Mosquito species in the northern Netherlands.

Student Number: S4116046

Name: Tim Radjiman

Research partner: Richard Barelds

Course: Research project Ecology & Evolution 2

Supervisors: Dr. Sebastian Lequime and PhD. Nadja Brait

Abstract

Background

Global warming has major impacts on mosquito ecology. It allows for longer breeding seasons for all mosquito species, and increases the chance that more tropical mosquito species will establish themselves in Northern Europe. This is a large risk for public health as mosquitoes are prominent vectors of a lot of pathogenic diseases e.g. West Nile Virus and Dengue Virus. Because of this it is important to keep track of where these tropical mosquitoes establish in Europe.

Methods

The question of what mosquito species are present in the northern Netherlands was tested. To do this BG-Sentinel traps were used with CO2 or human mimicking odor as attractant in the course of weeks (May) in two different locations (Zernike Campus and Leek). The mosquitoes captured were crushed to extract DNA which was then analyzed using genetic loci as COI and CQ11 for species recognition. Furthermore ANK2 and PK1 were used to quantify wolbachia populations.

Results

At least two different mosquito species were found, being *Culex pipiens* and *Aedes (Ochlerotatus) rusticus*. Furthermore a positive correlation between temperature and catch rate, a heavy preference for CO2 as an attractor, both culex biotypes and two Wolbachia haplotypes were found.

Conclusion

Overall only native mosquito species were found. An increase in day temperature has a positive effect on mosquito species abundance and mosquito catch rate. Native mosquito species prefer CO2 when compared to human odor. The conclusion drawn from this study is that in the northern Netherlands no invasive mosquito species are yet to be established.

1. Introduction

Global warming has many implications for society. It consists of many changes in local climates. One such change is increasing temperatures. When looking at Europe in context of global warming, we see a non-homogenous pattern of warming. With some areas reaching up to 4 degrees warmer averages when compared to 60 years ago. (David A Stainforth *et al.,* 2013)

This temperature change can increase the potential transmission of many diseases carried by mosquitoes. One of such viruses is for example West Nile virus (WNV). The potential transmission increases due to the fact that mosquitoes can increase their reproduction and number of blood meals per season. (Medlock JM et al., 2012) One study on the linkage of heat and a WNV outbreak found that minimum temperatures play an important role in the prevalence of WNV. (Paz S, 2006) Most vector-borne diseases are mainly transmitted between bird species and Culex mosquitoes. One of which being *Culex Pipiens* (*C. Pipiens*) , the northern house mosquito.

The species *C.pipiens* can be subdivided in biotypes. These being *Culex pipiens pipiens*, *Culex pipiens molestus* and sometimes hybrids between the two. What is most important to know is that these biotypes behave differently. *Culex pipiens pipiens* is known as an above ground form and mostly feeds on birds (ornithophilic). While *Culex pipiens molestus* is known as a more underground form which mostly feeds on mammals including humans (mammalophilic). (Zittra, C et al., 2016) Hybrids are a mix of the two and thus are a great bridge factor for vector-borne diseases With this it is clear that distinguishing the biotypes is of great importance. This however can only be done with molecular techniques as phenotypically these mosquitoes do not differentiate.

To identify between biotypes *C. Pipiens Pipiens* and *C. Pipiens Molestus* (hence forward called *C. Pipiens* and *C. Molestus* respectively) a microsatellite can be used, this being the locus CQ11. This can be done because it is known that microsatellite regions mutate at a very high rate. Because of the high mutation rate the two biotypes already have significantly different CQ11 loci, and thus can be differentiated. (Bahnck CM, Fonseca DM., 2006)

While this is true for the *Culex pipiens* biotypes there are also other mosquitoes present in Europe. One such mosquito which at least the females can also not be identified on a morphological base is *Culex Torrentium*. It is important to know if there are *Culex Torrentium* individuals present in mosquito populations, as it is speculated that this species is even better at transmitting WNV than *Culex pipiens*. (Bergman, A et al., 2021)

Species identification can be done using molecular methods. Using the mitochondrial cytochrome oxidase c subunit I (COI) which is a universal marker gene. A differentiation of *culex pipiens* and *culex torrentium* can be found, however only if the enzyme Bcl1 is

used for enzyme restriction. (Danabalan R et al., 2012)

Both *C. pipiens* and *C. torrentium* are important as different species or even biotypes can have a large influence on the overall Virome. Although ambiguous in their relation, Wolbachia and viral load have been shown to be linked. Wolbachia is an α-proteobacteria which commonly acts as a parasite in arthropods (Atyame CM et al., 2011) . In culex pipiens, Wolbachia is almost always present. While in *culex torrentium,* Wolbachia is almost never present. Thus researching Wolbachia interactions in *culex pipiens* could be vital to understand viral ecology in Culex. (Bergman, A et al., 2021)

