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Circadian rhythm and olfactory choices of *Culicoides* spp. in The Netherlands

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Abstract

Culicoides species (Diptera, Ceratopogonidae), or biting midges are an important vector of livestock viruses, such as blue tongue virus and African horse sickness virus. Besides, midges are identified as one of the main causes of allergic dermatitis, or sweet itch in horses.

Only little is known about *Culicoides* species in the Netherlands. Insight into the biology and ecology of these species could contribute to a better understanding of the spatial distribution of diseases vectored or caused by midges. Moreover, it can contribute to better control strategies.

The circadian rhythm and olfactory responses of *Culicoides* species in the Netherlands has been studied. Within both of the studies attention is also paid to the effect of some meteorological conditions on the catch size, on the effect of trap location on the catch size, rough comparisons between the efficacy of the Onderstepoort light trap and the Liberty Plus traps were made and the density and diversity of *Culicoides* species on the two test locations have been compared.

Results of the study on circadian rhythm show that there is a circadian rhythm in host-seeking activity of *Culicoides* species in the Netherlands. Most species were captured between 5pm and 11pm (41%). However, >25% of the species were captured during daylight hours. Of the seven different species found, *C. obsoletus* was the most common species, followed by *C. dewulfi* and *C. festivipennis*. Non of the species captured showed an unambiguous preference or aversion for one of the time periods. This study shows clear evidence of *Culicoides* diurnal activity.

Results of the study on olfactory responses show that there is a clear effect of the addition of 1-octen-3-ol to carbon dioxide as a bait for midges. Significantly higher catches were obtained with a combined bait of carbon dioxide and octenol, when compared to carbon dioxide alone, a combination of CO_2 and horse extract, or a combination of CO_2 and cattle extract. Between the other three traps no differences were found. Of the seven different species captured, *C. obsoletus* was the most abundant species found, followed by *C. dewulfi* and *C. festivipennis.*

No significant effect of meteorological conditions on the catch size was found, except for the period between 11am and 5pm. A significant negative correlation was found between wind speed and the number of captured midges in this period. Combining wind speed and saturation deficit classified correctly 74% of the observation.

No significant effect of trap location on the catch size was found.

Rough comparisons between the efficacy of the Onderstepoort light trap and the Liberty Plus traps as a sampling tool for midges gave contradictory results.

Comparisons on *Culiciodes* species density and diversity between the two test locations suggest similarity on diversity, but inequality on *Culicoides* abundance.

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1. Introduction

1.1 The importance of *Culicoides* species

Culicoides are small biting midges belonging to the family *Ceratopogonidae*. World wide, more than 1400 species have been identified (Mellor et al., 2000). In Europe about 700 species can be found, of which 130 species are obligate blood suckers attacking mammals (including human) and birds (Ducro, 2004).

The Netherlands contain about 25 different species. Van der Heijden (2007) describes how only two studies have investigated the presence of *Culicoides* species in the Netherlands, namely by Tatem et al. (2003) and by the Ministry of Agriculture (2006). In these studies the following species were found; *C. obsoletus; C. impunctatus; C. pulicaris; C. minutissimum; C. dewulfi*. Most individuals caught belong to the first two groups, suggesting that *C. impunctatus* and *C. obsoletus* are the most abundant *Culidoides* species in the Netherlands. However, Van der Heijden (2007) notes that only limited habitats are sampled in these studies. This might have influenced the results.

In a recent study by Takken et al. (2008), 15 *Culicoides* spp. are reported. The study aimed at the presence and distribution of *Culicoides* spp. in the Netherlands, focussing on wetland area, peat areas, floodplain areas and livestock farms. Eighty-eight percent of all *Culicoides* species consisted of the taxon *C. obsoletus* / *C. scoticus*.

For many years, in the Netherlands *Culicoides* spp. achieve their importance as one of the main causes of allergic dermatitis, or sweet itch in horses. Most studies on northern European midges are related to horses and sweet itch. Also this study was conducted at two horse farms, aiming at the problem of *Culicoides* hypersensitivity in horses. Only recently midges have received an increasing attention as a vector of livestock viruses such as bluetongue. It is expected that the importance of midges as a vector of viruses will increase (Conte et al., 2007; Mellor et al., 2000).

1.1.1 *Culicoides* as a cause of allergic dermatitis

Culicoides spp. are identified as being one of the main causes of allergic dermatitis, or sweet itch in horses. Sweet itch is caused by a hypersensitivity reaction to allergens in the saliva of *Culicoides* spp. and results from an over-vigorous response by the animal's immune system (The British Horse Society Welfare Department, 2005). Sweet itch is seen as a serious problem that affects thousands of horses and ponies across the world. Also in The Netherlands it is seen as a severe dilemma and the subject receives increasing attention.

Not all *Culicoides* species has been reported to cause allergic dermatitis. Ducro (2004) states that about five to ten *Culicoides* species in the Netherlands are able to cause an allergic reaction. Several studies identify *C. obsoletus* and *C. pulicaris* as being the most important species with respect to sweet itch (Anderson et al., 1991).

1.1.2 *Culicoides* as a vector of viruses

Culicoides spp. are able to vector several viruses of both human and animal. Especially as a vector of arbo-viruses of domestic livestock they achieve their importance (Mellor et al., 2000). Mellor et al. (2000) have listed the following *Culicoides*-borne viruses as being the eight most important; Oropouche virus; African Horse Sickness virus (AHSV); Bluetongue virus (BTV); Epizootic Hemorrhagic Disease virus; Palyam virus; Equine Encephalosis virus; Bovine Ephemeral Fever Virus; Akabane virus. These viruses mainly occur in tropic climates, since temperature is a limiting factor (Van der Heijden, 2007). However, in the summer of 2006 the south of the Netherlands was struck with an outbreak of the Bluetongue virus. This virus formerly only occurred in Europe around the Mediterranean Sea (Van der Heijden, 2007; Purse et al., 2005).

C. imicola is considered to be the most important vector of BTV and AHSV in Europe (Mellor, 1996). For so far, this species doesn't occur in Northern Europe, including the Netherlands. However, BTV has also been isolated from *C. obsoletus*, whereas AHSV has been isolated form both *C. obsoletus* and *C. pulicaris* (Conte et al., 2007). These species are very abundant in Europe, including the Netherlands. In The Netherlands BTV is found in *C. dewulfi* (Meiswinkel et al., 2008) and *C. chiopterus* (Dijkstra et al., 2008).

Literature point at the impact of climate change on the spread of diseases vectored by insects such as *Culicoides* spp. (Conte et al., 2007; Mellor et al., 2000; Wittman and Baylis, 2000; Van der Heijden, 2007). Conte et al. (2007) states that global warming might induce northward movement of potential vectors, as well as increase vectorial capacity.

1.2 The biology of *Culicoides* species

1.2.1 Life cycle

The lifecycle of *Culicoides* includes egg, four larval stages, pupa and adult. The duration of the life cycle depends on the species and climate conditions (Carpenter et al., 2006). The length varies from as little as seven days (tropical climate) to almost one year when larvae diapause during winter (Van der Heijden, 2007).

Except for a view species, female midges require a blood meal before maturing eggs. *C. impunctatus* is one of the autogenous species, producing a large egg batch in her first host-free period of ovigenesis. However, from the second ovigenesis the female requires a blood-meal (Carpenter et al., 2008). In general oviposition occurs within three to five days after feeding. The number of eggs per batch is regularly less than one hundred, but is highly variable with the size and quality of the blood meal. The eggs are banana-shaped and about 400 µm long and 50 µm wide. They are white when laid but darken rapidly. Except for the species who enter diapause, the eggs are not resistant to drying and usually hatch in two to seven days (Mellor et al., 2000).

The larval stage is the longest in the life cycle and can take up to several weeks, or even months when fourth-instar larvae diapause. However, the exact duration is highly dependent on species and ambient temperature. At higher temperatures, the duration of the larval stage decreases. Under favourable conditions the four larval stages can take as little as four to five days. Larvae have a worm-like body, are creamy white, and approximately two to five mm long. Larvae of *Culicoides* spp. are found in moist soils, including pools and streams, tree holes, saturated soils and mud, but also in animal dung and rotting fruit. *Culiciodes* larvae mostly feed on particles of vegetable matter. Some species are predaceous and feed on small organisms such as nematodes, protozoa and small anthropods.

Pupae are free floating or only loosely attached to the substrate. This stage is usually very brief, but does also depend on temperature and species. Generally this stage takes two to three days (Mellor et al, 2000).

The lifespan of an adult *Culicoides* species is highly dependent on ambient temperature, but usually short. Most adults survive less than three weeks, although sporadically their longevity can take up to 90 days (Mellor et al., 2000). Adults are most abundant near productive breeding sites, but will disperse to mate and to feed. The male peak density occurs approximately four weeks prior to that of females (Carpenter et al., 2008). In that way males are ready to mate when the females emerge from the pupal stage. In general mating occurs in flight when females fly into swarms of males (Kettle, 1962).

According to Kettle (1962), most species develop from egg to adult without any diapausing stage, producing one (e.g. *C. impunctatus*), or two (e.g. *C. obsoletus*) generations a year under temperate conditions. Mellor et al. (2000) state that under temperate conditions most species overwinter as fourth-instar larvae in diapause. Emergence of the over-wintering population occurs in early spring, depending on temperature. For example, the emergence of *C. impunctatus* occurs in early May in the Highlands of Scotland, whereas populations further south in the UK appear slightly earlier in late April (Carpenter et al., 2008).

1.2.2 Habitat

Culicoides spp. have been found in almost all parts of the world, except extreme Polar Regions, New Zealand, Patagonia, the Hawaiian Islands (Mellor et al., 2000) and Iceland. Both tropical and temperate regions seem to offer a suitable environment. Each species of *Culicoides* has its own habitat. Although these habitats can overlap, there are indications that even within one group, such as the *Obsoletus* Complex, differences between their breeding ecologies exist.

1.2.2.1 Oviposition site

The immature stages of almost all species (90%) require a wet soil (Mellor et al, 2000; Kettle, 1962; Conte et al., 2007). Breeding sites include pools, streams, tree holes, irrigation pipe leaks, saturated soil, animal dung, rotting fruit (Mellor et al, 2000), mud, reed sites and rain pools (Ulsu and Dik, 1999).

Kettle (1962) points out that despite the wide range of breeding habitats, each species usually is confined to one habitat. This particular habitat is related to the oviposition site of the species since larvae are unable to move very far. The preference of females to oviposit in companion can lead to high local concentrations of eggs and larvae (Kettle, 1962).

Kettle and Lawson (1952) have investigated the larval habitats of different *Culicoides* species in Great Britain. It turned out that *C. impunctatus* larvae were mostly found in oligotrophic (low nutrition, but high vegetation) bogland soils and at the edge of acid grassland. Larvae of *C. pulicaris* were found in swamps with the water level above the soil surface. Several species of the *Obsoletus* Complex were found to use cattle or horse dung as larvae habitat, but also leaf compost is being identified as a breeding site of this species. In the same research it turned out that *C. obsoletus* seems to prefer peaty soils as a breeding habitat and favours habitats that do not ever become waterlogged.

Conte et al. (2007) investigated the influence of biotic and abiotic factors on the abundance of *C. imicola* and the Obsoletus Complex in Italy. With respect to larvae habitat and oviposition site it turned out that *C. obsoletus* favour breeding habitats that are shielded from the sun and prefer to breed in forest leaf litter. The majority of the large collections of *C. obsoletus* came from municipalities with a higher humidity, caused by high evapotranspiration. Unlike *C. imicola*, *C. obsoletus* show to persist independently of soil type and terrain slope. This can be explained by the fact that whereas larvae of *C. imicola* require the surface layer, or A horizon (1-2 cm), larvae of *C. obsoletus* complete their development in the litter layer, or O horizon. In addition *C. obsoletus* larvae are able to tolerate significantly lower temperatures and persist on a wide variety of altitudes.

In a study by Blackwell et al. (1999), who investigated the spatial distribution of larvae of *C. impunctatus* in Scotland, a significant positive relationship was found between larval counts and soil PH, soil percentage organic content, soil percentage water content and the presence of mosses (*Sphagnum* spp.), rushes (*Juncus* spp.) and bog myrtle (*Myrica gale*). This study suggests that *C. impunctatus* has a very specific larval habitat.

1.2.2.2 Adults

The presence of appropriate breeding habitats is central to the prevalence of adult *Culicoides* spp. (Conte et al., 2007). This is because the development of larvae remains the key factor for a successful reproduction and persistence of the adult population.

As Conte et al. (2007) found large numbers of larvae of the Obsoletus Complex to develop in forest leaf litter, their results also suggest that adult populations of this complex favour a more shaded habitat with high green leaf densities.

Takken et al. (2008) studied the distribution and seasonal dynamics of *Culicoides* species in four different ecosystems in the Netherlands; wetland area, riverine systems, peat land areas and livestock farms. *C. impunctatus* was strongly associated with wetland and peat bog. This supports the observations on the species' ovipostition site by Kettle (1962), which are discussed earlier. According to this study, the Obsoletus Complex and the Pulicaris Complex are strongly associated with livestock farms.

Culicoides are poor flyers. In general *Culicoides* spp. remain in the vicinity of the breeding sites (Ducro, 2004). Though, the flight range of a species is dependent on local conditions such as the availability of hosts (Mellor et al., 2000). Long-range dispersal of *Culicoides* spp. is often associated with transport by wind (Mellor et al., 2000). In general, activity lessens during higher-speed winds and the mortality rate of adults increases. Almost all activity is suppressed at wind speeds greater than two to three knots (Ducro, 2004; Braverman, 1988). As a result, open areas with minimal tree shelter and absence of water-filled ditches or ponds are identified as being one of the most unsuitable habitats for *Culicoides* spp. Ducro (2004) states that the conservation of nature area and wetland in The Netherlands will lead to optimal ecological conditions for *Culicoides* species, suggesting a possible increase of biting midges in the future.

The prevalence of *Culicoides* spp. is also linked to altitude. In regions exceeding an altitude of 300 m, the incidence of midges is very rare (Ducro, 2004).

1.2.3 Feeding behaviour

For their energy source and metabolism adult *Culicoides* spp. mainly rely on the nectar of flowers. However, after mating most female midges require a blood meal for their eggs to develop fully. This blood-sucking or haematophagous behaviour involves feeding on warm-blooded animals, mostly mammals, such as cattle, horses, deer, but also sheep and human and sometimes even birds (Kettle, 1962). Once their prey has been located, the midge cuts through the skin with specialized mouthparts. The female midge injects a small amount of saliva, which contains proteins that prevent the blood from clotting. If the midge is undisturbed, it will drink for up to four minutes, by which the abdomen is distended and swollen (Hendry, 2000).

1.2.3.1 Host preference

There is a difference in host preference between the various *Culicoides* species. Each species has a range of hosts on which it will feed, one or more are preferred. Most of the species found in northern Europe prey on cattle, sheep, horses and deer. Only a few prey on human or domestic fowl and birds (Hendry, 2000). Blackwell et al. (1994) identified the blood meal sources of 246 female *C. impunctatus* in Scotland. With 38.4%, bovine hosts were most common, followed by deer (23%) and sheep (9.9%). According to Parker (1949), *C. impunctatus* was ten times more attracted to man than *C. pallidicornis*, *C. heliophilus*, or *C. obsoletus*.

