flowering period (Dorji et al., 2020; Johansson et al., 2013; Tun et al., 2021). Shifts in plant phenology are expected to have consequences for the acquisition of plant sugars by mosquitoes prior to winter, although the direction and size of the effect are unknown. Therefore, future studies should focus on identifying the interactions between mosquitoes and flowering plants and the effects on lipid build-up prior to winter.

Lipid depletion commenced in December, after which we observed a gradual decline in lipid content over the months. Between December and March, mosquitoes deplete approximately 30 % to up to nearly 50 % of their lipid reserves, depending on the year. These results corroborate the findings of Rozsypal et al. (2021), who reported that biotype *pipiens* mosquitoes accumulate enough lipid reserves to last more than one winter. Interestingly, lipid depletion was higher in sampling year 2, compared to sampling year 1. Mosquitoes collected in sampling year 1 were exposed to sub-zero temperatures, whereas the ones that were collected in sampling year 2 were not. Therefore, it remains unclear which cause underlies the differences we detected between the sampling years. It is unclear whether full depletion of lipid reserves at the end of the diapause period (when temperatures are relatively warm) will lead to termination of diapause period or to mortality. Furthermore, it remains unknown how survival rates of diapausing mosquitoes affect the size of the population in spring, and the consequences that may have on arbovirus epidemiology. The effects of long-term exposure to low temperatures on lipid utilization and survival need to be studied further. In order to do so, experimental studies are needed in which lipid utilization of diapausing mosquitoes is monitored under different temperature regimes.

4.3. Infection routes of diapausing mosquitoes

In our study, the vast majority of mosquitoes (97.5 %) were nulliparous, which is in line with results from other studies (Bergman et al., 2020; Nelms et al., 2013). However, we show that a very small proportion of mosquitoes is parous, which suggests they have taken a bloodmeal and produced eggs prior to winter. In theory, these mosquitoes can thus become infected as a result of taking an infectious bloodmeal prior to winter. This indicates that vertical transmission may not be the only route via which diapausing mosquitoes can become infected prior to winter. In order to exclude infection via blood-feeding, studies in which mosquitoes that are being screened for viruses are also tested on parity are necessary. However, such processes are laborious, and thus far, this has only been performed in one study on the persistence of SINV in biotype *pipiens* mosquitoes in Sweden (Bergman et al., 2020). Additionally, other infection routes are possible. It could be possible that mosquitoes become infected prior to winter via venereal transmission. However, evidence for venereal transmission of mosquito-borne viruses has only been shown for Zika virus and Chikungunya virus in *Aedes aegypti* mosquitoes, and not for any of the *Culex*-borne viruses (Geevarghese et al., 2010; Pereira-Silva et al., 2017). Therefore, we expect that vertical transmission remains the most likely infection route of diapausing mosquitoes.

5. Conclusion

Our study shows that anthropogenic habitats such as bunkers provide suitable overwintering locations for mosquitoes in diapause. As expected, the majority of the mosquitoes collected in our study were inseminated and nulliparous. This underscores that whenever infected mosquitoes in diapause are found, they are most likely infected via vertical transmission. However, infection via an infectious bloodmeal is not completely excluded. Furthermore, we show that body size and lipid depletion, both important parameters of mosquito survival, differ between years. The underlying causes for these differences remain unclear. Future experimental work can shed more light on the links between climatic effects, body size, lipid depletion, survival, and their consequences for mosquito population dynamics and virus transmission in the following summers.

CRediT authorship contribution statement

Rody Blom: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Jeroen Spitzen:** Writing – review & editing, Investigation. **Tessa de Haan:** Writing – review & editing, Investigation. **Constantianus J.M. Koenraadt:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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Data availability

Data will be made available on request.

References

Aardema, M.L., Olatunji, S.K., Fonseca, D.M., 2022. The enigmatic *Culex pipiens* (Diptera: Culicidae) species complex: phylogenetic challenges and opportunities from a notoriously Tricky Mosquito Group. Ann. Entomol. Soc. Am. 115 (1), 95–104. https://doi.org/10.1093/aesa/saab038.

Anderson, J.F., Main, A.J., Delroux, K., 2008. Extrinsic incubation periods for horizontal and vertical transmission of West Nile Virus by *Culex pipiens pipiens* (Diptera: Culicidae). J. Med. Entomol. 45 (3), 455.

