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Effects of Hibernation Site, Temperature, and Humidity on the Abundance and Survival of Overwintering *Culex pipiens pipiens* and *Anopheles messeae* (Diptera: Culicidae)

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Abstract

Knowledge of the hibernation site preferences and the factors which influence winter survival in these hibernation sites may enhance understanding of mosquito population dynamics after winter and how arboviruses persist in temperate regions. Our study quantified the number of adult overwintering mosquitoes in cellars and aboveground constructions and analyzed survival rates in relation to the environmental conditions in these sites. During the winters 2016/2017 and 2018/2019, 149 different constructions in Northwest Germany were sampled for mosquitoes. Mosquitoes were detected in 44% of the cellars and in 33% of the aboveground constructions. *Culex p. pipiens* Linnaeus was the most abundant species in cellars, whereas high numbers of *Anopheles messeae* Falleroni were collected from a single barn. Subsequently, an enclosure study was conducted during 2019/2020. Overwintering field-collected *Cx. p. pipiens* and *An. messeae* were divided into groups with or without fructose availability, and placed in cages with different man-made hibernations sites, where temperature and relative humidity were recorded hourly. For both species, increasing mean temperatures (5–16°C) but not mean relative humidity (58–94%) were correlated with winter mortality rates of the mosquitoes. The lipid measurements were greater and mortality rates were lower when both species were provided fructose. Larger specimens (determined by wing length) stored more lipids, and in *Cx. p pipiens*, but not in *An. messeae*, survival probability of large specimens was significantly greater than for small females. Mosquitoes showed a distinct pattern in the selection of overwintering sites, while temperature was an important driver for survival.

Key words: *Culex pipiens pipiens*, *Anopheles messeae*, Culicidae, hibernation site, winter mortality

In the last decade, mosquito-borne viruses have emerged or re-emerged in Central and Northern Europe, including West Nile (WNV), Batai (BATV), and Usutu (USUV) viruses (Jöst et al. 2010, 2011; Ziegler et al. 2020). USUV outbreaks have led to considerable reductions of local bird populations, particularly of European blackbirds (*Turdus merula*) (Lühken et al. 2017). Also rapidly increasing WNV detections have been observed in Austria and Germany since 2018 (Aberle et al. 2018, Pietsch et al. 2020). Although USUV infections are usually asymptomatic in humans (Cadar et al. 2017), WNV can cause symptomatic disease in humans and other mammals, especially equines. Around 20% of infected humans suffer from West Nile fever and 1% of the infections can lead to WNV neuroinvasive disease (Mostashari et al. 2001, Zou et al. 2010).

In temperate regions, transmission cycles of mosquito-borne pathogens are interrupted in winter, when most mosquitoes undergo facultative diapause initiated by decreasing day length and temperature (Denlinger and Armbruster 2014). Although members of the genus *Aedes* predominantly overwinter in the egg stage, several native mosquito species within the genera *Culex*, *Culiseta*, and *Anopheles* hibernate as adults (Schäfer and Lundström 2001, Denlinger and Armbruster 2014). Females emerging in late summer destined for diapause develop fat reserves and subsequently enter hibernation sites where they usually stay until spring (Sulaiman and Service 1983, Robich and Denlinger 2005). Cellars and other underground constructions are common overwintering sites for *Culex pipiens* s.l./*Cx. torrentium* and *Anopheles maculipennis* s.l. (Bergman et al. 2020, Dörge et al. 2020, Kampen et al. 2021). These taxa are considered important vectors for different mosquito-borne pathogens, e.g., *Cx. pipiens* s.l./*Cx. torrentium* for arboviruses such as WNV or SINV (Jansen et al. 2019, Lwande et al. 2019). During repetitive WNV outbreaks in North America, it has been observed that adult overwintering *Culex* may contribute to the establishment of WNV in temperate regions (Reisen and Wheeler 2019). Infected females may serve as a reservoir of WNV during the winter and initiate the transmission cycle to the following spring (Nasci et al. 2001, Anderson and Main 2006). Detections of WNV and SINV in overwintering *Cx. p. pipiens* (e.g., Czech Republic, Sweden, and Germany) indicate that the same mechanism also could promote the long-term establishment of arboviruses in Central Europe (Rudolf et al. 2017, Bergman et al. 2020, Kampen et al. 2021). This highlights the importance of understanding the overwintering ecology of native mosquito species.

The selection of appropriate hibernation sites is crucial for the survival of mosquitoes. Previous studies showed that anthropogenic sites frequently were used for hibernation by different mosquito species; e.g., *Cx. p. pipiens* can be found in high numbers in cellars and other underground constructions (Bergman et al. 2020, Dörge et al. 2020, Kampen et al. 2021). The relevance of aboveground constructions as hibernation sites for *Cx. p. pipiens* is unclear, but other taxa such as *An. maculipennis* s.l. have been observed in stables and sheds during winter (Weyer 1941). The microclimate in the hibernation sites can distinctly affect the winter survival of *Cx. p. pipiens*. Rozsypal et al. (2021) identified temperatures between 2 and 6°C as optimal for overwintering adults. Temperatures lower than 0°C led to death after several days and higher temperatures increased the metabolic rates and thus depleted the lipid reserves of *Cx. p. pipiens* females. Under unfavorable winter conditions, e.g., too high temperatures, mosquitoes may require additional energy to survive the winter. Mosquitoes occasionally leave their hibernation sites during winter (Onyeka and Boreham 1987) and seek energy sources (Reisen et al. 1986). Although nectar sources are scarce in winter, overwintering mosquitoes might be able to obtain energy from sugar-poor plant tissue, as is known from tropical and subtropical regions during the dry season (Müller and Schlein 2005).