Species that feed on the same host may select different parts of the body. The selection of these sites may be related to several factors such as visual and chemical properties of the host (De Jong and Knols, 1996). Braverman (1988) studied the preferred landing sites of *Culicoides* species on horses. He concluded that *C. schultzei* and *C. puncticollis* prefer to land on the belly, whereas *C. imicola* showed preference for the dorsal ridge. In this study the belly showed to be the warmest part of the body. Townley (1984), who also studied the preferred landing sites of *Culicoides* spp. on horses, found that *C. obsoletus*, *C. pulicaris* and *C. dewulfi* preferred landing on the mane and lower leg regions. He also found that midges that were biting on other areas such as the head, front and underside of the animal, tended to have smaller meals than midges that fed on the mane and lower leg regions.

1.2.3.2 The role of odour attractants and visual stimuli for host-seeking behaviour

With respect to host-seeking behaviour, both visual stimuli and odour recognition can play a role (Bishop et al., 2008). Midges have a well-developed olfactory sense, which is used to detect several chemicals of the body odour of mammals such as acetone and lactic acid. A variety of semiochemicals are likely to be involved in the complex process of host location (Blackwell et al., 1996). Several components of mammalian host-odour have proved to be significant attractive for ceratopogonids. Carbon dioxide, 1-octen-3-ol (a component of the body odour of ruminants) and acetone are demonstrated to be an attractant for host-seeking female *Culicoides* spp. Presumably these olfactory stimuli complement visual cues for the host-seeking midge (Mands et al., 2004).

The complementation of olfactory stimuli to visual cues is demonstrated by Bishop et al. (2008). Bishop et al. (2008) studied the attraction of *Culicoides brevitarsis* and *Culex annulirostris Skuse* to simulated visual and chemical stimuli from cattle. They suggest that the initial location of hosts by *C. brevitatsis* occurs by visual stimuli. Attraction to CO_2 is supposed to be a secondary stimulus, probably only occurring close to the host. They state that chemicals help the midge to recognize and come to the host, were landing is induced visually.

Several studies have investigated the efficacy of different host-odour baits for *Culicoides* species. Bhasin et al. (2001) studied the efficacy of 1-octen-3-ol, acetone, butanone and three different mixes of six phenolic compounds for *C. impunctatus* in Scotland. Highest catches were obtained with a bait combination of cow-urine, acetone and CO_2 or a combination of cow-urine, 1-octen-3-ol and CO_2. *C. impunctatus* was found to be extremely sensitive to CO_2. Results show synergism between the different odour baits.

Several studies have demonstrated the significance of the release rates of host-odour baits. Mands et al. (2004) found that the behaviour responses of *Culicoides* spp. to host odours are dose-dependent. Bhasin et al. (2001) concluded that higher concentrations than levels emitted by hosts reduces the effect or are repellent. Mands et al. (2004), who studied the efficacy of different host odour baits on various *Culicoides* species in Scotland, reveal that the optimal concentration of the odour bait depends on the characteristics of the animal host. They tested host odours from calf, water buffalo, red deer, sheep and pony. It was demonstrated that the water buffalo and red deer extracts were 10-fold more attractive than the other host extracts. Extracts from red deer and water buffalo were significant at a concentration level of 0.22 g/mL, whereas extracts from sheep, pony and calf were significant at a concentration level of 2.2 g/mL. They used a basic release rate of 2.1 mg/day in combination with 500 mL/min CO_2 and 6-8 mg/h 1-octen-3-ol. They decided upon these concentrations since former behavioural bioassays indicate that lower concentration did not lead to an attraction that was of any statistical significance. On the contrary, Bhasin et al. (2001) used single component odour at a release rate of 0.06 mg/h for 1-octen-3-ol, 23.00 mg/h for acetone and 5.96 mg/h for butanone. Although used as a single component, the release rate of 1-octen-3-ol is 100-fold smaller than the release rate of 1-octen-3-ol used in the experiment by Mands et al. (2004).

Besides host-odour, other chemicals can be involved in the process of host location. A volatile pheromone produced by parous female midges belonging to the species *C. impunctatus*, is suggested to be involved in the host-location behaviour of other female *C. impunctatus* (Blackwell et al., 1996). When a female has found a blood meal, she releases this pheromone, which acts as an attractant to other blood-seeking females. In combination with 1-octen-3-ol, it is found to be significantly attractive compared to 1-octen-3-ol alone (Blackwell et al., 1996). The study of Blackwell et al. (1996) does not refer to other species than *C. impuncatus*. It is unclear if other *Culicoides* species have a similar pheromone-based mechanism.

1.2.4 Circadian rhythm and the effect of meteorological conditions

The daily cycle, activity rates and host-seeking behaviour of *Culicoides* spp. are strongly influenced by meteorological conditions such as light intensity, temperature, wind speed, humidity, and rainfall (Mellor et al. 2000; Kettle, 1962; Blackwell, 1997; Kettle et al., 1998).

1.2.4.1 Circadian rhythm

Mellor et al. (2000) state that most species of *Culicoides* are crepuscular (they are primarily active during twilight, hence at dawn and dusk), or nocturnal. By falling light intensity their activity is triggered. However, complete darkness is suggested to suppress activity. This can lead to higher activity of certain species during moonlight than during darker periods.

The nocturnal activity of many *Culicoides* species might be explained by the risk of desiccation during day time when temperature are high and humidity is low. At night temperatures are relative low whereas humidity is relative high and the risk of dehydration is exploited (Mellor et al. (2000) .

A view other studies investigated the activity of adult midges during the 24-hours cycle. Braverman (1988) concluded that midges were mainly active from half an hour prior to half an hour after sunset. He focussed on *Culicoides* species in Israel, including *C. schultzei* and *C. puncticollis*. Also Ducro (2004), who has focussed on *Culicoides* species in the Netherlands, declares that midges are mainly active during dusk and dawn, especially when temperatures and humidity are high.

Kettle et al. (1998) studied factors affecting numbers of *Culicoides* spp. in truck traps in Australia. They concluded that time of day was the most important variable of influence. Other factors under study were between-day differences, annual cycles, lunar cycles, tidal cycles, wind speed and temperature. When trapping was carried out every hour for 24 hours, the general pattern of activity consisted of a peak at sunset, followed by reduced activity for the next 3-4 hours. Substantial activity occurred around midnight and in the period before sunrise. After sunrise activity sharply declined. The sunrise peak was larger than the sunset peak.
Kettle et al. (1998) state that the sunset peak is likely to be affected by wind speed, whereas the sunrise peak is likely to be affected by temperature. Significant differences were also found between species (e.g. *C. brevitarsis*, *C. marski* and *C. longior*), sexes and season. The interaction between season and time was highly significant. Further more, it is suggested that the response of the different species to time in the different seasons may differ slightly from each other.

1.2.4.2 The effect of meteorological conditions

Climate and weather have a big impact on the activity of *Culicoides* species. Adult midges may not be active on nights when meteorological conditions are bad (Mellor et al., 2000). Factors that influence *Culicoides* activity include light intensity, wind speed, temperature and rainfall. The factor wind speed is already shortly discussed in the paragraph on habitat.

Culicoides activity is positively correlated with temperature (Mellor et al., 2000; Kettle, 1962). The biting rate is correlated with the oviposition cycle, since most *Culicoides* females require a blood meal for the eggs to develop. High temperatures reduce the time required for the eggs to develop and thereby increase the biting rate. Each species has its own lower and upper temperature limit. According to Conte et al. (2007) the average minimum temperature of the Obsoletus Complex being active is 14.2 ºC, whereas that of *C. imicola* is 16.9 ºC. However, longevity of adult *Culicoides* spp. decreases significantly when temperature increases. On average adults live three times longer at 15 ºC than at 30 ºC (Wittman et al., 2002).

It is suggested that rainfall hinders the activity of most, maybe even all species (Murray, 1975, cited by Mellor et al., 2000). However, Blackwell (1997) found significantly positive relationships between rainfall and parous female catches and total female catches of the species *C. impunctatus*.
Rainfall does play an important role with respect to the abundance of *Culicoides* spp. in areas where temperatures are more suitable (Mellor et al., 2000) Mellor et al. (2000) refer to several studies that indicate a strong association with high levels of rainfall and great annual abundance in South Africa (Baylis et al., 1999), Isreal (Braverman and Galun, 1973), and Australia (Murray, 1995). The positive relation between rainfall and abundance is likely to be explained by improvement of breeding sites (Mellor et al. 2000), since the water content of soil is an important requirement for a suitable breeding habitat.

At higher temperatures, the survival rate of adult *Culicoides* spp. decreases. Besides temperature, moisture levels and wind speed can affect the survival rate (Mellor et al., 2000).

1.3 Research

Culicoides species are identified as being the main cause of allergic dermatitis in horses, a serious problem that affects thousands of horses and ponies across the world. Nonetheless, research on *Culicoides* spp. has lacked behind for years. Recently these biting midges receive increasing attention because of their role as a vector of arbo-viruses. Rapid progress is being made in the understanding of *Culicoides* biology. However, still little is known of its general ecology and its interaction with the environment including host animals.

Knowledge on the biology and ecology of *Culicoides* species in the Netherlands could contribute to a better understanding of the spatial distribution of sweet itch. Even more, it could provide some useful information on how to prevent and how to control *Culicoides* hypersensitivity in horses.

1.3.1 Research objective

The objective of this research is to gain insight into some aspects of the ecology of *Culicoides* species in The Netherlands. This study has emphasised on circadian rhythm and olfactory responses.

1.3.2 Research questions

To get more insight in some aspects of the ecology of *Culiciodes* species in The Netherlands, the following research questions are addressed:

1. What is the circadian rhythm of *Culicoides* species in The Netherlands?
2. Which of the four different host odour baits, including CO_2, CO_2 in combination with 1-octen-3-ol, CO_2 in combination with horse extract and CO_2 in combination with cattle extract, is most effective for *Culicoides* species in The Netherlands?

Circadian rhythm

Research on the circadian rhythm of *Culicoides* species might reveal data on their pattern of activity. A better understanding of diel periodicity of *Culicoides* activity might lead to suggestions on how to protect hypersensitive horses from blood-seeking females, for example by housing the animals at certain hours of the day.

Olfactory response

Data on the efficacy of different host odour baits provide the basis for further trapping experiments leading to control strategies for *Culicoides* species. Information on the differences between *Culicoides* species with respect to their preference for different host odours may contribute to a better understanding of feeding behaviour, host preference and spatial distribution of *Culicoides* spp.

Information on the ecology of *Culicoides* spp. in The Netherlands might also be useful in research on *Culicoides* species as a vector of arbo-viruses. For example, host preference is an important parameter in understanding the epidemiology of livestock diseases. Information on their daily cycle and on the efficacy of host odour baits can contribute to effective control strategies. Further more, this kind of information can be important with respect to predictions on the risk of disease outbreaks, vectored by *Culicoides* species. Such data is required since the sheer abundance and prevalence of *Culicoides* species makes effective vector control difficult (Mellor et al., 2000).

1.3.3 An unfinished study object

Originally, a third aspect of this study included the oviposition site of *Culicoides* spp. The related research question was:

* How does the oviposition site of *Culicoides* species in The Netherlands relate to ecological conditions?

Due to difficulties with the identification of the larval stages of *Culicoides* spp. and due to a lack of time, the study on ovipostion site has not been completed yet. This experiment will be continued next year. Information on this study, including a description of the research and results so far, can be found in Appendix IV.

2. Materials and methods

All data is obtained from field studies. Field studies started at the eighth of September 2008 and ended at the eleventh of October 2008.

2.1 Test location

The circadian rhythm of *Culicoides* species was studied at a horse stable in Nijkerk, a city in the centre of the Netherlands. The stable houses three horses. The horses are housed in an open barn and are able to go outside 24-h a day. No other types of animals are kept on the same pasture. Next to the stable there is a horse stable that houses approximately 50 horses indoors. There are no other farms in close proximity.

The olfactory response of *Culicoides* species was studied at a horse stable in Oirschot, a village in the south of the Netherlands. The stable houses approximately seventy Icelandic horses outside and is situated in a forest zone. There are no other types of animals on the farm and there are no other farms in close proximity. In 2005 a pilot study on *Culicoides* species has been carried out in this particular area. (Boet and Middelaar, 2005). The area is identified as an area with high numbers of *Culicoides spp.* More than ten percent of the horses on the farm (approximately ten horses) suffer from allergic dermatitis.

2.2 Sampling technique

For the experiment on circadian rhythm Mosquito Magnet® Liberty Plus traps were used. This suction trap is 85 cm high, covers one acre according to the producer and is powered by propane. It uses a 'counterflow technology', involving two concentric columns of air moving in opposite direction. The inner column of air is heated and releases 500 mL/min CO_2 as it is pumped out to act as an attractant. An additional attractant can be placed in the attractant carrier of the trap. An additional attractant that is often used with the Liberty Plus trap is 1-octen-3-ol. When CO_2 is pumped out it passes the attractant carrier and 1-octen-3-ol is released. The release rate of 1-octen-3-ol is 6-8 mg/h. The outer column of air acts to pull flying insects into a fine mesh polyester bag (www.mosquitomagnet.com).

Insects were collected alive. Collections were killed at -20 °C and transferred to 70% alcohol.

For the experiment on olfactory response similar traps were used as in the experiment on circadian rhythm. The collections were killed in a similar way too.

2.3 *Culicoides* identification

All collection obtained from the study on circadian rhythm were analysed for the presence and abundance of *Culicoides* spp. *Culiciodes* species are differentiated from each other by wing characters (Mellor et al., 2000). Determination of the different species occurred with the use of a stereo microscoop. *Culicoides* species were identified to the species level using standard protocols such as: Campbell, J.A., Pelham-Clinton, E.C., 1960; Taxonomic Review of the Britisch Species of *Culicoides Latreille* (Diptera Ceratopogonidae).

Collections obtained from the study on olfactory response were analysed similar to the analysis of the catches obtained from the study on circadian rhythm.

2.4 Statistical analysis

Data obtained from the experiment on circadian rhythm was analysed using the software programme SPSS (version 14.0). For analysis, catches (x) were transformed to ln(x+1) to reduce dependence of the variance on the mean and to achieve normality. All results were analysed using One-way ANOVA, followed by Fishers' LSD procedure, unless when described differently in chapter 3 *Research*. Analysis was done on the total number of captured *Culicoides* species and on the level of the individual species.

Data obtained from the experiment on olfactory response were analysed similar to the analysis of the data obtained from the experiment on circadian rhythm. To see if the host-species extract affected the proportion of *Culicoides* species collected, the different species composition in the different traps were compared (e.g. broader range of species or increased number of a particular species).

2.5 Additional measurements

On both of the test locations, a Tiny Tag® meteorological data logger (Gemini Data Loggers, Chichester, UK) was used to record humidity and temperature every ten minutes.

On both of the test locations, one Onderstepoort light trap was used for three sampling periods during the experiment to gain background information on the presence and abundance of *Culicoides* species in the vicinity under study.

3. Research

This research has focused on two main questions:

1. What is the circadian rhythm of *Culicoides* species in the Netherlands?
2. Which of the four different host odour baits, including CO_2, CO_2 in combination with 1-octen-3-ol, CO_2 in combination with cattle extract and CO_2 in combination with horse extract, is most effective for *Culicoides* species in The Netherlands?

