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The mammalogist's toolkit

The use of tools by mammals is an exceptional phenomenon (van der Grift 2010). By contrast *Homo sapiens* use tools for most of their activities. But what tools do we need for scientific mammal research? A brief historical review will help put this question in perspective. Toolkits contain important equipment, and this is true for mammalogists as well as craftsmen and the evolution of the contents of these kits over time is impressive. We cannot fairly apply the same scientific standards to early Cro-Magnon and to post-modern man. But there are some common traits of 'good science' that were shared by our prehistoric forebears: keen observation and passing acquired knowledge onto the next generation.

Since the dawn of mankind people have had a strong interest in vertebrates, with mammals perhaps triggering most of our curiosity. The numerous depictions from prehistoric caves clearly show this point. The few birds, if present at all, are sketchy and fish, amphibians or reptiles are scarce. The majority of rock art depicts beautifully carved or painted mammals which show that primitive man was keenly observant of anatomical details. The split hoofs in the numerous depictions of Artiodactyla are striking and the long slit of the preorbital gland in Cervidae is shown with a sense of utmost precision, while the variations in antler shape and size are almost as complete as current descriptions.

Alongside spears, bows and arrows, the toolkit in those days contained a range of tools for digging pits and strong nets, all to trap animals. At that time the goal of increasing knowledge about mammals was subordinate to consuming a rich source of protein and of fatty material that was sometimes stored for other purposes. The pictures of different mammals made by early *Homo sapiens* were a form of educational resource for passing on knowledge to future generations.

Nowadays the toolkit of modern mammalogists is much larger. Since the introduction of the metric system in 1799, and the almost worldwide acceptance of it, measurements and weights can unambiguously be identified; tools such as scales and calipers are a standard part of toolkits everywhere. Over time rifles replaced spears, bows and arrows and, since the early 1800s, cartridges and bullets have been used in hunting. These weapons also appeared in mammalogists' toolkits and were used regularly to collect mammals for natural history museums. For instance, Sir Alfred Russel Wallace brought back home a large number of specimens, of which 310 mammals, including 17 orang-utans, had been shot with a rifle (Wallace 1869). As recently as the second half of the last century Husson (1960) described a method - although rather shocking, as he mentions - for collecting bats in the tropics, shooting with the finest shot close to the centre of a bunch of bats, a method that avoided

damaging the skulls of the specimens. In 1897 the British inventor James Henry Atkinson invented the prototype mousetrap, called the "Little Nipper". Since then a wide array of snap traps has been designed. This ultimately led to the development of a specially designed 'museum snap trap' to collect small mammals as specimens for museums. With the growing interest in ecological studies, live traps have been designed, Havaharth^(R), Longworth^(R) and Sherman^(R), being some examples. Today's ecological studies would require up to several hundred small mammal traps, not so easily packed into a portable toolkit.

Electronic equipment, such as bat-detectors, has the great advantage of becoming smaller with the passing years. In the 1940s, Hooper needed a solid transport bike to cross London parks with his four kg Holgate detector to 'listen in the dark', as he described his activities (Boonman 1997). Now 21st century bat-detectors can be easily handheld, with options to listen and store ultrasonic batsounds.

Perhaps the most discrete steps in knowledge and the accompanying tools have been reached on the (sub) cellular and even molecular levels. And a bizarre phenomenon has emerged: the smaller the details, the bigger the instruments needed for proper analysis. Microscopes and electron microscopes can provide enough details to be able to study the numbers of chromosomes and other features within the cellular structures of mammals and other species. In 2011 the most promising equipment, seems to be Eppendorf^(R) tubes and related chemicals that open up new opportunities for further DNA studies. Proper DNA analysis in well-equipped laboratories can reveal a wide array of details. These can vary between determining the species of specimens, the family relations between individuals or genetic variation within populations.

Since prehistoric times, our way of depicting mammals has also evolved and developed.

Between 1566 and 1598, whales were regularly depicted in paintings which had obviously been correctly identified (Barthelmeß 1992). Since the eighteenth century realistic watercolour drawings and other paintings familiarized people around the world with local and distant mammal species. The unprecedented boom of photography and film since the end of the nineteenth century has also greatly contributed to our knowledge of mammalian life. Despite these technological advances we should not underestimate the work of modern artists, such as Paul Barruel, Helmuth Diller and Peter Twisk (figure 1). Their fine watercolours of mammals in typical postures add an extra dimension to written descriptions and can instantly provide details in a more direct way.

Mammalogy was initially a side product of humanity's main goal of acquiring proteins. Nowadays mammalogists do not need to be vegetarian, but they will only rarely consume discarded parts of objects of their study. Yet one can also overemphasize the importance of the toolkit. However, no matter how much equipment there is available (or how large it becomes), the most important point in science remains the amazement at the phenomena one encounters. This provides the basis for posing pertinent questions, undertaking good research and publishing the obtained results.

In this issue of Lutra, van den Brink et al. study isolated root vole (*Microtus oeconomus*) populations in the Netherlands. They used a wide array of tools to apply geometric morphometrics to vole skulls obtained from owl pellets. Their study also illustrates the increasing importance of applied DNA analysis, nowadays a standard tool in the mammalogist's toolkit. Cornelis, in his search for *Pipistrellus pygmaeus* flying around in a forest lane, couldn't have written his paper that records the addition of this species to the Dutch list without his bat-detector, an indispensable tool in modern research into

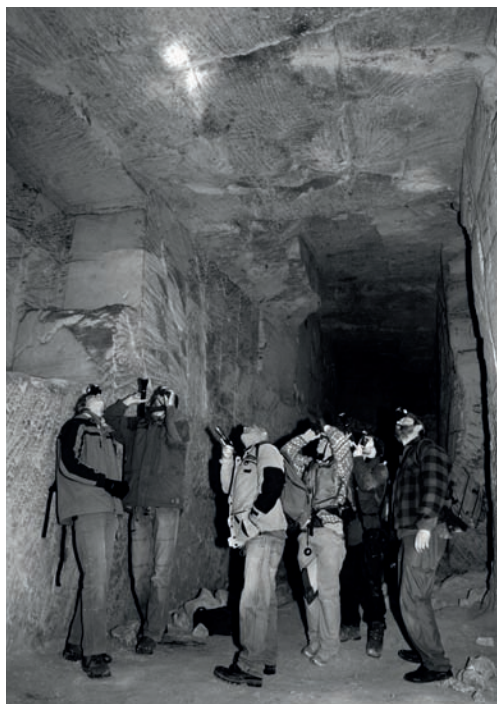


Photo 1. A torch and a pair of good eyes usually suffice to count hibernating bats. But to identify a bat hidden high up in a crack, a pair of binoculars may be required. *Photograph: B. Verboom.*

bats and their summer habitats. By contrast, hibernating bats can be investigated with simple tools: a torch and a pair of good eyes should do, as illustrated by Grol et al. in their study of the effects of a Christmas market on hibernating bats (photo 1). More sophisticated tools were used by Van Den Berge & Gouw to reveal the activities of the obscure pine marten (*Martes martes*) in a small and isolated forest complex. Numerous encounters with cetaceans have been described by sailors from their vessels at sea. Camphuysen & Krop describe the interactions between a female harbour porpoise and her calf, based on keen observations of the second author and his colleagues from a non-sailing object; however, an offshore gas production platform in this case can hardly be described as a tool.

In the second half of 2011 two new editors joined the board of *Lutra*, Jan Haelters and Eric Thomassen. Jan has dedicated most of his work to marine mammals along the Belgian coast and the Delta. Eric is a more general biologist with extensive field experience in almost all of Europe's countries, as well as on several other continents. Undoubtedly *Lutra* will benefit from their knowledge and skills in the years to come.

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Jan Piet Bekker



Figure 1. Edible dormouse (*Glis glis*) in a typical posture; watercolour drawing by Peter Twisk, also depicted (slightly adapted) in “Zoogdieren van de Benelux. Herkenning en onderzoek” by R. Lange et al. (1986).

The influence of a Christmas market on hibernating bats in a man-made limestone cave

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Abstract: It is generally acknowledged that human activity in bat hibernacula can disturb hibernating bats and that such activities need to be appropriately managed. The intensive commercial exploitation of limestone excavations during bats' hibernation period may be in conflict with the micro-environmental conditions that bats need to hibernate. The Fluweelengrot is a limestone quarry in the south of Limburg (the Netherlands). From 1997 onwards it has hosted a Christmas market that attracts over 100,000 people each November and December. The presence of so many visitors and fifty or so illuminated stalls drastically changes the microclimate of part of the cave system for 4-5 weeks. The parts of the cave system occupied by the Christmas market experience a temporary increase in substrate (ceiling) temperatures of 5-8 °C (with a maximum recorded temperature of 20.1 °C). To investigate the possible impact of the Christmas market on hibernating bats in the Fluweelengrot, we examined annual bat census data between the years 1980 and 2010. Data was divided into two periods: before the Christmas market (1980-1997) and during the Christmas market (1998-2010). From 1980 to 2010 seven species were found hibernating in the Fluweelengrot of which five were present in sufficient numbers to calculate trend indices. These were compared with the average indices in 89 other limestone quarries in south Limburg. For the whiskered/Brandt's bat (*Myotis mystacinus* and/or *M. brandtii*) and Geoffroy's bat (*Myotis emarginatus*), the trends in the number of individuals recorded in the Fluweelengrot were significantly less favourable than in other caves in south Limburg. In the absence of changes in other variables this suggests that the Christmas market has a negative impact on these species. Trends in the numbers of recorded pond bats (*Myotis dasycneme*), Daubenton's bats (*Myotis daubentonii*) and Natterer's bats (*Myotis nattereri*) did not differ significantly between the Fluweelengrot and other caves in the area. However a comparison of the distribution of these species of bats before (1990-1997) and during the Christmas market showed a significant shift in their distribution to parts further from the site of the Christmas market. The distribution of the whiskered/Brandt's bat and Geoffroy's bat was similar before and after the start of the Christmas market. Increasing commercial exploitation in a number of the marl caves in the south of Limburg is a cause of concern. Given the major importance of these caves as hibernacula for bats, including three species protected under Annex II of the EU Habitats Directive, we propose that impact assessment studies should be carried out to investigate the potential effects of human activities on hibernating bats in the caves.

Keywords: bats, Chiroptera, cave, quarry, Fluweelengrot, Christmas market, the Netherlands, human disturbance, census, temperature, population trend, distribution.

Introduction

Bat populations are affected by a wide range of stressful influences, one of these being human disturbance of caves that may be used

as either summer roosts or as hibernacula. Human disturbance in caves is known to have caused many population declines of cave-dwelling bats (Barbour & Davis 1969, Tuttle 1979, American Society of Mammalogists 1992, Johnson et al. 1998, Węgiel & Węgiel 1998). For this reason, one key aspect of bat conservation is to eliminate or control human

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entry into caves by, for instance, constructing a gate or fence at the entrance.

The Fluweelengrot is a subterranean limestone quarry in the municipality of Valkenburg in the south of the Province of Limburg, the Netherlands. It is known to have been used by hibernating bats at least since the annual bat censuses started in 1940. Since the middle of the 20th century the Fluweelengrot has also been a tourist attraction. During most of the year, there are daily guided tours of the historical carvings, wall paintings and sculptures found throughout the cave. Since November / December 1997, an annual Christmas market has been organised, running from late November until Christmas. This event attracts thousands of people each year and is thought to considerably disturb the bats that hibernate in the Fluweelengrot. This paper aims to investigate the influence of the Christmas market on the numbers of hibernating bats in the Fluweelengrot, by comparing the population trends of different bat species in the Fluweelengrot to those in similar caves in the south of Limburg, and by investigating the distribution of bats within the Fluweelengrot in relation to the Christmas market.

Hibernating bats frequently arouse to drink, copulate and/or feed. The frequency and purpose of natural arousals varies between and within species; e.g. feeding is uncommon in cave-dwelling bats (Boyles et al. 2006). Arousals from torpor bouts during hibernation are energetically expensive. Naturally occurring arousals from torpor may be responsible for the depletion of as much as 75% of the fat of hibernating bats (Thomas et al. 1990). Arousals may also be provoked by human disturbance. Bats can apparently get used to a low level of human activity during hibernation. However, excessive disturbance, leading to an increased frequency of arousal, may cause bats to abandon a site or decrease winter survival rates. Thus, visits to caves where bats hibernate should be kept to a minimum to reduce the risk of fat depletion caused by unnecessary arousals, which may

threaten the survival of the bats (Speakman et al. 1991, Thomas 1995, Mitchell-Jones et al. 2007, Boyles & Brack 2009).

The behavioural response of hibernating bats to disturbance is poorly documented. Thomas (1995) reported a significant increase in bat activity in a hibernation site in the hours following a visit, in spite of no bats being handled. This indicates that bats can be influenced by non-tactile disturbances even if they do not arouse immediately. The sensitivity of bats to human disturbance is also shown by several cases of recovery of bat populations (including species of *Myotis*, *Plecotus* and *Rhinolophus*) after human access to hibernation sites was reduced and / or bat banding ceased (Daan et al. 1982, Voûte & Lina 1986, Gaisler & Chytil 2002, Grol & Voûte 2010).

Human visitors to caves and underground quarries may impose different non-tactile stimuli to hibernating bats, such as light, sound and heat. Thomas (1995) assumed light and sound to be responsible for the increased flight activity detected after human visits. The Christmas market in the Fluweelengrot brings sound, light and heat to the cave, produced by the presence of thousands of visitors and by the power units used for lighting the cave. This is likely to change the microclimate of the Fluweelengrot, decreasing the humidity and increasing the ambient temperature.

Harmful effects of high ambient and substrate temperatures at roosts of hibernating bats have been reported by Humphrey (1978), Richter et al. (1993) and Martin et al. (2006). A microclimatic change in a hibernaculum may cause bats to arouse or lead to changes in the distribution of bats within a cave (Richter et al. 1993), and these responses may differ among species. Changes in the temperature may directly or indirectly act as stimuli to spontaneous arousals (Daan 1973). In an experimental setting Davis and Reite (1967) performed a stepwise increase in ambient temperature, between 5 °C and 10 °C they detected no arousals, but when the temperature was increased to 15 °C, four out of five

species studied responded by arousal from dormancy. Brenner (1974) did a controlled experiment with single hibernating bats (*Indiana myotis* *Myotis sodalis* and little brown myotis *Myotis lucifugus*) and found that *Indiana myotis* was aroused, and became active, at lower temperatures than little brown myotis. Such differences between species are reflected in the specific temperature zones that different species select for hibernation (Bezem et al. 1964, Daan 1973, Raesly & Gates 1987, Nagel & Nagel 1991, Brack 2007).