To evaluate the effects of hibernation site conditions on the abundance and survival of overwintering mosquitoes we conducted two successive studies. First, overwintering mosquitoes were collected from different anthropogenic sites, including cellars and aboveground buildings (“study 1”). Second, we conducted an enclosure study to analyze the effects of temperature, humidity, and fructose availability in these anthropogenic sites on the survival probability of the two dominant species collected in study 1, *Cx. p. pipiens* and *An. messeae* (“study 2”).

Material and Methods

Study 1: Screening Hibernation Sites

The study was conducted in North-West Germany with a focus on the cities of Bremen and Oldenburg, including its surrounding rural areas (districts: Ammerland, Aurich, Bremen, Cloppenburg, Cuxhaven, Oldenburg, Vechta, Wesermarsch). Mosquitoes were collected in cellars and unheated aboveground constructions. Aboveground constructions were classified by their size, dominant construction material, and access to mosquitoes and included barns (>50 m², wood and masonry, various open accesses), wooden sheds (<50 m², various open accesses), concrete or masonry sheds (<50 m², few open accesses), garages (<50 m², concrete, very little open access but one lockable gate), and carports (<50 m², wood, at least one side permanently open). In the winters of 2016/2017 and 2018/2019, 106 and 68 potential overwintering sites were sampled for mosquitoes, respectively. To check the consistency of hibernation site selection, 25 sites (7 cellars and 18 aboveground sites) screened during winter 2016/2017 were revisited in 2018/2019. Thirteen of these 25 sites were not colonized by mosquitoes in 2016/2017. Each overwintering site was visited once per winter between October and January. Mosquitoes were collected with handmade aspirators described by Vazquez-Prokopec et al. (2009). A strong flashlight was used to locate the resting mosquitoes. Collected mosquitoes were killed by freezing and were stored at −20°C until further analysis. All specimens were identified by morphological characters to the lowest level of taxonomic certainty (Mohrig 1969, Becker et al. 2020). *Culex pipiens* s.l./*Cx. torrentium* specimens were typed to species level using real-time PCR (Rudolf et al. 2013). *Culex* taxonomy followed the concept suggested by Harbach (2011). Apart from *Culex pipiens* s.l./*Cx. torrentium*, a subsample of *An. maculipennis* s.l. was identified to species by molecular typing following the qPCR assay of Lühken et al. (2016).

Mosquito abundance and biodiversity are strongly influenced by landscape factors (Zittra et al. 2017), including the number of mosquitoes in underground hibernation sites (Dörge et al 2020). Therefore, the Corine land cover classification of the surrounding landscape (buffer = 100 m) of each hibernation site was analyzed (Büttner et al. 2017). Hereby, eight different categories were aggregated into ‘urban’ (111: continuous urban fabric, 112: discontinuous urban fabric, 121: industrial or commercial units, 142: sport and leisure facilities) and ‘rural’ (211: nonirrigated arable land, 231: pastures, 242: complex cultivation patterns, 243: land principally occupied by agriculture, with significant areas of natural vegetation). The *Cx. p. pipiens* data was strongly overdispersed and zero-inflated. Hence, a zero-inflated generalized linear mixed model (GLMM) with negative binomial error distribution was fit to analyze the influence of the fixed effects of land cover (urban vs. rural), hibernation site type (cellar vs. aboveground site), size of the hibernation site (in square meter), and the study year (2016/2017, 2018/2019) on the number of *Cx. p. pipiens* collected per hibernation site. The effect of the five different types of aboveground constructions on the number of *Cx. p. pipiens* was analyzed by an additional GLMM excluding the data of the cellars. The other mosquito taxa were not collected in sufficient number to allow statistical analyses. Statistical analyses were performed in R (R Core Team 2020) using the package “glmmTBB” (Brooks et al. 2017).

Study 2: Survival in Hibernation Sites

The enclosure study was conducted between November 2019 and March 2020. On the 3 November 2019, overwintering mosquitoes were collected in a cellar and a barn at a rural site in north-west Germany (N53.461°, E8.360°), known for high numbers of overwintering *Cx. p. pipiens* and *An. messeae* (a palearctic member of the *An. maculipennis* complex) from study 1. Thereby, 736 *Cx. pipiens* s.l./*Cx. torrentium* and 296 *An. maculipennis* s.l. females were collected by aspirator and transferred into different insect cages (30 × 30 × 30 cm, Bioform, Nuremberg, Germany) while still in the field. One side of the cages consisted of a transparent plastic window, while the further five sides consisted of white insect mesh. The separation between the taxa was performed on-site, as *An. messeae* was almost exclusively found in the barn, and *Cx. pipiens* s.l./*Cx. torrentium* in the cellar. The cages were stocked with 30 (±3) *Cx. pipiens* s.l./*Cx. torrentium* and 19 (±2) *An. maculipennis* s.l., respectively. An additional 63 *Cx. pipiens* s.l./*Cx. torrentium* and 45 *An. maculipennis* s.l. were immediately frozen at −20°C as reference samples for lipid analysis. On the same day of mosquito collection, cages were installed at five cellars and six aboveground constructions. These hibernation sites were located in north-west Germany around Bremen and Oldenburg and were known for the presence of overwintering mosquitoes from study 1. Two cages containing either *An. maculipennis* s.l. or *Cx. pipiens* s.l./*Cx. torrentium* were placed in three cellars, three sheds, and a climate chamber (KMF 240, Binder, Tuttlingen, Germany). Due to the limited number of *An. maculipennis* s.l., the remaining five sites only contained cages with *Cx. pipiens* s.l./*Cx. torrentium*. At each site, one cage per mosquito species contained cotton pads (diameter = 6 cm, BioNaturel, Werdau, Germany) soaked in 8% fructose solution (Carl Roth, Karlsruhe, Germany), whereas the other cage did not contain fructose solution. All cages were equipped with pieces of cotton wool (approximate diameter: 8 cm) (Hartmann, Heidenheim, Germany) moistened with water to ensure minimum humidity (Koenraadt et al. 2019). Pads with fructose and watered cotton wool were replaced weekly. Each site was equipped with two data loggers (Hobo Pro v2 U23-001, Onset Computer Corporation, Bourne) that recorded hourly temperature and relative humidity inside and outside of the hibernation sites. The loggers outside were placed within solar radiation shields (Onset Computer Corporation, Bourne) and attached to free-standing trees at a height of 2 m. In addition, *An. maculipennis* s.l. and *Cx. pipiens* s.l./*Cx. torrentium* were kept in a climate chamber at a constant temperature (10°C) and relative humidity (75%), with one cage with and one cage without fructose pads. The constant environmental conditions in the climate chamber served as a control to test if the fluctuating temperature and relative humidity at the hibernation site affected mosquito survival. All sites and the climate chamber were checked at least once a week to collect dead mosquito specimens. At the end of the experiments on 13 March 2020, surviving mosquitoes were killed and stored at −20°C until further analyses.

