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Host preference of *Anopheles maculipennis s.l* in the Netherlands

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Minor Thesis
ENT-80424

March-July 2008

Summary

Malaria is one of the most infectious vector-borne diseases in the world today. In the Netherlands, the disease has successfully been eradicated, but the vectors which are able to transmit the malaria parasite *Plasmodium* are still present. Three species of the *Anopheles maculipennis* complex, *A. atroparvus, A. messeae* and *A. maculipennis sensu stricto*, are able to transmit the disease and still occur in the Netherlands. In order to establish whether these three mosquito species are still potential vectors of malaria in the Netherlands, their incidence and host preference was determined by establishing their presence on farms in several regions of the Netherlands and by assessing blood donors selected by these mosquitoes. In March and April all haematophagous mosquitoes present in a couple of animal shelters in the Netherlands were collected using oral plastic tube-aspirators and the specimens were stored in bottles containing 70 % ethanol. In the laboratory, the mosquito species identification was performed using PCR of the ITS2 region; identification of the origin of blood found in the midguts of the mosquitoes was performed using PCR of the mitochondrial cytochrome B gene.

During the experiment 88 specimens of *A. messeae*, 2 specimens of *A. atroparvus* and 1 specimen of *A. maculipennis* s.s were collected. On all samples defined as *A. messeae* another PCR and digestion experiment was performed to distinguish this species from its' subspecies *A. daciae*. The latter species, however, was not found in the samples of this study. Next to the three *Anopheles* species, several other haematophagous mosquitoes and flies were collected in the animal shelters. Blood meals of the collected mosquitoes were compared to positive controls of goat, human, pig, cow, dog, sheep and horse. From this analysis it was concluded that *A. messeae* seems to have a preference for sheep and cattle. From the other two anophelines only a few specimens were collected and no results were generated with the analysis of their bloodmeals. Not all samples gave satisfactory results with the blood meal analysis, making it difficult to draw some conclusions on the host preference of the three mosquito species of the *Anopheles maculipennis* complex in the Netherlands.

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Introduction

In recent years, several vector-borne diseases have re-emerged all over the world, causing not only health problems but also economical effects. Most outbreaks are due to changes in climate, human population or landscape. Malaria is one of the most infectious vector-borne diseases in the world today. Approximately 40 % of the world's population is at risk of malaria, the majority is living in the poorest countries. Every year, more than 500 million people are known to be infected with malaria and become very ill (WHO, 2007). Until 1945, endemic malaria occurred all over Europe, especially in the Mediterranean area. After a few outbreaks during the war, malaria eventually disappeared from Europe due to an eradication programme and since 1975, Europe is officially declared malaria-free (Kuhn *et al*, 2002). For this decrease in incidence, several reasons can be given: the application of insecticides and herbicides like DDT, the pollution of water, the availability of new drugs, improved housing, sanitation and also change of economical conditions (Van Seventer, 1969). The disease has successfully been eradicated from Europe, but the vectors, which are able to transmit the malaria parasite *Plasmodium*, are still present (Takken et al, 2002). A few cases of malaria are imported into Europe every year, mostly by tourists and immigrants from regions of the world where malaria is still endemic. This might, together with the expected climate-change enhance the risk for reintroduction of malaria into Europe (Martens *et al*, 2005)(Jetten & Takken, 1994).

Malaria is transmitted by mosquitoes belonging to the genus *Anopheles* (Order *Diptera*, Family *Culicidae, subfamily Anophelinae*). About four hundred and fifty *Anopheles* species have been described and approximately fifty of them can act as a vector in spreading the *Plasmodium* parasite, which causes the disease malaria in humans (Klowden, 2007). In the Netherlands only *A. atroparvus,* which is a member of the *Anopheles maculipennis* (s.l) complex, was known to spread Malaria. The *Anopheles maculipennis* complex consists of thirteen species, but in Western Europe, only three species of this complex are known, *A. atroparvus* van Thiel*, A. messeae* Falleroni *and A. maculipennis sensu stricto* Meigen.

The mosquitoes from this species complex are genetically distinct from each other, but morphologically appear to be similar, which is also called 'cryptic species' (Walton *et al*, 1999). The phenomenon of 'Anophelism without malaria', where the vector *A. maculipennis* occurred in certain areas of Europe but malaria did not, can be explained by the fact that *A. maculipennis* exists as a complex of species that differ in their ability to transmit malaria.
From the four known types of malaria parasites (*Plasmodium vivax, P. falciparum, P. malariae and P. ovale*), only *P. vivax* caused malaria in the Netherlands. *A. atroparvus* was the important transmitter of malaria to humans during outbreaks in Western Europe, in the Netherlands especially in the coastal area (Jetten & Takken, 1994). *A. messeae* was not considered to be such an important vector in Western Europe as it was in Eastern Europe. Next to mosquitoes of the *A. maculipennis* complex, two other anophelines can be found in the Netherlands, *A. claviger* and *A. plumbeus*. *A. claviger* used to be an important vector in Mediterranean areas and *A. plumbeus* is a rarely seen vector in western Europe (Takken *et al*, 2002). Recently a new (sub)species belonging to the *Anopheles maculipennis* complex, *A. daciae,* was discovered in Romania. After morphological inspection of the eggs and characterisation on molecular basis, it seemed to be a subspecies of *A. messeae* (Nicolescu *et al*, 2004). This subspecies was also found in the United Kingdom, Italy, Russia, Kazakhstan and could be expected to occur also here in the Netherlands.

Female mosquitoes take bloodmeals for the development of their eggs and next to that they feed on plant sugars which is the energy source for metabolic processes and host seeking flights (Klowden, 2007)(Takken & Knols, 1999). *Anopheles* mosquitoes are active in the evening to seek for large vertebrate hosts, of which they pick up information with their sensitive antennae and palps (Takken, 1991). Almost all anophelines seem to be attracted to CO_2, because this compound is present in the expired breath of vertebrates and therefore indicates the presence of a potential host for the blood-sucking mosquito. Some *Anophelines*, like *A. atroparvus*, are also attracted to human-specific odours, especially components of human sweat, like octenol, lactic acid and fatty acids (Jetten & Takken, 1994) (Dekker *et al*, 2002). After taking a blood meal from a vertebrate host, which can be taken indoors (Endophagy) or outdoors (Exophagy), the female will mostly rest indoors (Endophily) for a few days to digest the blood and develop the eggs (Day, 2005).

Host-seeking behaviour is inhibited when a female mosquito carries mature eggs, at this time she is ready for oviposition and she does not need any more blood. Eight days after the first blood meal, the first oviposition will take place (Jetten & Takken, 1994). Thereafter, eggs will mature in intervals of two days and each time a blood meal is needed. This means that every two (or more) days, the place where the blood meal was taken and the mosquito was resting, must be left to find a suitable place for oviposition (Clements, 1992).