Methods

Description of the site

The Fluweelengrot, also known as “Historische Grot” (Bels 1952, van Wijngaarden 1967), is a medium-sized, subterranean limestone excavation situated in the hills bordering the valley of the river Geul in the municipality of Valkenburg aan de Geul, in the Province of Limburg, the Netherlands. The geology of this part of the Netherlands consists of Cretaceous limestone, which is generally soft, with occasional layers of a more solid consistency. The Fluweelengrot is connected to several escape corridors, one of which leads to Valkenburg Castle (figure 1). Bats can enter the Fluweelengrot through several entrances. The tunnel (T in figure 1) is blocked by a brick wall in which a small hole was made to enable bats to fly in and out. The remains of the twelfth century Valkenburg Castle have been a tourist attraction since 1863. During restoration in 1937 a secret underground passage, hewn out of the rock beneath the castle, was discovered. This escape route gave direct access to the Fluweelengrot, which has a corridor system that is nearly 180 metres wide and 230 metres long and is practically horizontal (figure 2). The corridors of this cave are quite uniform in their height and width, both of which average around three metres. In 2010 a new emergency exit was created near tunnel T. Due to

their importance for hibernating bats, many of the marl excavations in south Limburg are listed as belonging to Natura 2000 sites. The Fluweelengrot is part of the ‘Geuldal’ Natura 2000 site.

Human activities in the Fluweelengrot

Ever since guided tours started to take place in the 1940s, the Fluweelengrot has a regular (almost) year-round flow of tourists, mainly visiting its wall paintings and sculptures. The number of visits per day varies over the year, but on busy days in spring, summer and autumn between 11 a.m. - 4.30 p.m., guided tours of approximately 50 minutes can start every half an hour, with a maximum of 40 people. In January, the cave is closed to the public, while employees prepare the cave for the season. In February, there are limited numbers of guided tours at 11 a.m. and 1 p.m. only. Guides carry a kerosene lamp.

For several decades the Fluweelengrot has also been used for recreational activities such as marlstone carving, climbing and ‘abseiling’, sporting events and weddings (there is a chapel) and as a location for television programmes. These activities mainly take place in spring, summer and autumn and less frequently in winter.

Since November/December 1997, an annual Christmas market has been held in the Fluweelengrot, lasting for about five weeks until the 23rd of December. This event attracts thousands of people each year. Visitors follow a circular route which has some fifty stalls, mostly set up in niches in the cave architecture (figure 2). The market is open from noon (10 a.m. at weekends) until 9.30 p.m. During the market, the greater part of the cave is illuminated and heated artificially using generators and electrical heating. During opening hours, the main entrance of the cave is blocked by two rows of plastic curtains to keep the warm and dry air inside. These curtains also block the main entrance for bats.

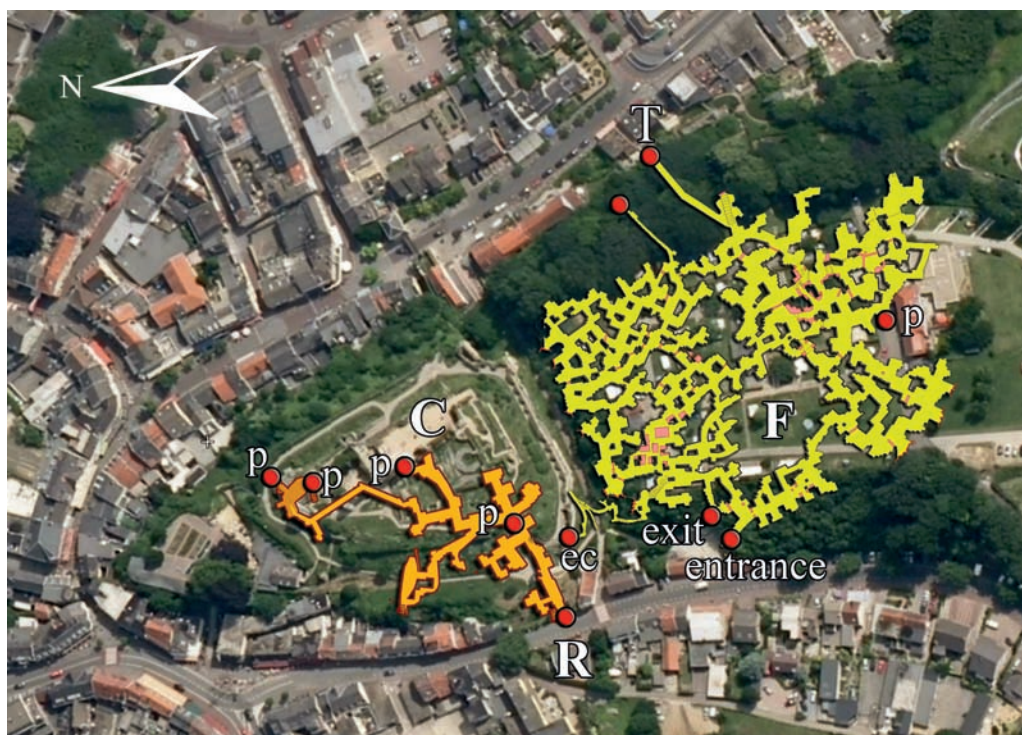


Figure 1. Aerial views of the Fluweelengrot (yellow) and the nearby 'Groeve Onder de Ruïne' (orange) cave systems. F = entrance of the Fluweelengrot, R = entrance of 'Onder de Ruïne' (under the ruin), C = ruins of Valkenburg Castle, p = shaft, T = tunnel, ec = escape route from the castle to the Fluweelengrot. Red dots indicate exit sites. Photo courtesy of Stevenhagen Geo Informatica.

The number of visitors of the Christmas market has increased from 95,000 in 1997 to a maximum of 128,000 in 2007 with an annual mean of 113,000. There has also been a sharp increase in the entrance fee over the years (from € 0.45 in 1997 to € 4.50 in 2011). As such the event has a considerable social and financial impact.

Survey methods

Bat counts have been carried out in the Fluweelengrot almost annually from 1940 onwards. To investigate the possible influences of the Christmas market on hibernating bats, the census data were divided into two periods: from 1980-1997, before the Christmas market began (BCM) and from 1998-

2010, during the years of the Christmas market (DCM). 1980 was taken as the start of the period BCM, because bat censuses were standardised at this time, enabling comparison of the counts between years. Every year the bat counts were carried out on January the 2nd or 3rd, when hibernating populations are assumed to be fairly constant (Daan 1973). The one exception was in 2010 when permission to do the census was granted for January 31 because the cleaning of the cave and removal of the Christmas decorations took place early in January. The counts were done with approximately the same group of people using the same method, systematically searching all accessible corridors for bats, using torches and binoculars. The exact location of each bat found was noted on a map, enabling a comparison of their distribution in



Figure 2. Map of the Fluweelengrot with the route of the Christmas market (red line). T = tunnel; bars and grey areas indicate the location of the Christmas stalls; B = pub. Courtesy of Stevenhagen Geo Informatica.

the cave in the BCM and DCM periods. Identification was done without handling the bats. Whiskered bats (*Myotis mystacinus*) and/or Brandt's bats (*Myotis brandtii*) were not distinguished, because of the difficulty in distinguishing between the two species in the field (e.g. Hanák 1970, Hoogenboezem 1982, Dietz & von Helversen 2004). For similar reasons, no distinction is made between brown long-eared bat (*Plecotus auritus*) and grey long-eared bat (*Plecotus austriacus*); both species are simply classified as 'long-eared bat (*Plecotus auritus/austriacus*)'. Bats that, for various reasons, could not be correctly identified were noted as 'species unknown'.

To identify any possible shifts in the bats' positions in the Fluweelengrot in relation to the Christmas market, we compared the distribution during the period 1990-1997 and the DCM period of five (groups of) species: pond bat (*Myotis dasycneme*), the whiskered/Brandt's bat, Natterer's bat (*Myotis nattereri*),

Geoffroy's bat (*Myotis emarginatus*) and Daubenton's bat (*Myotis daubentonii*).

Temperature measurements

Substrate temperatures of the ceiling in the Fluweelengrot were measured during a Christmas market (22 December 2009) and eleven days and five weeks after a Christmas market (3 January 2010 and 31 January 2010 respectively) (table 1). We used ceiling temperatures since most bats hibernate near the ceiling in the upper stratified air layers of the cave (de Wilde & van Nieuwenhoven 1954). It was not possible to take measurements outside the Christmas market area while it was in progress in December 2009. For comparison, we included ceiling temperatures taken during the census in 1990 (from just the southern half of the cave), seven years before the first Christmas market was organised (J.P. Bekker, unpublished data). Temperatures were measured at the same locations on all four dates, but fewer measurements were taken on 3 January 1990 ($n=69$; samples taken in the southern part of the cave only) and 22 December 2009 ($n=62$; parts of the quarry were inaccessible) than on 3 and 31 January 2010 ($n=118$).

The temperature samples from 1990 were read from a digital multipurpose thermometer, fixed on a bamboo stick and reaching up to 3 m; samples in 2009 and 2010 were taken with a Fluke 62 Mini Infrared Thermometer (Fluke Corporation, Everett, WA, USA).

Trend analysis

Trend analyses were performed by Poisson regression, using the TRIM programme (TREnds and Indices for Monitoring data; version 3.53; Pannekoek & van Strien 2001). Poisson regression was used to analyse count data, because the data was not normally distributed. TRIM computes whether a species has increased or decreased significantly,

Table 1. Ceiling temperature samples taken in (parts of) the Fluweelengrot in 1990, 2009 and 2010, compared with outside daily mean temperature (°C). External temperatures obtained from the Royal Netherlands Meteorological Institute (KNMI). ¹ samples in a part of the quarry only.

Date	<i>n</i>	Ceiling temp. (°C) Fluweelengrot			Outside temp. (°C) Maastricht		
		Minimum	Mean ±sd	Maximum	Minimum	Mean	Maximum
3 January 1990 ¹	67	2.2	8.6 ± 1.9	10.4	-2.3	0.9	2.9
22 December 2009 ¹	62	5.8	11.5 ± 5.3	20.1	0.2	1.4	2.6
3 January 2010	118	-4.2	9.8 ± 4.3	14.8	-6.7	3.9	0.8
31 January 2010	118	-2.2	7.6 ± 4.0	12.0	-1.5	0.3	1.7

remained stable or, if the change is uncertain. A trend of >1 denotes an increase and a trend of <1 denotes a decrease. We computed the trends before and after November/December 1997 (the start of Christmas market) and tested whether these differed between the Fluweelengrot and the other caves in south Limburg (*n*=89).

Indices were estimated for pond bats, whiskered/Brandt's bats, Daubenton's bats, Natterer's bats and Geoffroy's bats. As only small numbers of long-eared bat and greater mouse-eared bat (*Myotis myotis*) were found, reliable trends could not be established for these two species. Indices started from 1980, except for Natterer's bat and Geoffroy's bat, for which the index started in 1981 (as no Natterer's bats and Geoffroy's bats were found in the Fluweelengrot in 1980).

For temperature data and distribution of the bats, we used two-sided Student *t*-tests with a significance level of $\alpha=0.05$.

Results

Bat species and numbers

In the two periods (BCM and DCM) seven species were recorded. Daubenton's bat was the most abundant species in both periods with a yearly average of 32.1 (range 15-51) in the period BCM and 25.8 (range 16-39) in the period DCM (table 2; figure 3a).

The greater mouse-eared bat was the least frequently recorded species. Only single indi-

viduals were found in three years (1990, 1991 and 1996) in period BCM, and two and three individuals in 1998 and 1999 (period DCM). The number of long-eared bats was also low in both periods, with a maximum of two records per year over the whole period except for 2008 (DCM), when four long-eared bats were counted. In the period BCM no records were observed in six years, while in period DCM there were three years without records (table 2).

Pond bats were also only found in small numbers in both periods, with a yearly average of 3.7 in the period BCM (range 1-7) and 2.8 (range 0-8) in the period DCM (table 2; figure 3b). Numbers of the whiskered/Brandt's bat fluctuated in both periods, in the BCM period the average yearly number found was 5.9, with a range of 1-14. In the DCM period the yearly average was 5.6, with a range of 0-10 (table 2; figure 3c). 2003 was the only year when no specimens were found. Natterer's bat was recorded in eleven of the 18 census years in period BCM (average per year 1.6, range: 0-7). In period DCM, increasing numbers of this species were found, with an average of 10.4 bats per year and a range of 1-36 records in 2010 (table 2; figure 3d).

A similar trend was found for Geoffroy's bat. This species was either not recorded in the Fluweelengrot, or only in small numbers until the early 1990s, although the numbers increased towards the end of period BCM (average per year 2.9, range 0-12; table 2; figure 3e). In the DCM period, this species was recorded every year, with numbers varying from 5-23 and averaging 11.3 per year.

Table 2. Number of hibernating bats found in the Fluweelengrot in the BCM (1980-1997) and DCM (1998-2010) periods. Mmyo = greater mouse-eared bat, Plec = long-eared bat, Mnat = Natterer's bat, Mema = Geoffroy's bat, Mdas = pond bat, Mm/br = whiskered/Brandt's bat, Mdau = Daubenton's bat, indet = species unknown. Sum = total number of bats per year.