This chapter will give a description of the studies and the results that are obtained. First the experiment on circadian rhythm will be described, than the experiment on olfactory responses will be revealed.

3.1 Circadian rhythm

Within the study on circadian rhythm, attention is paid to several subjects other than the 24h activity pattern of *Culicoides* species. These subjects are:

- The effect of trap location on the numbers of captured *Culicoides* spp.
- The effect of some meteorological conditions on the number of captured *Culicoides* spp.
- The efficacy of the Liberty Plus traps compared to the Onderstepoort light trap

First the study on circadian rhythm will be described, after that the other subjects will be revealed.

3.1.1 Circadian rhythm

The aim of this study is to use baited suction traps to detail the flight activity of *Culicoides* species over replicated 24 h periods.

3.1.1.1 Research description

For this experiment three Mosquito Magnet® Liberty plus traps were used. Trapping was carried out 24 hours a day for a period of 15 days. Collections were made every six hours. During each 24 hour period the collecting net of the trap was emptied and changed according to the following time schedule: 11am; 5pm; 11pm; 5am.

The traps were placed in the proximity of the horses. The horses were unable to reach the traps. Traps were placed at > 50 m intervals from each other.

During the first five days CO_2 was used as bait. After the fifth day it was decided to add octenol as an additional attractant because numbers of captured *Culicoides* species were very low. This change in experimental setting does not influence the outcome when it comes to a relation between activity rate and time of the day, since similarity within the periods is maintained.

3.1.1.2 Results

Appendix I gives a complete overview of the data obtained from the experiment on circadian rhythm of *Culicoides* species. Appendix II includes tables showing the numbers per time period. Data from the three traps are pooled.

On the 29th of September between 5pm-11pm the number of captured *C.* spp. was extremely high (n=837) compared to the other numbers. It was decided to exclude the *Culicoides* samples from this day from the analysis and the results presented here in order to avoid the potential effects of sampling bias. Results of this day are included in Appendix I and II.

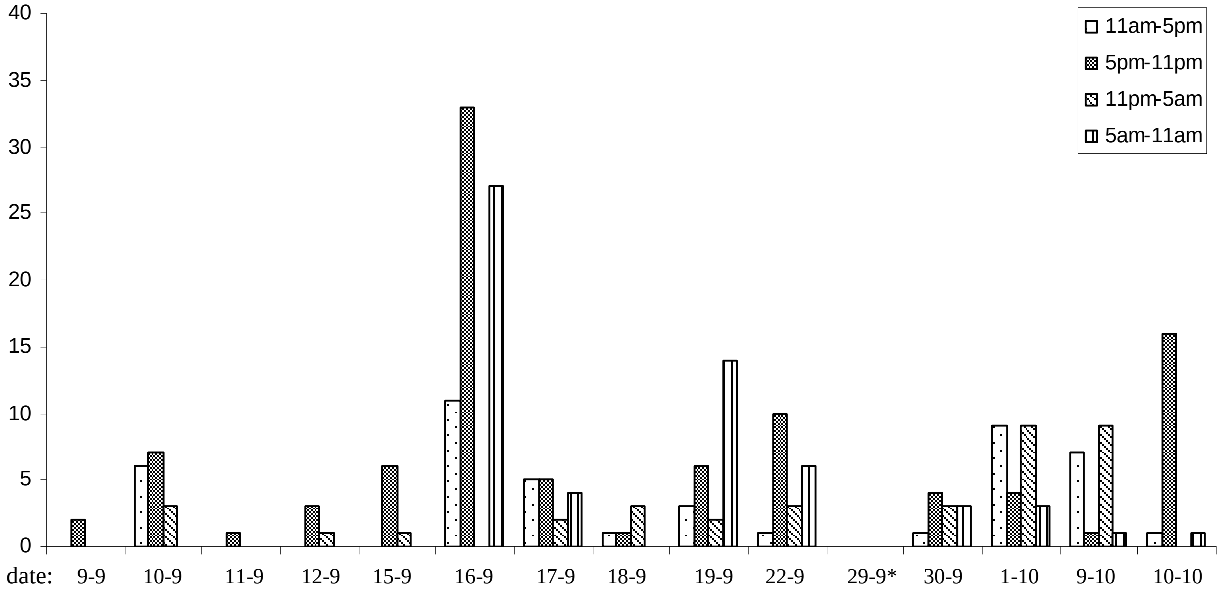
Table 3.1 shows the total number of captured *Culicoides* spp. during the experiment on circadian rhythm. *C. obsoletus* was the most abundant species found. *C. dewulfi* and *C. festivipennis* were captured in lower quantities, but still reasonably present. *C. chiopterus* and *C. punctatus* were captured in low amounts. *C. stigma* and *C. newsteadi* were only caught ones (Table 1.1).

Table 3.1 Total number of captured *Culicoides* spp.

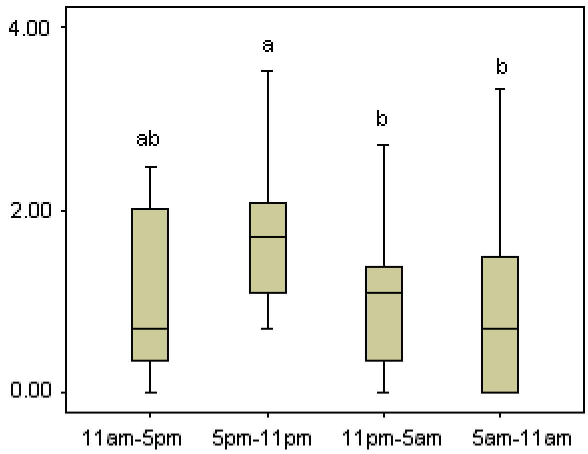
Species	Number
C. obsoletus	153
C. dewulfi	47
C. chiopterus	6
C. punctatus	7
C. stigma	1
C. festivipennis	24
C. newsteadi	1
Total number of *C.* spp.	239

Graph 3.1 (next page) shows the number of captured *Culicoides* spp. per six hour period per date. During all four time periods C*ulicoides* species were captured. From the 16th of September onwards, octenol was added as an additive attractant. Numbers of captured *C.* spp. seem to increase since this data point.

Highest numbers of midges were collected between 5pm and 11pm (average 7 ± 8.5). Lowest numbers of midges were collected between 11am and 5pm (average 3 ± 3.72) and between 11pm and 5am (average 3 ± 4.12). Numbers within groups are highly variable, especially in the period between 5pm and 11pm. Variances of all groups are similar (p=0.30). Numbers of the first and third group (11am-5pm and 11pm-5am) are not normally distributed (Graph 3.2).



Graph 3.1 Number of captured *Culicoides* spp. per six hour period per date.
* Data excluded, for results see appendices



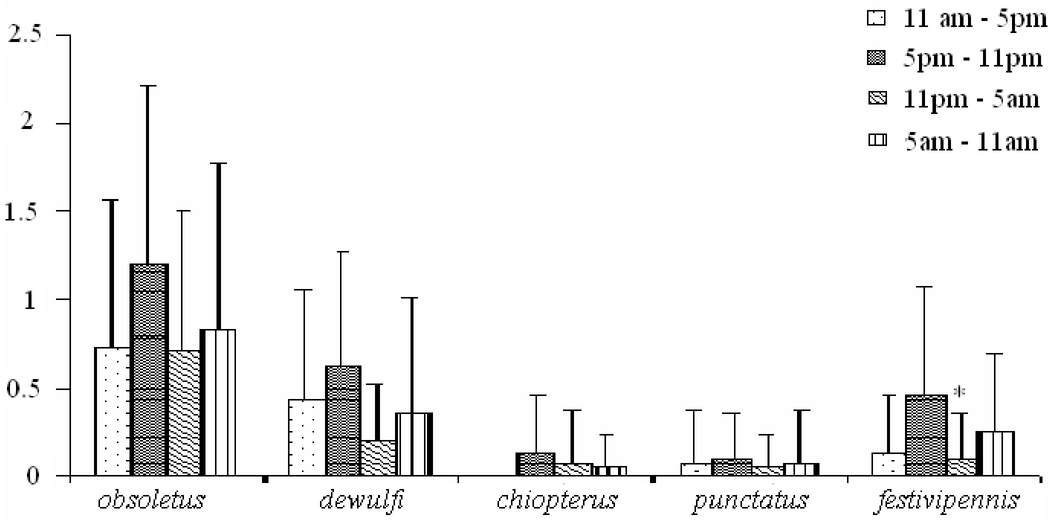
Graph 3.2 Boxplot of the total number of captured *C.* spp. in 2-log per time period

According to the statistical analysis there is no significant difference (p=0.098) between the total number of *Culicoides* spp. captured during the four different time periods. However, pair wise comparisons indicate significant differences between two groups (Graph 3.2).

There is a significant difference (p=0.035) between the number of captured *Culicoides* species in the period between 5pm-11pm and the period between 5am-11am. There is also a significant difference (p=0.035) between the catches in the period between 5pm-11pm and the period between 11pm-5am. During the period between 5pm-11pm significantly more *Culicoides* spp. were captured compared to the number of captured *C.* spp. in the two other periods. Besides, there is a trend toward higher numbers of *C.* spp. captured in the period between 5pm and 11am compared to the numbers of *C.* spp. captured during the periods between 11am-5pm (p=0.056). Between the other groups there was no significant difference in number of captured *Culicoides* spp, nor a trend towards higher numbers in one of the groups (Graph 3.2).

Graph 3.3 and Table 3.2 show the results of the analysis on the species level.

For *C. obsoletus*, *C. dewulfi* and *C. festivipennis* highest numbers were found between 5pm-11pm. The other species were captured in markedly lower quantities and do not show any differences between the time periods. None of the species shows an unambiguous preference or aversion for one of the time periods (Graph 3.3).



Graph 3.3 Mean catch size and stdv. of the individual *C.* species in 2-Log per time period.

Table 3.2 shows p-values of the analysis on the species level. According to the statistical analysis there is no significant difference between the numbers of any of the species captured during the four different time periods.

Table 3.3 p-values of the analysis on the species level

Species	p
C. obsoletus	0.412
C. dewulfi	0.285
C. chiopterus	0.562
C. punctatus	0.967
C. festivipennis	0.129

Using a method for pair wise comparisons, there is a trend (p=0.058) towards higher numbers of *C. dewulfi* captured during the period between 5pm-11pm compared to the number of *C. dewulfi* captured during the period between 11pm-5am. A significantly (p=0.034) higher number of *C. festivipennis* is captured during the period between 5pm-11pm compared to the number of *C. festivipennis* captured during the period between 11pm-5am. Also, there is a trend (p=0.05) towards higher numbers of *C. festivipennis* captured during the period between 5pm-11pm compared to the number of *C. festivipennis* captured during the period between 11am-5pm.

No statistical analysis is done for *C. stigma* and *C. newsteadi* since there was only one species captured per group.

3.1.2 The effect of trap location on the catches

The aim of this study is to reveal a possible effect of trap location on the number of *Culicoides* species captured during the experiment on circadian rhythm.

3.1.2.1 Research description

As described in paragraph 3.1.1.1 *Research description of the study on circadian rhythm*.

Trap A was located near the horse stables and surrounded by high vegetation. Trap B was located in horse pasture next to a barn. Trap C was located in horse pasture in open field.

3.1.2.2 Results

There is no significant difference (p=0.160) in number of captured *Culicoides* spp. between the three locations A, B and C. There is a trend (p=0.145 and p=0.071) toward lower numbers of *C.* spp. captured on location C, compared to the number of captured *C.* spp. on the other two locations.

3.1.3 The effect of some meteorological conditions on the catches

The aim of this study is to explore possible influences of some meteorological factors on any pattern observed during the experiment on circadian rhythm and to reveal a possible correlation between weather conditions and the number of *Culicoides* species captured during this experiment.

3.1.3.1 Research description

As described in paragraph 3.1.1.1 *Research description of the study on circadian rhythm*.

A Tiny Tag® meteorological data logger (Gemini Data Loggers, Chichester, UK) was used to record humidity and temperature every ten minutes. The data logger was placed next to one of the traps. Data on wind speed and sun hours were provided by the national meteorological service (KNMI, de Bilt, The Netherlands).

To analyse the correlation between the numbers of captured species and meteorological conditions, data obtained from the data logger are mediated per six hour time period. Only the test days are taken into account. Data on weather conditions during the days in-between the test days are excluded from the analysis. Correlation analysis is used to analyse the relation between *Culicoides* catches and individual meteorological variables. Linear regression is used to analyse the effect of a combination of various meteorological variables on the number of captured species.

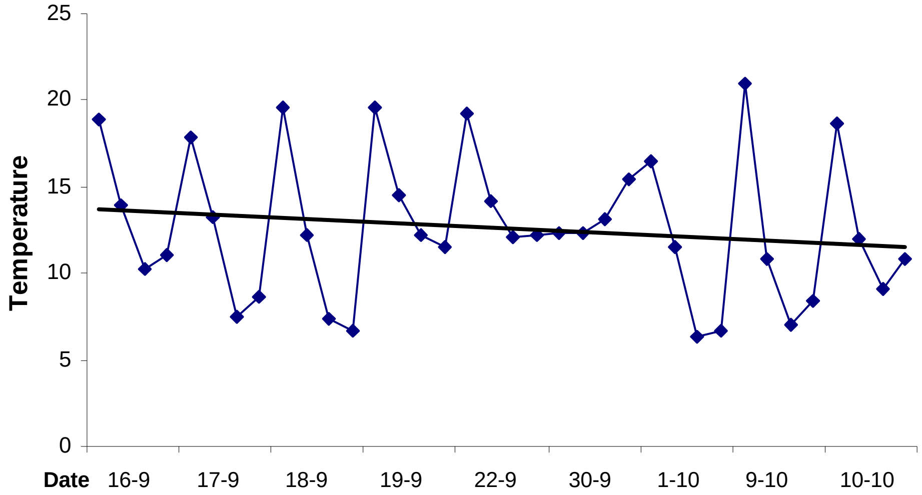
Saturation deficit is calculated using the formula $SD = (1 - RH/100) \times 4.9463 \times e^{(0.0621*T)}$ (Perret et al., 2000). Whereas RH = humidity (%) and T = temperature (°C).

For the analysis on the correlation between weather conditions and the number of *Culicoides* captured in the experiment on circadian rhythm, the catches without octenol were not taken into account. To analyse the correlation between the number of captured *C.* spp. and weather conditions a change in setting is undesirable.

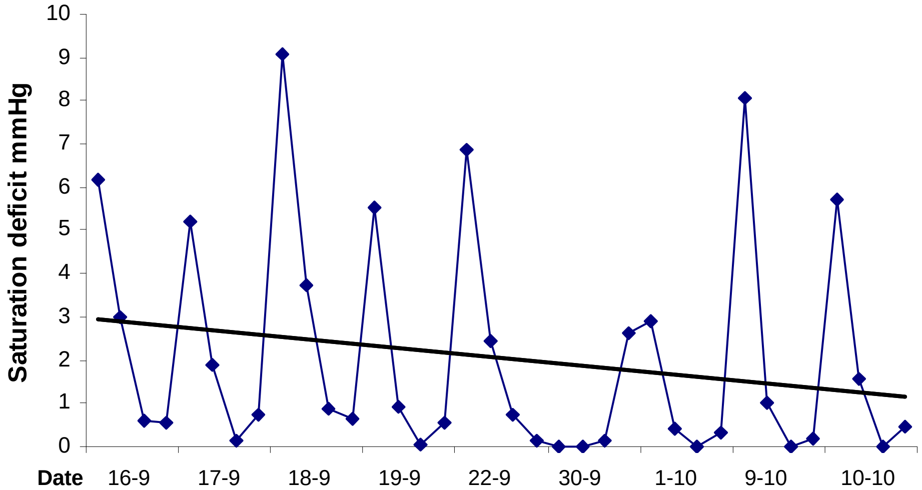
Special attention is paid on the results obtained during the period between 11am and 5pm.