Most *Anophelines* in Europe go into diapause during winter time. Diapause is induced by day length and temperature. Mosquitoes show individual variation in their reaction to these environmental factors. Diapause can be differentiated in complete diapause or incomplete diapause. Species that go into complete diapause as adults will not carry the infectious parasite *Plasmodium* into the next spring, but species that go into incomplete diapause continue transmitting the disease (Swellengrebel & de Buck, 1938). During complete diapause the females form a fat body and seek a cold and humid shelter, remaining completely inactive till spring. This type of diapause is showed by *A. maculipennis* s.s and *A. messeae* (Hackett *et al*, 1935). According to Jetten & Takken (1994), *A. maculipennis s.s* and *A. messeae* are in diapause from November till March-April. The oviposition of these species continues until the end of September. During incomplete diapause, the female also forms a fat body, but she remains active and continues taking blood meals, not for egg development but as substitution for nectar and she is only seeking shelter during cold weather. *A. atroparvus* shows this type of diapause, from September till March-April. The end of the diapause is determined by temperature (Jetten & Takken, 1994). According to this information from other studies, *Anopheles* mosquitoes are expected to be found in diapause in animal shelters approximately until April.

To study whether the three members of the *Anopheles maculipennis* complex are potential vectors of malaria in the Netherlands, their distribution and host preference in selected areas was investigated. Mosquitoes were collected in farming areas and the species composition of adult *Anopheles* mosquitoes was established. Their host preference was determined by analyzing the blood meals these mosquitoes took, using PCR of the mitochondrial cytochrome B gene. The study was expected to provide information about the host preference of *A. maculipennis s.s, A. atroparvus* and *A. messeae* in the Netherlands.

2. Material and Methods

2.1 Collection of mosquitoes from selected locations in the Netherlands

Mosquitoes were collected in animal shelters belonging to farms in three areas in the Netherlands indicated in figure 2.1. These animal shelters are indoor resting places for female *Anopheles* mosquitoes in diapause or when they have taken a blood meal (Day, 2005). Most suitable for the collection of mosquitoes were the older, wooden or stone, animal shelters with different animal species. The newer animal shelters are too open and clean and no mosquitoes were expected to be found.

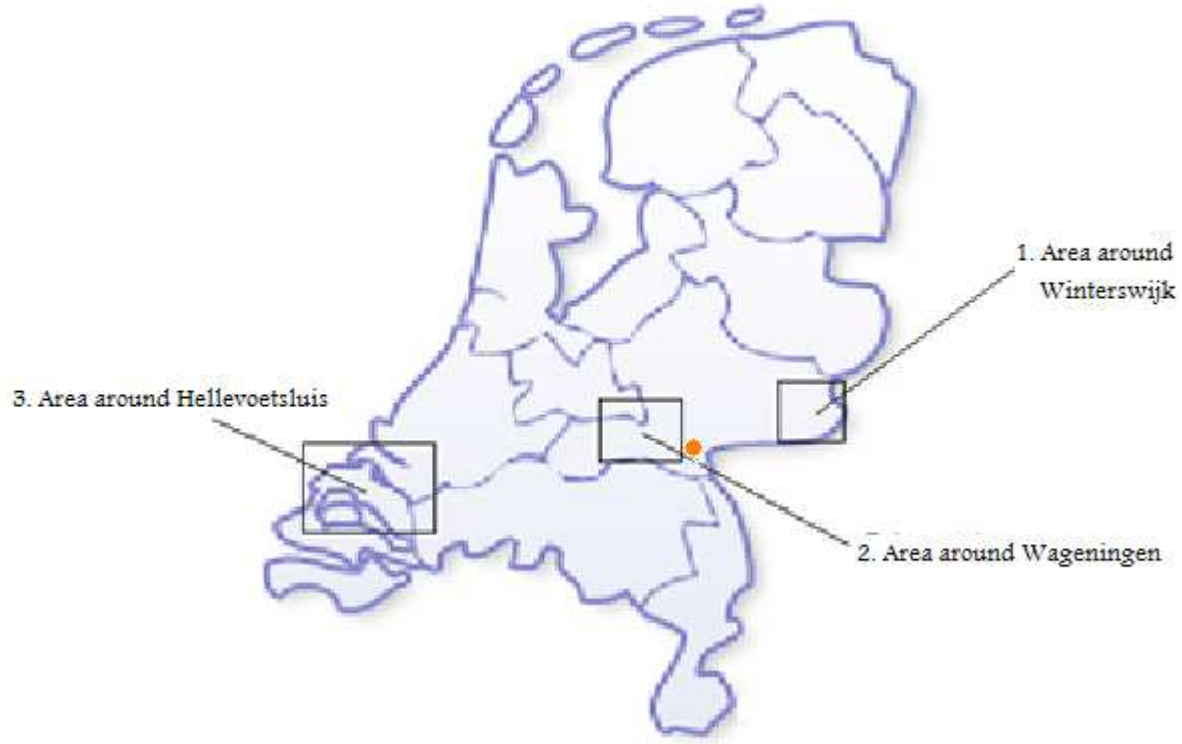

Figure 2.1. Map of the Netherlands, with the areas pointed out where mosquitoes were collected (red dot is indicating Lent).

In previous studies of Takken *et al*. (2002) and Swellengrebel & de Buck (1938) it was found that three mosquito species of the *Anopheles maculipennis* complex occur in different areas of the Netherlands; *A. atroparvus* was mostly found in the western part of the Netherlands because of its' preference for brackish water, *A. maculipennis sensu stricto* was mostly found in the eastern part of the Netherlands and *A. messeae* was found everywhere but certainly in the centre of the Netherlands. According to this information, three specific areas were chosen, as indicated in figure 2.1, for the collection of female mosquitoes.

Location one is located in the eastern part of the Netherlands, in the area around Winterswijk (51˚58'21.26"N, 6˚43'10.03"O). Location two is located in the centre of the Netherlands, in the area of the river Rhine south of Wageningen (51˚57'53.00"N,5˚39'43.29"O). Location three is located in the western part of the Netherlands, in the area around Hellevoetsluis, South Holland (51˚50'01.94"N, 4˚08'17.90"O). In these areas a number of suitable farms were selected, that varied in the amount and composition of animals, as shown in table 2.2. Mosquitoes were collected in March (collection 1) and April (collection 2). All mosquito species present were collected, including anophelines.

Table 2.2. Animal compositions at the different farms at the three locations

Locations	**Farms**	**Animals present (#)**
Location 1	Farm 1	Cattle(4), horses(3), goats(30), chickens(10), rabbits(6)
	Farm 2	Cattle(100), horses(2), dog(1)
	Farm 3	Horses(17), dog(1)
	Farm 4	Horses(8), dog(1)
	Farm 5	Cattle(8), horses(5), dog(1)
	Farm 6	Sheep(50), dog(1)
Location 2	Farm 1	Cattle(110), horses(1), dog(1)
	Farm 2	Goats(500), cattle(100), dog(1)
	Farm 3	Horses(15), sheep(40), dog(2)
	Farm 4	Cattle(100), sheep(80), dog(2)
Location 3	Farm 1	Horses (5), sheep(4), cattle(4), chickens(30), dog(1)
	Farm 2	Goats (650), dog (1)

In a fourth area in the eastern part of the country, in Lent near Nijmegen, mosquitoes were collected as well (once in June) as it was expected to collect *A. messeae*. During my study on *A. maculipennis s.l,* the Laboratory of Entomology received a mosquito from local residents of Lent, because aggressive mosquitoes made it impossible for them to sit or work outside. The mosquito received was identified as an anopheline species. Because of the nuissance they caused a study started in which, as a first step, mosquitoes were collected in that neighbourhood for the determination of the *Anopheles* species and blood meal analysis. Lent is a small village near Nijmegen, located next to the river Waal. Pastures with sheep and ponies are bordering the mostly free-standing houses.