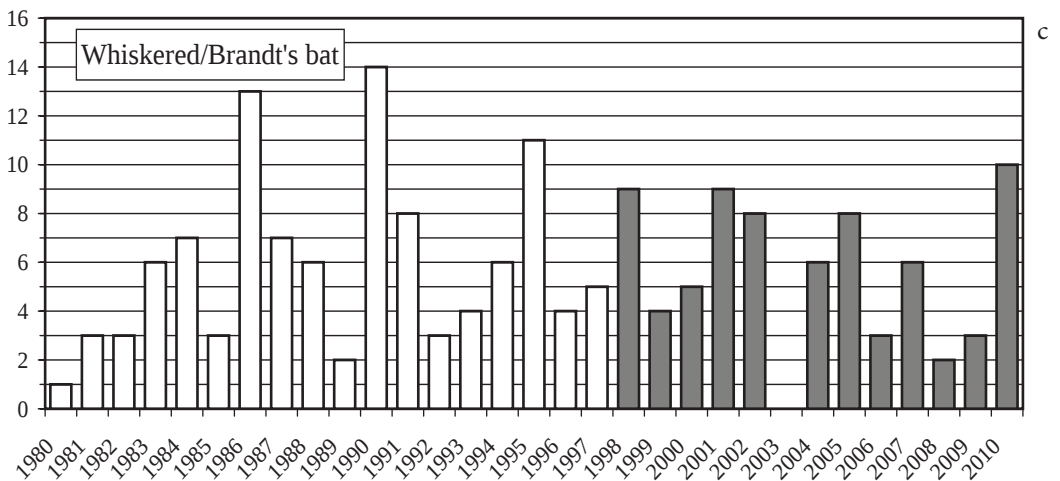
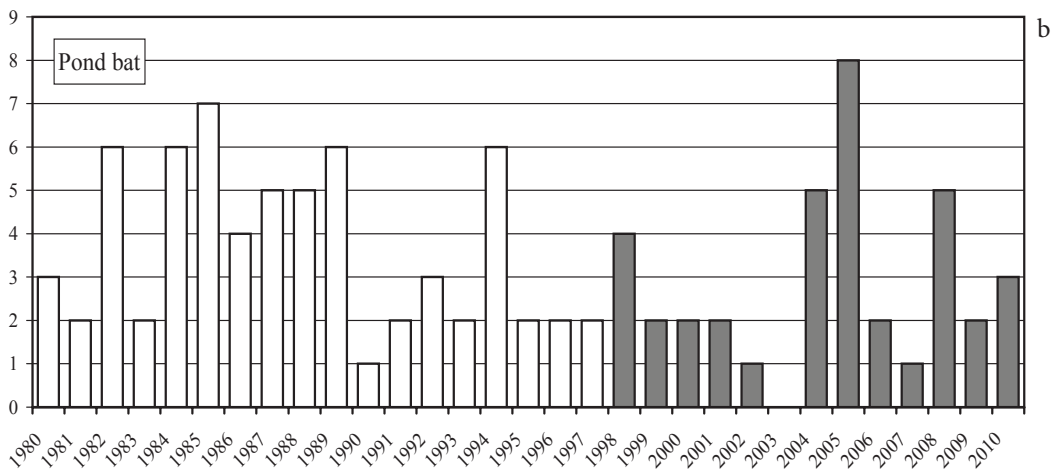
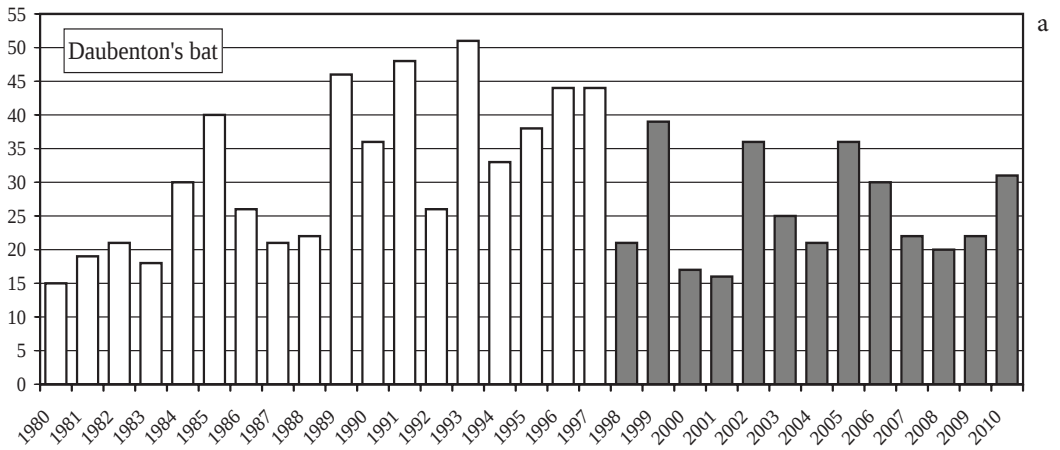
Year	Species								Sum
	Mmyo	Plec	Mdas	Mm/br	Mdau	Mnat	Mema	indet	
1980	0	1	3	1	15	0	0	5	25
1981	0	1	2	3	19	2	3	1	31
1982	0	2	6	3	21	0	0	2	34
1983	0	1	2	6	18	1	0	1	29
1984	0	0	6	7	30	0	0	2	45
1985	0	1	7	3	40	0	1	3	55
1986	0	2	4	13	26	0	0	3	48
1987	0	0	5	7	21	0	1	1	35
1988	0	0	5	6	22	0	2	4	39
1989	0	2	6	2	46	1	2	4	63
1990	1	0	1	14	36	3	0	4	59
1991	1	2	2	8	48	1	3	1	66
1992	0	1	3	3	26	2	2	4	41
1993	0	2	2	4	51	3	3	3	68
1994	0	2	6	6	33	5	6	6	64
1995	0	1	2	11	38	7	10	2	71
1996	1	0	2	4	44	2	7	1	61
1997	0	0	2	5	44	2	12	4	69
1998	2	0	4	9	21	7	12	1	56
1999	3	2	2	4	39	2	11	0	63
2000	0	1	2	5	17	6	7	0	38
2001	0	1	2	9	16	1	5	1	35
2002	0	1	1	8	36	14	12	3	75
2003	0	2	0	0	25	2	5	2	36
2004	0	1	5	6	21	5	10	0	48
2005	0	0	8	8	36	9	8	1	70
2006	0	2	2	3	30	13	19	2	71
2007	0	1	1	6	22	9	11	4	54
2008	0	4	5	2	20	12	7	2	52
2009	0	0	2	3	22	19	17	10	73
2010	0	1	3	10	31	36	23	3	107

Trends and indices

We used TRIM to compute trends for five bat species and compared indices in the Fluweelengrot to those in the other marl quarries in south Limburg ($n=89$). For whiskered/

Brandt's bat and Geoffroy's bat, there was a significant difference, with the increase in the numbers of both species being less in the Fluweelengrot than in other caves (table 3).

Before the Christmas market the whiskered/Brandt's bat in the Fluweelengrot showed an



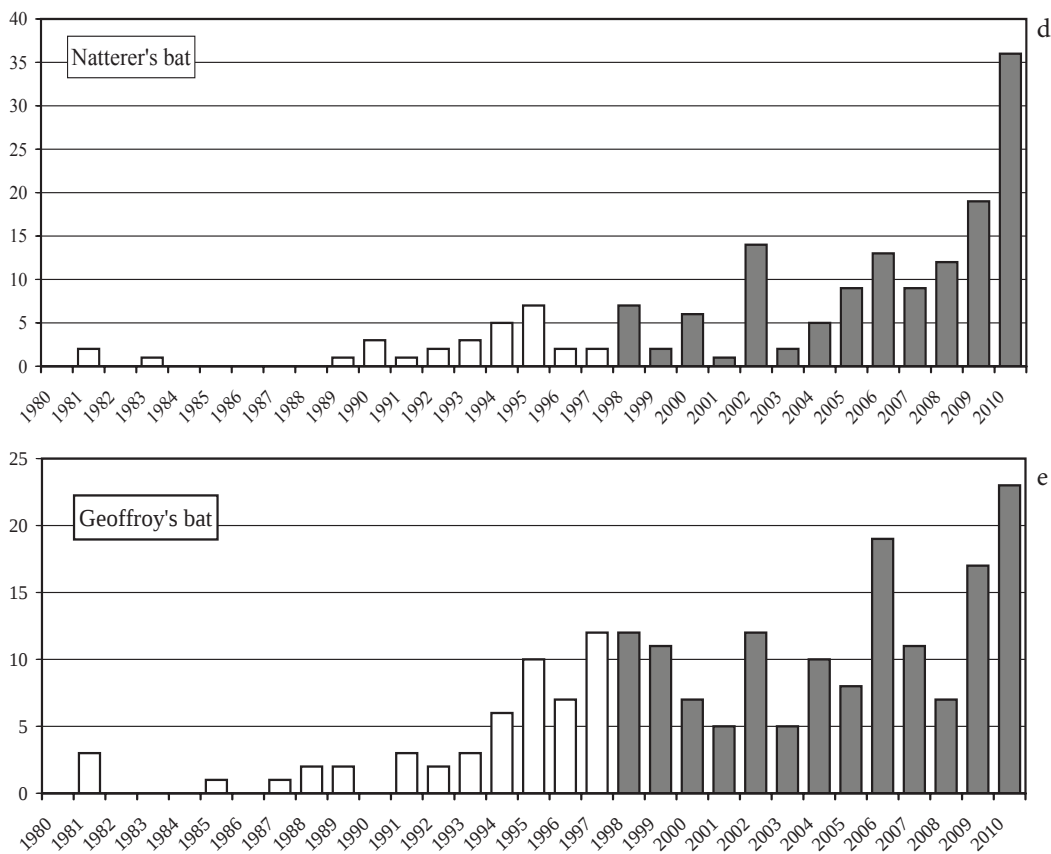


Figure 3. Number of Daubenton's bats (a), pond bats (b), whiskered/Brandt's bats (c), Natterer's bats (d) and Geoffroy's bats (e) from 1980 to 2010, before (white bars) and after (grey bars) the start of the Christmas market.

average annual increase of 3%, compared to a 5% increase in other caves (table 3). In the years after the first Christmas market took place (DCM), the index in the other south Limburg caves increased by 3% per year, while in the Fluweelengrot a decrease of 3% per year was found. Over the whole period, this resulted in a significantly smaller increase of the species in the Fluweelengrot ($P=0.02$) (table 3; figure 4a).

In the BCM period, the index of Geoffroy's bat in the Fluweelengrot showed a spectacular average increase of 21% per year: this compared to an average of 11% in the other south Limburg caves (table 3). In the DCM period, the increase in the Fluweelengrot was much smaller (4% per year), while numbers in the other caves continued to grow at an average

of 13% per year. Taking both periods together, the increase of Geoffroy's bats was significantly lower in the Fluweelengrot than in other caves ($P=0.007$) (table 3; figure 4b).

For the other three species, the trends in the Fluweelengrot did not differ from those in other caves. Daubenton's bat showed positive trends in the BCM period in both the Fluweelengrot (average annual increase 4%) and in the other south Limburg marl quarries (2%) (table 3; figure 4c), and negative trends for both in the DCM period (average annual decrease 5% and 1% respectively; table 3). The total decrease over both periods in the Fluweelengrot was not significantly different from that in the other south Limburg caves ($P=0.10$).

Table 3. Trend indices of the pond bat, whiskered/Brandt's bat, Daubenton's bat, Natterer's bat and Geoffroy's bat found in the Fluweelengrot and in other caves in south Limburg in the BCM (1980-1997) and DCM (1998-2010) periods, and probability *P* of differences between the indices over both periods in the Fluweelengrot and in other south Limburg limestone quarries (last column; Poisson regression).

Species	Period BCM		Period DCM		<i>P</i> _{BCM+DCM}
	Fluweelengrot	Other caves (<i>n</i> =89)	Fluweelengrot	Other caves (<i>n</i> =89)	
Daubenton's bat	1.04	1.02	0.95	0.99	0.10
Pond bat	0.97	0.99	1.00	1.04	0.21
Whiskered/Brandt's bat	1.03	1.05	0.97	1.03	0.02*
Natterer's bat	1.13	1.19	1.16	1.16	0.55
Geoffroy's bat	1.21	1.11	1.04	1.13	0.007**

For pond bat and Natterer's bat the trends over both periods, taken together, were similar in the Fluweelengrot and other caves (*P*=0.21 and 0.55 respectively) (figures 4c and 4b). Natterer's bat showed large increases (13-19%) in both periods in both the Fluweelengrot and the other caves in south Limburg.

Temperatures

The temperatures measured at four sampling dates in 1990 (3 Jan), 2009 (22 Dec) and 2010 (3 and 31 Jan) (table 1) were used to develop gradient maps, roughly showing the distribution of ceiling temperatures in the areas sampled (figures 5a-d). On 31 January 2010, 39 days after the Christmas market, a clear temperature gradient was visible in the cave, with temperatures increasing from below 0 °C in the entrance areas in the west and northwest to 10-12 °C in the southeastern part (figure 5d). During the Christmas market, on 22 December 2009, the ceiling temperatures in the area sampled were 5-8 degrees higher (at one location, where a pub was situated, a maximum of 20.1° C was measured) (figure 5b). Nine days after the Christmas market, on 3 January 2010, the figure is somewhere between the two, showing that the quarry was cooling down in the weeks after the Christmas market (figure 5c). Temperatures on 31 January 2010 were similar to those in Janu-

ary 1990, except for the western part of the quarry, where temperatures were lower in 2010, probably due to the long cold winter period (figures 5a and 5d).

Bat distributions within the quarry

A grid overlay was used to calculate the average location of different bat species in the Fluweelengrot over two periods: 1990-1997 and DCM (figure 6). The average location of three species of bat differed significantly between the two periods: pond bats (1990-1997: $x \pm sd = 42.76 \pm 27.42$, $y = 79.82 \pm 21.05$, $n=20$; DCM: $x = 33.89 \pm 21.83$, $y = 61.21 \pm 30.10$, $n=37$) (Student's *t*-test, *P*=0.02), Daubenton's bats (1990-1997: $x = 39.46 \pm 24.82$, $y = 61.94 \pm 24.48$, $n=320$; DCM: $x = 29.93 \pm 19.94$, $y = 56.66 \pm 24.00$, $n=336$) (*P*<0.001) and Natterer's bat (1990-1997: $x = 36.16 \pm 21.40$, $y = 55.60 \pm 26.29$, $n=25$; DCM: $x = 29.10 \pm 17.47$, $y = 48.51 \pm 27.26$, $n=135$) (*P*=0.04). These three species showed a shift towards the northwest of the quarry, away from the area most affected by the Christmas market (figures 6a-c). For whiskered/Brandt's bat and Geoffroy's bat, we found no difference between the two periods (whiskered/Brandt's bat: 1990-1997: $x \pm sd = 31.67 \pm 21.19$, $y = 58.21 \pm 27.11$, $n=55$; DCM: $x = 29.09 \pm 18.29$, $y = 55.67 \pm 19.28$, $n=73$; Geoffroy's bat: 1990-1997: $x = 41.81 \pm 19.13$, $y = 79.82 \pm 22.13$, $n=43$; DCM: $x = 41.21 \pm 17.28$, $y = 67.93 \pm 25.87$, $n=147$) (*P*>0.05) (figures 6d-e).

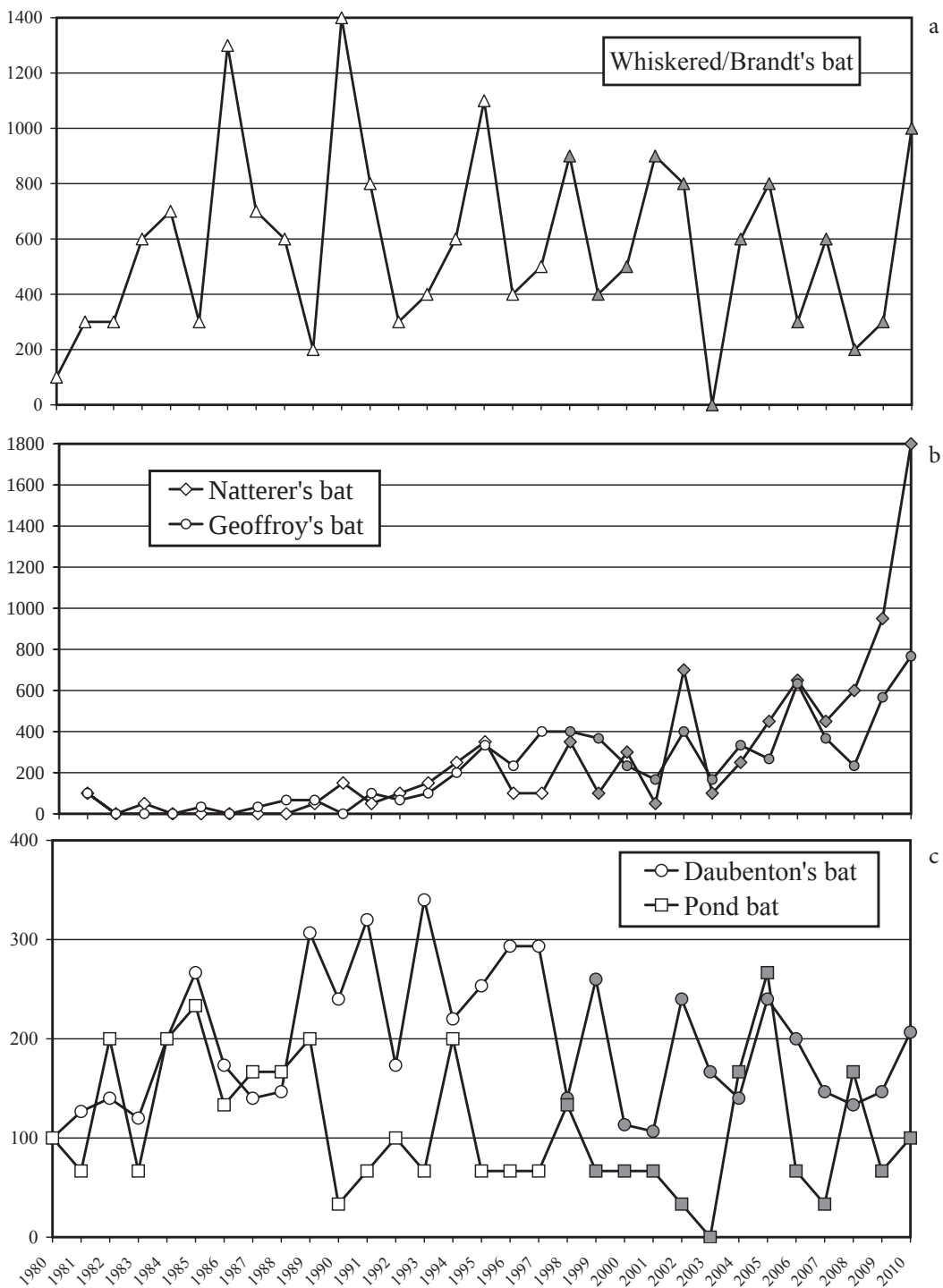


Figure 4. Trend indices of whiskered/Brandt's bat (a), Natterer's bat and Geoffroy's bat (b), and Daubenton's bat and pond bat (c) from 1980-2010. Dark grey points are years with a Christmas market.