3.1.3.2 Results

Graph 3.4 shows the mean temperature during the study period. Graph 3.5 shows the mean saturation deficit during the study period.



Graph 3.4 Mean temperatures during the study on circadian rhythm



Graph 3.5 Mean saturation deficit during the study on circadian rhythm

Table 3.4 shows the development of the individual variables during the experiment. There is no significant change in any of the variables during the experimental period. There is a trend towards a decreasing number of *C.* spp., an increase of wind speed, a decrease in saturation deficit and an increase in humidity. Overall, all variables are assumed to be considerably constant during the experimental period.

Table 3.4 Change in variables over time during the experiment

Variable	B	p
Culicoides spp.	-0.409	0.145
Temperature	-0.162	0.321
Wind speed	0.065	0.106
Saturation deficit	-0.139	0.166

Table 3.5 shows the correlation between the number of captured *Culicoides* species and individual meteorological variables. None of the variables show a significant correlation with the number of *C.* spp. captured.

Table 3.5 Correlation analyses of *Culicoides* species captured and the measured meteorological variables, showing the Pearson's correlation coefficient (r) and the associated probability (p)

	Temperature	Saturation deficit	Wind speed
r	0.112	-0.004	-0.179
p	0.541	0.982	0.295

Table 3.6 shows the contribution of combined meteorological variables to the number of captured *Culicoides* spp, using linear regression analysis. Numbers describe the associated probability.

None of the models show a significant correlation with the number of captured *Culicoides* species, neither does any of the individual variables within the models, except for temperature in model two. Model two, combining temperature, saturation deficit and wind speed, is most suitable but still only contribute as less as 6.9% to the final outcome.

Table 3.6 Structure of analysis assessing contribution of time and four meteorological variables to variation in number of captured *Culicoides* species and the associated probability. Columns: 1, all variables; 2-5, contribution of combined linear meteorological terms assessed by omission.

Source	1	2	3	4
Time of day	0.945			
Temperature	0.064	0.047	0.195	0.514
Saturation deficit	0.074	0.058	0.261	
Wind speed	0.069	0.064		
Significance of the model	0.273	0.156	0.426	0.514
Adjusted R-square	0.039	0.069	-0.007	-0.016

When focussing on the period between 11am and 5pm, the following results apply;

There is a significant negative correlation between wind speed and number of *Culicoides* species captured in the period between 11am and 5pm. None of the other meteorological individual variables shows a significant relationship with the number of captured species (Table 3.7).

Table 3.7 Correlation analyses of *Culicoides* species captured between 11am-5pm and the measured meteorological variables, showing the Pearson's correlation coefficient (r) and the associated probability (p)

	Temperature	Saturation deficit	Wind speed	Sun hours
r	0.076	-0.131	-0.688	-0.379
p	0.846	0.737	0.040	0.315

Table 3.8 shows the contribution of combined meteorological variables to the number of captured *Culicoides* spp. between 11am and 5pm, using linear regression analysis. Numbers describe the associated probability.

Model two and three are significantly correlated with the number of captured species. Within each of the models, several variables show a significant correlation with the number of captured *Culicoides* species. Model three, combining saturation deficit and wind speed is most suitable. Contribution to the final outcome is 74%.

Table 3.8 Structure of analysis assessing contribution of five meteorological variables to variation in number of captured *Culicoides* species between 11am-5pm and the associated probability. Columns: 1, all variables; 2-4, contribution of combined linear meteorological terms assessed by omission.

Source	1	2	3
Temperature	0.622	0.591	
Saturation deficit	0.400	0.347	0.019
Wind speed	0.027	0.009	0.003
Sun hours	0.865		
Significance of the model	0.086	0.027	0.007
Adjusted R-square	0.638	0.708	0.741

3.1.4 Comparisons between the Liberty Plus traps and the Onderstepoort light trap

The aim of this study is to gain background information on the presence and abundance of *Culicoides* species on the test location of the study on circadian rhythm using the Onderstepoort light trap and to make a rough comparison between the efficacy of the Liberty Plus traps and the Onderstepoort light trap.

3.1.4.1 Research description

As described in paragraph 3.1.1.1 *Research description of the study on circadian rhythm*.

One Onderstepoort light trap was used for three nights during the experiment on circadian rhythm. The light trap was placed in the horse stables. The horses were unable to reach the trap. The trap was operating between 5pm and 11am.

The Liberty Plus traps were not operating on the night of 13 September due to bad weather conditions and results of the third catch can not be used for comparisons between the traps. The light trap was operating between 5pm and 11am, similar to the sum of three time periods (5pm-11pm,11pm-5am and 5am-11am) of the Liberty Plus traps.

Differences between the traps were analysed using the Kruskal-Wallis test.

3.1.4.2 Results

Table 3.9 shows the total number of captured *Culicoides* species using the Onderstepoort light trap.

C. obsoletus is the most abundant species found. *C. dewulfi* and *C. punctatus* were captured in lower quantities, but still reasonably present. *C. chiopterus* and *C. festivipennis* were captured in the lowest amounts. No *C. stigma* and *C. newsteadi* were caught.

The number of captured species varies between the three nights. The average number of captured species per night is 144 (±83).

Table 3.9 Total number of captured *C*. spp. using the Onderstepoortval (5pm-11am)

Species	10/11sep	11/12sep	12/13sep	Total
C. obsoletus	85	217	77	379
C. dewulfi	5	8	8	21
C. chiopterus	3	3	2	8
C. punctatus	10	7	2	19
C. stigma	0	0	0	0
C. festivipennis	0	4	0	4
C. newsteadi	0	0	0	0
Total number of *C. spp.*	103	239	89	431

Table 3.10 shows the number of captured *C*. spp. using Liberty Plus traps compared to the number of captured species using the Onderstepoort light trap. Based on these two catches, the light trap is significantly (p=0.033) more effective than the Liberty Plus trap, baited in carbon dioxide. During the first night the light trap captured up to ten times more *Culicoides* spp. than the most successful Liberty Plus trap. In the second night the light trap captured 239 *Culicoides* spp., whereas no species were captured by all three Liberty Plus traps.

Table 3.10 Total number of captured *Culicoides* spp. Liberty Plus traps versus Onderstepoort light trap

	10/11 September 5pm-11am				11/12 September 5pm-11am			
	Light trap	LP1	LP2	LP3	Light trap	LP1	LP2	LP3
Total nr of *C.* spp.	103	0	9	1	239	0	0	0

3.2 Olfactory response

Within the study on olfactory responses of *Culicoides* species in the Netherlands, attention is paid to several subjects other than the main subject under study. These subjects are:

- The effect of trap location on the numbers of captured *Culicoides* spp.
- The effect of meteorological conditions on the number of captured *Culicoides* spp.
- The efficacy of the Liberty Plus traps compared to the Onderstepoort light trap

First the study on olfactory responses will be described, after that the other subjects will be revealed.

Finally, differences in diversity and density of captured *Culicoides* spp. between the two test locations, respectively situated in Nijkerk (circadian rhythm) and Oirschot (olfactory response) are revealed.

3.2.1 Olfactory response

The aim of this study was to use four baited suction traps to test the efficacy of four different host-odours as bait for *Culicoides* species. The different attractant under study were: CO_2, 1-octen-3-ol, horse extract and cattle extract.

3.2.1.1 Research description

For this experiment four Mosquito Magnet® Liberty plus traps were used. The first trap was baited with carbon dioxide. The second trap was baited with a combination of CO_2 and 1-octen-3-ol. The third trap used a combination of CO_2 and horse extract as bait and the fourth trap a combination of CO_2 and cattle extract. The additional attractant, respectively octenol, horse extract and cattle extract was placed in the attractant carrier of the traps.

The release rate of 1-octen-3-ol was 6-8 mg/h. 15 gram of hair of either horses or cattle were used to serve as animal extracts. The release rates of CO_2 and 1-octen-3-ol were determined by Mosquito Magnet® and a result of multiple studies (www.mosquitoemagnet.com). The release rate of cattle and horse extract were not determined. 15 gram of hair was used according to the size of the attractant carrier in the traps. Hair samples were changed every six days to optimize the bait.

The traps were placed in the proximity of the horses. The horses were unable to reach the traps. Traps were placed at > 50 m intervals from each other. Hence, one odour can not mask the effect of the other odour when stronger or more obvious (Bhasin et al., 2001).

The traps were placed randomly by the principles of Latin square. All traps were randomized every two days to minimize potential position biases.

The test period was sixteen days. There were eight replications of each treatment. Trapping was carried out 24 hours a day. Collection and randomization took place every other day in the afternoon. After each change, no trapping was carried out for a 5-minutes period. This allowed the dispersal of any untrapped *Culicoides* left over from the previous trial (Bhasin et al., 2001).

During the experiment one time the trap baited with cattle extract + CO_2 and one time the CO_2 baited trap fall out (dated 14 and 18 September). These test sessions were repeated after the experiment (26 and 28 September). To avoid positional bias it was decided to exclude all results dated 14 and 18 September from the analysis and use the results obtained from the replicas. Hence, exactly two replicas of each odour/location combination are used for the analysis.

3.2.1.2 Results

Appendix III gives a complete overview of the data obtained from the experiment on olfactory responses of *Culicoides* species.

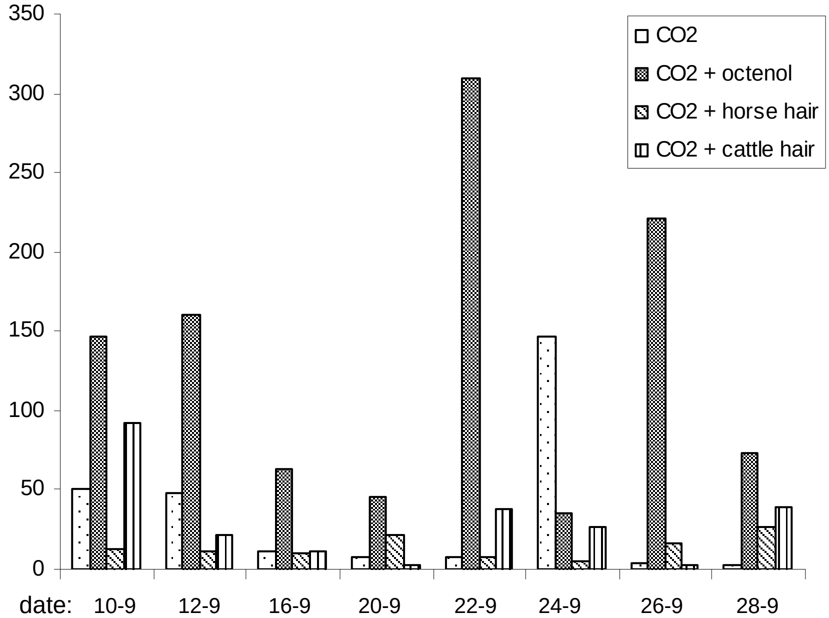
Table 3.11 gives an overview of the different species that were caught during the experiment. *C. obsoletus* is by far the most abundant species found. *C. dewulfi* and *C. festivipennis* were captured in lower quantities, nevertheless reasonably present. *C. punctatus* was captured in lower amounts. *C. chiopterus* and *C. stigma* were captured in low amounts. Only three *C. nubeculosis* were caught.

Table 3.11 Total nr. of captured *Culicoides* spp.

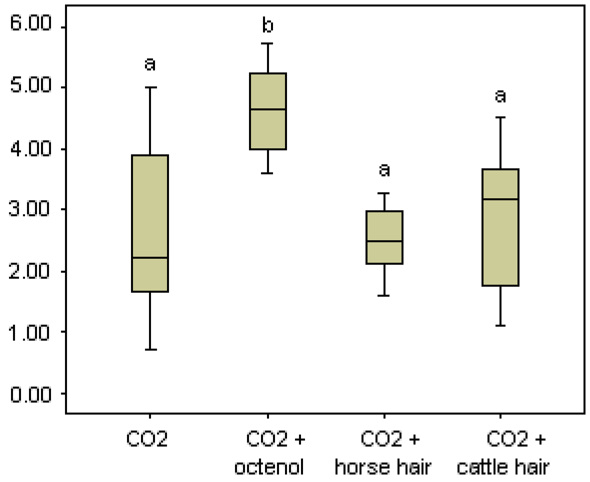
Species	Number
C. obsoletus	1187
C. dewulfi	230
C. chiopterus	16
C. punctatus	60
C. stigma	9
C. nubeculosis	3
C. festivipennis	173
Total number of *C. spp.*	1678

Graph 3.6 (next page) shows the number of captured *Culicoides* spp. per odour baited trap per catch. Every time, except for 24 September, the trap baited with CO_2 + octenol shows the highest number of captured *Culicoides* species (average 132 ± 99.4). Lowest numbers of midges were collected with the trap baited with CO_2 + horse extract (average 14 ± 7.3). The averages of the other traps were 35 ± 49.4 (CO_2) and 29 ± 29.6 (CO_2 + cattle extract).

Numbers within the groups are highly variable. The variances of the numbers captured using CO_2 + octenol or CO_2 + horse extract are smaller (p=0.032) than the variances of the numbers of species captured using CO_2 or CO_2 + cattle extract. Numbers within the first and last group (CO_2 and CO_2 + cattle extract) are not normally distributed (Graph 3.7)



Graph 3.6 Number of captured *Culicoides* spp. per odour baited trap per catch

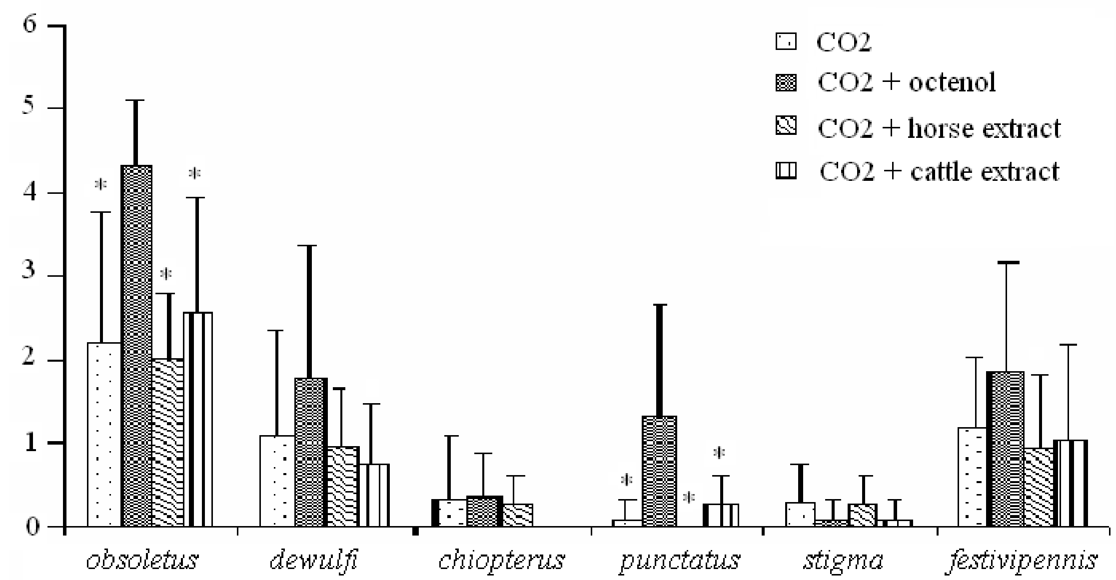


Graph 3.7 Boxplot of the total number of captured *C.* spp. per odour baited trap in 2-log

According to the statistical analysis there is a significant difference (p=0.001) in the total number of captured *Culicoides* spp. between the four odour baited traps. Pair wise comparisons indicate a significant difference between the number of captured *Culicoides* using a bait of CO_2 + octenol and all other three baits (Graph 3.6). Using the trap baited with CO_2 + octenol, significantly more *Culicoides* spp. were captured compared to the number of captured *C.* spp. using CO_2, CO_2 + horse extract, or CO_2 + cattle extract as a bait (respectively p=0.001; p<0.000; p=0.002). Between the other groups there was no significant difference in number of captured *Culicoides* spp, nor a trend towards higher or lower numbers in one of the groups.