Mosquitoes were collected from several locations around people's houses, for example the garden and an old cattle-shed now used for storage of rainwater. The mosquitoes were very aggressive and obviously very hungry since they tried to bite you constantly, also considering it was morning during the visit.

During two sessions in March and in April, the mosquitoes were collected in animal shelters using an oral, plastic tube-aspirator (hand-catch). The collections were transferred to small bottles containing 70% Ethanol until the samples were identified under the stereo microscope to exclude flies and other animal species. *Anopheles* mosquitoes are distinguishable from other mosquitoes by the equal length of the palps and proboscis, the interrupted hair row on the scutellum and the lack of bands on legs and abdomen. In the field they can be recognized by their resting position, which is with the abdomen sticking in the air, instead of parallel to the surface on which they are resting. The female mosquitoes were split up in two 1,5 ml Eppendorf tubes (the abdomen separated from the rest of the body). The abdomen was used for the analysis of the blood meal taken by the mosquito and the rest of the body was used to determine the specific species of the *A. maculipennis* complex.

2.2 Species composition of A. maculipennis s.l at the three different locations

The molecular species identification was performed by PCR using a protocol adjusted by Paaijmans (K. Paaijmans, unpublished data) to the one Proft *et al.* (1999) describes. In mosquitoes, the rDNA transcriptional unit is composed of an external transcribed spacer, an 18S subunit, an internal transcribed spacer 1 (ITS1), a 5.8S subunit, an ITS2 and a 28S subunit. The rDNA units are linked to each other by an intergenic spacer (IGS). The functional regions (18S, 5.8S and 28S) are highly conserved, but the transcribed (ITS1 and ITS2) and intergenic spacer (IGS) regions have high interspecific and low intraspecific variability, which makes them useful for PCR identification of morphologically similar species (Li & Wilkerson, 2007). The ITS2 region has been used successfully by Marinucci *et al* (1999), Linton *et al* (2001 and 2003), Patsoula *et al* (2007) and Collins *et al (*1996) for the identification of several mosquito species and this will also be the target region for the DNA amplification in this study.

The ITS2 region of the rDNA will be amplified by PCR using one universal forward and mosquito specific reverse primers.

Universal forward	5' TGTGAACTGCAGGACACATG 3'	-
A. atroparvus	5' CGTTTGGCTTGGGTTATGA 3'	117 bp productsize
A. messeae	5' GACGCCTCACGATGACCTT 3'	305 bp productsize
A. maculipennis s.s	5' TATTTGAGGCCCATGGGCTA 3'	410 bp productsize

DNA was isolated from the abdomen using the QiaAMP DNA Mini DNA Kit (Qiagen, Germany) according to manufacturer's instructions and it was used for species determination and blood meal analysis. Until the DNA extract could be used, it was stored in the -20 ˚C freezer.

For the species determination two different PCR reaction mixtures were made: the first one, using only the single leg or wing of one specimen of *A. maculipennis s.l.*, contained 5 µl 5x Green Buffer, 1.5 µl 25 mM $MgCl_2$, 0.5 µl dNTP's, 0.5 µl 25pmol/ µl *A. maculipennis sensu stricto* reverse primer, 0.5 µl 25pmol/ µl *A. atroparvus* reverse primer, 0.5 µl 25pmol/ µl *A. messeae* reverse primer, 0.5 µl 25pmol/ µl universal forward primer, 0.125 µl GoTaq Polymerase and 15.875 µl ddH_2O resulting in 25 µl/PCR tube. For the second PCR reaction mixture the same components were used in the same amounts except for the DNA that was added as 3 µl DNA extraction product instead of a part of the mosquito. To end with the same amount of PCR reaction mixture 12.075 µl of water was added instead of 15.075 µl.

A specific thermo profile (4 minutes at 94 °C, followed by 35 cycles at 94 °C for 1 minute, 50 °C for 1 minute and 72 °C for 2 minutes, the final extension step at 72 °C for 10 minutes) was followed in the PCR machine (Eppendorf Mastercycler Gradient) and the amplification products were visualized on an 1% agarose gel with ethidium bromide. The three *Anopheles* species produced different PCR products, visualised by distinct bands on the gel.

2.3 Distinguishing A. messeae and A. daciae

PCR

To be able to differentiate between *A. messeae* and its subspecies *A. daciae*, another PCR of the ITS2 region, followed by digestion with restriction enzyme BSTU1, was performed on all specimens that had previously (2.2) been identified as *A. messeae*. The PCR reaction mixture contained 10 µl 5x Green Buffer, 5 µl 25 mM $MgCl_2$, 0.5 µl dNTP's, 5 u µl 5 µM forward primer, 5 µl 5 µM reverse primer, 0.1 µl GoTaq Polymerase, 22.4 µl ddH_2O and 2 µl DNA extraction product, resulting in 50 µl/PCR tube (this procedure was developed by Dr. Y Linton, BMNH, London and kindly provided to us).

A specific thermo profile (2 minutes at 94 °C, followed by 35 cycles at 94 °C for 30 seconds, 57 °C for 1 minute and 72 °C for 30 seconds, the final extension step at 72 °C for 10 minutes) was followed in the PCR machine.

Digestion

The resulting PCR products were digested at 60 °C during three hours using the enzyme BSTU1, cutting at 109 bp (only for *A. messeae*) and 332 bp (for *A. messeae* and *A. daciae*). The mixture for the digestion contained 13.5 µl ddH_2O, 2 µl Buffer2, 0.5 µl Enzyme BSTU1 and 4 µl PCR product, resulting in 20 µl/PCR tube. Digestion products were visualized on an 1% agarose gel containing ethidium bromide. *A. messeae* should produce two bands of 109 bp and 332 bp, *A. daciae* should only produce one band of 332 bp.

2.4 Bloodmeal analysis of anopheline spp. in the Netherlands

For the identification of the blood meals taken by the *A. maculipennis* mosquitoes and other haematophagous mosquitoes, the method (slightly adapted) described by Kent & Norris (2005) was used. This method is based on a multiplex PCR of the mitochondrial cytochrome B gene, a method also used by Molaei *et al.* in 2007 for the determination of the host feeding pattern of *Culex quinquefasciatus* and by Boakye *et al.* in 1999 to identify human blood meals from black flies. Several (animal) species, like sheep, horse, dog, cow, pig, goat and also human, were expected to be found in the bloodmeals taken by the collected *Anopheles* mosquitoes.

The universal reverse and the animal-specific forward PCR primers for these animal species were selected from the study by Kent & Norris (2005) and the ones not used in their study were developed with Vector NTI database, using alignments of the cytochrome B gene. Specific primers (5'-3') shown in table 2.3 were all used in PCR mixtures for the amplification of our extracted DNA samples.