Anderson, J.F., Main, A.J., Cheng, G., Ferrandino, F.J., Fikrig, E., 2012. Horizontal and vertical transmission of West Nile Virus Genotype NY99 by *Culex salinarius* and Genotypes NY99 and WN02 by *Culex tarsalis*. Am. J. Trop. Med. Hyg. 86 (1), 134–139. https://doi.org/10.4269/ajtmh.2012.11-0473.

Atkinson, D., 1994. Temperature and organism size—a biological law for ectotherms? Adv. Ecol. Res. 25, 1–58. https://doi.org/10.1016/S0065-2504(08)60212-3.

Bakonyi, T., Haussig, J.M., 2020. West Nile virus keeps on moving up in Europe. pii=2001938 Eurosurveillance 25 (46). https://doi.org/10.2807/1560-7917.ES.2020.25.46.2001938.

Barr, J.S., Estevez-Lao, T.Y., Khalif, M., Saksena, S., Yarlagadda, S., Farah, O., Shivere, Y., Hillyer, J.F., 2023. Temperature and age, individually and interactively, shape the size, weight, and body composition of adult female mosquitoes. J. Insect Physiol. 148, 104525. https://doi.org/10.1016/j.jinsphys.2023.104525.

Becker, N., Petrić, D., Zgomba, M., Boase, C., Madon, M. B., Dahl, C., Kaiser, A., 2020. Mosquitoes: Identification, Ecology and Control (3rd ed.). Springer International Publishing. https://doi.org/10.1007/978-3-030-11623-1.

Bergman, A., Dahl, E., Lundkvist, Å., Hesson, J.C., 2020. Sindbis Virus Infection in Non-Blood-Fed Hibernating *Culex pipiens* Mosquitoes in Sweden. Viruses 12 (12), 1441. https://doi.org/10.3390/v12121441.

Blom, R., Schrama, M.J.J., Spitzen, J., Weller, B.F.M., Van Der Linden, A., Sikkema, R.S., Koopmans, M.P.G., Koenraadt, C.J.M., 2023. Arbovirus persistence in North-Western Europe: are mosquitoes the only overwintering pathway? One Health 16, 100467. https://doi.org/10.1016/j.onehlt.2022.100467.

Blom, R., Krol, L., Langezaal, M., Schrama, M., Trimbos, K.B., Wassenaar, D., Koenraadt, C.J.M., 2024. Blood-feeding patterns of *Culex pipiens* biotype *pipiens* and *pipiens/molestus* hybrids in relation to avian community composition in urban habitats. Parasit. Vectors 17 (1), 95. https://doi.org/10.1186/s13071-024-06186-9.

Briegel, H., 1990. Metabolic relationship between female body size, reserves, and fecundity of *Aedes aegypti*. J. Insect Physiol. 36 (3), 165–172. https://doi.org/10.1016/0022-1910(90)90118-Y.

Brugman, V.A., Hernández-Triana, L.M., Medlock, J.M., Fooks, A.R., Carpenter, S., Johnson, N., 2018. The role of *Culex pipiens* L. (Diptera: Culicidae) in virus transmission in Europe. Int. J. Environ. Res. Public Health 15 (2), 389. https://doi.org/10.3390/ijerph15020389.

Ciota, A.T., Matacchiero, A.C., Kilpatrick, A.M., Kramer, L.D., 2014. The effect of temperature on life history traits of *Culex* mosquitoes. J. Med. Entomol. 51 (1), 55–62. https://doi.org/10.1603/ME13003.

Colpitts, T.M., Conway, M.J., Montgomery, R.R., Fikrig, E., 2012. West Nile virus: biology, transmission, and human infection. Clin. Microbiol. Rev. 25 (4), 635–648. https://doi.org/10.1128/CMR.00045-12.

Detinova, T.S., 1962. Age-grouping methods of diptera of medical importance with special reference to some vectors of malaria. WHO Monograph Series 47.

Dohm, D.J., Sardelis, M.R., Turell, M.J., 2002. Experimental Vertical Transmission of West Nile Virus by Culex pipiens (Diptera: Culicidae). J. Med. Entomol. 39 (4), 640–644. https://doi.org/10.1603/0022-2585-39.4.640.

Dörge, D.D., Cunze, S., Schleifenbaum, H., Zaenker, S., Klimpel, S., 2020. An investigation of hibernating members from the *Culex pipiens* complex (Diptera, Culicidae) in subterranean habitats of central Germany. Sci. Rep. 10, 10276. https://doi.org/10.1038/s41598-020-67422-7.