Graph 3.8 and table 3.12 show the results of the analysis on the species level.

Especially within the group of *C. obsoletus* and *C. punctatus* the number of captured species is much higher using CO_2 + octenol as bait when compared to the other baits that are used. Also for *C. dewulfi* and *C. festivipennis*, there is a trend towards higher numbers for CO_2 + octenol, compared to the other baits. The other species do not show such a tendency. However, numbers within these groups are much lower (Graph 3.8).



Graph 3.8 Mean catch size and stdv. of the individual *C.* species in 2-Log per odour baited trap.

Table 3.12 shows the p-values of the statistical analysis per species. There is a significant difference in the number of captured *C.* spp. between the four different odour baits within the group of *C. obsoletus* and *C. punctatus*. Pair wise comparisons point out that within both of the groups significantly more species were captured using CO_2 + octenol as a bait, compared to the number of *C.* spp. captured by all of the other baits.

There is no significant difference in the number of captured *C.* spp. between the four odour baits within the group of *C. dewulfi*, *C. chiopterus*, *C. stigma* and *C. festivipennis*. Using a method for pair wise comparisons, no significant differences were found between any of the baits within all of the groups. However, there is a trend towards higher numbers of *C. dewulfi* captured using CO_2 + octenol as a bait, compared to the number of *C. dewulfi* captured using CO_2 + horse hair (p=0.144) or CO_2 + cattle hair as a bait (p=0.08). For *C. festivipennis* similar results were found (p=0.091 for CO_2 + octenol compared to CO_2 + horse hair and p=0.123 for CO_2 + octenol compared to CO_2 + cattle hair).

No statistical analysis is done for *C. nubeculosis* since there were only three species captured.

Table 3.12 p-values of the analysis on the species level

Species	p
C. obsoletus	0.002
C. dewulfi	0.305
C. chiopterus	0.429
C. punctatus	0.003
C. stigma	0.421
C. festivipennis	0.308

3.2.2 The effect of trap location on the catches

The aim of this study is to reveal a possible effect of trap location on the number of *Culicoides* species captured during the experiment on olfactory responses.

3.2.2.1 Research description

As described in paragraph 3.2.1.1 *Research description of the study on olfactory responses*.

The results of 14 and 18 September are included in the analysis on the effect of trap location.

Trap position A was in the horse pasture. Trap position B was located next to the paddock (<1.5m). Trap position C and D were located between pastures at a distance of >10 m from the horses.

3.2.2.2 Results

There is no significant difference (p=0.151) in number of captured *Culicoides* spp. between the four locations A, B, C and D.

Pair wise comparisons show a significant higher number of captured *Culicoides* species on location B, compared to location C (p=0.028). There is a trend (p=0.117) towards higher numbers of *C.* spp. captured on location B, when compared to the numbers captured on location D.

3.2.3 The effect of some meteorological conditions on the catches

The aim of this study is to explore possible influences of some meteorological factors on the number of captured *Culicoides* species during the experiment on olfactory responses.

3.2.3.1 Research description

As described in paragraph 3.2.1.1 *Research description of the study on olfactory responses*.

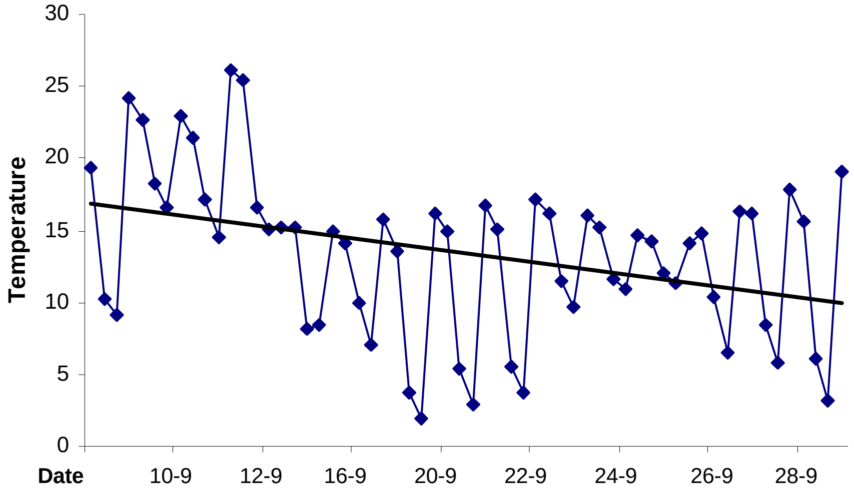
A Tiny Tag® meteorological data logger (Gemini Data Loggers, Chichester, UK) was used to record humidity and temperature every ten minutes. The data logger was placed next to one of the traps. Data on wind speed, sun hours and rainfall were provided by the national meteorological service (KNMI, de Bilt, The Netherlands).

To analyse the correlation between the numbers of captured species and meteorological conditions, data obtained from the data logger are mediated per two day period. Correlation analysis is used to analyse the relation between *Culicoides* catches and individual meteorological variables. Linear regression is used to analyse the effect of a combination of various meteorological variables on the number of captured species.

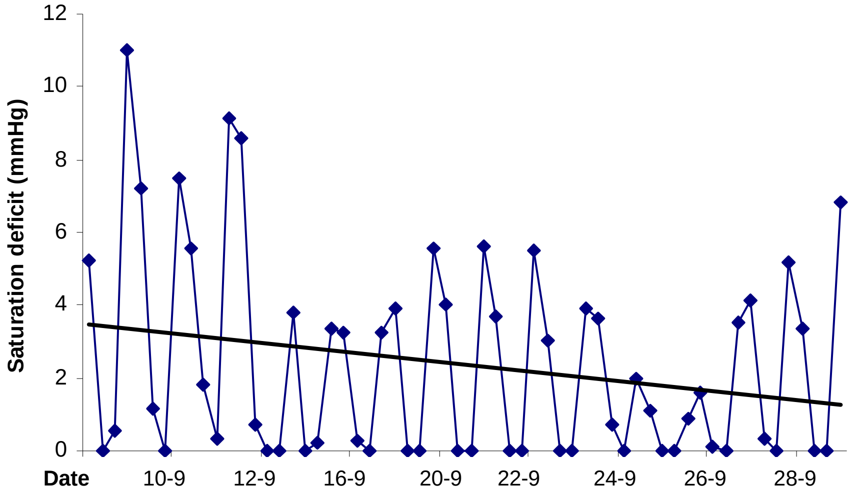
Saturation deficit is calculated using the formula $SD = (1 - RH/100) \times 4.9463 \times e^{(0.0621*T)}$ (Perret et al., 2000). Whereas RH = humidity (%) and T = temperature (°C).

3.2.3.2 Results

Graph 3.9 shows the mean temperature during the study period. Graph 3.10 (next page) shows the mean saturation deficit during the study period. Measurements were mediated per six hours time period, resulting in four values per day.



Graph 3.9 Mean temperatures during the study on olfactory responses



Graph 3.10 Mean saturation deficit during the study on olfactory responses

Table 3.13 shows the development of the individual variables during the experiment. There is a significant decrease in saturation deficit during the experimental period. There is a trend towards an decrease in temperature during the experimental period. The other variables do not show a significant change or a trend towards an increase or a decrease during the experiment.

Table 3.13 Change in variables over time during the experiment

Variable	B	p
Culicoides spp.	-0.048	0.637
Temperature	-0.935	0.059
Wind speed	-0.108	0.314
Saturation deficit	-0.198	0.042
Hours of sunshine	0.281	0.639
Hours of rainfall	-0.063	0.753

Table 3.14 shows the correlation between the number of captured *Culicoides* species and individual meteorological variables. None of the variables show a significant correlation with the number of *C.* spp. captured.

Table 3.14 Correlation analyses of *Culicoides* species captured and the measured meteorological variables, showing the Pearson's correlation coefficient (r) and the associated probability (p)

	Temperature	Saturation deficit	Wind speed	Sunshine	Rainfall
r	0.371	0.516	0.091	0.478	-0.387
p	0.366	0.191	0.830	0.231	0.343

Table 3.15 shows the contribution of combined meteorological variables to the number of captured *Culicoides* spp, using linear regression analysis. Numbers describe the associated probability. None of the models show a significant correlation with the number of captured *Culicoides* species. Temperature and rainfall are the two variables showing the highest correlation with the number of species captured. Only temperature shows a significant correlation with the number of captured species when combined with saturation deficit, sunshine and

rainfall (model two). Model one, combining temperature, saturation deficit, wind speed, sunshine and rainfall is most suitable. Contribution to the final outcome is 72%.

Table 3.15 Structure of analysis assessing contribution of five meteorological variables to variation in number of captured *Culicoides* species and the associated probability. Columns: 1, all variables; 2-4, contribution of combined linear meteorological terms assessed by omission.

Source	1	2	3	4
Temperature	0.074	0.041	0.086	0.077
Saturation deficit	0.109	0.084	0.222	
Wind speed	0.404			
Sunshine	0.222	0.145		
Rainfall	0.083	0.054	0.064	0.074
Significance of the model	0.187	0.100	0.133	0.120
Adjusted R-square	0.722	0.713	0.508	0.401

3.2.4 Comparisons between the Liberty Plus traps and the Onderstepoort light trap

The aim of this study is to gain background information on the presence and abundance of *Culicoides* species on the test location of the study on olfactory responses using the Onderstepoort light trap and to make a rough comparison between the efficacy of the Liberty Plus traps and the Onderstepoort light trap.

3.2.4.1 Research description

As described in paragraph 3.2.1.1 *Research description of the study on olfactory responses*.

One Onderstepoort light trap was used for three test periods of two days each during the experiment on olfactory responses. The light trap was placed in the horse stables. The horses were unable to reach the trap. The trap was operating 24h a day.

3.2.4.2 Results

Table 3.16 shows the total number of captured *Culicoides* species using the Onderstepoort light trap.

C. obsoletus is the most abundant species found. Low numbers of *C. dewulfi, C. chiopterus* and *C. festivipennis* were captured. None of the other species that were captured during the experiment on olfactory responses using odour baited Liberty Plus traps, were captured using the Onderstepoort light trap for six nights.

Table 3.16 Total number of captured *C.* spp. using the Onderstepoort light trap

Species	14-16sep	16-18sep	18-20sep	Total
C. obsoletus	13	18	2	33
C. dewulfi	3	0	0	3
C. chiopterus	0	2	0	2
C. punctatus	0	0	0	0
C. stigma	0	0	0	0
C. festivipennis	2	0	0	2
C. nubeculosis	0	0	0	0
Total number of *C. spp.*	18	20	2	40

Table 3.17 shows the number of captured *C.* spp. using the baited Liberty Plus traps from the experiment on olfactory response compared to the number of captured species using the Onderstepoort light trap.

Based on these three catches, the light trap is significantly (p=0.033) less effective than the Liberty Plus trap baited with CO_2 + octenol (LP2). There is no significant difference between the light trap and the other three baited Liberty Plus traps (p=0.676; p=0.714 and p=0.461 respectively).

Table 3.17 Total number of captured *Culicoides* spp. Liberty Plus traps versus Onderstepoort light trap

	Light trap	LP1	LP2	LP3	LP4
16 September	18	12	63	10	*m*
18 September	20	*m*	34	17	11
20 September	2	7	45	22	2

LP1=CO_2; LP2=CO_2 + octenol; LP3=CO_2 + horse extract; LP4=CO_2 + cattle extract
m =missing value

3.2.5 Comparisons between the two test locations in Nijkerk and Oirschot

The aim of this study is to compare *Culicoides* species density and diversity on the two test locations of the two experiments discussed in this study.

3.2.5.1 Research description

The numbers and the various *Culicoides* species captured in Nijkerk (test location of the experiment on circadian rhythm) were compared to the numbers and the various *Culicoides* species captured in Oirschot (test location of the experiment on olfactory responses).

Numbers compared came from Liberty Plus traps using the same bait during the same period. This means that from 8 until 16 September results obtained in Nijkerk are compared to the results of the CO_2-baited trap in Oirschot, and from 16 September until 10 October results obtained in Nijkerk are compared to the results of the trap baited with CO_2 + octenol in Oirschot.

3.2.5.2 Results

Table 3.18 (next page) shows the number of species captured on the two test locations.

During the first period the number of *Culicoides* species captured per day in Oirschot was eight times higher than the average number of *Culicoides* spp. captured per day per trap in Nijkerk.

During the second period the number of *Culicoides* species captured per day in Oirschot was approximately three times higher than the average number of *Culicoides* spp. captured per day per trap in Nijkerk.

Table 3.18 Comparison of the total number of *Culicoides* spp. captured per Liberty Plus trap per day per location

	Experiment circadian rhythm **Location Nijkerk**	Experiment olfactory response **Location Oirschot**
Bait	CO_2	CO_2
Date	08 September - 16 September	08 September - 16 September
Nr. of catch days	5	8
Nr. of *C.* spp. captured	30	130
Nr. of captured *C.* spp. per day	6	16
Nr. of traps	3	1
Nr. of species per trap per day	**2**	**16**
Bait	CO_2 + octenol	CO_2 + octenol
Date	16 September - 01 October	16 September - 01 October
Nr. of catch days	7	12
Nr. of *C.* spp. captured	403	719
Nr. of captured *C.* spp. per day	58	60
Nr. of traps	3	1
Nr. of species per trap per day	**19**	**60**

Table 3.19 shows the individual species captured as a percentage of the total number of *Culicoides* spp. captured per location. Numbers are very much alike. On both of the location *C. obsoletus* was by far the most abundant species found, followed by *C. dewulfi* and *C. festivipennis*. The other species were found in much lower quantities. In Nijkerk one *C. newsteadi* was found, which was not found in Oirschot. In Oirschot three *C. nubeculosis* were found, this species was not found in Nijkerk.