The legs of each specimen were removed and used for molecular species identification using the PCR assays from Rudolf et al. (2013) for *Cx. pipiens* s.l./*Cx. torrentium* and Lühken et al. (2016) for *An. maculipennis* s.l. Each specimen was individually typed to species level. Apart from *An. messeae* and *Cx. p. pipiens* as dominant species, the molecular assays revealed the presence of three *An. maculipennis* s.s. Meigen, three *An. atroparvus* Van Thiel, and nine *Cx. torrentium* Martini specimens. Due to the low number of specimens, these three species were removed from statistical analyses.

The right wing of each specimens was mounted in Euparal (Carl Roth, Karlsruhe, Germany) on a microscope slide and photographed under 20× magnification with a stereomicroscope (Leica M205 C, Leica Microsystems, Wetzlar, Germany). The images were used to measure the wing length from the distal end of the alula to the wing tip by means of Fiji image processing software (Schindelin et al. 2012) as bioscience bundle of ImageJ (Schneider et al. 2012).

The remaining mosquito body without the legs and the right wing was used to determine the lipid content following the protocol of Van Handel (1985). Each mosquito body was dried at room temperature for 24 h in a desiccator, weighted (±0.001 mg) with a microbalance (Sartorius Cubis MSA3.6-000-DM, Sartorius, Göttingen, Germany), and subsequently homogenized in a 2 ml tube with metal beads by means of a mixer mill at 25 Hz (MM 200, Retsch, Haan, Germany). Total lipids then were extracted in chloroform:methanol (1:1) and the supernatant evaporated at 90°C. After cooling to room temperature, the remaining lipids were dissolved in sulfuric acid and heated for further 10 min. Finally, vanillin reagent was added and absorbance was measured spectrophotometrically at 525 nm. Lipid content of the samples was quantified by using a standard curve prepared with soybean oil (Vandemoortele, Ghent, Belgium).

For the reference mosquitoes frozen at the beginning of winter, the relationship between wing size and absolute lipid content for each species was analyzed with a linear model. The statistical significance of the effects of the mean temperature and relative humidity per hibernation site and fructose availability on the lipid content per dry weight of *An. messeae* and *Cx. p. pipiens* specimens was tested by Analysis of Variance (ANOVAs). Species-specific differences in the mean dry weight and the mean total lipids between *An. messeae* and *Cx. p. pipiens* were tested by Welch's *t*-tests. Kaplan–Meier survival analyses were used to calculate the proportion of surviving mosquitoes as a function of time from 3 November 2019 to 13 March 2020 (130 d). In a pre-analysis, we checked whether mean temperature or the temperature fluctuations expressed as standard deviation per site had a greater effect on the mosquito survival. Therefore, a Cox proportional regression model including both temperature variables was applied to compare their explanatory power. Subsequently, the temperature variable with lower standard error and lower *p*-value was integrated into the final model. In the final model, the effects of the variables wing length, temperature, and relative humidity, as well as the categorical variable fructose availability (yes or no) on the survival rates of *Cx. p. pipiens* and *An. messeae* were estimated by Cox proportional hazards regressions models. Because not all sites could be equipped with cages of *An. messeae*, two Cox regressions were calculated for *Cx. p. pipiens*; one referred to all sites, whereas the second only included the sites with *An. messeae* and *Cx. p. pipiens* to directly compare the effects of the analyzed variables on the survival rates between species. Statistical analyses and visualization were performed in R (R Core Team 2020), including the package 'survival' (Therneau and Lumley 2014).

Results

Study 1: Screening Hibernation Sites

In winter 2016/2017, 1,839 mosquito specimens were collected, including 1,581 *Cx. p. pipiens* (1,574 females, 7 males), 4 female *Cx. torrentium*, 1 female *Cx. territans* Walker, 42 *Cs. annulata*/*subochrea* (40 females, 2 males), and 211 female *An. maculipennis* s.l. In winter 2018/2019, 758 *Cx. p. pipiens* (757 female, 1 male), 5 female *Cx. torrentium*, 104 *An. maculipennis* s.l. (103 females, 1 male), and 6 *Cs. annulata*/*subochrea* (5 females, 1 male) (Table 1) were collected. No blood-engorged females were observed during both winters.