One more effect of increasing temperature is an increase in invasive mosquito species. Like Culex these mosquitoes can also benefit from long warm periods. Invasive species include *Aedes albopictus*, *Aedes aegypti*, *Aedes japonicus*, *Aedes atropalpus* and *Aedes koreicus*. These species do not only carry more vector-borne diseases like Chikungunya, Dengue and yellow fever virus (Schaffner F et al., 2013). They are also able to outcompete native mosquito species. These invasive Aedes mosquitoes are already known to be established in south- Europe. Although they were also found in the Netherlands, they are not yet established there. To understand the spread of these mosquitoes it is crucial to have more data on where they have been found.

Because of the significance both invasive and native mosquitoes have on human health, it is important to monitor what species are abundant in ‘established gray areas’ like the Netherlands.

This leads to the purpose of this project, it is to find out what species can be found in the Northern Netherlands which doubles as the main research question. Our hypothesis is that we expect to find mostly *Culex pipiens*, which would be subdivided into mostly *Culex pipiens pipiens* and some *Culex pipiens molestus* and hybrids with no invasive mosquito species. Following this, relevant questions relating to temperature, species preference, culex biotypes, Wolbachia and morphology will also be answered. For this we used both CO2 and odor traps on several days to capture mosquitoes. Morphological analysis was performed. Followed by DNA processing, what then will be used in multiple PCR’s to acquire COI, CQ11, ANK2 and PK1 products which are used to identify species and Wolbachia genotypes. COI products were also used for Sanger sequencing, but only for unidentified species. We found at least two different mosquito species, a positive correlation between temperature and catch rate, a heavy preference for CO2 as an attractor, both culex biotypes and two Wolbachia haplotypes. We conclude that no invasive mosquito species have been found in Groningen as of this moment.

Material & Methods

Mosquito collection

Mosquitoes are collected using BG-Sentinel traps (Biogents) containing either bottled CO2 or human mimicking odor (Biogents) as an attractant. These traps were laid out at the Zernike campus animal facility, and

were turned on from 17:00 to 9:00. The traps were active for a period of 3 to 4 weeks, but only on days when the daytime temperature exceeded 10°C. To compare the CO2 and odor catch rates, the two traps were placed 10 meters apart, both containing one of the attractants. To avoid location bias, the two lures were switched around on a regular basis. A third trap was used using only the CO2 as an attractant at a sampling location in Leek. Furthermore a BG-Pro trap (Biogent) was used in the forest next to Zernike campus to enlarge the sampling sites. This last trap used a mixture of 7g yeast, 100g sugar and 1L water to produce CO2, rather than using bottled CO2. Manual catching of mosquitoes was also done in this forest. After the mosquitoes were collected they were frozen for at least 15 minutes. As soon as they all perished, they were identified using a light microscope and a reverse identification key for mosquito species, made by the ECDC. All mosquitoes were put in individual tubes, and pliers were cleaned with alcohol after each mosquito to avoid cross contamination. Tubes containing mosquitoes that did not appear to be *C. pipiens* were marked, so they could later be easily recognized, and used for further analysis. Samples that were not processed immediately were frozen away at -80°C to preserve RNA.

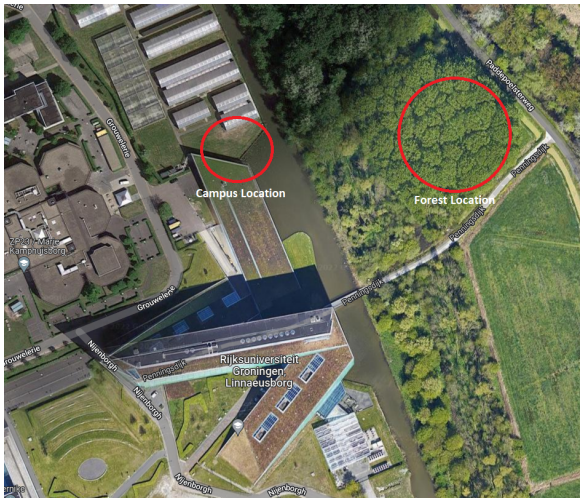

Figure 1: Capture locations Groningen

DNA/RNA extraction

To extract the mosquito DNA and RNA the QIAamp Viral RNA Mini Kit (Qiagen) To extract the DNA/RNA 2ml cryotubes and 1.5 ml eppis were used. Before starting extractions the RNA station and pipettes were cleaned using RNAse away, afterwards ~10 1mm glass beads and 140µl PBS were added to the cryo tubes. The mosquito samples were put into individual cryo tubes (while still on ice), and these samples were crushed using the standard program on the bead beater twice (still on ice). The resulting mixture was centrifuged for 90 seconds at 10000rpm and transferred to a new 1.5ml eppi. Nucleic acids were extracted according to the protocol (RNA Virus mini kit). DNA/RNA mix was eluted in a 60 µl AVE buffer. The final product was then split into two tubes both containing 30 µl. One of each was stored at -80°C to preserve RNA. The remaining tubes were stored at -20°C and used for further analysis.