Dorji, T., Hopping, K.A., Meng, F., Wang, S., Jiang, L., Klein, J.A., 2020. Impacts of climate change on flowering phenology and production in alpine plants: The importance of end of flowering. Agr Ecosyst Environ 291, 106795. https://doi.org/10.1016/j.agee.2019.106795.

DWHC, 2022. Merelsterfte door usutuvirus, vogelmalaria en parasieten. https://dwhc.nl/merelsterfte-door-usutuvirus-vogelmalaria-en-parasieten/.

Field, E.N., Shepard, J.J., Clifton, M.E., Price, K.J., Witmier, B.J., Johnson, K., Boze, B., Abadam, C., Ebel, G.D., Armstrong, P.M., Barker, C.M., Smith, R.C., 2022. Semi-field and surveillance data define the natural diapause timeline for *Culex pipiens* across the United States. Commun. Biol. 5, 1300. https://doi.org/10.1038/s42003-022-04276-x.

Fyie, L.R., Tronetti, H.R., Gardiner, M.M., Meuti, M.E., 2023. Potential for urban warming to postpone overwintering dormancy of temperate mosquitoes. J. Therm. Biol 115, 103594. https://doi.org/10.1016/j.jtherbio.2023.103594.

Geevarghese, G., Ghodke, Y., Sudeep, A., Arankalle, V., Mavale, M., Gokhale, M., Parashar, D., Mishra, A.C., 2010. Venereal Transmission of Chikungunya Virus by Aedes aegypti Mosquitoes (Diptera: Culicidae). Am. J. Trop. Med. Hyg. 83 (6), 1242–1244. https://doi.org/10.4269/ajtmh.2010.09-0577.

Giglia, G., Agliani, G., Munnink, B.B.O., Sikkema, R.S., Mandara, M.T., Lepri, E., Kik, M., Ijzer, J., Rijks, J.M., Fast, C., Koopmans, M.P.G., Verheije, M.H., Gröne, A., Reusken, C.B.E.M., Van Den Brand, J.M.A., 2021. Pathology and Pathogenesis of Eurasian Blackbirds (Turdus merula) Naturally Infected with Usutu Virus. Viruses 13, 1481. https://doi.org/10.3390/v13081481.

Goddard, L.B., Roth, A.M.Y.E., Reisen, W.K., Scott, T.W., 2003. Vertical Transmission of West Nile Virus by Three California Culex (Diptera: Culicidae) Species. J. Med. Entomol. 40 (6), 743–746. https://doi.org/10.1603/0022-2585-40.6.743.

Haba, Y., Mcbride, L., 2022. Origin and status of *Culex pipiens* mosquito ecotypes. Curr. Biol. 32 (5), R237–R246. https://doi.org/10.1016/j.cub.2022.01.062.

Hahn, D.A., Denlinger, D.L., 2007. Meeting the energetic demands of insect diapause: nutrient storage and utilization. J. Insect Physiol. 53 (8), 760–773. https://doi.org/10.1016/j.jinsphys.2007.03.018.

Ibáñez-Justicia, A., Smitz, N., Blom, R., Vanderheyden, A., Jacobs, F., Meganck, K., Gombeer, S., Backeljau, T., Koenraadt, C.J.M., Griep, J.S., De Meyer, M., Stroo, A., 2022. *Anopheles maculipennis* Complex in The Netherlands: First Record of *Anopheles daciae* (Diptera: Culicidae). Diversity 14, 636. https://doi.org/10.3390/d14080636.

Jaenson, T.G.T., 1987. Overwintering of *Culex* mosquitoes in Sweden and their potential as reservoirs of human pathogens. Med. Vet. Entomol. 1 (2), 151–156. https://doi.org/10.1111/j.1365-2915.1987.tb00336.x.

Johansson, J., Bolmgren, K., Jonzén, N., 2013. Climate change and the optimal flowering time of annual plants in seasonal environments. Glob. Chang. Biol. 19, 197–207. https://doi.org/10.1111/gcb.12006.

Kampen, H., Tews, B.A., Werner, D., 2021. First Evidence of West Nile Virus Overwintering in Mosquitoes in Germany. Viruses 13, 2463. https://doi.org/10.3390/v13122463.