Table 2.3. Primer configuration of prospective host animals of blood feeding mosquitoes

Goat (894F)	5' CCTAATCTTAGTACTTGTACCCTTCCTC 3'	132	bp	productsize
Human (741F)	5' GGCTTACTTCTCTTCATTCTCTCCT 3'	334	bp	productsize
Pig (573F)	5' CCTCGCAGCCGTCATCTC 3'	453	bp	productsize
Cow (121F)	5' CATCGGCACAAATTTAGTCG 3'	561	bp	productsize
Dog (368F)	5' GGAATTGTACTATTATTCGCAACCAT 3'	680	bp	productsize
Sheep	5' GCATTTGCTTAATTTTACAGATTC 3 '	912	bp	productsize
Horse	5' CCCACTAATTAAAATCATCAATC 3 '	1000	bp	productsize
Reverse (1025)	5' GGTTGTCCTCCAATTCATGTTA 3'	-		

To prevent cross annealing with non-target templates, the animal specific forward primers were designed with at least 5-13 nucleotide differences from homologous regions in the other animal sequences. The expected PCR products were designed to differ in size (approximately 100 bases) at the agarose gel.

The PCR reaction mixture contained 5 µl 5x Green Buffer, 1.5 µl 25mM $MgCl_2$, 0.5 µl dNTP's, 2 µl 25pmol/ µl universal reverse primer, 2 µl 25pmol/ µl forward primer goat, 2 µl 25pmol/ µl forward primer human, 2 µl 25pmol/ µl forward primer pig, 2 µl 25pmol/µl forward primer cow, 2 µl 25pmol/ µl forward primer dog, 2 µl 25pmol/µl forward primer sheep, 2 µl 25pmol/µl forward primer horse, 0.125 µl GoTaq polymerase, 4.875 µl ddH_2O and 1 µl DNA extraction product from the abdomen of the mosquito, resulting in 25 µl/PCR tube.

After amplification in a PCR machine, using a specific program (5 minutes at 95°C, followed by 35 cycles at 95°C for 1 minute, 50°C for 1 minute and 72°C for 1.5 minute, the final extension step at 72° for 7 minutes), the PCR products were visualized on an 1 % agarose gel with ethidium bromide.

DNA samples of the different species (goat, sheep, horse, cow, human, dog and pig), were used as positive controls. These samples were collected by DNA extraction from meat bought at the butcher (for sheep, cow and pig), from a tube of blood received from a veterinarian (goat) and from hairs (human, horse and dog).

3. Results

3.1 Tests

Several tests were carried out before the collection of the mosquitoes could start. One test was performed to find out if the mosquitoes' blood could still be used for blood meal analysis when they were stored in 70% ethanol, in -20 ˚C freezer or in silica gel. The results of bloodmeal analysis with these storage methods were compared to bloodmeal analysis using fresh mosquitoes. It was useful to have some information about this before the mosquitoes were collected and stored. For these experiments, mosquitoes (with blood from humans and cows) from the mosquito cultures at the Laboratory of Entomology were used and for each storage method several mosquitoes were used. From these tests could be concluded that there is no difference in results on bloodmeal analysis when mosquitoes are stored in ethanol, at -20 ˚C or when fresh mosquitoes are used. Blood meals from mosquitoes that were stored on silica gel after feeding did not give any result. The results of these tests are shown in table 3.1.

Table 3.1. Results of blood meal identification following different methods of storage after the blood meal

Storage method	Human blood	Cow blood
Fresh mosquitoes	+	+
In Ethanol	+	+
at -20˚C	+	+
In Silica gel	-	-

Another test was performed to check whether the protocol and primers ordered for the bloodmeal analysis worked and if multiple blood meals from different animals taken by one mosquito could be visualized on the gel. For these tests different positive controls were used. From these tests it was concluded that most primers, except those of birds, and the protocol worked well. Unfortunately not all primers could be combined in one mixture; goat, human, pig, cow and dog could be combined, but sheep and horse could not be combined with the other five. This resulted in a new protocol where two PCR reaction mixtures were made per mosquito sample. Figure 3.2 shows the results of the test to check whether the

protocol worked with the positive controls. From these tests could also be concluded that multiple blood meals taken by one mosquito could be visualized on one gel.

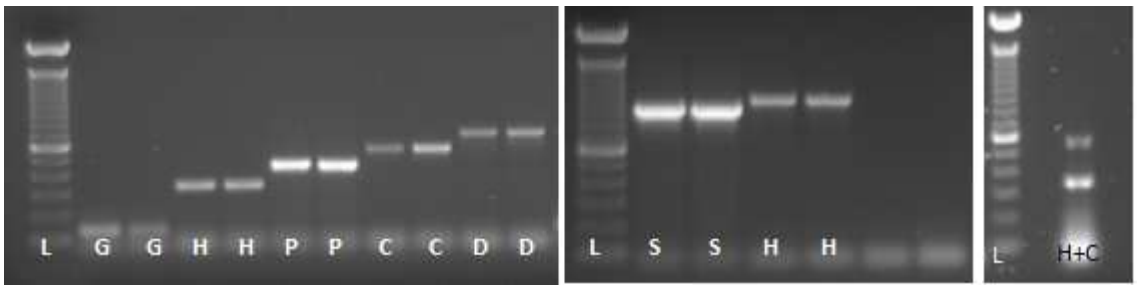

Figure 3.2. Result of the test to check if the protocol worked with the positive controls. From left to right: DNA Ladder, 2x goat, 2x human, 2x pig, 2x cow, 2x dog, DNA Ladder, 2x sheep and 2x horse, DNA ladder, mixture of cow and human in one sample.

3.2 Mosquito collections from several locations in the Netherlands

The numbers of mosquitoes collected varied much per location and collection date, as indicated in table 3.3. Next to the *Anophelines*, two other haematophagous mosquito species, *Culiseta annulata* and *Culex pipiens* and a few flies were collected. Collection date does not seem to influence the amount of collected specimens. In total 91 specimens of *Anopheles maculipennis s.l* and 68 specimens of *Anopheles plumbeus* were collected. Furthermore 60 specimens of *Culiseta annulata*, 9 specimens of *Culex pipiens* and 152 flies were collected. From the two haematophagous mosquito species the bloodmeals were analyzed as well.

Table 3.3. Mosquito collections from several locations in the Netherlands

Location	Collection	A. maculipennis s.l	A. plumbeus	Culiseta annulata	Culex pipiens	Anisopodidae	Chironomydae	Tipulidae	Piophilidae	Total
1	March	0	0	7	0	4	0	0	19	30
	April	4	0	2	6	10	1	4	6	33
2	March	6	0	13	1	27	0	7	6	60
	April	7	0	13	0	9	0	1	4	34
3	March	5	0	11	0	2	9	1	4	32
	April	69	0	14	0	1	36	0	1	121
4	June	0	68	0	2	0	0	0	0	70
	Total	91	68	60	9	53	46	13	40	**380**

3.3 Species composition of A. maculipennis s.l at three different locations

The majority of *A. maculipennis s.l*. collected were identified as *A. messeae*. As can be seen in table 3.4, very few *A. atroparvus* and *A. maculipennis sensu stricto* were collected. Two specimens of *A. atroparvus* were found at a farm in the western part of the Netherlands. Only one specimen of *A. maculipennis sensu stricto* was found in the eastern part of the Netherlands. *A. messeae*, with 88 specimens, was the most regularly found mosquito at each farm.