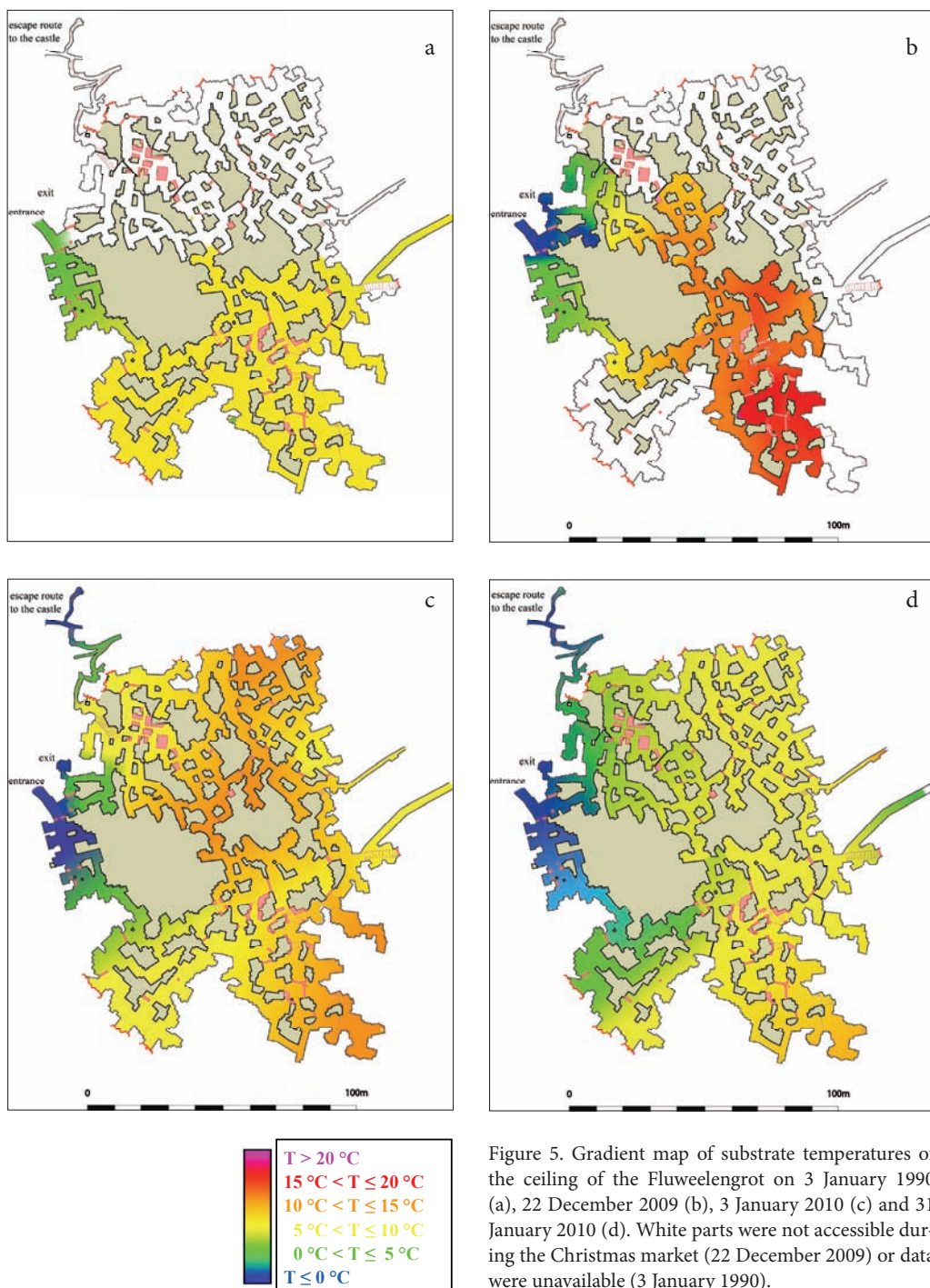


Figure 5. Gradient map of substrate temperatures of the ceiling of the Fluweelengrot on 3 January 1990 (a), 22 December 2009 (b), 3 January 2010 (c) and 31 January 2010 (d). White parts were not accessible during the Christmas market (22 December 2009) or data were unavailable (3 January 1990).

Discussion

We investigated the presumed disturbance to hibernating bats in the Fluweelengrot caused by the annual Christmas market. Temperatures during the Christmas market were considerably higher than before and after the event and we expected this to create a considerable disturbance for bats hibernating in the cave. We did not collect data on changes in humidity and sound levels, so the actual nature of the disturbance and the relative importance of various potential stimuli remain unclear. However, we can safely assume that the presence of many thousands of people, lighting and electrical power units during the Christmas market also play a role and that the Christmas market is a major cause of disturbance to hibernating bats in, at least part, of the Fluweelengrot.

Our study shows that the numbers of whiskered/Brandt's bat and Geoffroy's bat in the Fluweelengrot declined in relation to other limestone caves in Limburg. This indicates that the Christmas market had a negative influence on these species. For three other species, pond bats, Daubenton's bats and Natterer's bats, there was no evidence of such a relationship. However, between 1998 and 2010, after the first Christmas market, these latter three species showed a shift towards the north western parts of the cave, away from the Christmas market area. Although we do not know the underlying causes of the distributional changes, these results suggest that a number of individuals of these species moved possibly to avoid the influence of the Christmas market.

For practical reasons, temperature samples after 1997 were restricted to one winter only. The conclusions that we can draw from this data are therefore limited and do not offer insights into the possible long-term effects of the annual temperature rise that takes place during the Christmas market. We recommend the collection of microclimatic data on a regular basis (e.g. every three to five years)

to see if the microclimate of the cave is changing (e.g. becoming warmer), which in the long run may negatively affect its suitability as a bat hibernaculum.

We are aware of the limitations of our method for investigating the distribution of bats. The two-dimensional projection of the bats' distribution that we used is a simplification of the actual distribution, as bats also locate themselves at different heights. In our opinion, however, this method is sufficient for the conclusions that we have drawn here.

The population trends of whiskered/Brandt's bat and Geoffroy's bat in the Fluweelengrot may be related to other factors. However, we have no indications of other human activities (e.g. the number or pattern of visitors during the year) or other conditions in the Fluweelengrot that would influence hibernating bats having changed since 1980, except for the introduction of the Christmas market.

The Christmas market in the Fluweelengrot is a form of intensive commercial exploitation, which is not a unique phenomenon in the marl quarries in south Limburg. A similar Christmas market, with even more visitors, is organised annually in the nearby Gemeentegroeve. Like the Fluweelengrot, this cave is home to hibernating Geoffroy's, pond and greater mouse-eared bats, all Habitats Directive Annex II-species. The impact of this event on the bats in this quarry has not yet been studied. In several other quarries in Limburg the number and diversity of sporting and other recreational activities, including cave biking, quad riding, abseiling and archery have increased over the last few decades. Guided tours and marl carving have been taking place in the Fluweelengrot, since the 1940s (Sluiter & van Heerdt 1957), but the levels of intensity between 1980 and 2010 seems to be relatively unchanged. Compared to these activities, the Christmas market is a major source of disturbance. Furthermore, the Christmas market coincides with the bats' hibernation period, while activities that take

place between spring and autumn have less effect. The fact that the Christmas market is set up after the bats have settled for hibernation, makes this an unpredictable source of disturbance, which the bats can not anticipate by choosing locations away from the market area.

In spite of the potential threats, increases have been observed in the population levels of several species of bats in quarries in south Limburg, at least since the early 1980s (e.g. Dijkstra et al. 2006, Verboom 2006, Grol & Voûte 2010). These trends do not just reflect the increasing number of hibernacula investigated over the years, but are, at least partly, thought to be real population increases (Dijkstra et al. 2006). There may be various species specific reasons for these positive trends. The banning of certain pesticides and wood preservatives, improvements in the conditions in hibernacula for bats, and even climate change may all have played a role (e.g. Limpens et al. 1997, Dijkstra et al. 2006). The effects of ceasing the practice of banding bats in their hibernacula, recognised as a major mortality factor and a cause of decline of several bat species (Sluiter et al. 1971, Daan 1980, Voûte et al. 1980, Daan et al. 1982, Baker et al. 2001, Dietz et al. 2006) is unlikely to play a role here. This was practised in the marl caves in Limburg from the 1930s on, but was abandoned in 1959. Thereafter, identification in hibernacula has been done without handling the bats. Some species appear to have benefited from these measures, enabling the numbers of hibernating bats to increase over the last decades. In the light of these developments, increasing human activity in a number of these bat hibernacula, and the intention of the provincial and local governments to further intensify tourism in the region, including in the quarries, is a cause of concern (e.g. ZKA Markt & Beleid & NIB Consult 2001).

Disturbance of bats in their hibernacula can affect bats in different ways. Arousals that occur in addition to natural arousals during a winter, e.g. as a consequence of human distur-

bance, increase fat depletion and reduce bats' chances of surviving the hibernation period. Johnson et al. (1998), for instance, found that Indiana bats (*Myotis sodalis*) in a hibernaculum with few visits lost less weight than those in a more frequently visited hibernaculum (average number of winter visits: 5.5 and 378 respectively). This supports the argument that arousals during the hibernation have a negative effect on the bats' condition, with possible consequences for their survival rates and reproduction success in the following spring (Boyles & Brack 2009).

A bat may respond to arousal from unnatural conditions in several ways. It may remain in the same location and hide deeper in its crevice, or it may move to another location, either in the same or in another hibernaculum. Our study showed a negative trend (compared to other quarries) in the populations of whiskered/Brandt's bat and Geoffroy's in the Fluweelengrot after 1997, but did not show a change in their distribution throughout the cave. On the other hand, while there were no discernible differences in the population levels of pond bat, Daubenton's bat and Natterer's bat, there was evidence of these species having moved in a north westerly direction in the quarry. These results suggest that some species of bats remained in the Fluweelengrot (albeit in different locations), while others moved to other hibernacula or found hiding places where they were invisible for the researchers.

One problem with surveys of hibernacula is that the relationship between the number of bats recorded and the actual number of bats present is not always clear. Punt & van Nieuwenhoven (1957), for instance, marked bats of different species (brown long-eared bat, lesser horseshoe bat *Rhinolophus hipposideros* and several *Myotis* species) in a quarry with radioactive bands, and found that between 20 and 45% of the bats were hidden in invisible locations. Bats in deep crevices may be overlooked, especially in complex sites like marl caves, where cracks and crevices can be deep and hard to inspect. It is possible that some

of the bats in the Fluweelengrot moved deeper into cracks and crevices as a reaction to the Christmas market and were overlooked during the counts. This could explain the trends in the numbers of recorded whiskered/Brandt's and Daubenton's bats. For Geoffroy's bat this is not a very likely explanation, as this species does not usually hide in cracks and fissures, but prefers to hang freely on ceilings, often in a dome-shaped space (Bezem et al. 1964).

Displacements within a hibernaculum do occur throughout the winter season. Some bats are found in exactly the same location for several months, whereas some individuals are recorded at a certain locality on only one visit (Daan & Wichers 1968). Studies of bats hibernating in south Limburg's quarries suggest species specific differences in this behaviour. According to ter Horst & van Nieuwenhoven (1958), Geoffroy's bat has long and uninterrupted hibernation bouts of ten weeks or longer, while Daubenton's bat and Natterer's bat may remain in torpor for periods of around eight weeks and whiskered/Brandt's bats for six weeks. Other species were found to wake up more frequently. Presumably, bat species with more frequent periodical arousals also exhibit a higher displacement activity (Daan & Wichers 1968).