Kilpatrick, A.M., Kramer, L.D., Campbell, S.R., Alleyne, E.O., Dobson, A.P., Daszak, P., 2005. West Nile virus risk assessment and the bridge vector paradigm. Emerg. Infect. Dis. 11 (3), 425–429. https://doi.org/10.3201/eid1103.040364.

Kingsolver, J.G., Huey, R.B., 2008. Size, temperature, and fitness: three rules. Evol. Ecol. Res. 10, 251–268. https://doi.org/10.17615/jtzc-v174.

KNMI, 2021a. Daggegevens van het weer in Nederland. https://www.knmi.nl/nederland-nu/klimatologie/daggegevens.

KNMI, 2021b. Jaar 2020. https://www.knmi.nl/nederland-nu/klimatologie/maand-en-seizoensoverzichten/2020/jaar.

KNMI, 2022. Jaar 2021. https://www.knmi.nl/nederland-nu/klimatologie/maand-en-seizoensoverzichten/2021/jaar.

Koenraadt, C.J.M., Möhlmann, T.W.R., Verhulst, N.O., Spitzen, J., Vogels, C.B.F., 2019. Effect of overwintering on survival and vector competence of the West Nile virus vector *Culex pipiens*. Parasit. Vectors 12 (147). https://doi.org/10.1186/s13071-019-3400-4.

Koenraadt, C. J. M., Münger, E. D., Spitzen, J., Altundag, S., Oude Munnink, B. B., Sikkema, R. S., Schrama, M., Blom, R. (In prep.). Overwintering of Usutu virus in diapausing mosquitoes in The Netherlands.

Lima, C.A., Almeida, W.R., Hurd, H., Albuquerque, C.M., 2003. Reproductive aspects of the mosquito *Culex quinquefasciatus* (Diptera: Culicidae) infected with *Wuchereria bancrofti* (Spirurida: Onchocercidae). Mem. Inst. Oswaldo Cruz 98 (2), 217–222. https://doi.org/10.1590/S0074-02762003000200009.

Lyimo, E.O., Takken, W., 1993. Effects of adult body size on fecundity and the pre-gravid rate of *Anopheles gambiae* females in Tanzania. Med. Vet. Entomol. 7 (4), 328–332. https://doi.org/10.1111/j.1365-2915.1993.tb00700.x.

Mitchell, C.J., 1983. Differentiation of Host-Seeking Behavior from Blood-Feeding Behavior in Overwintering *Culex Pipiens* (Diptera: Culicidae) and Observations on Gonotrophic Dissociation. J. Med. Entomol. 20 (2), 157–163. https://doi.org/10.1093/jmedent/20.2.157.

Mohammed, A., Chadee, D.D., 2011. Effects of different temperature regimens on the development of *Aedes aegypti* (L.) (Diptera: Culicidae) mosquitoes. Acta Trop. 119 (1), 38–43. https://doi.org/10.1016/j.actatropica.2011.04.004.

Nasci, R.S., Savage, H.M., White, D.J., Miller, J.R., Cropp, B.C., Godsey, M.S., Kerst, A.J., Bennett, P., Gottfried, K., Lanciotti, R.S., 2001. West Nile Virus in Overwintering *Culex* Mosquitoes, New York City, 2000. Emerg. Infect. Dis. 7 (4), 742–744. https://doi.org/10.3201/eid0704.010426.

Nelms, B.M., Macedo, P.A., Kothera, L., Savage, H.M., Reisen, W.K., 2013. Overwintering Biology of Culex (Diptera: Culicidae) Mosquitoes in the Sacramento Valley of California. J. Med. Entomol. 50 (4), 773–790 https://doi.org/10.1603%2Fme12280.

Nikolay, B., 2015. A review of West Nile and Usutu virus co-circulation in Europe: How much do transmission cycles overlap? The Royal Society of Tropical Medicine and Hygiene 109, 609–618. https://doi.org/10.1093/trstmh/trv066.

Onyeka, J.O.A., Boreham, P.F.L., 1987. Population studies, physiological state and mortality factors of overwintering adult populations of females of *Culex pipiens* L. (Diptera: Culicidae). Bull. Entomol. Res. 77 (1), 99–111. https://doi.org/10.1017/S0007485300011585.

Oude Munnink, B.B., Münger, E., Nieuwenhuijse, D.F., Kohl, R., Linden, A.V.D., 2020. Genomic monitoring to understand the emergence and spread of Usutu virus in the Netherlands, 2016–2018. Sci. Rep. 10, 2798. https://doi.org/10.1038/s41598-020-59692-y.