COI barcoding

For COI barcoding a 25µl reaction PCR mix as shown in Table 1 was used. This is a

standard COI reaction mix, with the addition of MgCl2 for better results. The primers used were LCO1490F (5'-GGTCAACAAAT CATAAAGATA-3') and HCO2198R (5'-TA AACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al, 1994). All but the dNTP and Taq were mixed and vortexed, after which the dNTP was added, and only right before the strips were loaded the Taq would be added. All of this was done on ice. PCR cycling conditions are shown in Table 2. The resulting PCR product was used in gel electrophoresis and digested with Bcl1 enzyme.

Table 1: COI PCR master mix

reagents	25 µl reaction
10x Standard Taq reaction buffer	2.5µl
10mM dNTP	0.5µl
10µM forward Primer	1µl
10µM reverse Primer	1µl
Template DNA	1µl
Taq DNA Polymerase	0.5µl
50mM MgCl2	1µl
PCR grade water	17.5µl

Table 2: Cycler conditions PCR COI:

temp°C	time	cycles
95	5'	
95	1'	30
50	1'	30
72	1'	30
72	10'	
10	-	

CQ11 microsatellite

For microsatellite CQ11 a standard 20µl reaction PCR mix as shown in Table 3 was used. The primers used were pipCQ11R (5'-CATGTTGAGCTTCGGTGAA-3'), molCQ11R (5'-CCCTCCAGTAAGGTATC AAC-3') and CQ11F (5'-GATCCTAGCAAG CGAGAAC-3') as part of a multiplex PCR (Bahnck et al, 2006). The same order of mixing as in COI was applied. To validate the different biotypes, a PCR mix with only one of the reverse primers was used, as this could distinguish between the Culex biotypes and the hybrid. PCR cycling conditions are shown in Table 4. The resulting PCR product was used in gel electrophoresis. Samples were identified as *C. pipiens pipiens* (180 bp), *C. pipiens molestus* (250 bp) or hybrids when there is presence of both fragments (Bahnck and Fonseca 2006).

Table 3: CQ11 PCR master mix

reagents	20µl reaction
10x Standard Taq reaction buffer	2µl
10mM dNTP	0.4µl
10µM molCQR, CQ11F	0,3µl
10µM PipCQR	0.2µl
Template DNA	2µl
Taq DNA Polymerase	0.2µl
PCR grade water	12.85µl
50mM MgCl2	0.8µl
BSA	0.15µl

Table 4: Cycling conditions PCR QC11

temp °C	time	cycles
95	5'	
94	30‘'	40
54	30“	40
72	40“	40
72	5'	
10	-	

Wolbachia primers

To determine if, and how many Wolbachia genotypes were present in the captured Culex mosquitoes, the loci ANK2 and PK1 were used. Both ANK2 and PK1 reactions were the same (table 5), only the primers were switched. For the PK1, pk1F (5'-CTTCTTCTGTGAGTGTACGT-3') and pk1R (5'-TCCATATCGATCTACTGCGT-3') primers were used, while ANK2 used ank2F (5'-CCACTACATTGCGCTATAGA-3') and ank2R (5'-ACAGTAGAACTACACTCCTC CA-3') primers (Atyame et al, 2011). The same mixing order as in the COI and CQ11 was used once again, all whilst working on ice. The resulting PCR products were put on gel electrophoresis and later digested using HinfI enzyme for ANK2, and TaqI enzyme for PK1 (see Table 8).