Overall, mosquitoes were detected in 44% (29/66 sites) of the investigated cellars and in 33% (39/120 sites) of the aboveground constructions. The presence or absence of overwintering mosquitoes was confirmed for 25 sites studied in both winters (17% of all sites). The collection data of *Cx. p. pipiens* was highly skewed, i.e., most specimens were detected in few hibernation sites in the two winters (Fig. 1). Significantly more *Cx. p. pipiens* were collected in cellars compared to aboveground structures (GLMM, coefficient = 3.30, $z = 6.80$, $p < 0.001$): mean = 34.9 ± 90.1 (standard deviation) versus mean = 1.5 ± 4.9 mosquitoes per hibernation site. The surrounding land cover, the size of the hibernation site, and the study year had no significant effect on the number of mosquitoes collected per hibernation site. With respect to aboveground constructions, the number of

Table 1. Total number of specimens per mosquito taxon collected in two winter seasons

Mosquito taxon	2016/2017 Aboveground (69 sites)	2016/2017 Underground (37 sites)	2018/2019 Aboveground (42 sites)	2018/2019 Underground (26 sites)
Cx. p. pipiens	104	1477	37	721
Cx. torrentium	1	3	2	3
Cx. territans	1	0	0	0
Cs. annulata/subochrea	16	26	3	3
An. messeae	0	1	80	0
An. daciae	1	0	0	0
An. maculipennis s.l.	212	2	24	0

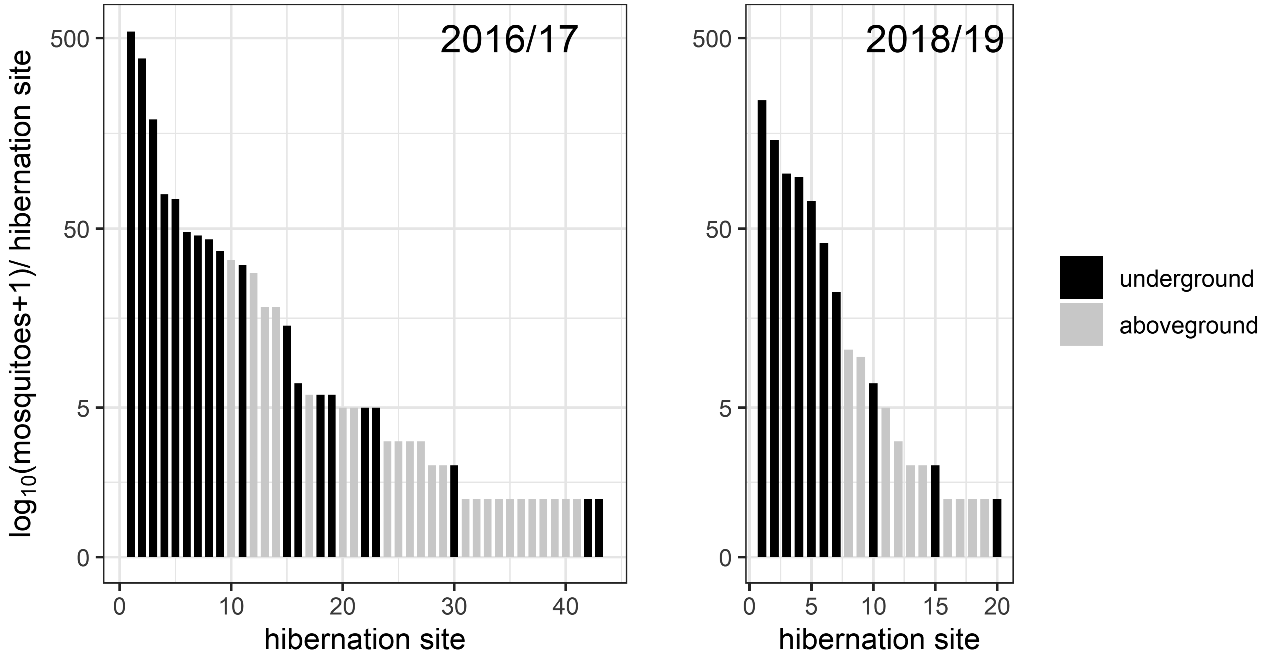

Fig. 1. Number of *Cx. p. pipiens* per hibernation site in a logarithmic scale. Each bar represents one hibernation site. Sites without *Cx. p. pipiens* were excluded from the figure.

Cx. p. pipiens within concrete or masonry sheds (mean = 3.3 ± 8.4, no. of sites = 18), barns (mean = 2.2 ± 5.2, no. of sites = 13), garages (mean = 1.4 ± 3.9, no. of sites = 24), and carports (mean = 0.7 ± 1.6, no. of sites = 11) was significantly higher compared to wooden sheds (mean = 0.4 ± 1.0, no. of sites = 39) (GLMM, coefficient = 1.83, z = 2.15, p = 0.032).

Ninety-seven percent of *An. maculipennis* s.l. was collected at a single barn in a rural landscape close to the North Sea. This barn was sampled in both winters and provided 306 out of total 314 *An. maculipennis* s.l. (205 specimens in 2016/2017 and 101 specimens in 2018/2019). All analyzed *An. maculipennis* s.l. (77 specimens from 2018/2019) from this barn were identified as *An. messeae*. By contrast, only two *An. messeae* were collected in two cellars close to this barn (<50 m distance), although these two cellars were colonized by high numbers of overwintering *Cx. p. pipiens* (more than 50 specimens in both winters).

Study 2: Survival in Hibernation Sites

During the enclosure study, conducted from the 3 November 2019 to 13 March 2020, mean temperatures inside the hibernation sites ranged from 6 to 16°C, and mean relative humidity ranged from 58 to 94%. The mean temperatures outside the hibernation sites ranged from 6 to 7°C and the mean relative humidity ranged between 84 and 92% (Supp Table S1 [online only]).