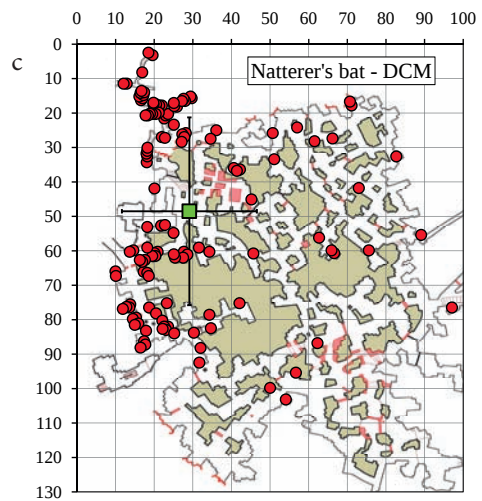
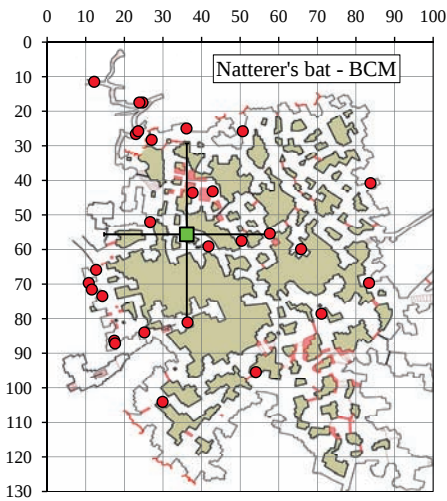
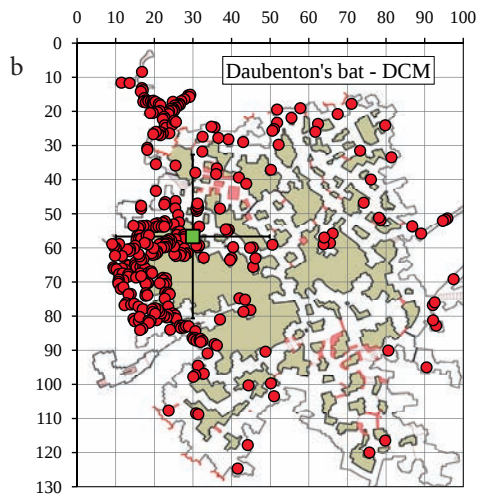
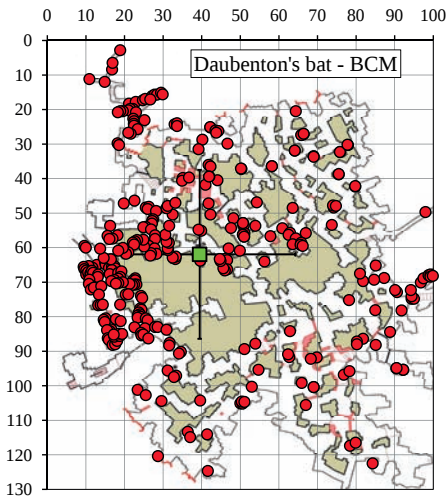
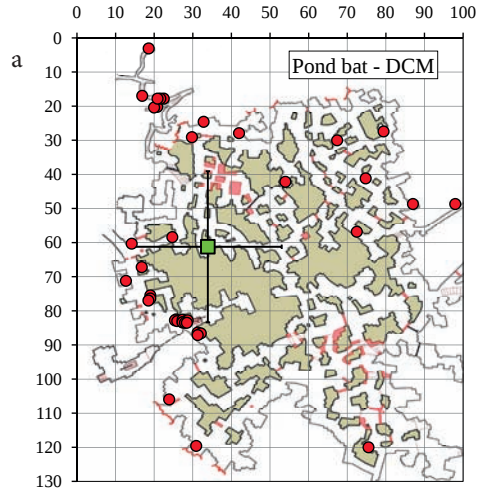
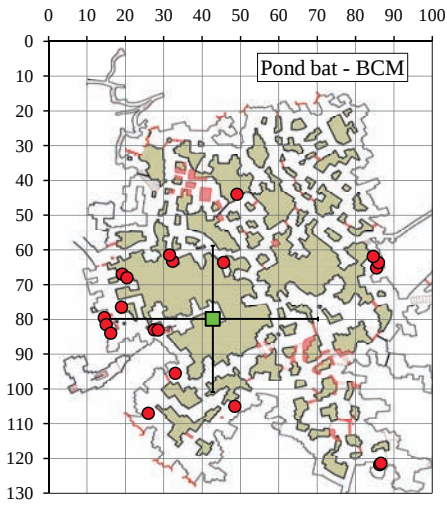
Hibernating bats are found in a range of different positions which offer different degrees of protection; from very exposed hanging positions to narrow crevices and holes. It is clear that the position influences exposure to microclimatic factors. Climatic adaptation is an important factor influencing the distribution of bats (Bezem et al. 1964, Daan & Wichers 1968). For some bats the temperature zone that they select within hibernacula is an important survival factor (Ransome 1968).

As yet, there is no consensus about the role and relative importance of different non-tactile stimuli in provoking bat arousals. De Wilde & van Nieuwenhoven (1954) tried to find which stimuli, caused by humans visiting a limestone quarry in south Limburg, bats in hibernation were sensitive to. They concluded

that warm air rising from gasoline lamps always had an effect; rays of light pointed at the animals at a distance of about 30 cm caused arousal in some cases and the sounds of human voices never aroused hibernating bats. By contrast, laboratory experiments by Speakman et al. (1991) on six common British species indicated that, under experimental conditions, non-tactile disturbances only lead to arousals in few cases, and caused only minimal increases in energy expenditure. They suggested that it may not be necessary to prevent non-tactile disturbances; although they pointed out that their interpretation of responses to non-tactile stimuli may not apply in natural conditions. The results of Speakman et al. (1991) are contradicted by Thomas (1995), who argued that light and the sound of human voices provoked an increase of flight movements of little brown myotis and northern myotis (*Myotis septentrionalis*) in a mine. While only a small proportion of the bats responded directly to the non-tactile stimuli caused by humans, these arousals propagated through the hibernating bat population, leading to increased flight activity of some bats up to 8.5 hours after the human visits.

The results of our study stress the need to further investigate the possible impact of recreational and other commercial activities in the caves of south Limburg. Many of these are major hibernacula for bats, and may house hundreds of bats each winter, including important numbers of species protected under Annex II of the EU Habitats Directive, i.e. Geoffroy's bats, pond bats and greater mouse-eared bats. We propose that impact assessment studies should be carried out to investigate the potential effects of human activities on bats in the caves. Activities that take place during the winter period may in particular pose serious threats to hibernating bats in these important hibernacula.

Acknowledgements: We would like to thank the members of the Telgroep Utrecht for their valuable participation in collecting data over the years. We appreciate



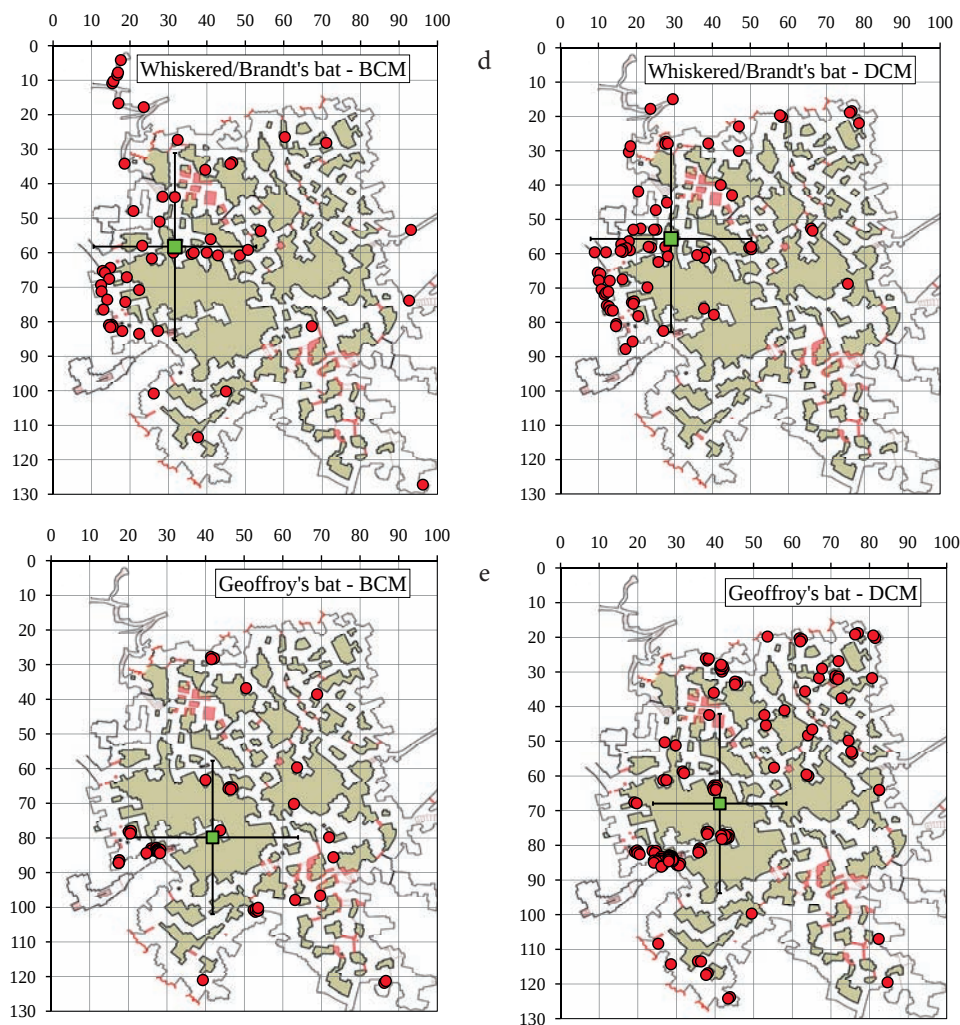


Figure 6. Map of the Fluweelengrot with the locations of pond bats (a), Daubenton's bats (b), Natterer's bats (c), whiskered/Brandt's bats (d) and Geoffroy's bats (e) in the period 1990-1997, here referred to as BCM (left), and DCM (1998-2010) (right) periods, with mean (green square) and standard deviation.

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Samenvatting

Effecten van een kerstmarkt op overwinterende vleermuizen in een mergelgroeve

Sinds november/december 1997 vindt in de Fluweelengrot, een mergelgroeve in de zuidlimburgse gemeente Valkenburg, jaarlijks een kerstmarkt plaats. Over een periode van vier tot vijf weken bezoeken elk jaar meer dan 100.000 mensen deze markt, die bestaat uit circa 50 stalletjes die zijn opgesteld langs een route door de groeve. Uit metingen in de winter van 2009-2010 bleek dat de temperatuur tijdens de kerstmarkt in een deel van de groeve 5-8 °C hoger lag (tot maximaal 20,1 °C) dan ruim een maand erna. We onderzochten de mogelijke effecten van deze potentiële bron van verstoring op de aantallen en verspreiding van vleermuizen in de groeve. In totaal werden in de onderzoeksperiode 1980-2010 tenminste zeven soorten vleermuizen aangetroffen. Vale vleermuizen (*Myotis myotis*) en grootoorvleermuizen (*Plecotus auritus/austriacus*) werden in zeer kleine aantallen aangetroffen. Voor de andere vijf soorten zijn trendanalyses gedaan. Hieruit bleek dat de trends van de baard-/Brandt's vleermuis (*M. mystacinus/Brandtii*) en de ingekorven vleermuis (*M. emarginatus*) over 1980-2010 significant achterbleven bij de gemiddelde trend van deze soorten in 89 andere Limburgse mergelgroeven. We onderzochten tevens de verspreiding van waarnemingen van genoemde vijf soorten over de Fluweelengrot. Drie soorten, de watervleermuis (*Myotis daubentonii*), de meervleermuis (*M. dasycneme*) en de franjestaart (*M. nattereri*), bleken na de eerste kerstmarkt in november/december 1997 significant meer in noordwestelijke richting te zijn waargenomen dan in de voorafgaande periode 1990-1997. Dit komt vooral door een toename van het aantal waarnemingen in een deel van de groeve buiten het kerstmarktgebied. De resultaten suggereren een negatieve invloed van de kerstmarkt op de vleermuizen in de groeve. Omdat we alleen temperatuur hebben gemeten, is niet bekend welke factoren (temperatuur, luchtvochtigheid, licht, geluid) een rol hebben gespeeld. Gezien het grote belang van de Limburgse mergelgroeven als winterverblijf voor vleermuizen, adviseren we om nader onderzoek te doen naar

de potentiële effecten van de toenemende toeristische druk op overwinterende vleermuizen in de groeven.

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First recording of the soprano pipistrelle (*Pipistrellus pygmaeus*) in the Netherlands

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Abstract: In the early 1990s, the soprano pipistrelle (*Pipistrellus pygmaeus*) was recognised as a separate (cryptic) species. It is now considered to be widespread in Europe, but was unknown in the Benelux countries until 1998, when the first bioacoustic recording was reported in Belgium. This paper reports the first confirmed record of *Pipistrellus pygmaeus* in the Netherlands verified using bioacoustics. Intermediate pulse intervals of echolocation calls preceding songflight calls and the bandwidth of songflight calls are proposed as new characteristics that can be used to discriminate between *Pipistrellus pygmaeus* and *Pipistrellus pipistrellus*.

Keywords: *Pipistrellus pygmaeus*, soprano pipistrelle, first recording, the Netherlands.

Introduction

Amongst the bat species known to be present in the Netherlands are the two pipistrelle species: Nathusius' pipistrelle (*Pipistrellus nathusii* (Keyserling & Blasius 1839)) and common pipistrelle (*Pipistrellus pipistrellus* (Schreber 1774)). The sibling species of the latter, the soprano pipistrelle (*Pipistrellus pygmaeus* (Leach 1825)), is known to be a rather common species in the countries bordering the Benelux, but had not yet been recorded within the Netherlands (Jones & van Parijs 1993, Sattler 2003) (although Dietz et al. (2007) includes the Netherlands in the species' distribution area). *Pipistrellus pygmaeus* was first reported to be present in Belgium in 1998 (Kapfer et al. 2007, Dekeukeleire 2010).

It seemed just a matter of time before *Pipistrellus pygmaeus* would be found in the Netherlands and several unconfirmed observations have been reported. One of the author's own recordings made at the end of 2007 in a forest lane near Leersum in the Langbroekerwetering seemed to contain faint traces of

Pipistrellus pygmaeus calls, from a bat probably flying at some distance. In 2008 and the following years, I was able to make closer, distinctive recordings on many occasions at the same location in Leersum, confirming the presence of this species in the Netherlands.

Since then there have been a number of confirmed recordings of *Pipistrellus pygmaeus* all over the Netherlands, in Groningen, Den Haag, and near Utrecht and Zeewolde. In Zundert, near the border with Belgium, a bat captured in July 2011 was morphologically identified as *Pipistrellus pygmaeus*. Last but not least, I recorded two *Pipistrellus pygmaeus* bats calling at the same time near Leersum in September 2011.

This paper, however, will focus on the bat that was recorded in Leersum in 2007.

As they have evolved, European microchiropteran bats have developed different kinds of echolocation calls, with frequencies generally between 10 and 180 kHz. Their calls can be of almost constant frequency (CF), frequency modulated (FM), or a combination of both; pulse lengths of regular echolocation calls range from a few microseconds to 80 ms. Bat species use the pulse characteristics that most suit their needs. Aerial hawkers, flying fast in

open spaces, tend to use low, long quasi-constant frequency (qCF) calls allowing them to detect prey and obstacles at long ranges. By contrast gleaners such as *Myotis* and *Plecotus* species that scan foliage for prey at a slow pace, tend to use short, steep FM calls. Pulse characteristics such as maximum and minimum frequency, pulse duration and inter pulse interval, usually differ between species, although some overlap is possible (Barataud 1996, Boonman et al. 2008, Skiba 2009).

Bats also produce sounds for intra and inter-species communication, to attract or repel other bats. The pulses of these types of calls are often lower in frequency, broad banded, longer in duration, more varied and are often repeated quickly in succession. Lower frequencies are used because these are less attenuated by air and thus travel further. These social calls can be emitted when the bat is stationary (e.g. from a tree hole) or whilst flying (songflight calls). The characteristics of these social calls are also often species dependent, although overlaps can also occur (Barataud 1996, Pfalzer 2002, Skiba 2009).

Depending on the quality of recording and the openness of the environment, several European species can be successfully identified on the basis of the characteristics of their echolocation pulses, songflight or social calls, using proper analysis software (Barataud 1996, Pfalzer 2002, Skiba 2009). These characteristics were heavily utilised within this survey (see below).

The two pipistrelle species present in the Netherlands are, in general, easily distinguished on the basis of the frequency of maximum energy of their qCF pulses: for *Pipistrellus nathusii* this is around 38 kHz and for *Pipistrellus pipistrellus* it is around 46 kHz (Jones & van Parijs 1993, Barataud 1996, Skiba 2009). Deviations from these values are possible, resulting in some overlap of the frequencies of these species, which makes discrimination more problematic. *Pipistrellus pipistrellus* calls can also occasionally end above 50 kHz, creating a possible overlap with the calls of

Pipistrellus pygmaeus, which has a frequency of maximum energy that is commonly between 53 and 57 kHz (Jones & van Parijs 1993).