Table 5: Wolbachia PCR master mix (ANK2 and PK1)

reagents	25µl reaction
10x Standard Taq reaction buffer	2.5µl
10mM dNTP	0.5µl
10µM Primer F	1.875µL
10µM Primer R	1.875µL
Template DNA	1µl
Taq DNA Polymerase	0.1µl
PCR grade water	16.25µl
50mM MgCl2	0.75µl
BSA	0.15µl

Table 6: Cycling conditions PCR Wolbachia (ANK2 and PK1)

temp °C	time	cycles
95	5'	
94	30‘'	36
52	30“	36
72	1-1.5 ‘	36
72	2'	
10	-	

Enzyme restriction on COI products and Wolbachia products

Enzyme restriction was done on both the COI products and the ANK2 and PK1 products, given that they gave a good result on the gel. The enzymes used for this were Bcl1 (COI), HinfI and TaqI (Wolbachia) respectively. Enzyme restriction on COI products allows for gel electrophoresis differentiation between *C. pipiens* and *C. torrentium*. While ANK2 and PK1 enzyme restriction allows for gel electrophoresis differentiation between Wolbachia geno- and haplotypes. Master mix for COI enzyme restriction is shown in table 7, and the ANK2/PK1 enzyme restriction master mix is shown in table 8. All three enzyme products are then incubated for one hour; BclI enzyme restriction incubation was done at 50°C, HinfI enzyme restriction incubation was done at 37°C, and TaqI enzyme restriction incubation was done at 65°C.

Table 7: Enzyme reactions BclI

reagents	30µl reaction
Buffer G	2µl
BclI	0.5µl
PCR grade water	22.5µl
Template DNA	5µl

Table 8: Enzyme reactions ANK2/PK1

reagents	20µl reaction
Buffer R/TaqI	2µl
BSA	0.2µl
HinfI/TaqI	0.5µl
PCR grade water	12.3µl
Template DNA	5µl

Gel electrophoresis

Gel electrophoresis is used to visualize the PCR products, as it allows for the identification of, and the differentiation between samples. Gel electrophoresis was used for COI, CQ11, ANK2, PK1, BclI, HinfI and TaqI products. To create the gel agarose MP (multi-purpose) and 1x TAE was used, the amount of agarose was either 1 or 2%, depending on what product it was for (see table 9). The mixture was placed in the microwave for 3 minutes, while shaking once in between to ensure proper dissolving. If strings were noticed in the mixture after this time it was heated up until they disappeared. After cooldown it was poured into a gel chamber containing a comb, of which the size was depending on the amount of samples. After pouring the gel it was given 20 minutes to set. When the gel was set the comb could be removed, and the gel could be loaded. 5µl PCR product and 1µl Orange G, were mixed, after which this was loaded onto the gel. One of the chambers was loaded with a ladder to quantify the base length of the PCR products. For this either a 100bp ladder or a smart ladder were used, based on the sample (see table 9). After loading the gel was run using electrophoresis as shown in table 9, after which the gel was stained using ethidium bromide for 20 minutes, and de-stained using a 1x TAE solution for 20 minutes, both while slightly shaking. After the gel had been destained it was visualized using UV radiation.

Table 9: Configuration of gel electrophoresis per product type

Product type	COI	CQ11	ANK2/ PK1	BclI/HinfI/TaqI
Agarose %	1%	2%	2%	2%
V	120	100	100	100
mA	200	200	200	200
Time (min)	15	40	30	30
Ladder	Smart ladder	Smart ladder	100 bp ladder	100 bp ladder

Sanger Sequencing of COI products

To quantify what species were caught, sanger sequencing was used. 20 µl of each of the samples to be sequenced were sent together with both of the COI primers to the company BaseClear. When the result came back the edges of the sequences were trimmed in FinchTV (version 1.4.0), after which they were BLASTed (blastn)

For statistical analysis the Spearman's rank correlation coefficient was calculated and Chi-square tests were performed. All figures except pictures were created using Google spreadsheets.

3. Results

This section is about the results gathered in our research. Traps were set up in a period of 3 weeks, for a total of 13 days. Overall 67 mosquitoes were caught.

Morphology

Morphological analysis was done to determine what was caught to be *Culex pipiens*. This had to be done considering CQ11, ANK2 and PK1 PCR reactions do not work on most other mosquito species with the primers used. To determine the species the mosquito specimens were frozen for 15 minutes. Afterwards they were examined under a normal light microscope using a reverse mosquito key. To determine if a mosquito was *Culex pipiens*, abdomen pattern, size, color and a round abdomen end were checked. Certain mosquitoes that lacked these criteria were sanger sequenced to confirm if they were a different species. Certain *Aedes* mosquitoes were also sanger sequenced as these species could not be determined based solely on the identification key. It was found that *Culex pipiens* had some difference in phenotypic expression, as it was found that there was a variety in size, color and pattern. But this might not be caused by a difference in genotype. Concluding, the different Culex pipiens phenotypes found might be due to one being a juvenile see figure 3, and one having had a blood meal see figure 4.

Figure 2: Example of what a typical *Culex pipiens pipiens* looks like. Note the color, pattern and rounding of the abdomen.

Figure 3: *Culex pipiens pipiens*, which does not have *culex pipiens pipiens* characteristics like size and abdomen pattern.