Table 3.4. Species composition at three different locations

Location	***A. messeae***	***A. atroparvus***	***A. maculipennis sensu stricto***
1	3	0	1
2	14	0	0
3	71	2	0

The pictures of the agarose gels taken following a PCR procedure to determine the species composition of the collected *Anopheles* mosquitoes, gave mostly similar results. As also can be seen in figure 3.5, that *A. messeae* was without doubt the most common species.

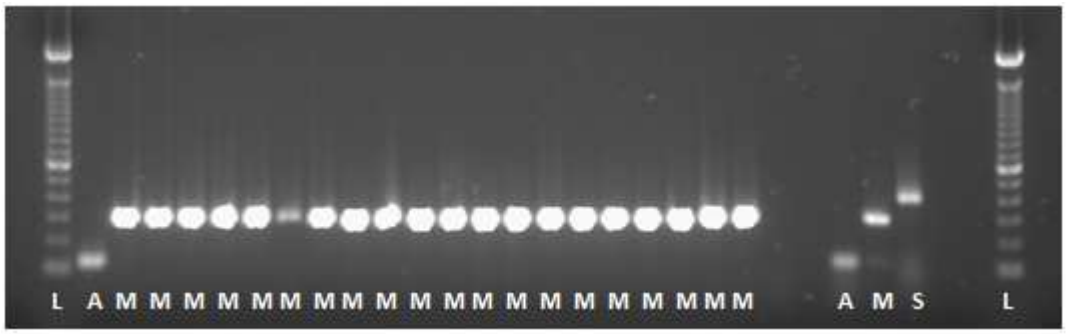

Figure 3.5. Species determination on this gel with one sample *A. atroparvus (A)*, other samples *A. messeae (M)* and three positive controls (*Atroparvus(A), Messeae(M) and Maculipennis sensu stricto(S)*). The samples on this gel were collected in the western part of the Netherlands.

3.4 Distinguishing A. messeae and A. daciae

All samples previously defined as *A. messeae* were used in another PCR and digestion experiment to distinguish *A. messeae* from its subspecies *A. daciae*. The pictures of the gels all showed the same result as indicated in figure 3.6 below.

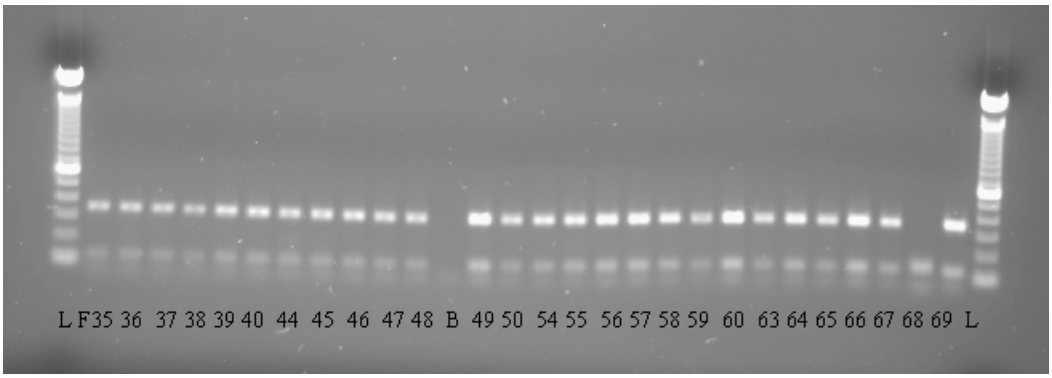

Figure 3.6: Distinguishing (sub)species *A. messeae* and *A. daciae* on this agarose gel after PCR and digestion. All samples (except for sample 68, which was apparently not totally digested) show two bands, indicating *A. messeae*.

All samples previously defined as *A. messeae*, still seemed to be *A. messeae* after a different PCR and digestion experiment, specifically designed to distinguish between A. messeae and A. daciae (Nicolescu et al, 2004). Only one high band (332bp) would indicate the presence of *A. daciae.* All samples showed 2 bands (109 and 332 bp), indicating *A. messeae*. Number 68 in figure 3.6 shows only one low (109) band, which could indicate another *Anopheles* species then *messeae* or *daciae*, but in previous experiments with DNA extract and leg/wing this sample was defined to be *Anopheles messeae*.

3.5 Bloodmeal analysis of anophelines in the Netherlands

Blood meals taken by the collected haematophagous mosquitoes were analyzed using the multiplex PCR with primers of animal species which are suitable hosts of the mosquitoes. As shown in Table 3.7 some animals are more preferred than others. *Anopheles messeae* seems to prefer sheep, cattle, humans and dogs, *A. plumbeus* seems to be attracted to sheep, humans and dogs and *Culiseta annulata* seems to have a preference for sheep and cattle. From Culex *pipiens, Anopheles maculipennis s.s* and *Anopheles atroparvus* only a few specimens were collected and with the analysis of their bloodmeals no good results were generated.

Table 3.7: Animal origin of blood meals taken by the different haematophagous mosquitoes.

Mosquito Species (#)	Sheep	Horse	Human	Cow	Goat	Dog	Pig	No result
Culiseta annulata(60)	18	1	0	18	2	0	1	29
Culex pipiens (9)	0	0	1	1	0	0	2	6
Anopheles messeae (88)	34	1	5	17	0	10	0	43
Anopheles maculipennis s.s (1)	0	0	0	0	0	0	0	1
Anopheles atroparvus (2)	0	1	0	0	0	0	0	1
Anopheles plumbeus (68)	39	0	6	0	0	6	0	23

Figure 3.8 and 3.9 are examples of pictures of agarose gels taken after multiplex PCR, not with many but with quite clear results. Figure 3.8 shows the results after a PCR, performed with five primers for goat, human, pig, cow and dog. Figure 3.9 shows the results after a PCR of the same samples as used for figure 3.8, now performed with two primers for sheep and horse.

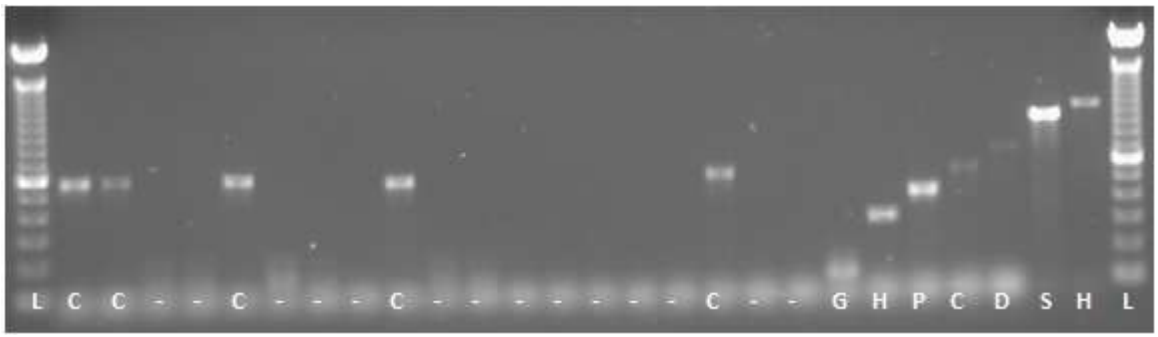

Figure 3.8: Example of a picture of an agarose gel after multiplex PCR with primers for Goat, Human, Pig, Cow and Dog. The seven sample on the right are the positive controls (Goat, Human, Pig, Cow, Dog, Sheep, Horse). The five visible samples were identified as cow(C).