Overall, 57% (409/726) of *Cx. p. pipiens* specimens and 72% (211/293) *An. messeae* specimens survived until the end of the study period. At sites with both species, 68% (322/474) of *Cx. p. pipiens* survived the winter, indicating a similar winter mortality of *An. messeae* and *Cx. p. pipiens*. In the pre-analysis using temperature variables only, mean temperature per site had a statistically significant effect on the survival of *Cx. p. pipiens* (coef = 0.28, exp(coef) = 1.28, p < 0.001), whereas the standard deviation of the temperature had no statistically significant effect (coef = 0.02, exp(coef) = 1.02, p = 0.725). Therefore, mean temperature per site was included as variable in the final cox regression model. Herein, the survival probability of *Cx. p. pipiens* significantly decreased with increasing mean temperature with an exponential coefficient of 1.25 (Table 2), i.e., holding the other covariates constant, a temperature increase of 1 degree increases the hazard of death during the study period by 25%. Survival probability of *Cx. p. pipiens* was significantly higher at sites with higher relative humidity and within cages with sugar availability (see Table 2 for model coefficients and p-values). In total 59% (212/357) of the *Cx. p. pipiens* specimens died in the cages without fructose pads, while 28% (105/369) of the specimens died

Table 2. Output of the Cox regression model for the survival rates of *An. messeae* and *Cx. p. pipiens* in dependence of the microclimatic temperature and relative humidity as well as fructose availability and wing length

Explanatory variables	Coefficient	Standard error	*p* Value	Exp (coefficient)	95% Confidence interval for exp (coefficient)
An. messeae					
Sugar availability (without sugar)	3.19	0.39	**<0.001**	24.23	11.22–52.31
Mean temperature per site	0.42	0.08	**<0.001**	1.53	1.30–1.79
Mean humidity per site	−0.02	0.02	0.2	0.98	0.94–1.01
Wing length per specimen	−0.52	0.44	0.24	0.59	0.25–1.41
Cx. p. pipiens (referring to sites together with *An. messeae*)					
Sugar availability (without sugar)	1.12	0.2	**<0.001**	3.07	2.09–4.51
Mean temperature per site	0.22	0.06	**<0.001**	1.25	1.11–1.40
Mean humidity per site	−0.01	0.02	0.57	0.99	0.96–1.02
Wing length per specimen	−0.86	0.29	**0.003**	0.42	0.24–0.74
Cx. p. pipiens (referring to all sites equipped with cages of *Cx. p. pipiens*)					
Sugar availability (without sugar)	1.26	0.14	**<0.001**	3.53	2.71–4.61
Mean temperature per site	0.26	0.03	**<0.001**	1.29	1.22–1.38
Mean humidity per site	−0.01	0.01	0.08	0.99	0.97–1.00
Wing length per specimen	−0.51	0.20	**0.01**	0.60	0.40–0.89

For *Cx. p pipiens*, two Cox regression models were conducted, one refers to all sites and one solely includes the sites, which were also equipped with *An. messeae* allowing a direct comparison of the results between both species. Significant *p*-values ($p < 0.05$) in bold.

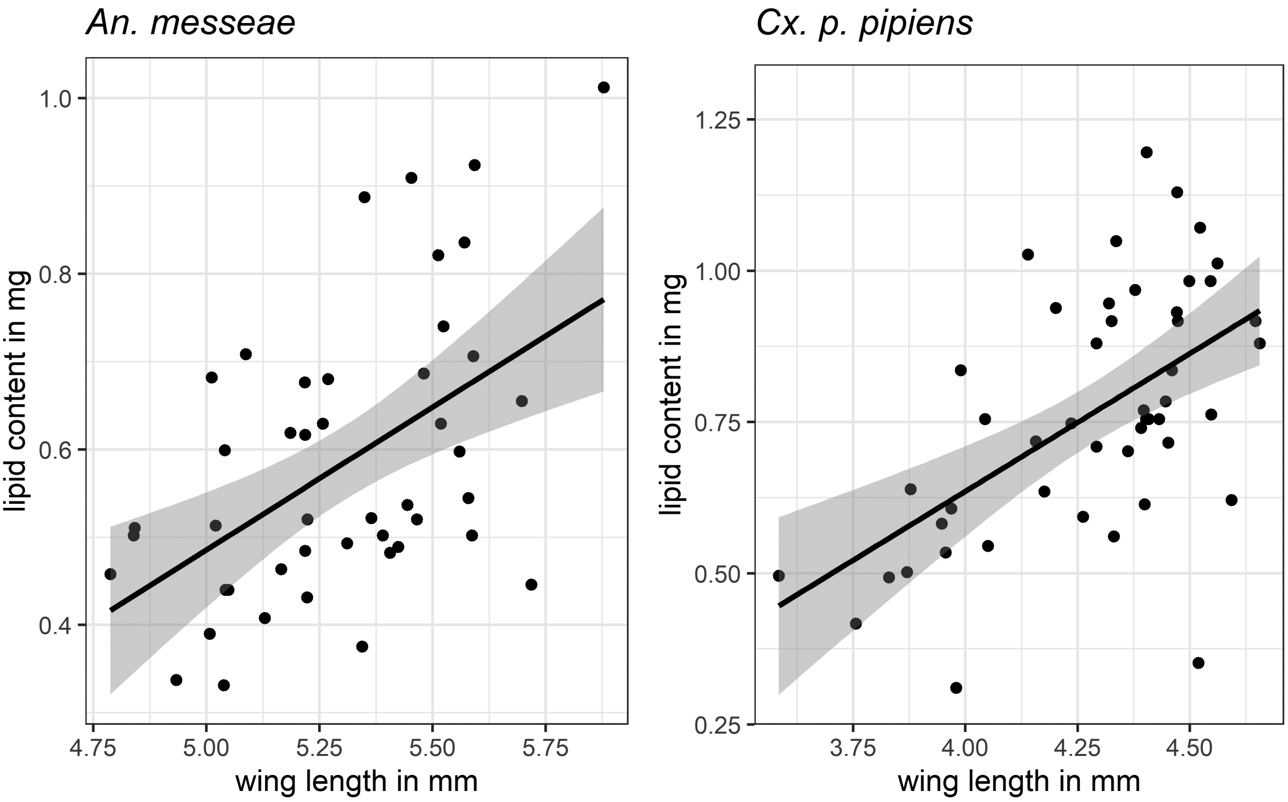

Fig. 2. Relationship between wing length and lipid content of the reference mosquitoes collected at the beginning of the study winter. Linear models with 95% confidence interval in grey show a significant increase of lipid content with increasing wing length for *An. messeae* ($R^2 = 0.27$) and for *Cx. p. pipiens* ($R^2 = 0.32$).

in the cages with fructose. The wing lengths of *Cx. p. pipiens* ranged from 3.19 to 4.94 mm and were significantly correlated with survival probability, whereby larger specimens had a greater survival probability than smaller females.