Figure 4: *Culex pipiens pipiens* which lacks the abdominal pattern and color of typical *Culex pipiens pipiens*.

Figure 5: *Aedes Ochlerotatus rusticus*, which was very prevalent in the forest sampling location.

Temperature-catch rate

The temperature experiment was done to understand to what extent certain species of mosquitoes would be active at temperature thresholds, and to what extent they would be present. This experiment can also show whether day or night temperature is most important to predict mosquito abundance. The procedure consisted of laying CO2 and odor traps every day when the temperature was above 10°C. A clear trend in which the mosquitoes' catch rate is correlated to day and night temperature becomes present upon looking at the data (Figure 6). The correlation of this trend is significant: Capture/Day Temp r= 0.83, p=<0.001, Capture/Night Temp r= 0.62, p=0.027. There also appears to be a certain threshold of the day temperature, where there are no captures below roughly 15°C. Furthermore the amount of different species present appear to be elevated at higher temperatures. Concluding, mosquito activity and species abundance seem to peak at higher temperatures, and day temperature seems to be a better predictor for mosquito abundance than the nighttime temperature. This is relevant for conducting mosquito diversity screenings, higher temperatures increase the chances of catching more mosquito species.

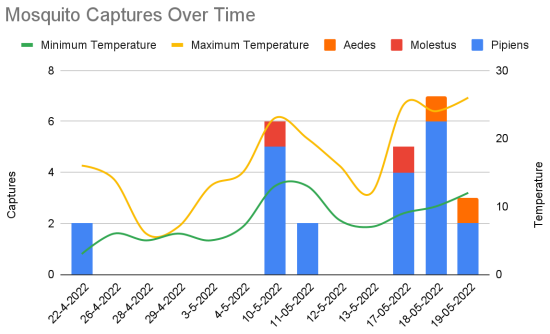

Figure 6: Mosquito captures over time, higher mosquito catch rate and higher mosquito species abundance with increasing temperature, temperature on the right Y-Axis, Captures on the left Y-Axis. Dates on the X-Axis
Capture/Day Temp r= 0.83, p=<0.001, Capture/Night Temp r= 0.62, p=0.027. The statistical analysis used is the spearman's rank correlation coefficient.

Odor-CO2

Comparing artificial human mimicking odor and CO2 as mosquito attractant was done to understand mosquito species preference in attractant. The experiment was done by interchanging CO2 and odor in two separate traps that were 10 meters apart for several days. From all mosquitoes caught, none were attracted to the human mimicking odor, instead, all mosquitoes that were caught were attracted by CO2 as seen in figure 7. Concluding, the species caught had no preference to artificial human odor, but a strong preference for CO2. The preference in attractors is a relevant topic for public health, as CO2 is also exhaled by other animals, and therefore the risk of mosquitoes carrying animal originated diseases increases, when compared to

mosquitoes that would mostly be attracted to human odor. However these mosquitoes are less likely to spread pathogenic diseases between humans.

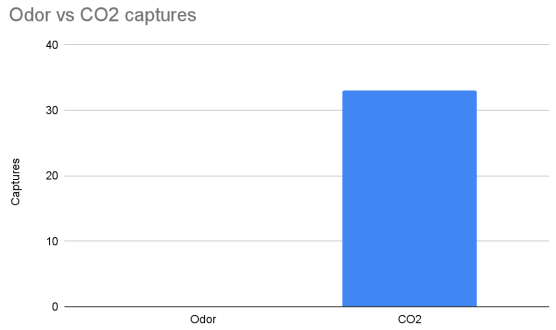

Figure 7: Odor compared to CO2 catch counts
All mosquitoes caught were attracted to CO2,
None were attracted to human mimicking odor
Captures on the Y-Axis
Treatments on the X-Axis

Pipiens-Molestus Ratio

This experiment was done to see what percentage of the *C. pipiens* population was *C. pipiens molestus* and what percentage were hybrids. The experiment was conducted using two sampling sites: site Zernike campus and site Leek. The captured mosquitoes had their DNA extracted and the CQ11 analyzed. It can be observed that the Leek molestus proportion was higher than Zernike campus. The Leek population was found to be 14.3% see figure 8 and 9. A Chi square test was done to test whether the pipiens-molestus ratio found in this experiment is significantly higher than the expected 3.25% (Zittra et al 2016). X^2: 9.123, df= 2. p=0.01. On the other hand, the Zernike campus did not differ significantly from the expected value X^2:1.005, df= 2. p=0.6049. Concluding, Leek seems to have a higher *C. pipiens molestus* ratio when compared to Zernike campus. This is relevant to public health due to the fact that a more homogenous distribution of *C. pipiens pipiens* and *C. pipiens molestus* can increase the chance of potential hybridization. This leads to more bridge vector individuals for avian pathogens distributing in humans.