Table 3.19 The individual species found as a percentage of the total number of *Culicoides* spp. captured per location

	Location	
Species	Nijkerk (%)	Oirschot (%)
C. obsoletus	65.6	70.7
C. dewulfi	17.9	13.7
C. chiopterus	2.3	1.0
C. punctatus	2.7	3.6
C. stigma	0.4	0.5
C. festivipennis	10.7	10.3
C. newsteadi	0.4	-
C. nubeculosis	-	0.2
Total	100	100

4. Discussion

This study attempted to gain insight into the circadian rhythm and olfactory responses of *Culicoides* species in The Netherlands, thereby looking at several other factors. This chapter will discuss the outcomes of this study considering circadian rhythm, olfactory response, effects of trap location on the number of captured species, effects of some meteorological conditions on the number of captured species, comparisons between the efficacy of the Liberty Plus traps and the Onderstepoort light trap and comparisons between the diversity and density of the captured *Culicoides* species on the two test locations, respectively situated in Nijkerk and Oirschot.

4.1 Circadian rhythm of *Culicoides* species

This experiment used baited suction traps to study the circadian rhythm of *Culicoides* spp. Carbon dioxide and octenol are frequently used as an attractant for (blood-seeking) female *Culicoides* species (Blackwell et al., 1996). Hence, the circadian rhythm in host-seeking activity of *Culicoides* spp. is revealed, rather than the general circadian rhythm of *Culicoides* spp. However, it is this part of the population that is of interest when it comes to sweet itch, or the transmission of viruses.

This study shows that there is a circadian rhythm in host-seeking behaviour of *Culicoides* species found in the Netherlands. Most species were captured during the period between 5pm and 11pm (41% of the total catch). However, >25% of the captured *Culicoides* spp. were caught during daylight hours. 19% of all species were caught between 11am and 5pm. None of the species captured showed an unambiguous aversion for one of the time periods.

These results are partly consistent with the findings by Service (1971). He studied the adult flight activity of some British *Culicoides* species, including *C. obsoletus* and *C. punctatus*. According to his results, numbers of captured species increase significantly between 6pm and 9pm, after which they dropped again. However, the experiment of Service (1971) showed almost none activity during the rest of the day, whereas this experiment shows that patterns of activity are not restricted to this particular time period.

Only two studies were found suggesting similar activity patterns. Reuben (1963) proposed a general increase in activity of *C. impunctatus* around sunset, but otherwise flight was evenly spread over 24 hours. His findings were objected by Blackwell (1997), who found that female *C. impunctatus* have a bimodal pattern of activity, with peaks at dawn and dusk. Recently, Balenghien et al. (2008) reported *Culicoides* diurnal activity during a study on *C. obsoletus* / *scoticus*, *C. dewulfi* and *C. chiopterus* as a vector of the bluetongue virus in north eastern France. The study was performed in October 2007. Using a mouth aspirator on the belly of a sheep between 10am and 6pm, highest catches were found between 10am and 11am. Using a backpack operator during three afternoons, a total number of 1076 *Culicoides* species were collected. Balenghien et al. (2008) suggest clear evidence of *Culicoides* diurnal activity.

Previous reports on the flight activity of *Culicoides* species have mainly focussed on neotropical species, which are mostly found to be crepuscular of nocturnal (Mellor et al., 2000). Only little has concentrated on northern European species other than *C. impuncatus*. Dik and Ergül (2006) studied the nocturnal flight activity of twelve

Culicoides spp., including *C. obsoletus* and *C. punctatus*. They found that *Culicoides* species fly at night and their numbers decrease in the morning. However, this study did not account for daylight hours.

Recently, van der Rijt et al. (2007) studied the daily cycle of *Culicoides* species in the Netherlands. They found that all of the *Culicoides* species captured (mainly *C. obsoletus,* followed by *C. pulicaris*) were most active at sunset, less so at sunrise and only a few species were captured in the afternoon or at night. However, samples were only taken when it was dry and when there was little or no wind. This might have caused sampling biases.

Conflicts between results presented here and results from previous studies can be explained by differences between *Culicoides* species under study, but also by difference between environmental conditions, meteorological conditions and trapping methods. Results from experiments on neotropical species do not just apply for northern European species. Any clarification of differences between the study by van der Rijt et al. (2007) and results presented here are two-folded.

First of all, Van der Rijt et al. (2007) used a tent trap and in our experiment suction traps are used. Different trapping methods can suggest different activity patterns and meteorological conditions can affect the efficacy of trapping methods in different ways (Blackwell, 1997). Secondly, Van der Rijt et al. (2007) only sampled during dry and windless days, whereas in this study sampling occurred under all weather conditions. The activity of *Culicoides* spp. is influenced by meteorological conditions. Conflicts of results might be explained by the exclusion of certain weather conditions in one of the experiments.

This study was performed in late season. Seasonality influences *Culicoides* activity (Kettle et al., 1998). Difference between seasons is influenced by meteorological conditions, day length and other factors that cycle annually. Kettle et al. (1998) studied factors affecting the activity of *Culicoides* species in Australia. They found that the interaction between season and time was highly significant, indicating that at a particular time, catch depend on the season. Results found here may not fully apply for springtime or summer. Also Balenghien et al. (2008), which reported diurnal activity of northern European *Culicoides* spp., conducted their study in late season.

It is presumed that *Culicoides* host-seeking behaviour is highly correlated with the gonothrophic development stage of females. Balenghien et al. (2008) found that of the 1076 *Culicoides* spp. captured during the afternoon using a backpack aspirator near haystacks, 92% were parous females and 80% were gravid females. They suggest these midges were probably seeking for a breeding site. The age structure of the collected females is not investigated in the present study. There might be a relation between the diurnal host-seeking activity and gonotrophic development stage of *Culicoides* spp. It is assumed that mainly host-seeking nulliparous and parous female midges are attracted to odour baited traps. However, no distinction is made between these two groups during the experiment. Non-baited suction traps can be used to reveal any possible relation between diurnal activity and sex-ratio / developmental stage, including female midges from all developmental stages and male midges.

This work shows that there is a circadian rhythm in host-seeking activity of *Culicoides* species in the Netherlands. However, this work also shows that a significant number of *Culicoides* spp. is active during daylight hours. Hence, recommendations to stable *Culicoides* hypersensitive horses at night in order to avoid allergic dermatitis do not apply. Moreover, current European rules for stocking livestock indoors at night in order to avoid bluetongue transmission are invalid. European rules are based on the ecology of *C. imicola,* which is considered to be the most important vector of blue tongue disease (Mellor, 1996). *C. imicola* is found to be active during night times. Current legislation does not account for the diurnal behaviour of indigenous species such as *C. obsoletus*. However, BTV has recently been isolated from this species, which means current rules are unfounded.

A final point of consideration is the sudden outbreak of *Culicoides* species on the 29th of September. On the 29th of September (test day 11) between 5pm and 11pm the number of captured *Culicoides* spp. was extremely high compared to the other catches. The number of captured species was 837, almost 200 times higher than the average catch of the experiment (n=4.44). In order to avoid the potential effect of sampling bias, it was decided to exclude the *Culicoides* samples from this day from the analysis. When including this number, assumptions on normality and equal variances would be invalid and non-parametric tests would be required. Since these tests do not facilitate the comparison between groups, parametric test are preferred.

There is no evident explanation for the extremely high number of captured *Culicoides* species on the 29th of September. However, the weather conditions were favorable, with relatively high temperatures on the night before the catch and relatively high humidity on the day of the catch. All species were captured in one single trap. The other traps did not show such high numbers. Maybe, the particular trap was situated near an oviposition site of midges and because weather condition were positive, a complete batch of larvae hatched into adult midges, subsequently looking for a host and finding the baited trap. Another possibility is that a host animal has passed the trap followed by thousands of midges.

4.2 Olfactory choices of *Culicoides* species

This study shows that there is a clear effect of addition of octenol to carbon dioxide, when it comes to olfactory responses of *Culicoides* species.

A significant higher number of *Culicoides* species was captured using a combination of octenol and CO_2 as a bait, compared to CO_2 alone, but also compared to a combination of horse hair and CO_2 and a combination of cattle hair and CO_2. Trap catches were respectively 3.8, 9.5 and 4.5-fold greater. Nearly 63% of all captured *Culicoides* spp. was caught with the trap baited with octenol + CO_2.

Whereas the differences between the numbers of captured species per odour baited trap were significant for *C. obsoletus* and *C. punctatus*, differences were less obvious for the other species. There is a similar trend towards a preference for octenol + CO_2 in the group of *C. dewulfi* and *C. festivipennis,* but the other species did not show such a preference at all. While in some cases numbers might be too low to show any clear contrasts, differences in olfactory responses between the various species could also be based on physiological differences. Response and attraction to particular odour extracts vary between *Culicoides* species due to antennal receptor profiles (Mands et al., 2004). When the receptor profiles for a certain chemical are absent, the species will not respond to the bait. Natural host preference is another important factor of influence. Although receptors to locate certain semiochemicals are present, species might not respond due to their natural preference for certain hosts (Mands et al., 2004). Little research is done on antennal receptor profiles of *Culicoides* spp.

1-octen-3-ol is present in a variety of leguminous plants, but it is also a component of the body odour of ruminants (Bhasin et al., 2001). *C. obsoletus* and *C. punctatus* have a preference for cattle as their natural host. This might explain the significance in number of species captured using octenol + CO_2. The fact that a combination of cattle hair and CO_2 did not had the same effect, is probably caused by another reason.

Various studies outline the effect of a shift in concentration on the efficacy of the bait (Blackwell et al., 1996; Bhasin et al., 2001). Bhasin et al. (2001) showed that there is an increase of the effective length of the plume with each substantial increase in the volume of CO_2 released. They also showed that the sensitivity of *C. impunctatus* across a range of CO_2 levels is very high. Similar results were found by Mands et al. (2004) who tested various animal odours on different *Culicoides* species. Results showed that positive behavioural responses to host odours were dose dependent. Extracts of water buffalo and red deer were found to be more active than extracts of sheep, pony and calf.

Failure of the host extracts (cattle hair and horse hair) to increase the yield of *Culicoides* species may be explained by a low dose. The release rates of the animal extracts are undetermined and possibly very low. In order to increase the efficacy of odour baits based on animal extracts, samples should be concentrated or replaced by purified or synthetic components.

Previous reports on the olfactory response of *Culicoides* species already found remarkable differences in the effectiveness of different odour baits. Blackwell et al. (1996) studied the olfactory response of *C. impunctatus*. They found that octenol increased female *C. impuncatus* catches when compared with control catches. When using a combination of octenol with a volatile hormone produced by parous female midges, additive effects were recorded. Bhasin et al. (2001) tested the efficacy of several putative attractants for *C. impunctatus*. Highest catches were obtained with triple bait combinations, using cow urine + acetone + CO_2 or using cow urine + octenol + CO_2. Compared to CO_2 alone, these combinations increased trap catches 22 and 24-fold respectively. Mands et al. (2004) studied the effect of adding hexane extracts of hair samples of five different host animals to a combined attractant of octenol and CO_2. The effect was studied on the behavioural response of six different species, including *C. impunctatus*, *C. pulicaris*, *C. albicans* and *C. delta*. The addition of sheep and red deer extracts had a negative effect, whereas the addition of calf, pony and water buffalo extracts had an additive effect. Only water buffalo significantly enhanced the trap catches of both *C. impunctatus* and *C. pulicaris*. Differences between the olfactory responses of various species under study were established.

Synergism between CO_2 and 1-octen-3-ol has being recorded for numerous *Culicoides* species (Bhasin et al., 2001), as well as for mosquitoes (Takken and Kline, 1989). In this study synergism could not be revealed since octenol has not been tested in isolation. Also the effect of different release rates is not been investigated. To maximize trap catches such information will be of great importance. However, it has been proved that the addition of octenol to CO_2 has a clear effect on *Culicoides* catches. This suggests that further research on odour baits for *Culicoides* spp. might enhance the efficacy of the Liberty Plus trap significantly, rendering it an efficient sampling tool for *Culicoides* spp. 24 hours a day. Attention should be paid on increasing the affectivity of the bait without selecting for a particular species of *Culicoides*. Hence, cocktails of semiochemicals might be more effective than single chemicals.

Lastly, a small remark on the analysis of the results should be made. To reduce dependence of the variance on the mean and to achieve normality, catches (x) were transformed to ln(x)+1. Although normality could be assumed, equal variances could not be assumed (p=0.032). Hence, a non-parametric test should have been used. However, it was decided to use One-way ANOVA since this test facilitates the comparison of two groups when followed by Fisher's LSD procedure. Results obtained when using a non-parametric test (Kruskall Wallis), were similar to the results presented in chapter 3.

4.3 Effects of trap location on the catches

There was no significant difference in number of captured *Culicoides* species between the three trap locations in Nijkerk. However, there was a trend towards lower numbers on location C, compared to the two other locations.

There was also no significant difference in number of captured *Culicoides* species between the four different trap locations in Oirschot. However, a clear trend toward higher numbers on location B was shown, when compared to location C and D.

There are various factors that can cause differences of species abundance between the locations. To draw any conclusions, these various factors, including the influence of meteorological conditions on the trap, type of vegetation near the trap, trap position with regard to the hosts, etcetera need to be investigated. However, some speculations can be made.

In Nijkerk, trap C was located in the open field. On the contrary, trap A was located near the stables and surrounded by high vegetation and trap B was located next to a barn. Possibly, trap C had a higher exposure to meteorological conditions such as wind and rain, compared to the other traps. This might have caused lower numbers of captured species.

In Oirschot, trap position B was located next to the paddock (<1.5m), whereas trap position A was in the pasture and trap position C and D were located between pastures at a distance of >10 m from the horses. During the experiment over 30 horses lived in the paddock 24 hours a day. Approximately 20 horses went out in the pasture during day times. Compared to the pasture, the number of horses per square meter was much higher in the paddock. The high numbers of captured species on location B, compared to location C and D is probably related to the trap position with regard to the horses.

Randomization of the traps during an experiment is crucial to avoid sampling biases. Host animals provide a strong stimuli for midges. An optimal trap position contributes to successful sampling and is very important when it comes to efficient control strategies for *Culicoides* species.

4.4 Effects of meteorological conditions on the catches

This study suggests a possible correlation between temperature, saturation deficit and wind speed with catch size. However, results were not consistent.

Results did not show any correlation between the number of captured *Culicoides* species and individual meteorological conditions when looking at the total catch per day (study on circadian rhythm), or at the total catch per two days (study on olfactory response). These results did also not show any significant correlation with one of the models combining various meteorological factors.

However, when focussing on the number of species captured during the period between 11am and 5pm, a significant negative correlation is found between wind speed and number of captured species. Also the combination of the three variables temperature, saturation deficit and wind speed, showed a significant correlation with the catch. Combining wind speed (ranging from 0.9 m/s till 4.4 m/s) and saturation deficit (ranging from 2.64 till 9.10) gave the most optimal significant model with a contribution of 74% to the final outcome. Temperature, ranging from 15.4 ºC till 21 ºC was of minor importance. This result is consistent with former studies, suggesting temperatures below 10 ºC (Mellor et al., 2000) and beyond 24 ºC (Kettle, 1962) suppressing *Culicoides* activity. Temperatures found here fit within this range, and therefore are assumed to have little effect on the catches.