The survival probability of *An. messeae* was significantly lower at sites with higher mean temperatures, but mean relative humidity had no significant influence on survival probability. The survival rates of *An. messeae* were significantly higher in cages with sugar availability: 50% (73/145) of the *An. messeae* specimens died in the cages without fructose pads, whereas only 6% (9/148) died in cages with fructose. The wing length of *An. messeae* ranged from 4.46 to 6.16 mm, but had no statistically significant influence on the survival probability (see Table 2 for all coefficients and *p*-values).

For the reference mosquitoes sampled at the beginning of winter, the dry weight of *An. messeae* (mean: 1.619 ± 0.344 mg [standard deviation]) was significantly greater than for *Cx. p. pipiens* (mean: 1.397 ± 0.300 mg) (*t*-test, $t = 3.43$, df = 87.58, $p < 0.001$). However, the total amount lipids measured in *An. messeae* (mean: 0.584 ± 0.162 mg) was significantly lower than in *Cx. p. pipiens* (mean: 0.761 ± 0.204 mg) (*t*-test, $t = -4.92$, df = 100.93, $p < 0.001$). The lipid content of *Cx. p. pipiens* (linear model, coef = 0.46, df = 46, $R^2 = 0.31$, $p < 0.001$) and of *An. messeae* (linear model, coef = 0.32, df = 43, $R^2 = 0.26$, $p < 0.001$) was positively correlated with wing length (Fig. 2), indicating wing length was a reliable proxy for the amount of lipids stored before entering the hibernation site.

In both mosquito species, the mean lipid content per dry weight of specimens surviving the winter was lower than in reference specimens (*Cx. p. pipiens* specimens: 233 µg/mg; *An. messeae*: 192 µg/mg). The lowest amounts of lipids were measured in dead specimens (Fig. 3). For *An. messeae*, the mean temperature at the hibernation sites (ANOVA, $F_{1,279} = 38.14$, $p < 0.001$) and fructose availability (ANOVA, $F_{1,279} = 38.83$, $p < 0.001$) significantly influenced the lipid content of the specimens, whereas the mean relative

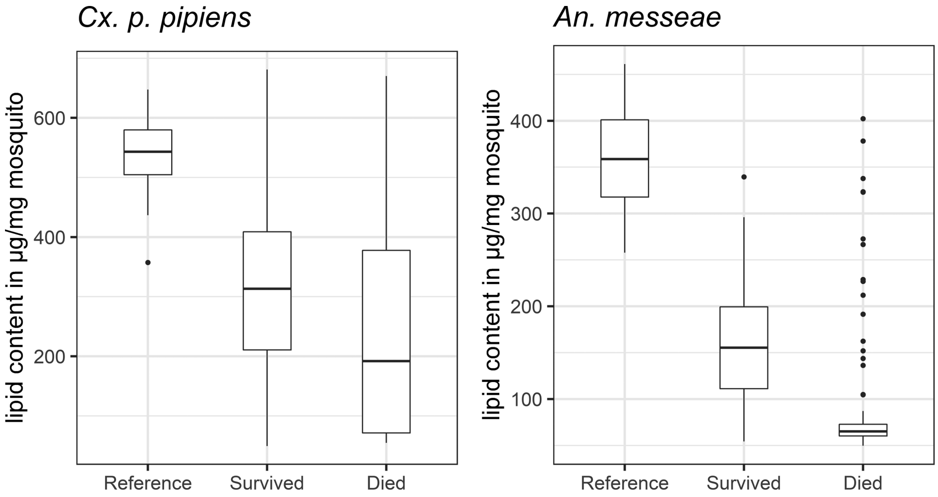

Fig. 3. Boxplots show the variation of lipid content (in µg) per dry mosquito weight given for the reference specimens collected at the beginning of winter and the specimens that died or survived during the winter.