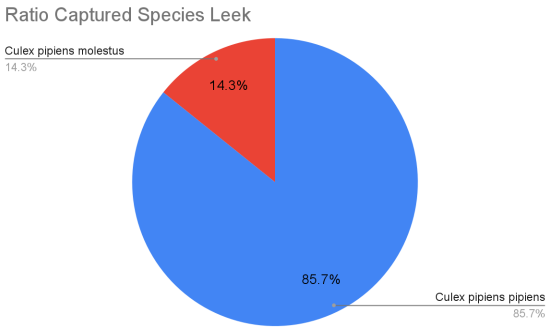

Figure 8: distribution of *C. pipiens pipiens* and *C. pipiens molestus* in Leek, Molestus distribution differs significantly from expected 3.25%. For this we used a Chi-square test: X^2: 9.123, df= 2. p=0.01

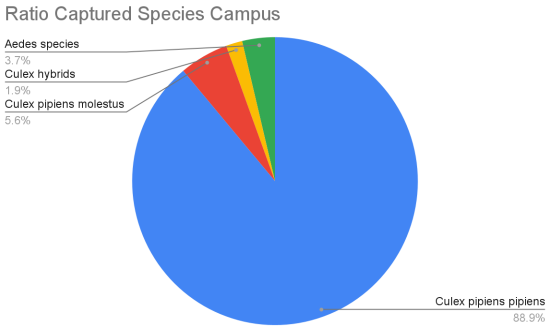

Figure 9: distribution of mosquito species on Campus, Distribution of Culex biotypes does not differ significantly from expected 3.25% For this we used a Chi-square test: X^2:1.005, df= 2. p=0.6049

Wolbachia genotyping

Wolbachia genotyping was done to quantify what strains of wolbachia are present in *C. pipiens*. To answer this, the locus ANK2 and PK1 which are part of the Ankyrin domain genes in Wolbachia were used. HinfI and TaqI enzymes were applied to the ANK2

and PK1 products respectively. These were then used in gel electrophoresis as shown in figure 10. The bands for both observed enzymes could then be compared with the lookup table (Dumas et al, 2013), in which the HinfI: a (313 bp), b (217, 195, 98 bp), c (293, 217 bp), d (217, 195 bp) and e (415 bp). And the TaqI a/e (903,430 bp), b (669, 665 bp), c (851, 498 bp) and d (497, 251, 107 bp) bands could be used to tell the haplo- and genotype. Using this, 2 different haplotypes were observed in Wpip-II. Concluding, there is high probability of wolbachia haplotype diversity in *C. pipiens* populations in Groningen, given the small sample size already shows diversity. This is relevant for studying viral ecology, as it is unknown what wolbachia haplotypes have an effect on viral load in *C. pipiens*.

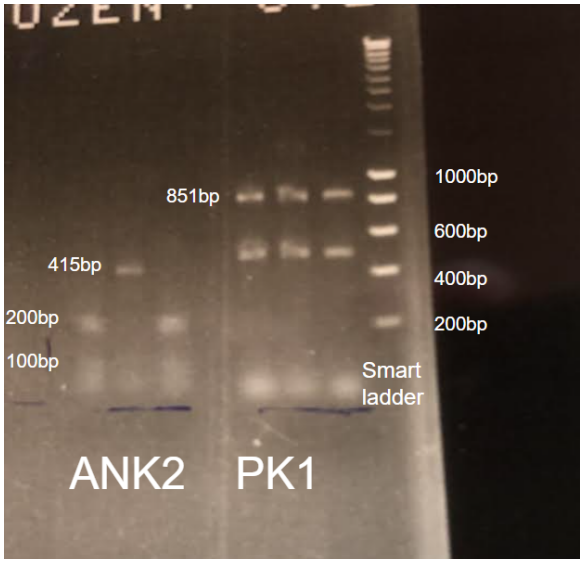

Figure 10: Enzyme digested product of ANK2 and PK1. ANK2 locus has base pair diversity which implies a different wolbachia haplotype.

4. Discussion

Result summary

It was found that morphological identification can be done between Aedes and Culex, but certain Culex species cannot be identified this way as expected. Higher temperatures increase catch rate. All mosquitoes were caught using CO2, no mosquitoes were caught using human odor. Which infers that at least native mosquito species are more attracted to CO2 than human odor. Culex biotypes ratio is in favor of C.pipiens, while in Leek specifically C.molestus increases in ratio. Which might be due to Leek being located in an agricultural area. Lastly two different Wolbachia haplotypes were found.