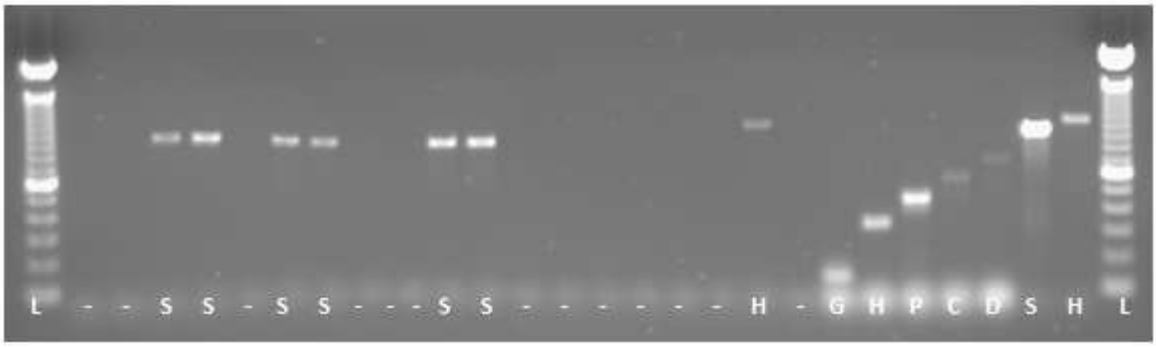

Figure 3.9: Example of a picture of an agarose gel after multiplex PCR with primers for Sheep and Horse. The seven samples on the right are again the positive controls (Goat, Human, Pig, Cow, Dog, Sheep, Horse). The seven positive samples were identified as sheep(S) and horse(H).

3.6 Anopheles plumbeus: an unusual vector near the river

After determination under the stereomicroscope in the laboratory, most (68 of 70) anopheline mosquitoes collected in Lent turned to be *A. plumbeus*, a species not very common in the Netherlands. As can be seen in table 3.10, the specimens were collected at several points around the residents' houses, like the pond, shed of the café and the garden. Three different mosquito species were collected, but *A. plumbeus* appeared to be the most abundant. This species does not belong to the *A. maculipennis* complex and therefore the species identification could not be performed using the PCR used in this study . However, the blood meals these *A. plumbeus* mosquitoes took, could be analyzed using the multiplex PCR again. The results (shown in table 3.7) of the analysis of the blood meals of *A. plumbeus* indicate a preference for sheep, humans and dogs.

Table 3.10. Mosquito collections from several locations around people's houses in Lent

Location	***A. plumbeus***	***Culex pipiens***	***Anisopodidae***
Pond	0	1	0
Shed of the cafe	37	1	0
Garage/Garden	12	0	0
Taxus hedge	19	0	3

A specific study about this *Anopheles plumbeus* plague in Lent has been performed by Frans Jacobs. In this research, the mosquito population is monitored in space and time, to find the centre of the pest, their oviposition places and eventually stop the plague that's bothering the local residents of Lent.

4. Discussion

The selection of farms suitable for the collection of mosquitoes was made while driving through the three selected areas, resulting in different numbers of farms per location. These differences are probably caused by the variation in time spent on each farm. On the farms in the western part of the Netherlands (two farms), many mosquitoes were visible on the walls resulting in a long time spent at the farm. On the farms in the eastern part of the Netherlands (six farms), not a lot of mosquitoes were collected resulting in a shorter period of time spent at the farms. Nowadays, farmers are more specialized to one type of animal, making it more difficult to find animal shelters with a mixture of various species. Also, the animal shelters used these days are open, clean and not made of wood, which are all characteristically not much preferred by the *Anopheles* mosquitoes. The date of collection (March or April) does not seem to influence the number of mosquitoes found. At some locations more mosquitoes were collected in March and at other locations more mosquitoes were collected in April.

The mosquitoes were mainly situated on the walls of the animal shelters, making it not difficult to catch them with the oral plastic-tube aspirator. The collected mosquitoes were transferred to bottles containing 70% ethanol, which preserves them well until processing in the laboratory. All mosquitoes and flies could be identified under the stereo-microscope and on the ones identified as *Anopheles*, a PCR of the ITS2 region was performed to distinguish the three different species. Per sample, the PCR was performed twice; once with a leg or wing and once with a DNA extraction product of the entire mosquito, to check for mistakes, contamination and failure of the PCR. This PCR for species identification gave results on all collected samples.

A. atroparvus was, according to previous studies of Jetten & Takken (1994) and Swellengrebel and de Buck (1938), expected to occur more frequently in the western part of the Netherlands, since this species of the *A. maculipennis* complex prefers to deposit its eggs in brackish water. Two specimens were found at a farm in the western part of the Netherlands. *A. maculipennis sensu stricto* was expected to occur in the eastern part of the Netherlands, but only one specimen was found.

A. messeae was expected to occur all over the Netherlands and was indeed with 88 specimens the most regularly found mosquito. The differences in number of collected species are very large and this could indicate that the geographic distribution of *A. atroparvus* and *A. maculipennis s.s* has diminished in this area. Also some Culex pipiens and Culiseta annulata were collected, especially the former is interesting because of its' capability of transmitting the West Nile virus (Hamer *et al*, 2008).

From the new PCR and digestion experiment with the mosquitoes previously defined as *A. messeae* could be concluded that *A. daciae* was not present in the samples collected on the three locations in the Netherlands. From a study performed by Linton *et al* (unpublished data) it was known that with digestion by the enzyme BSTU1, *A. daciae* could be distinguished from *A. messeae*, viewed on an agarose gel by different bands. All my samples showed two bands indicating *A. messeae*, but there was no positive control of *A. daciae* available. Assuming that the test worked well, there are indeed no *A. daciae* present in the collected samples.

Nowadays, there are several methods available to identify the source of vertebrate blood in the intestinal tracts of mosquitoes. Some examples that were widely used in the past are the precipitin method, agar gel diffusion test and ELISA (Washino and Tempelis, 1983). These methods require the preparation of sera against blood of each expected host species and pre-adsorption steps are necessary to eliminate cross-reactions (Haouas *et al*, 2007). Furthermore, these methods are not specific enough to distinguish between meals taken from members of closely related host species (Oshagi *et al*, 2005). PCR, which is a more recently discovered molecular-based assay, has several advantages over the older methods to identify the animal species in the bloodmeals. It increases the efficacy and reliability of bloodmeal identifications, detecting also very small blood meals. Because of these advantages was chosen to use PCR of the mitochondrial cytochrome B gene for the detection of the animal species in the bloodmeals of the mosquitoes.