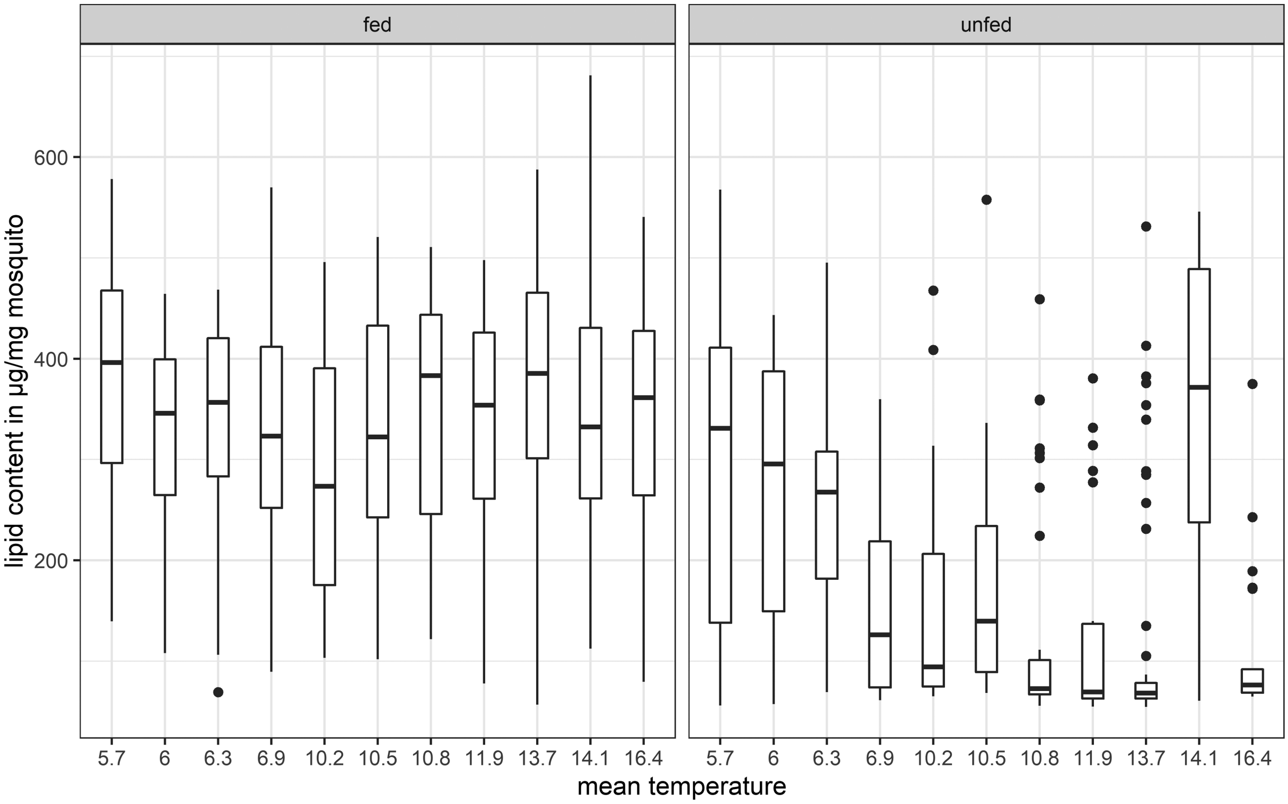

Fig. 4: Boxplots show the variation of lipid content (in µg) per mg dry weight of *Cx. p. pipiens* plotted as a function of the mean temperature per hibernation site. Results are presented separately for cages with (fed) and without (unfed) fructose.

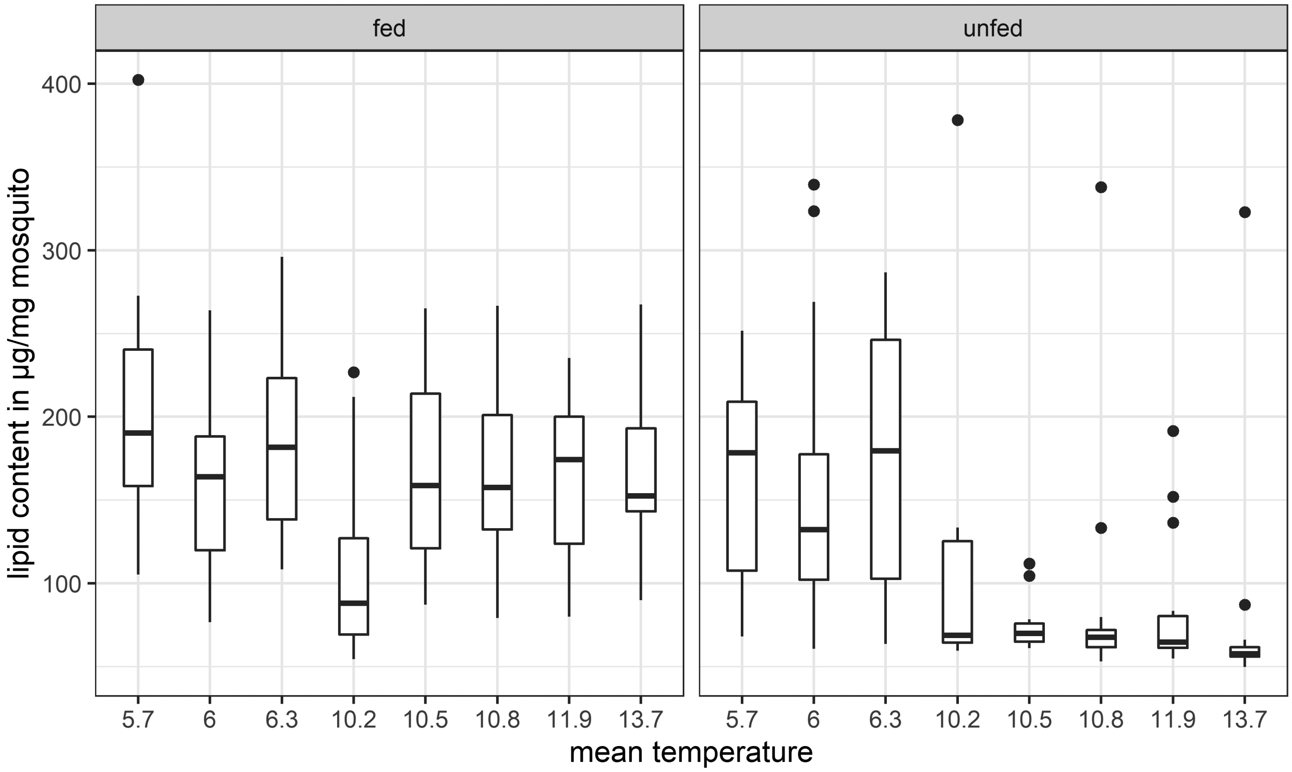

Fig. 5. Boxplots show the variation of lipid content (in µg) per mg dry weight of *An. messeae* plotted as a function of the mean temperature per hibernation site. Results are presented separately for cages with (fed) and without (unfed) fructose.

humidity did not. For *Cx. p pipiens*, the mean temperature per hibernation site (ANOVA, $F_{1,707} = 8.36$, $p = 0.003$), the mean relative humidity (ANOVA, $F_{1,707} = 4.18$, $p = 0.041$), and fructose availability (ANOVA, $F_{1,707} = 242.17$, $p < 0.001$) significantly influenced the lipid content per specimen. As visually inspected for *Cx. p. pipiens* (Fig. 4) and for *An. messeae* (Fig. 5), higher mean temperatures at the hibernation sites distinctly reduced the lipid content of the specimens in cages without fructose, but seemed to have little influence on the lipid content for specimens in cages with fructose.