Morphology

Morphological identification was found to be quite difficult as expected. There is however a clear difference between *Culex* mosquitoes and *Aedes* mosquitoes. Between the found Culex samples, morphological analysis does not help with identifying the species. Some phenotypic variation was found in two of the Culex samples see figure 3 and 4). But these features might be due to one being a juvenile mosquito, while on the other hand the mosquito might have had a blood meal which causes the black dot. To quantify what mosquito species are present, only molecular methods seem to be reliable.

Temperature-catch rate

Temperature is important in predicting mosquito populations. Higher temperatures allow mosquitoes to copopulate for a longer time, which in turn increases the mosquito population (Medlock JM et al., 2012). This project indicates that mosquito abundance is correlated with night and day temperature. Day temperature was found to be more correlated with mosquito abundance. We also found an indication that species abundance also increases with temperature although no statistical tests were conducted for this. These findings add to the already existing information on mosquito temperature relations. (Medlock JM et al., 2012, Paz S, 2006) Although this project did not have a large sample size it still shows a positive trend. Our hypothesis to the question, 'to which point does temperature influence the catch rate of mosquitoes?' was that increased temperature would cause an increase in catch rate seems to be correct based on our results. The next research question based on temperature-catch rate relations could be about species specific catch rates. To what extent does temperature influence invasive *Aedes* species abundance for example.

Odor-CO2

Human odor and CO2 have been shown to be good attractors for mosquitoes (Zhao, Z et al., 2022, Smallegange, R.C, et al., 2010). This attraction depends on the species. With *Aedes* being more attracted to odor (Zhao, Z et al., 2022) and *Culex* more attracted to CO2.(Smallegange, R.C, et al., 2010) We found that no mosquitoes were attracted to the human mimicking odor, while all mosquitoes caught, including some *Aedes* mosquitoes were attracted to CO2. This could be due to the fact that the *Aedes* odor attraction might only be for invasive *Aedes* species, (Zhao, Z et al., 2022) and might not be the case for native *Aedes ochlerotatus rusticus* mosquitoes. Because we only caught *Culex pipiens* and *Aedes ochlerotatus* to our knowledge, it can be concluded that these two species do not prefer human odor and are heavily attracted to CO2. Our hypothesis for the question: if there is a difference in mosquito species attraction when comparing CO2 to human odor, which was that *Aedes* should be attracted to human odor, while *Culex* should be attracted to CO2 seems to be incorrect. But because we only had a few *Aedes* mosquitoes we can not say for certain that they are not attracted to human odor. On the other hand, *Culex* mosquitoes certainly are more attracted to CO2 all culex caught were with CO2, none were attracted to odor. *Aedes ocheloratus* and *Culex pipiens* are only a small fraction of the total mosquito species list in Europe. This raises the question: If there are any native mosquito species that are attracted to human odor in Europe? Sampling in the forest location might give more insight on how the *Aedes Ochlerotatus rusticus* react to odor, as these mosquitoes are very abundant in this location.

Pipiens-Molestus ratio

Culex pipiens pipiens (C.pipiens) is known to feed on birds (ornithophilic). While *Culex pipiens molestus (C.molestus)* is known to

feed on mammals including humans (mammalophilic). (Zittra, C et al., 2016)

It was found in our study that in Leek there are more *C.molestus* mosquitoes than expected. This might be due to Leek being in an agricultural area which is known to have greater *C.molestus* ratios when compared to urban areas (Bergman, A et al., 2021). Although this might be true, our findings might indicate that in The Netherlands there is a different ratio between *C.pipiens* and *C.molestus*, when compared to other countries. This might lead to more hybridization between the two biotypes in The Netherlands which leads to a larger bridge vector in The Netherlands for known human-pathogenic viral diseases. This leads to a new research question, What is the ratio of *C. pipiens* and *C. molestus* in The Netherlands.

Wolbachia genotyping

Our results suggest that there is definitely genetic variation in the Wolbachia population in Groningen. Our study only found two Wolbachia haplotypes, but this is probably due to a very low sample size of successful Wolbachia enzyme restrictions. Although the sample size was low, the fact that two wolbachia haplotypes were found suggests that genetic variation in the *Culex pipiens* wolbachia population is large. The wolbachia haplotypes found do correlate with previous *Culex pipiens* wolbachia work. (Dumas et al, 2013) Due to a low sample size, a larger study about the wolbachia population in The Netherlands might turn out to be different then our findings, and it would give more insight into the viral ecology of *Culex pipiens.*

COI experiment & Limitations

Although most of the research was successful, some limitations have also been found. The COI experiment was such a limitation as we could not get the PCR reaction right. This was probably due to handling issues. Because of the lack of successful COI products the ratio of *Culex pipiens* and *Culex torrentium* could not be measured. Due to this the theory that the species Culex torrentium is hardly ever infected with Wolbachia could also not be measured.