The study was carried out in the Langbroekerwetering area, located in the centre of the Netherlands, between the Kromme Rijn river and the Utrechtse Heuvelrug (figure 1). It contains several estates and a mosaic of orchards, wooded banks, watercourses, woodlands and pastures. On the instigation of the Utrecht provincial government a three-year research survey, led by Eric Jansen, was started in 2007 by the Dutch Mammal Society and a local nature conservation group called the 'Vereniging Natuur en Milieu Wijk bij Duurstede'. Many volunteers, including the author, took part in this project which aimed to investigate the use of this unique landscape by bats. One of the survey's first goals was to determine the bat species present in the area.

On 23 August 2007 I made a recording of songflight calls, from a *Pipistrellus nathusii* and from what was assumed to be a *Pipistrellus pipistrellus*. Only later that year in October, closer examination of the spectrogram showed a couple of very faint pipistrelle-like qCF pulses of 6 ms long, ending at 56 kHz, with an intermediate pulse interval (IPI) of around 80 ms. The bandwidth of one set of songflight calls seemed to be wider and higher in frequencies than what one would normally expect of *Pipistrellus pipistrellus*. This finding was discussed with two bat experts, Herman Limpens and John Mulder, but it was concluded that there was not enough data to positively identify the calling bat as *Pipistrellus pygmaeus*.

In 2008, my first visit to the area was at 02:00 a.m. on July 5, and after a few minutes I recorded new qCF calls ending at around 55 kHz. On further visits, similar pulse sequences were recorded, many of which were as loud as the typical echolocation calls from *Pipistrellus pipistrellus* flying nearby. Other bat workers working in the field were able to confirm these observations.

On 16 July and 6 September 2008 an attempt was made to capture the calling bat with mist-

nets, but without success. During the remainder of 2008, I recorded qCF pulse sequences ending above 55 kHz during all my visits to the area except for one, when the weather was bad. In the following years, I recorded similar sequences, culminating in the recording of simultaneous songflight calls with similar characteristics from two bats near Leersum in September 2011.

For comparison purposes, personal recordings of *Pipistrellus pipistrellus* bats near Leersum as well as of *Pipistrellus pygmaeus* bats in the UK are used in this article.

Materials and methods

This survey used heterodyne bat detectors (D100 and D200; Pettersson Elektronik AB, Sweden) which allow for some bat species producing qCF calls to be readily identified in the field. For species that produce FM pulses, e.g. *Myotis* species, time expansion (TE) bat detectors (D240x, Pettersson Elektronik AB, Sweden) were used. On one occasion an Anabat detector (Titlley Scientific, Australia) was used with a PDA attached, showing real-time Anabat division graphs. The use of mistnets provided additional data when TE recordings were inconclusive or when other information was desired.

A D240x TE bat detector (307 kHz sample rate, 8 bit resolution) was used to make 3.4 second long recordings. During replay, these recordings were slowed down 10 times by the detector and recorded as TE recordings onto a solid state Transcend T.sonic 520 wav recorder, with a sample rate of 32 kHz and 16 bit resolution. An Edirol R09-HR recorder was also used, with a sample rate of 44.1 kHz and 16 bit resolution. Both recorders stored information in lossless format (i.e. as wav files, not as compressed MP3s). The Edirol allowed more tuning of the input signal level and had better recording characteristics than the T.sonic. However, this was barely noticeable during analysis of the recordings since the resulting performance of the D240x, during

replay, was lower than those of both recorders.

Two software programs, Cool Edit 96 (Syntrillium Software Corporation, USA) and Bat-Sound (Pettersson Elektronik AB, Sweden) were used for spectrogram and power spectrum analysis. Characteristics such as start and end frequency, maximum and minimum frequency, frequency of maximum energy, pulse duration, IPI and pulse type were measured in an attempt to identify the species that were the source of the recordings.

Results

Songflight calls

Most recordings made in Leersum after mid-July 2007 contained songflight calls interspersed with echolocation calls. Songflight activity was especially high around midnight, gradually shifting forward in the evening as a year progressed. These calls consisted of either three ($n=9$), but more often four syllables ($n=29$), with increasing intensity and duration. The first syllable often had a smaller bandwidth (mean=17.1 kHz, $sd=4.3$) and all but the last one had a hook-like appearance in the spectrograms, with the last syllable ending in a downward sweep (figure 2). The pitch of the syllables in the calls decreased slightly¹.

The songflight calls covered frequencies from 48.4 (mean=41.2 kHz, $sd=3.9$) down to 18.0 kHz (mean=21.1 kHz, $sd=1.8$) (table 1). The median frequency of maximum intensity was 22.2 kHz ($n=38$); the median bandwidth was 23.8 kHz (figure 3). Songflight calls were in general preceded and followed by an echolocation pulse with median IPI lengths of 40.0 ms and 92 ms, respectively (figures 4 and 5).

Similar measurements were taken from

¹ A comparison of the songflight calls of the two bats in Leersum in 2011 showed that the syllables in the calls of the second bat were slightly increasing instead of decreasing, thus making it possible to distinguish the bats from each other.

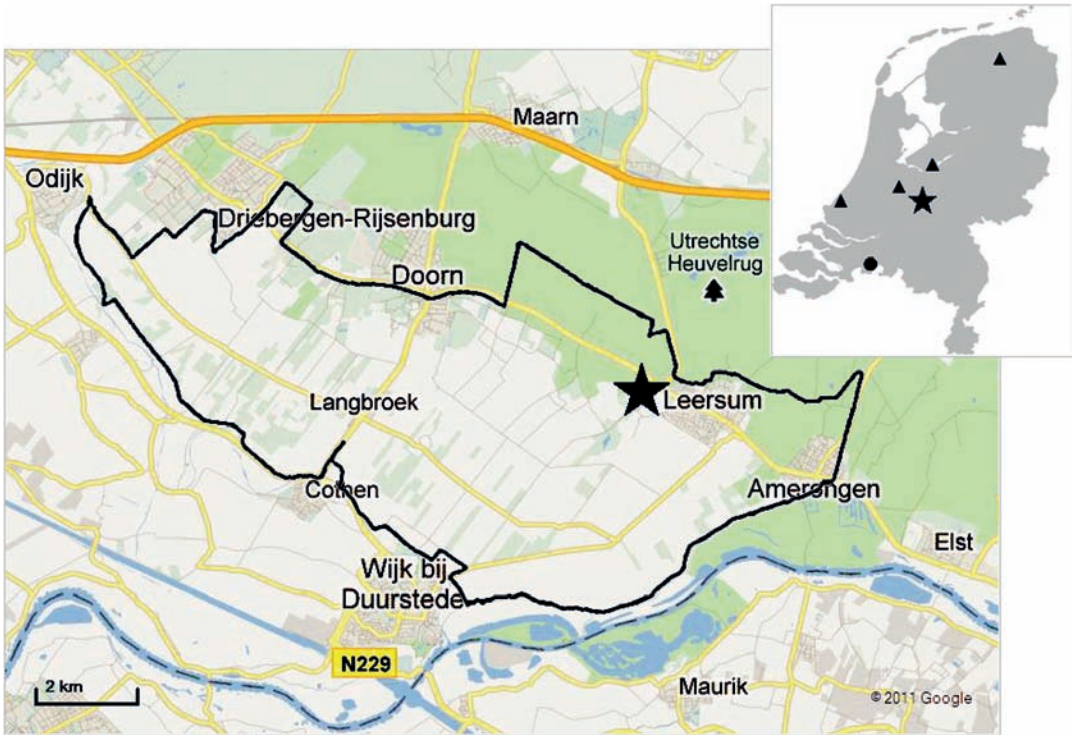


Figure 1. Location of the first confirmed record of the soprano pipistrelle (*Pipistrellus pygmaeus*) (asterisk) in 2007 in the Langbroekerwetering (bounded area) in the centre of the Netherlands. The insert shows other locations of recorded *Pipistrellus pygmaeus* until September 2011 in the Netherlands (triangles: bioacoustic identification; circle: morphological identification).

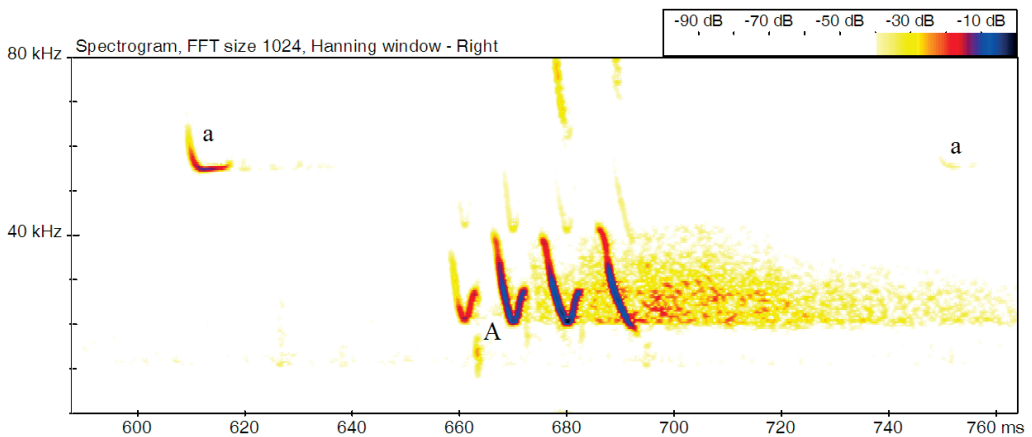


Figure 2. Spectrogram of an echolocation call (a: duration: 8.7 ms, minimum frequency: 54.5 kHz) followed, after 48 ms, by a songflight call (A: four syllables) of the Leersum bat; the recording was made on 22 August 2008, 00:41 a.m., Leersum.

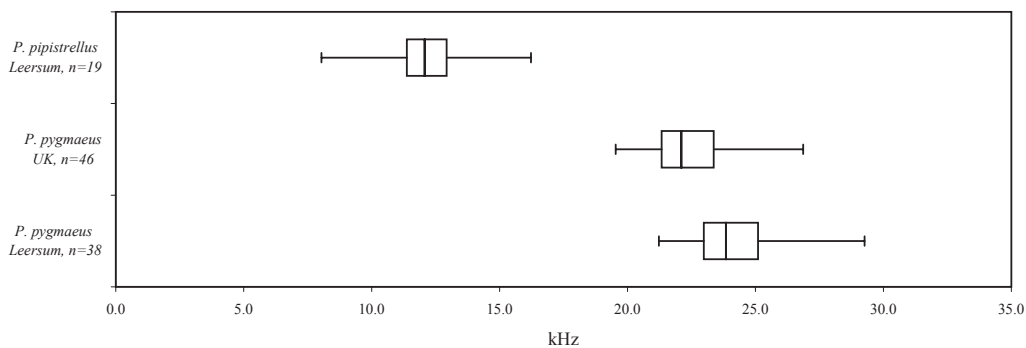


Figure 3. Boxplot showing the bandwidth (median, 25% and 75% percentiles min and max) of songflight calls.

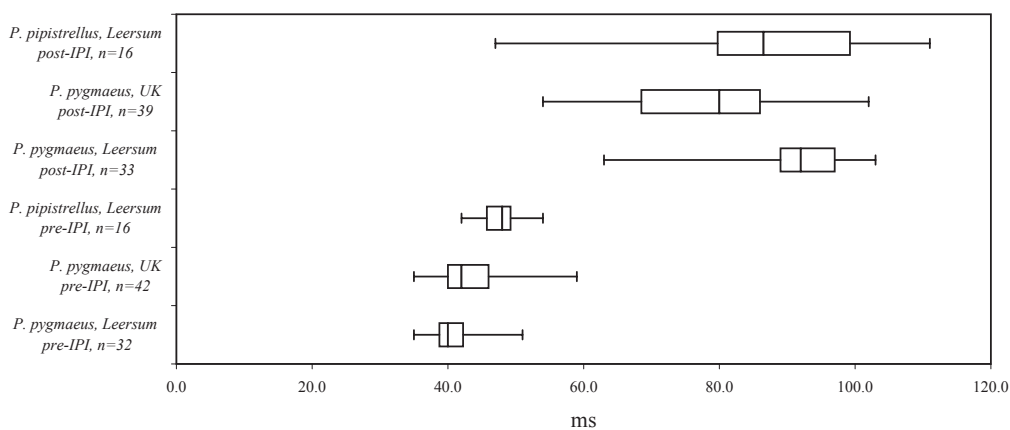


Figure 4. Intermediate pulse intervals (median, 25% and 75% percentiles, min and max) of echolocation calls immediately preceding (pre-IPI) and following (post-IPI) songflight calls.

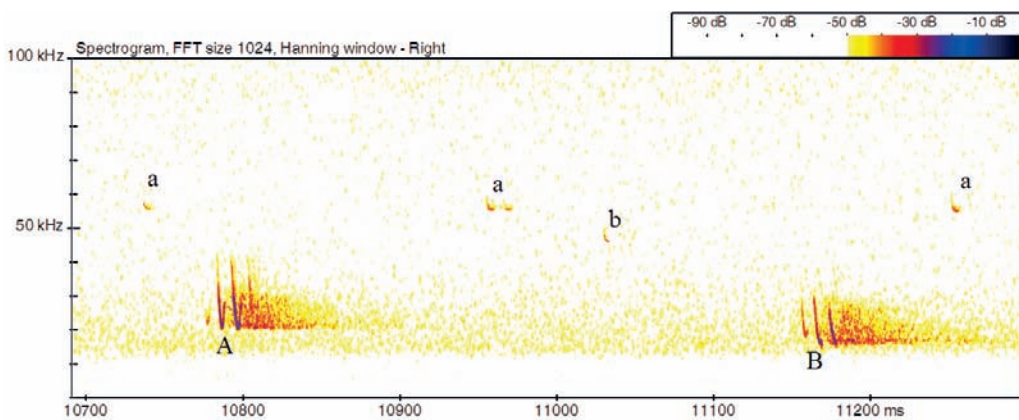


Figure 5. Spectrogram of songflight calls A, B (three syllables) and echolocation calls a, b of the Leersum bat (A, a) and a *Pipistrellus pipistrellus* (B, b); 15 August 2008, 00:13 a.m., Leersum.

Table 1. Number of songflight calls containing 2, 3 or 4 syllables, and the start and minimum frequencies (median, min, max) of complete songflight calls. Authors recordings: Llanthony, Powys, May 2008; Trentishoe, Devon and Ebbesbourne, near Salisbury, August 2009.

	Number of calls <i>n</i> syllables long			Starting frequency (kHz) Median (Min-Max)	Minimum frequency (kHz) Median (Min-Max)
	2	3	4		
<i>Pipistrellus pygmaeus</i> near Leersum	0	9	29	41.9 (28.4-48.4)	20.8 (18.0-27.4)
<i>Pipistrellus pygmaeus</i> in the UK ¹	1	31	14	40.0 (28.5-44.7)	19.8 (16.4-22.8)
<i>Pipistrellus pipistrellus</i> near Leersum	1	9	9	26.5 (19.5-33.6)	16.3 (13.6-21.4)

recordings of *Pipistrellus pipistrellus* bats flying near Leersum and *Pipistrellus pygmaeus* bats in the UK (Llanthony, Powys, May 2008; Trentishoe, Devon and Ebbesbourne, near Salisbury, August 2009).