No significant relationship was found with sun hours, referring to light intensity. Similar results were found by Blackwell (1997), who also did not found any relation between illumination and number of *C. impunctatus* caught. On the contrary, Kettle (1962) describes how light intensity plays an important role for *C. barbosia*. However, according to Kettle (1962) also for *C. furens* illumination seems to be less important.

When looking at the other periods, significant results were found for the period between 5am and 11am. Combining temperature and wind speed resulted in a model contributing 71% to the final outcome showing a significant correlation ($p=0.01$) with the number of captured species. Whereas temperature was found to be of minor importance in the period between 11am and 5pm, in this period temperature, now ranging from 6.6 ºC till 13.2 ºC is a significant factor. On the contrary, saturation deficit, ranging from 0.12 till 0.75 was of minor importance. High relative humidity is assumed to be important to *Culicoides* spp. due to their small size and hence, high rates of desiccation. At these levels, humidity is optimal and any effect of a small change in saturation deficit on *Culicoides* activity is negligible (Mellor et al., 2000).

There was no significant correlation found between the number of captured *Culicoides* species and weather conditions during the two other periods. In the period between 5pm and 11pm, temperature ranged from 10.8 ºC till 14.5 ºC and saturation deficit ranged from 0 till 3.75. In the period between 11pm and 5am, temperature ranged from 6.4 ºC till 12.3 ºC and saturation deficit ranged from 0 till 0.87. Ranges of wind speed were similar in all four periods. Although the effect of saturation deficit is expected to be minor within these ranges, temperature and wind speed could have had an effect based on literature and former results. Further more, while the effect of wind speed was significant during the period between 11am and 5pm, the p-values of the relationship between this individual factor and number of captured species during the other periods were respectively 0.916, 0.960 and 0.597.

This study suggests a possible correlation between meteorological conditions and catch size. However, results were not conclusive and differences in the significance of the correlation between various time periods were found. This suggest the idea that *Culicoides* species might be more sensitive to small variations in weather conditions during certain hours of the day, compared to other time periods. However, the correlation between weather factors and catch size was considered to be a minor part of this study and excessive research is needed to confirm such speculations.

In addition, it is recommended to integrate the correlation between meteorological factors and *Culicoides* catches in all studies on daily cycle. Most species show marked fluctuations through though 24 hours. These variations might be correlated with a change in weather conditions (Kettle et al., 1998).

Finally, it is assumed that activity drops down at temperatures below 10 °C (Mellor et al., 2000; Kettle, 1962). However, this study shows that at average temperatures of below 10 °C still numerous *Culicoides* spp. are active. Muijskens (2008) studied the over wintering behaviour of *Culicoides* species. She also suggests *Culicoides* activity at outdoor temperatures below 10 °C. However, she focussed on livestock farms assessing *Culicoides* activity in stables, where temperatures are assumed to be higher than outdoors.

During this study there were several factors that might have influenced the outcome and should be taken into account. These factors are:

- Data on temperature and humidity were obtained from data loggers that were placed near the test traps. Data on wind speed, sun hours and rain fall were provided by the national meteorological service (KNMI, de Bilt, The Netherlands). Data recorded by KNMI might differ from the actual values on microclimate level. Small differences might have influenced the results.

- Except for saturation deficit in the experiment on olfactory response, none of the weather conditions changed significantly during the experiment. Negative results may reflect the limited ranges over which the study was conducted.

- Meteorological conditions can affect the efficacy of trapping as well as the activity rate of *Culicoides* spp. Suction traps may operate less efficiently at higher wind speeds because their ability to draw in air is lessened.

- There may have been other factors that have influenced the number of *Culicoides* spp. captured but which are not recognized as such. Such factors might influence the results, leading to misinterpretations when it comes to correlation between weather conditions and *Culicoides* caught. For example, the effects of moonlight or moon phase were not investigated in the present study. According to Kettle e al. (1998) these factors have a significant effect on *Culicoides* activity. Also the effect of ecological conditions may not be underestimated.

- The number of captured species could have been to low, or the experimental period could have been to short for any reliable analysis.

- Correlations between *Culicoides* activity and meteorological conditions vary between species (Mellor et al., 2000). In this study species are pooled.

- The effects of meteorological conditions are studied under the presence of carbon dioxide and octenol. Hence, results represent odour induced behaviour. *Culicoides* activity might be triggered by the attractants.

4.5 Comparisons between the efficacy of the Liberty Plus traps and the Onderstepoort light trap

Differences between the number of species captured with the Onderstepoort light trap and the Liberty plus traps were assessed. Results found on the two test locations were not similar.

In Nijkerk, a significant higher number of species was captured using the light trap, compared to the number of captured species using the Liberty Plus traps. Similar results were found by Takken et al. (2008) who made a rough comparison between the Liberty Plus trap and the Onderstepoort light trap. However, in Oirschot the number of *Culicoides* species captured with the Liberty Plus trap baited with CO_2 + octenol was significantly higher than the catches of the light trap, whereas no differences were found between the light trap the other three Liberty Plus traps.

The trapping method influences the catches. The level of attractiveness to different traps depends on the species. Using a baited trap, blood seeking females which respond to the bait are selected. Using a light trap, only positively photo tactic flying insects are attracted. The efficacy of the Liberty Plus trap for the *Culicoides* species captured in the present study is unknown (Takken et al., 2008). However, Mands et al. (2004) describes that this trap is an effective sampling tool for *C. impunctatus.*

Overall, the light trap is described as a very effective sampling tool for *Culicoides* species, being more effective than any other trapping method (Blackwell, 1997). However, light traps are only effective during night time hours. Considering the diurnal activity of several *Culicoides* species, baited traps have the advantage of being effective 24 hours a day. The unexpected results found in Oirschot regarding the comparison between the light trap and the Liberty Plus traps might be influenced by that fact, since comparisons are based on 48 hour catches, including day and night time hours. However, other factors might have influenced the catches too. Possibly the location of the light trap had a negative effect on the number of species captured. The light trap was placed near the stables housing three horses, at a distance of about 200 m from the other traps and the paddock with the other horses. The load of horses in the paddock might have had a very strong power of attraction on *Culicoides* species, manipulating the efficacy of the light trap (see paragraph 4.3 ‘*Effects of trap location on the catches*’).

Comparisons between the two different traps are based on three replicas. Only one light trap is been used, placed on a single location. For an accurate comparison, more replicas are required. Also, several light traps need be used on various locations to avoid sampling biases.

4.6 Comparisons between the diversity and density of *Culicoides* species captured on the two locations situated in Nijkerk and Oirschot

Culicoides species diversity was similar for the two test locations. *Culicoides* densities appear higher on the test location in Oirschot, compared to the test location in Nijkerk.

On both locations, *C. obsoletus* was found to be the most abundant species. *C. obsoletus* is proven to be capable of causing allergic dermatitis in horses (Anderson et al., 1991). Also *C. chiopterus*, *C. dewulfi*, *C. punctatus* and *C. nubeculosis* are suspected or proven to be allergenic. Almost 90% of all species captured on both of the locations are associated with sweet itch.

Recently, Takken et al. (2008) studied the population dynamics of *Culicoides* spp. in the Netherlands. They suggest that the taxonomic group *Culicoides obsoletus*, including *C. obsoletus / C. scoticus*, *C. dewulfi*, *C. chiopterus*, as well as the group *Culicoides pulicaris*, including *C. punctatus*, were strongly associated with farms. These finding are consistent with the species found in the present study. However, on both of the locations also high numbers of *C. festivipennis* were found. *C. festivipennis* is an opportunistic feeder, feeding on birds as well as on mammals. In former studies, this species is described as a minority, almost not associated with livestock farms (Takken et al., 2008; Meiswinkel et al., 2008).

Morphological differences between species from the Obsoletus Complex are very little. In former studies, species of this complex, including *C. obsoletus*/*C scoticus*, *C. dewulfi* and *C. chiopterus*, may not always have been differentiated from each other, but included under the taxon *C. obsoletus*.

Since the experimental setup was not equal for the two locations, accurate comparisons between population densities can not be made. There seems to be a higher density of *Culicoides* species in Oirschot compared to Nijkerk. Differences in abundance can have many causes. To study the differences between the *Culicoides* populations in Nijkerk and Oirschot, it would be interesting to compare the ecological systems on the two locations, including habitat type and geographical and ecological characteristics.

It is unknown if the catches are representative for the *Culicoides* population in the vicinity under study.

5. Conclusions

Circadian rhythm

This study shows that there is a circadian rhythm in host-seeking activity of *Culicoides* spp. found in the Netherlands. Most species were captured in the period between 5pm and 11pm. However, a considerably amount of species was captured during daylight hours. None of the species found showed an unambiguous aversion for one of the time periods.

This study suggests clear evidence for *Culicoides* diurnal activity. Hence, recommendations to stable *Culicoides* hypersensitive horses at night to avoid allergic dermatitis do not apply. Current European rules for stocking livestock indoors at night to avoid bluetongue transmission are invalid.

This study was performed in late season. It is assumed seasonality influences *Culicoides* activity. Results found here may not apply for early spring or summer.

The age structure of the female *Culicoides* captured is not investigated. There might be a relation between diurnal host-seeking behavior and the gonotrophic developmental stage of *Culicoides* spp.

Olfactory responses

This study shows that there is a clear effect of addition of octenol to carbon dioxide as bait for *Culicoides* spp. The addition of horse hair and cattle hair to CO_2 did not increased the catches compared to CO_2 alone. Failure of the host extracts to increase the catches might be explained by a low dose.

C. obsoletus and *C. punctatis* showed a preference for a bait combination of octenol and CO_2, compared to the other baits tested. The preference of the other species captured was less significant. Differences in olfactory responses might be based on physiological differences or difference in host preference between the various species.

In this study synergism between the odours is not revealed. Also the effect of different release rates is not investigated. To maximize trap catches such information is very important.

Further research on odour baits for *Culicoides* spp. might enhance the efficacy of the Liberty Plus traps significantly, rendering it an efficient sampling tool for *Culicoides* spp. 24 hours a day. Attention should be paid on increasing the affectivity of the bait without selecting for a particular species of *Culicoides*.

Trap location

This study suggests that trap position influences the sampling efficacy of the trap. Hence, randomization of the traps is crucial to avoid sampling biases during tests on the efficacy of trapping methods. An optimal trap position contributes to the efficiency of the control strategy.

Meteorological conditions

This study suggests a possible correlation between temperature, saturation deficit and wind speed with numbers of *Culicoides* species captured. No significant relation between sun hours and catch size was found. Results assessing the correlation between meteorological factors and captured species were not consistent and differences in the significance of the correlation between various time periods were found. Several factors might have influenced the results.

Onderstepoort light trap

Results assessing the difference in efficacy between the Liberty Plus traps and the Onderstepoort light trap were contradictory. In Nijkerk, the light trap seemed significantly more effective than the Liberty Plus traps. In Oirschot opposite results were found. The location of the light trap in Oirschot might have influenced to results, leading to a limited catch size and misleading results. For an accurate comparison between the traps, sufficient replicas are required as well as the use of several light traps on various locations.

Culicoides diversity and density

Culicoides species diversity was similar for the two test locations, respectively situated in Nijkerk and Oirschot. On both locations *C. obsoletus* was found to be the most abundant species, followed by *C. dewulfi* and *C. festivipennis*. Other species found were *C. chiopterus*, *C. punctatus*, *C. stigma*, *C. nubeculosis* and *C. newsteadi*.

Culicoides densities appear higher on the test location in Oirschot, compared to the test location in Nijkerk. Since the experimental setup was not equal for the two locations, accurate comparisons between the population densities could not be made.

6. Recommendations for future work

Only little is known about *Culicoides* species in the Netherlands. For accurate strategies against *Culicoides* hypersensitivity in horses, or against the transmission of arbo-viruses in livestock, insight into the ecology of indigenous *Culicoides* species is required. This study attempted to gain insight into the circadian rhythm and olfactory responses of *Culicoides* species in the Netherlands. Results gave rise to the following recommendations for future research:

Research on the circadian rhythm of *Culicoides* species, including their activity pattern during springtime and summer. This study gave some first evidence of *Culicoides* diurnal activity. More research is needed to confirm and to complete these results. Also, this study is performed in late season. To be able to give accurate advice to prevent horses from sweet itch and with respect to European legislation for the transmission of bluetongue, it is crucial to know whether or not the same activity patterns can be found across all seasons.

Research on the correlation between meteorological conditions and *Culicoides* activity. Results in this field have not always found to be consistent and more research could contribute to a better understanding. Also the effect of weather conditions on *Culicoides* diurnal activity would be an interesting study object. This study can be complementary to research on the effect of seasonality on diurnal activity.

Research on the optimization of odour baits for *Culicoides* spp. This study indicates that the submission of certain odours can lead to a significant increase of the catch. Research on olfactory responses of *Culicoides* species and the efficacy of various odour baits can contribute to effective control strategies 24 hours a day. Attention should be paid on increasing the affectivity of the bait without selecting for a particular species of *Culicoides*. Synergism between the odours and the effect of different release rates should be taken into account.

Research on factors that influence *Culicoides* species abundance and trap efficacy in a microhabitat. This study suggests that the position of the trap contributes to the efficacy of the trap. Insight into the various factors that influence species abundance and trap efficacy in a microhabitat can contribute to the optimization of control strategies by optimizing the trap position. Factors of interest include type of vegetation near the trap, trap position with regard to the host, barns and other fundamental objects, the influence of meteorological conditions on the trap at a certain position, etc.