The bloodmeals of all collected mosquitoes were analyzed using a PCR with multiple primers. Unfortunately not all the selected primers could be combined in one reaction mixture; the primers for goat, human, pig, cow and dog could be used together, but sheep and horse could not be combined with any of the others. Possibly, cross-annealing of the designed primers was not successfully prevented. This resulted in two different PCR mixtures for each sample, which is not quite optimal. Unfortunately not all samples gave good results after blood meals were analyzed using the PCR test and some of the bands could not be visualized on the agarose gel. An explanation for this result could be that there was not enough blood present in the mosquito, but given the advantage that PCR can even detect the smallest blood meals, this might not be the correct explanation. Another explanation could be that the mosquito took a blood meal of an unexpected animal of which there were no primers in the reaction mixture or the simple fact that something went wrong during the PCR .

Many samples gave good results. The pictures of the agarose gels, taken after the multiplex PCR of the bloodmeals, could be analyzed without a lot of problems. Mostly, when the size specific bands were visible on the agarose gel, they were characteristic enough to determine which species occurred in the bloodmeal. The specimens from this study identified as *Anopheles messeae* seem to prefer sheep, cattle, humans and dogs, *A. plumbeus* seems to be attracted to sheep, humans and dogs and *Culiseta annulata* seems to have a preference for sheep and cattle. From Culex *pipiens, Anopheles maculipennis s.s* and *Anopheles atroparvus* only a few specimens were collected and with the analysis of their blood meals no good results were generated. The universal preference for cattle was predictable (from the results of Takken *et al*, 2002), but the preference for sheep was not expected, because of the thick fur of the animal. As expected, a relationship can be seen between the animal species present on the farm or in the neighbourhood of the farm and the animal species found in the bloodmeals taken by the mosquitoes. For example, on a farm in the middle of the country (farm 4 at location 2), hundred cows and eighty sheep were present. Analysis of the blood meals taken by the mosquitoes collected at this farm, revealed a host-preference for sheep and cow. This kind of relationship between animal species present at the farm and animal species found in the blood meal, was seen on most farms.

Surprisingly most of the mosquitoes collected in Lent were recognized as *Anopheles plumbeus*, a rarely seen mosquito in the Netherlands. This species is hibernating as a larva, it preferably breeds in water-filled holes of trees or in old car-tires and is capable of transmission of the malaria parasite *P. falciparum (*Krüger *et al,* 2001*)*. The female *A. plumbeus* mosquitoes are biting during daytime as well as in the evening, explaining the unusual aggressiveness of the mosquitoes when they were collected in the morning. Most mosquitoes were collected in the shed of the pub where rainwater was stored, it seemed to be an excellent breeding site for these mosquitoes. Against expectations, no *A. plumbeus* mosquitoes and larvae were found near the pond, which periodically flooded with water from the river Waal. Analysis of the mosquitoes' bloodmeals indicated a host-preference for sheep, humans and dogs. The presence of human blood in the mosquito can be explained by the fact that the mosquitoes were very hungry and aggressive, which resulted in biting humans. The presence of blood from sheep and dog in the collected mosquitoes could be explained by the presence of these animals in the area.

5.Conclusion

In this study, the main goal was to investigate if the three members of the *Anopheles maculipennis* complex are potential vectors of malaria in the Netherlands. The species composition of adult *Anopheles* mosquitoes was established by collecting mosquitoes from farms from several regions in the Netherlands and their host preference was determined by analyzing the blood meals these mosquitoes took. Using PCR of the ITS2 region, the collected mosquitoes were identified molecularly, resulting in 88 specimens of *A. messeae*, 1 specimen of *A. maculipennis s.s* and 2 specimens of *A. atroparvus*. *A. daciae* was not identified among the *A. messeae* specimens. Using the recently discovered PCR of the mitochondrial cytochrome B gene, the blood meals taken by these collected haematophagous mosquitoes were analyzed resulting in a preference for sheep and cattle for *Anopheles messeae*, a preference for sheep, humans and dogs for *A. plumbeus* and a preference for sheep and cattle for *Culiseta annulata*. From Culex *pipiens, Anopheles maculipennis s.s* and *Anopheles atroparvus* only a few specimens were collected and the analysis of their blood meals gave no results. In general the host-preference of most mosquitoes found, seemed to be sheep and cattle. Furthermore a relationship can be seen between the presence of certain animal species on a farm and animal species found in the blood meals of the mosquitoes collected at this farm. Unfortunately not all samples gave satisfactory results on the blood meal analysis, making it difficult to draw conclusions on the host preference of the three mosquito species of the *Anopheles maculipennis* complex in the Netherlands.

Acknowledgements

I would like to thank several people for all the help during my minor thesis. I would like to thank Frans for all his help on the farms and his explanations in the laboratory. I would like to thank Willem Takken for his supervision and valuable comments. I also would like to thank Patrick for his support and patience in the laboratory. Thanks to all employees of the department for the great working environment and the nice time I had at Entomology.

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Protocol 1: DNA extraction (tissue protocol Qia Amp DNA mini kit)

1. Cut up to 25 mg of tissue (up to 10 mg spleen) into small pieces. Place in a 1.5 ml microcentrifuge tube, and add 180 µl of Buffer ATL.

2. Add 20 µl proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.

3. Briefly centrifuge the 1.5 ml micro-centrifuge tube to remove drops from the inside of the lid.

4. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml micro-centrifuge tube to remove drops from inside the lid.

5.Add 200 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml micro-centrifuge tube to remove drops from inside the lid.

6.Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.*

7. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.*

8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

9. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

10. Place the QIAamp Mini spin column in a clean 1.5 ml micro-centrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

11. Repeat step 10

Protocol 2: PCR-protocol for identification of the different species of *Anopheles maculipennis s.l* using mosquito DNA extracts/leg

1.Check if sufficient dNTP and primer solutions are present. Make new solutions from stock if necessary: Un/mes/atr/macu: 1 µl stock + 3 µl ddH_2O

Preparing samples

2.Defrost reagents and primers on ice. Keep GoTaq polymerase in freezer.
3.Make the PCR mix (per sample: 5 µl 5x Green Buffer, 1.5 µl 25 mM $MgCl_2$, 0.5 µl dNTP's, 0.5 µl 25pmol/ µl *A. maculipennis sensu stricto* reverse primer, 0.5 µl 25pmol/ µl *A. atroparvus* reverse primer, 0.5 µl 25pmol/ µl *A. messeae* reverse primer, 0.5 µl 25pmol/ µl universal forward primer, 0.125 µl GoTaq Polymerase and 15.875 µl ddH_2O resulting in 25 µl/PCR tube). Prepare enough PCR-mix for the samples, positive and negative controls.
4.After adding the GoTaq polymerase, mix PCR-mix thoroughly with a vortex.
5.Label 100 µl Eppendorf tubes and add 22 µl (with DNA extract)/25 µl (with leg/wing) to each tube
6.Mix well by pipetting up and down several times.
7.PCR-machine, program (4 minutes at 94 °C, followed by 35 cycles at 94 °C for 1 minute, 50 °C for 1 minute and 72 °C for 2 minutes, the final extension step at 72 °C for 10 minutes).