Echolocation pulses

The echolocation pulses from the Leersum bat for sequences without songflight calls showed a bimodal distribution of IPIs. The main peak of IPIs occurred around 82 ms (*n*=48), with a minor second maximum occurring at around 157 ms (*n*=6) (figure 6). The median frequency of the maximum energy of echolocation pulses in sequences without songflight calls was 56.5 kHz, the corresponding median minimum frequency was 56.0 kHz (*n*=60) (figure 7). The mean pulse duration was 5.0 ms (*n*=60, *sd*=1.0). Echolocation pulse durations tended to be longer in sequences containing songflight calls than in sequences without, but this difference was not further investigated. Similar measurements were obtained from recordings made of *Pipistrellus pipistrellus* bats flying near Leersum.

Discussion

As the Leersum bat has not been captured, a morphological determination (Dietz & von Helversen 2004) could not be performed, hence

identification was based solely on the characteristics of the recordings. Measured values for the frequency with the maximum energy, minimum frequency and pulse duration of the Leersum bat are all typical of *Pipistrellus pygmaeus*, especially as the minimum frequency was never observed to be lower than 52 kHz and always maintained normal pulse durations of at least 5 ms (cf. Boonman et al. 2008, Pfalzer 2008, Skiba 2009). *Pipistrellus pipistrellus* is also known to be able to issue qCF calls ending above 52 kHz which means that misidentification is possible (Wicht et al. 2003). On these results alone it is possible that the Leersum bat could be a *Pipistrellus pipistrellus*. However, one would expect the pulse duration to be much shorter than 5 ms and the call intensity to be lower. Unfortunately, Wicht et al. (2003) did not report on exact pulse durations and intensity for the two reported *Pipistrellus pipistrellus* bats. These could have been misidentified as *Pipistrellus pygmaeus* solely on the basis of their echolocation calls above 56 kHz.. The authors also did not report whether these two bats were also able to produce normal *Pipistrellus pipistrellus* echolocation calls, ending at around 46 kHz. Thirdly, the bats were released ‘from the hand’, which could have influenced call characteristics in several ways, compared to a bat recorded in its natural habitat during normal flight behaviour.

The Leersum bat in this study is clearly distinct from *Pipistrellus pipistrellus* by virtue of several characteristics, although for two char-

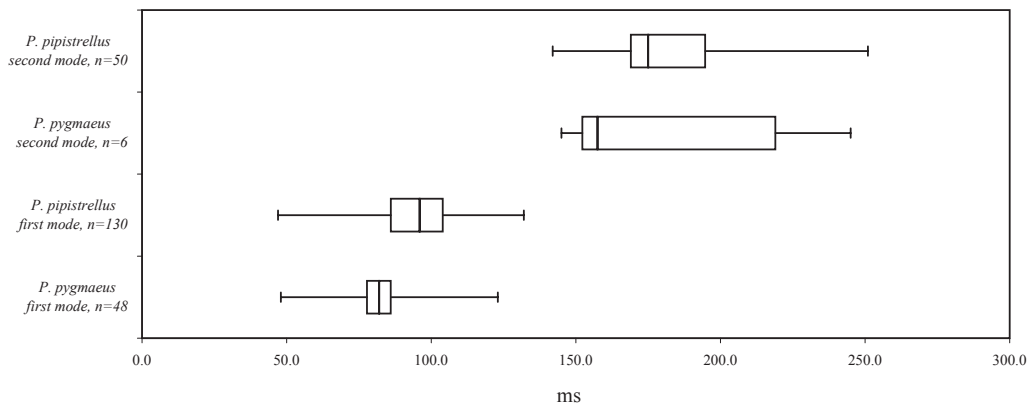


Figure 6. Bimodal distribution of intermediate pulse intervals (median, 25% and 75% percentiles min and max) of echolocation pulses in recordings without songflight calls of bats in Leersum.

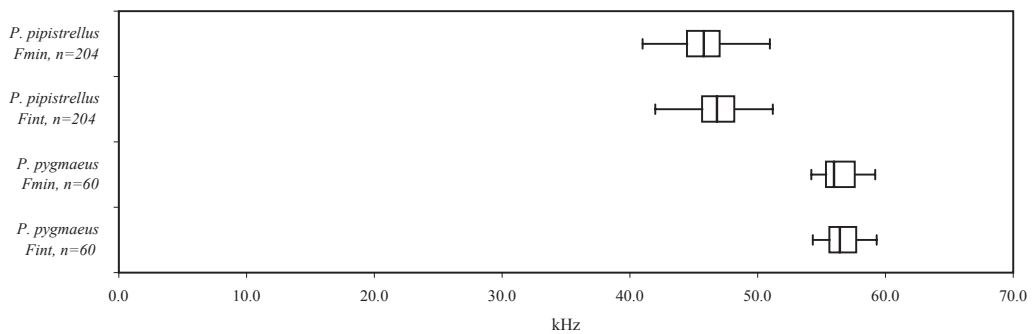


Figure 7. Minimum frequency (Fmin) and frequency of maximum intensity (Fint) (median, 25% and 75% percentiles, min and max) of echolocation pulses in recordings without songflight calls of bats in Leersum.

acteristics there are similarities (figures 3, 4, 6 and 7). Firstly, the overlaps in the plots for the IPIs of an echolocation pulse immediately following a songflight call (post-IPIs) indicate that this characteristic should not be used for differentiation (figure 4). Secondly, the overlap in the box plots for the second peak of IPIs of echolocation pulses in recordings without songflight calls makes identification seem inconclusive (figure 6). However, the overlap, or less well pronounced difference between the two species in the typical values for this second mode of IPIs of echolocation pulses, is well known (Boonman et al. 2008, Skiba 2009).

By contrast the plots for the IPIs of an echolocation pulse preceding a songflight call strongly indicate that these pre-IPIs can very

well be used as a differentiation characteristic (figure 4). Wingbeats are strongly synchronised with the emission of echolocation calls (Wong & Waters 2001). This might also be true for songflight calls, which are more intense and have a much longer duration than echolocation calls, and need to be integrated into a bat's call behaviour. Before a bat can issue a songflight call, it first issues an echolocation pulse and listens for echoes to ascertain that it will not encounter any obstacles during its flight. An IPI of 40 ms will cover about seven metres. The bat then issues a songflight call of roughly 30 ms and starts to listen for responses from any other bats, while trying to postpone the issuing of the next echolocation pulse for as long as possible. This could explain the large

range of measured post-IPI values. The small range of pre-IPI values seems to suggest that bats have a preferred wing position and stroke direction for making a songflight call and its preceding echolocation pulse. Wong and Waters (2001) report that intermediate pulse intervals of 40-50 ms were the result of echolocation pulses issued at the beginning of the downstroke and at the end of the upstroke in the same wingbeat. The pre-IPI values measured in this article seem to suggest that this is also the case for the songflight call and its preceding echolocation call.

The recorded songflight calls of the Leersum bat had a wide range of bandwidths and the frequency of maximum energy was above 20 kHz, which is typical of *Pipistrellus pygmaeus*. Those of *Pipistrellus pipistrellus* are less wide and lie below 20 kHz (Barlow & Jones 1997, Pfalzer 2008, Skiba 2009). On the other hand, the majority of observed numbers of syllables (four) in the songflight calls of the Leersum bat is more typical of *Pipistrellus pipistrellus*; although some overlap does occur between the two species (Barlow & Jones 1997, Pfalzer 2008, Skiba 2009, personal recordings in the UK) (table 1).

Unfortunately, measurements of other characteristics of the songflight calls from the Leersum bat cannot be compared to the values reported by Pfalzer (2008) for the two species. For *Pipistrellus pygmaeus* Pfalzer only reports on calls consisting of three syllables, whereas the bat in my study mainly uses four. For *Pipistrellus pipistrellus*, Pfalzer made no differentiation between the numbers of syllables in a call, which makes statistical evaluation rather dependent on the distribution of the number of syllables in his set of calls. I suspect pipistrelle songflight call characteristics are highly dependent on the number of syllables in the call; e.g., the total call duration largely depends on the number of syllables.

None of the recordings (until September 2011) showed two bats calling at the same time with qCF calls ending at around 55 kHz. This suggests that all the call sequences were

probably obtained from a single, isolated bat resident near Leersum. Furthermore, these calls were only recorded within a relatively small area around Leersum. This means that it is unlikely that there is a nearby *Pipistrellus pygmaeus* colony. The large number of songflight calls issued after mid-July indicates that this bat is probably a male. For the same reason, the second bat observed in September 2011 also appears to be male.

It is not yet known why no other recordings had been made of *Pipistrellus pygmaeus* in the Netherlands, nor how this first bat came to be resident near Leersum. Studies have shown that *Pipistrellus pygmaeus* prefers riparian habitats, but there is no valid explanation why the species is so rare in the Netherlands. The increasing number of observations seems to indicate that *Pipistrellus pygmaeus* is finally starting to colonise the Netherlands. My very recent observation of a second male near Leersum could be an indication of that.

Conclusion

The bandwidth of songflight calls and the inter pulse intervals of echolocation calls preceding songflight calls seem to be useful characteristics for identifying *Pipistrellus pygmaeus*. Taken together with other echolocation and songflight call characteristics, the first recorded bat near Leersum in 1997 can be identified as *Pipistrellus pygmaeus*, which has been confirmed by others (H. Limpens, G. Jones, personal communication). Hence, an accidental recording of a bat on 23 August 2007 finally resulted in the recording of *Pipistrellus pygmaeus* as a new species for the Netherlands, an unforeseen spin-off of the Langbroekerwetering bat project.

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Samenvatting

De eerste waarneming van de kleine dwergvleermuis (*Pipistrellus pygmaeus*) in Nederland

Na afloop van een vleermuisinventarisatie op 23 augustus 2007 op het landgoed Broekhuizen bij Leersum in de Langbroekerwetering maakte ik in de omgeving extra Time Expansion opnames met een batdetector. Tijdens een spectrogramanalyse in oktober 2007 ontdekte ik enkele zeer zwakke, maar lange *Pipistrellus*-echolocatiepulsen met een eindfrequentie van boven de 55 kHz in een van de opnames, met daarnaast enkele sociale geluiden die typisch zijn voor *Pipistrellus*. In 2008 werden tijdens aanvullende bezoeken aan het gebied opnieuw opnames van pulsreeksen gemaakt met eindfrequenties van boven de 55 kHz, ook door andere vleermuisonderzoekers. Ook in de jaren daarna, tot aan het maken van de definitieve versie van dit artikel in oktober 2011, konden in Leersum dergelijke opnames gemaakt worden. Aan de hand van kenmerken zoals (minimum, maximum, piek-) frequentie, pulslengte en pulsintervallengte kon voor het eerst de aanwezigheid van een kleine dwergvleermuis (*Pipistrellus pygmaeus*) in Nederland worden bevestigd.

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Hot spot for pine marten (*Martes martes*) and first record of a natal den in Flanders (Belgium)

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Abstract: From 2000 to 2011, pine marten (*Martes martes*) reproduction has been regularly recorded in a small forest complex of about 250 hectares in the north of the Province of East Flanders (Flanders, Belgium). The local habitat is characterised by a mix of forest types, in which coppice stands and fen forest patches are prevailing on substantial surfaces, combined with many small satellite woodlands spread out around the margin. The home range size of a radio-collared breeding female, recorded from August 2010 to April 2011, appeared to be very small (<1 km²), elsewhere in Europe only found in the pristine forest of Białowieża (Poland) which is home to a very dense pine marten population, or in 'a-typical' habitat composed of hedged farmland with small forest fragments in the French Ardennes. However, the location studied here appears to be peculiar not only as it is a small and fragmented habitat for pine marten, but even more as it is an isolated hot spot in an open landscape, far from any massive forest complex that holds a vital pine marten population. The home range was located in the most compact centre of the forest complex and was intensively used throughout the whole study period. Remarkably, a 20 metre broad channel was bisecting the home range, not at all being a barrier although it can only be crossed over by active swimming. This finding should be noted with respect to possible attempts of diminishing predation risks from martens (e.g. to rare breeding birds) by creating landscape 'barriers' such as broad ditches or even channels. Given the preliminary results of telemetry and camera trapping, the pine marten's social system remains unclear to some extent in situations where suitable habitat is very limiting and the presence of direct neighbours is obscure.

Keywords: pine marten, *Martes martes*, Sinaai, Flanders, Belgium, home range, natal den, fragmentation, isolation, telemetry, camera trapping.

Introduction

Pine martens (*Martes martes*) are very rare in Flanders and the southern provinces of the Netherlands and their prolonged presence during recent decades is not well understood (Van Den Berge et al. 2000). In order to get more insight in the situation, an on-going project to collect and autopsy traffic victims from all over Flanders was started in 1998. Additionally, camera trapping has been successfully tested and potential pine marten habitats have been systematically scanned for the presence of the species since 2008 (Van Den Berge 2009). Inte-

grating all the available information (including chance sightings) from these programmes shows that pine martens are settled and breeding in at least three locations in Flanders: one in the Waasland region in the north of the Province of East Flanders and two in the Antwerp part of the Kempen region (figure 1). Recently, local reproduction has also been recorded at one location in the southern border zone of the Netherlands (Wijsman 2009). While the locations in the Kempen are in a quite well-forested landscape across the national border, the one in Waasland, situated at the other side of the river Schelde, is characterised by only small and scattered woodlands, far from any massive forest or large woodland complex (figure 1). In this respect, it is surprising to find a breed-

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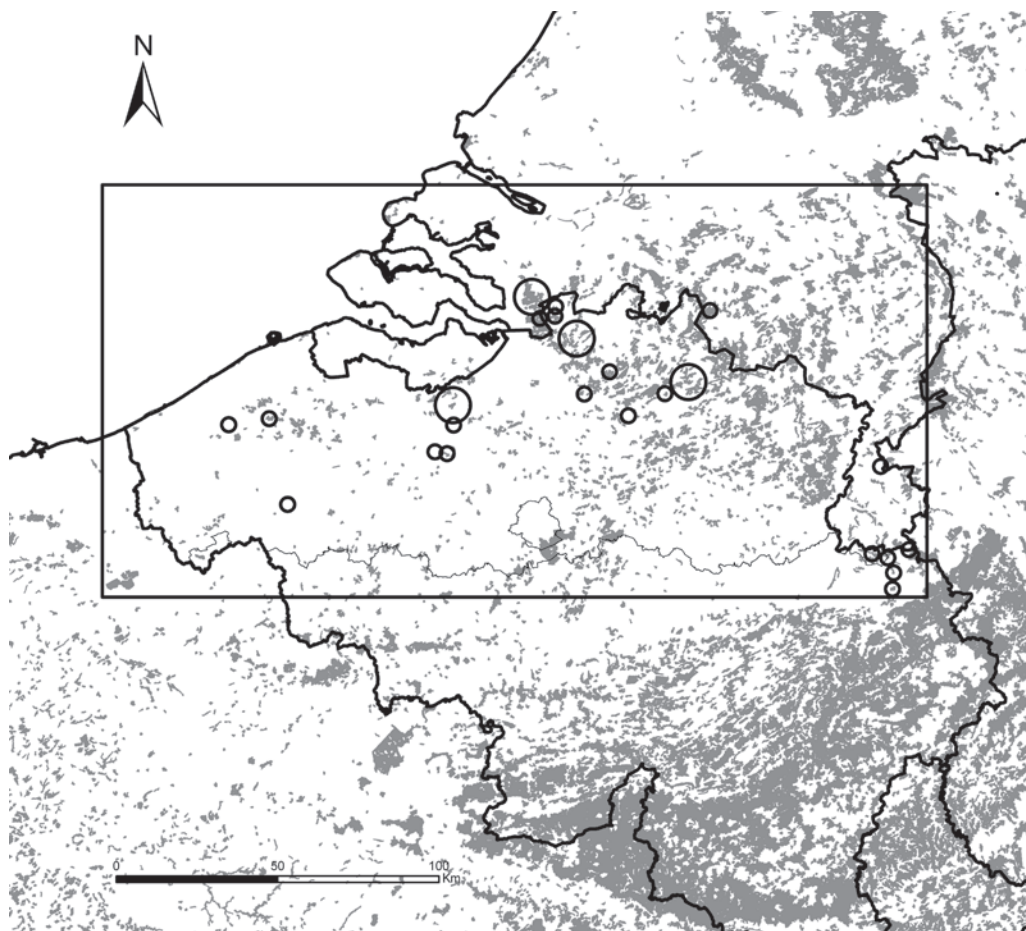


Figure 1. Reports of pine marten from 2000–2011 in Flanders (Belgium) and the southern Netherlands, related to the presence of forest (in grey) according to the CORINE land cover survey (2006). Circles indicate locations of (combinations of) traffic kills, camera trapping results and reliable sightings; large circles indicate reproduction sites, with the most western location being Sinaai. Flemish data sourced from the INBO carnivore databank, Dutch data from van der Lans et al. (2006) and H. Wijsman (communicated by his Boommarker Nieuwsbrief), and Walloon border data from R. Janssen (personal communication).

ing population has been there for more than ten years. This article gives an overview of our findings at this location, called Sinaai.