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Appendices

Appendix I Data set experiment 1 Circadian rhythm

Table 1.1 Total number of captured *Culicoides* spp. per six-hour period (1)

Nr.	date	time	total nr *C.*spp.	*C. obsoletus*	*C. dewulfi*	*C. chiopterus*	*C. punctatus*	*C. stigma*	*C. festivipennis*	*C. newsteadi*
1	9-sep	11:00-17:00	0							
2		17:00-23:00	2			1				1
3	10-sep	23:00-05:00	0							
4		05:00-11:00	0							
5		11:00-17:00	6		6					
6		17:00-23:00	7	2	4				1	
7	11-sep	23:00-05:00	3		1	2				
8		05:00-11:00	0							
9		11:00-17:00	0							
10		17:00-23:00	1		1					
11	12-sep	23:00-05:00	0							
12		05:00-11:00	0							
13	14-sep	11:00-17:00	0							
14		17:00-23:00	3		1				2	
15	15-sep	23:00-05:00	1		1					
16		05:00-11:00	0							
17		11:00-17:00	0							
18		17:00-23:00	6	2	1				3	
19	16-sep	23:00-05:00	1	1						
20		05:00-11:00	0							
21		11:00-17:00	11	6	3				2	
22		17:00-23:00	33	22	4	2			5	
23	17-sep	23:00-05:00	0							
24		05:00-11:00	27	17	6	1	2		1	
25		11:00-17:00	5	4	1					
26		17:00-23:00	5	4	1					
27	18-sep	23:00-05:00	2	2						
28		05:00-11:00	4	2	1				1	
29		11:00-17:00	1		1					
30		17:00-23:00	1	1						
31	19-sep	23:00-05:00	3	2	1					
32		05:00-11:00	0							

Table 1.1 Total number of captured *Culicoides* spp. per six-hour period (2)

Nr.	date	time	total nr *C.*spp.	*C. obsoletus*	*C. dewulfi*	*C. chiopterus*	*C. punctatus*	*C. stigma*	*C. festivipennis*	*C. newsteadi*
33	21-sep	11:00-17:00	3	2	1					
34		17:00-23:00	6	4			1		1	
35	22-sep	23:00-05:00	2	1	1					
36		05:00-11:00	14	8	4				2	
37		11:00-17:00	1		1					
38		17:00-23:00	10	5	4				1	
39	23-sep	23:00-05:00	3	1			1	1		
40		05:00-11:00	6	4					2	
41	29-sep	17:00-23:00	837	787	31		11		7	1
42	30-sep	23:00-05:00	14	13					1	
43		05:00-11:00	2	1					1	
44		11:00-17:00	7	5					2	
45		17:00-23:00	4	4						
46	1-okt	23:00-05:00	3	2					1	
47		05:00-11:00	3	3						
48		11:00-17:00	1	1						
49	8-okt	11:00-17:00	9	8					1	
50		17:00-23:00	4	4						
51	9-okt	23:00-05:00	9	9						
52		05:00-11:00	3	2	1					
53		11:00-17:00	7	5			2			
54		17:00-23:00	1				1			
55	10-okt	23:00-05:00	9	8					1	
56		05:00-11:00	1	1						
57		11:00-17:00	1	1						
58		17:00-23:00	16	14	2					
59	11-okt	23:00-05:00	0							
60		05:00-11:00	1	1						

Appendix II Data set experiment 1 Circadian rhythm - numbers per time period

Table 2.1 Total number of captured *Culicoides* spp. per six hour period

11am-5pm

Catch no.	total nr *C.* spp.	*C. obsoletus*	*C. dewulfi*	*C. chiopterus*	*C. punctatus*	*C. stigma*	*C. festivipennis*	*C. newsteadi*
1	0	0	0	0	0	0	0	0
2	6	0	6	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	11	6	3	0	0	0	2	0
7	5	4	1	0	0	0	0	0
8	1	0	1	0	0	0	0	0
9	3	2	1	0	0	0	0	0
10	1	0	1	0	0	0	0	0
11	7	5	0	0	0	0	2	0
12	1	1	0	0	0	0	0	0
13	9	8	0	0	0	0	1	0
14	7	5	0	0	2	0	0	0
15	1	1	0	0	0	0	0	0

5pm-11pm

Catch no.	total nr *C.* spp.	*C. obsoletus*	*C. dewulfi*	*C. chiopterus*	*C. punctatus*	*C. stigma*	*C. festivipennis*	*C. newsteadi*
1	2	0	0	1	0	0	0	1
2	7	2	4	0	0	0	1	0
3	1	0	1	0	0	0	0	0
4	3	0	1	0	0	0	2	0
5	6	2	1	0	0	0	3	0
6	33	22	4	2	0	0	5	0
7	5	4	1	0	0	0	0	0
8	1	1	0	0	0	0	0	0
9	6	4	0	0	1	0	1	0
10	10	5	4	0	0	0	1	0
11	837	787	31	0	11	0	7	1
12	4	4	0	0	0	0	0	0
13	4	4	0	0	0	0	0	0
14	1	0	0	0	1	0	0	0
15	16	14	2	0	0	0	0	0

11pm-5am

Catch no.	total nr *C.* spp.	*C. obsoletus*	*C. dewulfi*	*C. chiopterus*	*C. punctatus*	*C. stigma*	*C. festivipennis*	*C. newsteadi*
1	0	0	0	0	0	0	0	0
2	3	0	1	2	0	0	0	0
3	0	0	0	0	0	0	0	0
4	1	0	1	0	0	0	0	0
5	1	1	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
7	2	2	0	0	0	0	0	0
8	3	2	1	0	0	0	0	0
9	2	1	1	0	0	0	0	0
10	3	1	0	0	1	1	0	0
11	14	13	0	0	0	0	1	0
12	3	2	0	0	0	0	1	0
13	9	9	0	0	0	0	0	0
14	9	8	0	0	0	0	1	0
15	0	0	0	0	0	0	0	0

5am-11am

Catch no.	total nr *C.* spp.	*C. obsoletus*	*C. dewulfi*	*C. chiopterus*	*C. punctatus*	*C. stigma*	*C. festivipennis*	*C. newsteadi*
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	27	17	6	1	2	0	1	0
7	4	2	1	0	0	0	1	0
8	0	0	0	0	0	0	0	0
9	14	8	4	0	0	0	2	0
10	6	4	0	0	0	0	2	0
11	2	1	0	0	0	0	1	0
12	3	3	0	0	0	0	0	0
13	3	2	1	0	0	0	0	0
14	1	1	0	0	0	0	0	0
15	1	1	0	0	0	0	0	0

Appendix III Data set experiment 2 Olfactory response

Table 3.1 Total number of captured *Culicoides* spp. per odour baited trap

CO_2

date	total nr *C.*spp.	*C.obsoletus*	*C. dewulfi*	*C. chiopterus*	*C.punctatus*	*C. stigma*	*C. nubeculosis*	*C.festivipennis*
10-sep	51	43	4	1	0	0	0	3
12-sep	48	38	2	7	1	0	0	0
14-sep	19	6	3	0	0	2	0	8
16-sep	12	7	1	0	0	2	0	2
18-sep	*m*	*m*	*m*	*m*	*m*	*m*	*m*	*m*
20-sep	7	1	1	0	0	0	0	5
22-sep	7	4	1	0	0	0	0	2
24-sep	147	81	50	0	0	1	0	15
26-sep	4	3	0	0	0	0	0	1
28-sep	2	0	0	0	0	1	0	1

CO_2 + octenol

date	total nr *C.*spp.	*C.obsoletus*	*C. dewulfi*	*C. chiopterus*	*C.punctatus*	*C. stigma*	*C. nubeculosis*	*C.festivipennis*
10-sep	147	99	3	0	28	0	1	16
12-sep	160	141	0	2	17	0	0	0
14-sep	313	260	2	0	4	0	0	47
16-sep	63	52	7	0	0	0	0	4
18-sep	34	14	17	1	0	0	0	2
20-sep	45	23	0	0	4	0	0	18
22-sep	310	237	38	2	0	0	0	33
24-sep	36	28	2	0	6	0	0	0
26-sep	221	123	87	1	1	1	0	8
28-sep	73	64	4	0	0	0	0	5

CO_2 + horse hair

date	total nr *C.*spp.	*C.obsoletus*	*C. dewulfi*	*C. chiopterus*	*C. unctatus*	*C. stigma*	*C. nubeculosis*	*C.festivipennis*
10-sep	13	8	2	1	0	0	0	2
12-sep	11	8	0	1	0	1	1	0
14-sep	34	20	4	3	1	1	0	5
16-sep	10	2	1	0	0	1	0	6
18-sep	17	8	5	0	0	0	0	4
20-sep	22	13	4	0	0	0	0	5
22-sep	7	6	1	0	0	0	0	0
24-sep	5	1	4	0	0	0	0	0
26-sep	17	10	5	0	0	1	0	1
28-sep	26	19	0	1	0	0	0	6

CO_2 + cattle hair

date	total nr *C.*spp.	*C.obsoletus*	*C. dewulfi*	*C. chiopterus*	*C.punctatus*	*C. stigma*	*C. nubeculosis*	*C.festivipennis*
10-sep	92	56	5	0	0	0	0	31
12-sep	21	18	2	0	0	0	1	0
14-sep	*m*	*m*	*m*	*m*	*m*	*m*	*m*	*m*
16-sep	11	5	2	0	0	1	0	3
18-sep	2	0	1	0	0	0	0	1
20-sep	3	3	0	0	0	0	0	0
22-sep	38	35	0	0	1	0	0	2
24-sep	27	25	1	0	1	0	0	0
26-sep	3	0	0	0	1	0	0	2
28-sep	39	34	3	0	0	0	0	2

Appendix IV The unfinished study on the oviposition site of *Culicoides* spp.

Due to difficulties with the identification of the larval stages of *Culicoides* spp. and due to a lack of time, the study on ovipostion site has not been completed yet. This appendix includes a description of this study and shows the results so far.

Research

Research question

How does the oviposition site of *Culicoides* species in The Netherlands relate to ecological conditions?

Importance of the study:

There is a lack of knowledge on the breeding habitats of *Culicoides* species in Europe (Conte et al., 2007). The difficulty of finding breeding habitats is an important constraint on control measures available, since application of larvicides is not a practical alternative. In order to find a successful control strategy, but also to predict the effect of ecological factors as well as the impact of climate change on *Culicoides* species, it is essential to understand ecological factors that influence their seasonal and regional abundance level.

Materials and Methods

The aim of this study was to take soil samples from three different soil types to detail the oviposition site of *Culicoides* species in the Netherlands. The soil types under study were pasture ground, forest ground, and the muckheap, which represent possible microhabitats for *Culicoides* larvae.

Experimental protocol

The oviposition site of *Culicoides* spp. was studied at the same location where the study on olfactory responses of *Culicoides* spp. was conducted.

The different soil types under study included pasture ground, forest ground and the muckheap. With respect to pasture ground and forest ground, three different sites each type were selected. All sites were near the horse living. Of each site, a pooled sample of ten sub samples were collected. The sub samples were taken randomly and scattered over each site. With respect to the muckheap, three samples were taken.

Samples were collected at noon. A soil-drill was used to collect the samples. Samples of 10 cm in depth were taken. Since *C. obsoletus* are recognized to oviposit in forest leaf litter (Kettle, 1962), the top layer was included. The samples were collected in labelled plastic bags to avoid dehydration. Each sample was investigated for larvae in the laboratory.

Extraction technique

The technique that was used in this experiment to recover the larvae from the soil was based on sieving and flooding by magnesium sulphate solution.

First, the Oostenbrink elutriator was used to separate the larvae from the heaviest soil particles. In the elutriator an upward water stream makes larvae and other fine particles float, whereas heavier particles sink to a lower part of the apparatus. The undercurrent water stream was started at a rate of 1000 ml/min, and was reduced to 800 ml/min during the second part of the extraction.

The suspension was sieved true four 45 µm sieves to remove the most finest particles.

A centrifugal flotation method was used for the extraction of active as well as inactive larvae from the debris that was washed from the sieves. 409 grams of $MgSO_4$ per litre water was used to obtain a solution with a gravity of 1.20. The suspension was centrifuged for three minutes at 3000 rpm. The supernatant was sieved true a 25 µm sieve.

A detailed description of the technique that was used can be found in Bezooijen, J., van, 1999; Methoden en technieken voor nematologie (p.43-48; 52-55).

Larvae identification

Little information on the identification of *Culicoides* larvae is available. It was intended to use the protocol by Kettle, D.S., Lawson, J.W.H., 1952; The early stages of British biting midges *Culicoides Latreille* (Diptera Ceratopogonidae) and allied genera. However, this has failed.

The protocol by Stehr, F.W., 1987; Immature Insects volume 1 and volume 2, has found to be very helpful in the identification of larvae to the level of the Lower Diptera.

Statistical analysis

It was intended to analyse the effect of the different soil types on larval numbers using one-way ANOVA, followed by Fishers' LSD procedure.

Results

Table 4.1 (next page) shows the total number of larvae found per sample site. A total number of six larvae from the Lower Diptera were found. Three larvae were found in the soil samples coming from pasture ground. All larvae were found on the same parcel. No larvae were found in the soil samples from forest ground. Two larvae were found in the samples coming from the muckheap. Both larvae were found in the same sample. One larvae was found in the extra sample, consisting of moistness from the muckheap.

Table 4.1 Numbers of larvae found per sample site

Sampling site	Numbers of larvae found[1]
Pasture 1	-
Pasture 2	3
Pasture 3	-
Forest 1	-
Forest 2	-
Forest 3	-
Muckheap 1	-
Muckheap 2	-
Muckheap 3	2
Extra sample	
Moistness of muckheap	1
Total	**6**

[1] Larvae found belong to the genus Lower Diptera.
Identification to the species level is not performed.

Discussion

Although the study on oviposition site has not been finished yet, some remarks can already be made.

The number of larvae of Lower Diptera found in this experiment is extremely low. A total of 300 cm^3 pasture ground, 300 cm^3 forest ground and 300 cm^3 of muckheap is sampled. Only three Diptera larvae were found in pasture ground and only two in the muckheap. In forest ground, no larvae were found at all. One larvae was found in the extra sample, consisting of 200 ml moistness from the muckheap. Regarding the high abundance of adult *Culicoides* species and other insects in the vicinity under study, higher numbers of larvae were expected. However, the efficacy of the sampling and extraction techniques that are used are unknown.

Little research is done in *Culicoides* larvae. Only a few studies describe sampling and extraction techniques to recover the larvae from the soil. Although some techniques are described to be effective, e.g. the flotation technique with sugar solution (Blackwell and King, 1997), there is no unanimity about the most optimal technique.

The extraction technique that is used in this experiment is based on sieving and flooding techniques with magnesium sulphate solution. This method is being describes by Kettle (1962) as an effective and non selective extraction technique. However, using this technique the samples remained very contaminated and could not be used for further determination. To get a clear sample, the Oostenbrink elutriator was used as a first step, after which the other techniques were applied.
The Oostenbrink elutriator is normally used to extract nematodes from different soil types, including manure. It is assumed *Culicoides* larvae can be extracted in a similar way when the technique is adjusted for their size and weight, being larger and heavier than the average nematode. However, this technique is not proven to be effective for *Culicoides* larvae and might have influenced the results.

The identification of the extracted larvae turned out to be extremely difficult. Species identification of *Culicoides* larvae have been described (Kettle and Lawson, 1952), but require considerable skill in insect morphology. Having no experience in the identification of larvae, it was impossible to identify any larvae to the species level. Hence, other techniques were required. Frequently larvae are reared for identification purposes. However, this method is not always successful and very time consuming.

Several studies describe the use of molecular identification methods of *Culicoides* species by polymerase chain reaction (Raich et al., 1993; Perrin et al., 2006; Mathieu et al., 2007; Nolan et al., 2007). Species-specific primers have been developed for several species, including species of the *C. obsoletus* complex (Nolan et al., 2007; Mathieu et al., 2007), for the *C. pulicaris* complex (Nolan et al., 2007) and for C. *festivipennis* and *C. nubeculosis* (Perrin et al., 2006).

Molecular analysis is successfully used for the identification of adult, larval and pupal stages of *Culicoides* spp. (Raich et al., 1993; Mathieu et al., 2007). Specimens of *C. scoticus* and *C. dewulfi* were analysed from different geographical localities in Europe and no intraspecific variations was observed (Nolan et al., 2007). Former developed primers are assumed to be suitable for the identification of larvae found during the present study. Since only a small amount of the larvae is required for DNA isolation, the rest of the sample can be used to test other primers in case of a negative result.

<u>Conclusion</u>

Due to difficulties with the identification of the larval stages of *Culicoides* spp., the study on oviposition site has not been completed yet.

The number of larvae of Lower Diptera found in this experiment is extremely low. The efficacies of the sampling and extraction techniques that are used are unknown. Species identification of *Culiciodes* larvae requires considerable skill in insect morphology. Molecular analysis will be used to identify the larvae found in the present experiment.

Research on the optimization of sampling and extraction techniques for *Culicoides* larvae is required. Only little research is done on *Culicoides* larvae. A good understanding of the materials and methods needed to perform any research on *Culicoides* larvae is a first requisite.