Pereira-Silva, J.W., Nascimento, V.A.D., Belchior, H.C.M., Almeida, J.F., Pessoa, F.A.C., Naveca, F.G., Ríos-Velásquez, C.M., 2017. First evidence of Zika virus venereal transmission in *Aedes aegypti* mosquitoes. Mem. Inst. Oswaldo Cruz 113 (1), 56–61. https://doi.org/10.1590/0074-02760170329.

Rijks, J., Kik, M., Slaterus, R., Foppen, R., Stroo, A., Ijzer, J., Stahl, J., Gröne, A., Koopmans, M., Van der Jeugd, H., Reusken, C. (2016). Widespread Usutu virus outbreak in birds in the Netherlands, 2016. *Eurosurveillance*, *21*(45), pii=30391. https://doi.org/10.2807/1560-7917.ES.2016.21.45.30391.

Robich, R.M., Denlinger, D.L., 2005. Diapause in the mosquito *Culex pipiens* evokes a metabolic switch from blood feeding to sugar gluttony. Proc. Natl. Acad. Sci. 102 (44), 15912–15917. https://doi.org/10.1073/pnas.0507958102.

Robich, R.M., Rinehart, J.P., Kitchen, L.J., Denlinger, D.L., 2007. Diapause-specific gene expression in the northern house mosquito, *Culex pipiens* L., identified by suppressive subtractive hybridization. J. Insect Physiol. 53 (3), 235–245. https://doi.org/10.1016/j.jinsphys.2006.08.008.

Rozsypal, J., Moos, M., Rudolf, I., Košťál, V., 2021. Do energy reserves and cold hardiness limit winter survival of *Culex pipiens*? *Comparative Biochemistry and Physiology*. Part A 255, 110912. https://doi.org/10.1016/j.cbpa.2021.110912.

RStudio Team, 2023. RStudio: Integrated Development for R (Version 2023.06.0) [Computer software]. RStudio, PBC. www.RStudio.com.

Rudolf, I., Betá, L., Bla, H., Venclíková, K., Straková, P., Mendel, J., Bakonyi, T., Schaffner, F., Nowotny, N., 2017. West Nile virus in overwintering mosquitoes, central Europe. Parasit. Vectors 10, 452. https://doi.org/10.1186/s13071-017-2399-7.

Rudolf, I., Sikutova, S., Sebesta, O., Mendel, J., Malenovsky, I., Kampen, H., Medlock, J., Schaffner, F., 2020. Overwintering of *Culex modestus* and other mosquito species in a reedbed ecosystem, including arbovirus findings. Journalof the American Mosquito Control Association 36 (4), 257–260. https://doi.org/10.2987/20-6949.1.

Sanburg, L.L., Larsen, J.R., 1973. Effect of photoperiod and temperature on ovarian development in *Culex pipiens pipiens*. J. Insect Physiol. 19 (6), 1173–1190. https://doi.org/10.1016/0022-1910(73)90202-3.

Sauer, F.G., Timmermann, E., Lange, U., Lühken, R., Kiel, E., 2022. Effects of Hibernation Site, Temperature, and Humidity on the Abundance and Survival of Overwintering *Culex pipiens pipiens* and *Anopheles messeae* (Diptera: Culicidae). J. Med. Entomol. 59 (6), 2013–2021. https://doi.org/10.1093/jme/tjac139.

Sauer, F.G., Lange, U., Schmidt-Chanasit, J., Kiel, E., Wiatrowska, B., Myczko, Ł., Lühken, R., 2023. Overwintering *Culex torrentium* in abandoned animal burrows as a reservoir for arboviruses in Central Europe. One Health 16, 100572. https://doi.org/10.1016/j.onehlt.2023.100572.

Sikkema, R. S., Schrama, M., van den Berg, T., Morren, J., Munger, E., Krol, L., van der Beek, J. G., Blom, R., Chestakova, I., van der Linden, A., Boter, M., van Mastrigt, T., Molenkamp, R., Koenraadt, C. J., van der Brand, J. M., Oude Munnink, B. B., Koopmans, M. P., van der Jeugd, H. (2020). Detection of West Nile virus in a common whitethroat *(Curruca communis)* and Culex mosquitoes in the Netherlands, 2020. *Eurosurveillance*, *25*(40), pii=2001704. https://doi.org/10.2807/1560-7917.ES.2020.25.40.2001704.