Another limitation is the timing of the experiment which is also important. This is due to the fact that invasive mosquitoes are more active later in the year. And later in the year higher day temperatures will be reached, which correlates to higher catch rates of mosquitoes.

Conclusion

To conclude, our study showed that in May there are at least two mosquito species present depending on location, which being *Culex pipiens* and *Aedes ochlerotatus rusticus*. It also showed that the mosquito population in the northern Netherlands mainly consisted of *Culex pipiens*, although this can change depending on sampling site e.g. forest or urban areas. It was also found that temperature does correlate with mosquito abundance, and potentially mosquito species diversity. Native mosquito

species seem to have a heavy preference for CO2 as an attractant when compared to human odor. When looking at *C.molestus* and *C.pipiens* it seems that agricultural areas have a large influence on the ratio between the two. This study also showed that there is indication of wolbachia haplotypic diversity in the *Culex pipiens* population of Groningen. And morphological traits can differ in the *Culex pipiens* complex, although it is not known if these traits have any effect on the fitness of the individual. Lastly our study showed no signs of invasive *Aedes* species being established in the Northern Netherlands.

References

1. David A Stainforth *et al* 2013 *Environ. Res. Lett.* **8**034031(https://iopscience-iop-org.proxy-ub.rug.nl/article/10.1088/1748-9326/8/3/034031)

2. Paz S (2006) The WNV outbreak in Israel (2000) from a new perspective: the regional impact of climate change. International Journal of Environmental Health Research 15:1–13

3. Zittra, C., Flechl, E., Kothmayer, M. *et al.* Ecological characterization and molecular differentiation of *Culex pipiens* complex taxa and *Culex torrentium* in eastern Austria. *Parasites Vectors* 9, 197 (2016). https://doi.org/10.1186/s13071-016-1495-4

4. Bahnck CM, Fonseca DM. Rapid assay to identify the two genetic forms of Culex (Culex) pipiens L. (Diptera: Culicidae) and hybrid populations. Am J Trop Med Hyg. 2006 Aug;75(2):251-5.

5. Schaffner F, Medlock JM, Van Bortel W. Public health significance of invasive mosquitoes in Europe. Clin Microbiol Infect. 2013 Aug;19(8):685-92. doi: 10.1111/1469-0691.12189. Epub 2013 Apr 10. PMID: 23574618.

6. Atyame CM, Delsuc F, Pasteur N, Weill M, Duron O. Diversification of Wolbachia endosymbiont in the Culex pipiens mosquito. Mol Biol Evol. 2011 Oct;28(10):2761-72. doi: 10.1093/molbev/msr083. Epub 2011 Apr 22. PMID: 21515811.

7. Bergman, A., Hesson, J.C. *Wolbachia* prevalence in the vector species *Culex pipiens* and *Culex torrentium* in a Sindbis virus-endemic region of Sweden. *Parasites Vectors* 14, 428 (2021). https://doi.org/10.1186/s13071-021-04937-6

8. Danabalan R, Ponsonby DJ, Lintoni YM. A critical assessment of available molecular identification tools for determining the status of Culex pipiens s.l. in the United Kingdom. J Am Mosq Control Assoc. 2012 Dec;28(4 Suppl):68-74. doi: 10.2987/8756-971X-28.0.68. PMID: 23401945.

9. Smallegange, R.C., Schmied, W.H., van Roey, K.J. *et al.* Sugar-fermenting yeast as an organic source of carbon dioxide to attract the malaria mosquito *Anopheles gambiae*. *Malar J* 9, 292 (2010). https://doi.org/10.1186/1475-2875-9-292

10. Medlock JM, Hansford KM, Schaffner F, et al. A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options. Vector Borne Zoonotic Dis. 2012;12(6):435- 447. doi:10.1089/vbz.2011.0814

11. Zhao, Z., Zung, J.L., Hinze, A. *et al.* Mosquito brains encode unique features of human odour to drive host seeking. *Nature* 605, 706–712 (2022). https://doi.org/10.1038/s41586-022-04675-4

12. Dumas, E., Atyame, C.M., Milesi, P. et al. Population structure of Wolbachia and cytoplasmic introgression in a complex of mosquito species. BMC Evol Biol 13, 181 (2013). https://doi.org/10.1186/1471-2148-13-181

13. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994 Oct;3(5):294-9. PMID: 7881515.