Preparing the gel

8.Prepare a 1.0% gel by adding 100ml TAE buffer to 1.0 gram agarose in an Erlenmeyer. Place in a microwave for 2 minutes to dissolve the agarose.
9.Add 3.5 µl ethidium bromide and mix well. Be careful: Ethidium bromide is mutagenic.
10.Place gel combs in a tray and pour the gel in gently. Remove air bubbles with a pipette tip.
11.Leave the gel to dry for approximately 30 minutes.

Gel electrophoresis

12.Take gel combs out of the gel and place the gel in an electrophoresis tray filled with TAE buffer (3.5 µl ethidium bromide added to each 100 ml TAE buffer). Make sure the slots are the negative (black pole). The DNA samples will run towards the positive pole.
13.Take a piece of parafilm and put 4 µl droplets of loading buffer on it for each sample, when you do not have a green coloured buffer.
14.Take 10 µl of the sample and mix it well with the loading buffer with a pipette. Pipette this mixture in slots of the gel.
15.Pipette 4 µl of a DNA ladder in the first and last slot.
16.Switch on machine on 80 V and leave to run for 50 min.

Photographing the gel

17.Take the gel out of the electrophoresis tray and make a picture using UV light and the system in the molecular lab.

Protocol 3: Protocol for PCR and digestion to distinguish *A. messeae* from *A. daciae*

1.Check if sufficient dNTP and primer solutions are present. Make new solutions from stock if necessary: forward and reverse primer: 1 µl stock + 3 µl ddH_2O
ITS2 5.8s(Long): 5'- ATC ACT CGG CTC GTG GAT CG- 3'
ITS2 28S: 5'- ATG CTT AAA TTT AGG GGG TAG TC -3'

Preparing samples
2.Defrost reagents and primers on ice. Keep GoTaq polymerase in freezer.
3.Make the PCR mix (per sample: 10 µl 5x Green Buffer, 5 µl 25 mM $MgCl_2$, 0.5 µl dNTP's, 5 µl 5 µm Forward primer, 5 µl 5 µm Reverse primer, 0.1 µl GoTaq Polymerase and 22.4 µl ddH_2O resulting in 50 µl/PCR tube). Prepare enough PCR-mix for the samples, positive and negative controls.
4.After adding the GoTaq polymerase, mix PCR-mix thoroughly with a vortex.
5.Label 100 µl Eppendorf tubes and add 48 µl PCR-mixture and 2 µl of DNA extraction product to each tube.
6.Mix well by pipetting up and down several times.
7.PCR-machine, program (2 minutes at 94 °C, followed by 35 cycles at 94 °C for 30 seconds, 57 °C for 1 minute and 72 °C for 30 seconds, the final extension step at 72 °C for 10 minutes).

Digestion
8. Defrost enzyme and buffer on ice.
9. Make the digestion mix (per sample: 2 µl 10xbuffer 2, 0.5 µl BSTU1 enzyme and 13.5 µl ddH_2O). Prepare enough digestion-mix for the samples and negative controls.
10. Mix thoroughly with a vortex.
11. Label 100 µl Eppendorf tubes and add 16 µl digestion-mixture and 4 µl of DNA extraction product to each tube.
12. Mix well by pipetting up and down several times.
13. Incubate at 60 °C for three hours.

Preparing the gel
14.Prepare a 1.0% gel by adding 100ml TAE buffer to 1.0 gram agarose in an Erlenmeyer. Place in a microwave for 2 minutes to dissolve the agarose.
15.Add 3.5 µl ethidium bromide and mix well. Be careful: Ethidium bromide is mutagenic.
16.Place gel combs in a tray and pour the gel in gently. Remove air bubbles with a pipette tip.
17.Leave the gel to dry for approximately 30 minutes.

Gel electrophoresis
18.Take gel combs out of the gel and place the gel in an electrophoresis tray filled with TAE buffer (3.5 µl ethidium bromide added to each 100 ml TAE buffer). Make sure the slots are the negative (black pole). The DNA samples will run towards the positive pole.
19.Take a piece of parafilm and put 4 µl droplets of loading buffer on it for each sample, when you do not have a green coloured buffer.
20.Take 8 µl of the sample and mix it well with the loading buffer with a pipette. Pipette this mixture in slots of the gel.
21.Pipette 4 µl of a DNA ladder in the first and last slot.
22.Switch on machine on 80 V and leave to run for 50 min.

Photographing the gel

23.Take the gel out of the electrophoresis tray and make a picture using UV light and the system in the molecular lab.

Protocol 4: PCR protocol for bloodmeal analysis by cytochrome B gene

1.Check if sufficient dNTP and primer solutions are present. Make new solutions from stock if necessary: 1 µl stock + 3 µl ddH_2O

Preparing samples

2.Defrost reagents and primers on ice. Keep GoTaq polymerase in freezer.

3.Make PCR mix 1: per sample: 5 µl 5x Green Buffer, 1.5 µl 25 mM $MgCl_2$, 0.5 µl dNTP's, 2 µl primer goat, 2 µl primer pig, 2 µl primer cow, 2 µl primer human, 2 µl primer dog, 2 µl primer reverse, 0.125 µl GoTaq Polymerase and 4.875 µl ddH_2O resulting in 25 µl/PCR tube.

Make PCR mix 2: per sample: 5 µl 5x Green Buffer, 1.5 µl 25 mM $MgCl_2$, 0.5 µl dNTP's, 2 µl primer sheep, 2 µl primer horse, 2 µl primer reverse, 2 µl primer reverse, 0.125 µl GoTaq Polymerase and 10.875 µl ddH_2O resulting in 25 µl/PCR tube.

Prepare enough PCR-mix for the samples, positive and negative controls.

4.After adding the GoTaq polymerase, mix PCR-mix thoroughly with a vortex.

5.Label 100 µl Eppendorf tubes and add 24 µl PCR mix 1 or 2 to each tube and add 1 µl DNA extract.

6.Mix well by pipetting up and down several times.

7.PCR-machine, program (2 minutes at 95 °C, followed by 35 cycles at 95 °C for 1 minute, 50 °C for 1 minute and 72 °C for 1 minutes, the final extension step at 72 °C for 7 minutes).

Preparing the gel

8.Prepare a 1.0% gel by adding 100ml TAE buffer to 1.0 gram agarose in an Erlenmeyer. Place in a microwave for 2 minutes to dissolve the agarose.

9.Add 3.5 µl ethidium bromide and mix well. Be careful: Ethidium bromide is mutagenic.

10.Place gel combs in a tray and pour the gel in gently. Remove air bubbles with a pipette tip.

11.Leave the gel to dry for approximately 30 minutes.

Gel electrophoresis

12.Take gel combs out of the gel and place the gel in an electrophoresis tray filled with TAE buffer (3.5 µl ethidium bromide added to each 100 ml TAE buffer). Make sure the slots are the negative (black pole). The DNA samples will run towards the positive pole.

13.Take a piece of parafilm and put 4 µl droplets of loading buffer on it for each sample, when you do not have a green coloured buffer.

14.Take 10 µl of the sample and mix it well with the loading buffer with a pipette. Pipette this mixture in slots of the gel.

15.Pipette 4 µl of a DNA ladder in the first and last slot.

16.Switch on machine on 80 V and leave to run for 50 min.

Photographing the gel

17.Take the gel out of the electrophoresis tray and make a picture using UV light and the system in the molecular lab.