Sim, C., Denlinger, D.L., 2008. Insulin signaling and FOXO regulate the overwintering diapause of the mosquito *Culex pipiens*. Proc. Natl. Acad. Sci. 105 (18), 6777–6781. https://doi.org/10.1073/pnas.0802067105.

Streng, K., Holicki, C. M., Hesson, J., Graham, H., Chandler, F., Krol, L., Blom, R., Münger, E., Koenraadt, C. J. M., Schrama, M., Van der Jeugd, H., Lundkvist, Å., Koopmans, M. P. G., van der Poel, W. H. M., & Sikkema, R. S. (in prep.). Sindbis in the Netherlands: Virus detection in a resident bird and seropositivity in birds and horses.

Tun, W., Yoon, J., Jeon, J.-S., An, G., 2021. Influence of Climate Change on Flowering Time. J. Plant Biol. 64 (3), 193–203. https://doi.org/10.1007/s12374-021-09300-x.

Tyndale-Biscoe, M., 1984. Age-grading methods in adult insects: A review. Bull. Entomol. Res. 74 (3), 341–377. https://doi.org/10.1017/S0007485300015637.

Van Handel, E., 1985. Rapid determination of total lipids in mosquitoes. J. Am. Mosq. Control Assoc. 1 (3), 302–304.

Vázquez, A., Jiménez-Clavero, M.A., Franco, L., Donoso-Mantke, O., Sambri, V., Niedrig, M., Zeller, H., Tenorio, A., 2011. Usutu virus – potential risk of human disease in Europe. pii=19935 Eurosurveillance 16 (31). https://doi.org/10.2807/ese.16.31.19935-en.

Vilibic-Cavlek, T., Petrovic, T., Savic, V., Barbic, L., Tabain, I., Stevanovic, V., Klobucar, A., Mrzljak, A., Ilic, M., Bogdanic, M., Benvin, I., Santini, M., Capak, K., Monaco, F., Listes, E., Savini, G., 2020. Epidemiology of Usutu Virus: The European Scenario. Pathogens 9 (699). https://doi.org/10.3390/pathogens9090699.

Vlaskamp, D. R., Thijsen, S. F., Reimerink, J., Hilkens, P., Bouvy, W. H., Bantjes, S. E., Vlaminckx, B. J., Zaaijer, H., van den Kerkhof, H. H., Raven, S. F., & Reusken, C. B. (2020). First autochthonous human West Nile virus infections in the Netherlands, July to August 2020. *Eurosurveillance*, *25*(46), pii=2001904. https://doi.org/10.2807/1560-7917.ES.2020.25.46.2001904.

Vogels, C.B.F., Van De Peppel, L.J.J., Van Vliet, A.J.H., Westenberg, M., Ibañez-Justicia, A., Stroo, A., Buijs, J.A., Visser, T.M., Koenraadt, C.J.M., 2015. Winter activity and aboveground hybridization between the two biotypes of the west nile virus vector *Culex pipiens*. Vector-Borne and Zoonotic Diseases 15 (10), 619–626. https://doi.org/10.1089/vbz.2015.1820.

Vogels, C.B.F., Möhlmann, T.W.R., Melsen, D., Favia, G., Wennergren, U., Koenraadt, C.J. M., 2016. Latitudinal diversity of *Culex pipiens* biotypes and hybrids in farm, peri-Urban, and wetland habitats in Europe. PLoS One 11 (11), e0166959.

Vogels, C.B.F., Göertz, G.P., Pijlman, G.P., Koenraadt, C.J.M., 2017. Vector competence of European mosquitoes for west Nile virus. Emerging Microbes Infect. 6 (11), e96.

Washino, R.K., 1977. The physiological ecology of gonotrophic dissociation and related phenomena in mosquitoes. J. Med. Entomol. 13 (4), 381–388. https://doi.org/10.1093/jmedent/13.4-5.381.

Yeap, H.L., Endersby, N.M., Johnson, P.H., Ritchie, S.A., Hoffmann, A.A., 2013 Jul. Body size and wing shape measurements as quality indicators of *Aedes aegypti* mosquitoes destined for field release. Am J Trop Med Hyg 89 (1), 78–92. https://doi.org/10.4269/ajtmh.12-0719.