Discussion

Mosquitoes were found in various overwintering sites, including cellars and different types of unheated aboveground constructions. For *Cx. p. pipiens*, cellars were a key hibernation site. This agreed with studies from Sweden with high numbers of *Cx. p. pipiens* found in cellars (Bergman et al. 2020) and from China, which documented the highest densities of the closely related *Cx. pipiens pallens* Coquillett in cellars in comparison with other potential man-made hibernation sites (Liu et al. 2016). Naturally, *Cx. p. pipiens* probably overwinter in caves (Zittra et al. 2019, 2021; Dörge et al. 2020). These studies on caves in central Germany and Austria reported a small proportion of *Cx. p. molestus* (~1%), whereas we detected none, but *Cx. p. molestus* is probably much rarer in northern Germany (Rudolf et al. 2013). In the lowlands of the North Germany Plain, where our study was conducted, natural caves were absent. Here, cellars provided the most important underground hibernation sites and probably could be considered as a substitute overwintering habitat for *Cx. p. pipiens*. However, aboveground constructions also were accepted as hibernation sites by *Cx. p. pipiens*, particularly concrete or masonry sheds and garages. These sites provided similar characteristics as cellars (e.g., microclimate) and were preferred over wooden sheds or carports. Because overwintering mosquitoes were easy to target and can be detected in high concentrations in the hibernation sites, Liu et al. (2016) and Kobayashi et al. (2012) suggested that their removal may provide a complementary mosquito control technique. In our study, we detected high numbers of *Cx. p. pipiens* in certain cellars in rural and urban environments. Considering the greater variety and quantity of cellars or similar underground sites in urban areas, targeting overwintering *Cx. p. pipiens* for mosquito control purposes might be more realistic in rural landscapes than in urban areas. The lower availability of underground sites in the investigated rural area may concentrate overwintering *Cx. p. pipiens*, which would simplify the efficient reduction of the mosquito population. The efficiency of targeting overwintering mosquitoes for control measures may deserve further investigation: e.g., establishing the link between the survival of the overwintering population and the size of the following summer population.

Anopheles maculipennis s.l., predominantly represented by *An. messeae*, was almost exclusively collected in a single barn. The cellars around this barn were not colonized by *An. maculipennis* s.l., indicating that they may prefer aboveground constructions as hibernation sites, which is in agreement with observations by Weyer (1941). *Cx. torrentium*, *Cx. Territans*, and *Cs. annulata/subochrea* were not collected in sufficient numbers to derive clear preferences for certain types of hibernation sites. Interestingly, *Cx. torrentium* could only be detected sporadically, although collection data from the summer would suggest a high abundance in northern Germany (Rudolf et al. 2013, Hesson et al. 2014). In line with studies from Sweden (Jaenson 1987, Bergman et al. 2020) and from England (Service 1968), *Cx. torrentium* is strongly underrepresented or completely absent in man-made hibernation sites. The fact that *Cx. torrentium* is probably one of the major vectors of WNV and SINV in central and northern Europe (Jansen et al. 2019, Lwande et al.

2019), calls for studies to find their primary hibernation sites, which would allow further evaluation for the role of *Cx. torrentium* in the persistence of mosquito-borne viruses.

The enclosure study confirmed that cellars and different aboveground constructions allowed the survival of overwintering mosquitoes. When comparing *An. messeae* and *Cx. p. pipiens*, the parameters studied affected the winter survival of both species in a similar manner. Highest survival rates were observed at hibernation sites with mean temperatures less than 7°C, which also were associated with the highest amount of remaining lipids in the mosquitoes. Therefore, our results were largely consistent with Rozsypal et al. (2021), who suggested an optimum temperature between 2 and 6°C for the survival of overwintering mosquitoes. However, due to the extraordinary warm winter, our data itself does not allow a conclusion about minimum temperature, which can be tolerated by overwintering mosquitoes. The winter 2019/2020 was the second warmest ever recorded in Germany with temperatures 3.9°C above the reference years 1961–1990 (Imbery et al. 2021). In line with observations from caves (Mammola et al. 2019), the abiotic conditions inside the hibernation site were strongly dependent on the outdoor temperature. Hence, rising winter temperatures due to global warming would increase the energy consumption of overwintering mosquitoes, probably affecting winter mortality or overwintering behavior, e.g., by shortening the diapause period. In a warmer winter, mosquitoes may need to leave their hibernation sites earlier in the year to seek for energy sources, e.g., winter-flowering plants, and begin blood-feeding as known from studies in California, USA (Reisen et al. 1986, Nelms et al. 2013). This particularly affects *An. messeae*, which stored a distinctly lower amount of lipids before entering the hibernation sites in comparison with *Cx. p. pipiens*. Accordingly, the effect of fructose availability in this study had, although significant for both species, a greater influence on the survival of *An. messeae*. In addition, as shown for *Cx. p. pipiens*, larger specimens have an advantage under high winter temperatures because they can store more lipids.

In conclusion, *Cx. p. pipiens* and *An. messeae* were detected strongly concentrated in certain man-made constructions, which were regularly used as hibernation sites. Although *Cx. p. pipiens* were frequently found in cellars, *An. messeae* probably preferred aboveground constructions, such as barns, as hibernation sites. Both, cellars and aboveground constructions, provided abiotic conditions, which allowed the survival of mosquitoes. Temperatures below 7°C were associated with the lowest mortality and metabolic rates. However, many hibernation sites were characterized by mean temperatures above 7°C in 2019/2020. In such an extraordinary warm winter, mosquitoes benefit from fructose availability to survive the winter, in particular *An. messeae*, which stores less lipids than *Cx. p. pipiens* before entering the hibernation sites.

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Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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