Methods and results

Study area

The Sinaai area consists of a complex of small woods and field woods ranging from 1–2 hec-

tares to about 100 hectares in size, which are surrounded by arable land and meadows. The shortest distance to other more or less compact forests (of at least 100 hectares) in the region ranges from 3 to 8 km. The complex lies in the Moervaart Depression, an alluvial landscape of quaternary origin that has been developed within the Flemish Valley north of Gent. In historical times, it was predominantly in use as meadow land, drained by a dense network of ditches and water channels of varying

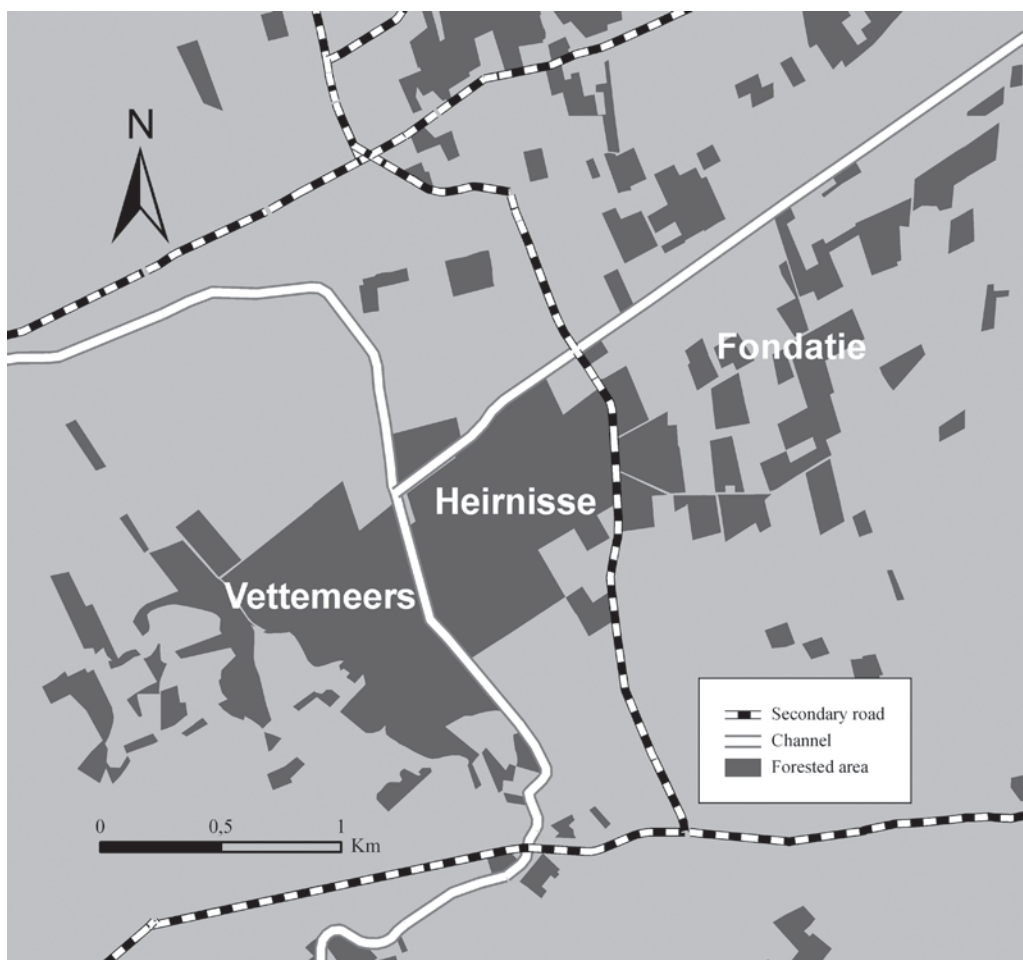


Figure 2. The sub-areas of the pine marten reproduction site Sinaai in the north of the Province of East Flanders (Flanders, Belgium).

dimensions. From the end of the 18th century onwards there was some small scale afforestation which gave the area its current characteristics (Baeté et al. 2004). One can identify three sub-areas (figure 2). The central part, called the Heirnisse, is bordered on two sides by small channels, the Moervaart in the west and the Stekense Vaart in the north. East of the Heirnisse is the Fondatie, the two are separated from each other by a secondary road. The Fondatie is a more open sub-area with only scattered woods. To the west of the Heirnisse, i.e. on the other side of the Moervaart channel, there is the Vettemeers. Both the Heirnisse and

the Vettemeers are relatively extensive forests. The Heirnisse became a strict (non-intervention) forest reserve in the 1990s and is owned by the Flemish government. In the other two areas a private nature reserve is being established. However, the land in the Vettemeers is still mostly in private ownership.

The Heirnisse is characterised by stands of coppices: mainly alder (*Alnus* sp.), birch (*Betula* sp.) and hazel (*Corylus avellana*), mixed with older Canadian poplars (*Populus x canadensis*), some small stands of matured oaks (*Quercus robur*) and middle-aged Corsican pine (*Pinus nigra*). There is a widespread network of ditches



Photo 1. The Heirnisse at Sinaai is characterised by stands of coppice mixed with fen forest, sedge and reed beds. Photograph: INBO.

and much land is covered by bramble thickets. Locally fen forest, sedge and reed beds are prevailing (photo 1). After the area was designated as a forest reserve an attempt was made (in 2004) to eliminate all American oak (*Quercus rubra*) coppice and American black cherry (*Prunus serotina*), leaving all the trunks on the spot and resulting in several quasi clear-cut bramble patches totalling about two hectares. In 2010 it was decided to scrape all the dead wood together and create massive wood heaps. The Vette-meers and Fondatie originate from the same historical complex and have similar forest stands, although these have been substantially desiccated in recent decades, resulting in dry ditches, an absence of marsh vegetation and more vertically structured forest stands. Both areas are locally interspersed with small to medium-sized clusters of spruce (*Picea abies*) and contain some artificial ponds. Unlike the Heirnisse there are some inhabited parcels. The whole complex contains about 250 hectares of forest, mostly concentrated in the

Heirnisse and the Vette-meers, separated from each other by the Moervaart channel.

The presence of pine marten

Beside some information based on oral history from local people, the first concrete record of pine martens being present at Sinaai dates from the 7th of July 2000, when a young male was found as traffic kill (Van Den Berge et al. 2000, Van Den Berge & De Pauw 2003). With a baculum (os penis) weight of only 0.12 g, this animal obviously appeared to be born in spring 2000 according to Broekhuizen & Müskens (2000b), still living in the parental territory at that time according to Broekhuizen & Müskens (2000a), and so proving the first and definitive record of reproduction in Flanders. On the 5th of June 2004 another traffic kill was found on the same section of road: an adult female, clearly lactating. However, as the dead body was gravely damaged, neither the uterus



Figure 3. Home range measured as the minimum convex polygon (MCP 100) of a female breeding pine marten from the 4th of August 2010 till the 1st of April 2011 at the Sinaai location. Black dots: telemetry fixings indicating inactivity. White dots indicate activity (with minimum intervals of 24 hours).

nor the ovaries could be inspected for recent gestation. As false gestation is known to occur in martens (Broekhuizen & Müskens 2000b), lactation itself could not be considered as further proof of local reproduction. Moreover, based on three independent tooth sections for cementum aging by a specialised laboratory (Matson's Lab, Montana, USA), this female should only be one year old – i.e. born in spring 2003 – being too young to have had offspring. Nevertheless, two days after the finding of the dead female, a local naturalist observed two or three young pine martens playing in the shrubs and succeeded in filming them. So, most probably there were two adult females in the same

reproduction territory that year.

On the 1st of June 2005, we ourselves made a prolonged chance observation of a fleeing adult female with at least two young, climbing into an oak tree and making themselves rigid on the branches of it.

During the following years, the continued presence of pine martens seemed likely as there were regular findings of scats and prey remnants (especially of middle sized birds and eggs), although confusion with polecat (*Mustela putorius*) or stone marten (*Martes foina*) could not always be excluded. According to local naturalists, polecat used to be a 'common' inhabitant in the area, and proof of the presence of

stone marten was provided by the finding of a traffic kill near the Fondatie, in spring 2011. In autumn 2007 the first pine marten was photographed by a camera trap (Trailmaster TM550) in the Heirnisse (using valerian oil as a lure) and in the following summer (July) another type of camera trap (Moultry M40) registered a pine marten in the Fondatie. By contrast, we did not succeed in recording any pine martens in any of the surrounding forested areas of the Waasland region (Kloosterbos, Puyenbroek and Heidebos) despite intensive effort using up to five cameras together (minimum one/km²).

In 2008 and 2009, the presence of marten scats and prey remnants was very noticeable in both the Heirnisse and the Fondatie, suggesting increased (territorial) activity and probable reproduction. Therefore, in the summer of 2009 the camera trapping technique (Moultry M40/D40/I60; Spypoint IR8; Reconyx HC600) was combined with a feeding place (fruit, honey, peanut butter) in both these sub-areas to detect the presence of young animals. This plan appeared to be very successful and resulted during several weeks in plenty of photographs and videos of up to three pine martens together: apparently an adult female with her two young (photo 2). The last family pictures date from mid-September, after which camera trapping was only occasionally successful.

In the early spring of 2010 an exhaustive attempt was made to survey all possible natal den sites, especially tree cavities, in both the Heirnisse and the Fondatie (Conings 2010). However, pine marten activity in spring seemed much less than in the preceding two years and the inspection of all known tree cavities (93 in the Heirnisse and 63 in the Fondatie) remained negative. Camera trapping resulted in pictures of just one single adult marten, suggesting there was no successful breeding in 2010.

With the exception of the Sinaai location, there is hardly any other recent information about even the temporary presence of pine marten in East Flanders (figure 1). Besides a reliable chance observation of a pine marten



Photo 2. Still from a video-trap movie (Moultry I 60) recorded on the 12th of September 2009 in the Heirnisse at Sinaai: two young pine martens foraging at the foreground on a feeding place and the adult female passing in the background (left). *Photograph: INBO.*

hunting a squirrel in the city park of Lokeren on the 31st of August 2008 (some five km from the Sinaai location), only one other traffic victim has ever been found. On the 18th of March 2010, a sub-adult male of about eleven months old was killed in the municipality of Kalken, about 15 km from the Sinaai location. His throat patch pattern appeared to be different from the young animals frequently photographed at the Sinaai location in 2009, suggesting another breeding location (Van Den Berge 2010). However, a subsequent camera trapping session in the neighbouring Berlare forest during the summer of 2010 remained unsuccessfully, although some observations suggest there was local reproduction in this forest complex in 2000 and 2001.

Radio telemetry and natal den

On the 4th of August 2010 we succeeded in catching alive an adult female pine marten at the Sinaai location which we radio collared and then recorded telemetrically for the next eight months (radio-collar Televilt 151 MHz band, Telonics TR-4 receiver with a H-aerial) to the early spring of 2011. Because of the low level of tooth abrasion, the animal was estimated to be in its second year of life, i.e. born

in spring 2009. According to the nipple physiognomy, no young had ever been breastfed.

During the whole telemetry period the radio-collared marten was located by triangulation two to four times a week, mainly by day, with minimum time intervals of 24 hours. According to the signal interval, it was possible to distinguish between the activity of the marten (active or resting). When the animal was active and moving around, the first fixing was selected.

In total, 123 successful fixing days were realised (figure 3) and the spread of the fixing locations soon became quite predictable, indicating a stable home range size. According to Stier (2000) and Schröpfer et al. (1989), pine marten home ranges can be characterised by the distribution of the fixings during the resting phases, i.e. mainly by day. Apart from that, at least 32% of the telemetry records during day time indicated spontaneous activity, being well spread all over the fixings. So, to interpret home range position, the lack of a substantial number of nocturnal fixings should not be a problem.

As a fact, the most striking finding was the position of the home range, which was located on both sides of the Moervaart channel, combining the sub-areas of the Heirnissee and the Vette-meers during the whole investigation period. In absence of any bridge or other construction, the 20 metre broad channel could only be crossed over by voluntary swimming, which must have occurred at least 41 times. By contrast we found no indication that the secondary road bordering the Heirnissee was ever crossed as we did not record any visit to the Fondatie during the telemetry period, even though it is known that this sub-area was inhabited by pine marten in 2008 and 2009.

In small woods or scattered forest complexes, the application of the minimum convex polygon method (MCP) to calculate the home range surface can result in an important overestimation by including parts of unforested and unused land (Stier 2000, Mergely 2007). However, given the concrete posi-

tion of the fixings, which were almost all concentrated in the compact forest core (figure 3), this method appears quite advantageous here, and moreover it allows for comparison with other studies. So, enclosing all the fixings (MCP100), home range totals only 0.92 km², whereas in the Vette-meers the home range border appears to be amply situated within the sub-area, i.e. not coinciding with its irregular borders or including any substantial unforested land.

Day hides could only be looked for when the marten was in the Heirnissee, as unfortunately we had no permission to enter the strictly private properties of the Vette-meers. Day hides were only looked for by spot-check, as they soon appeared to be almost always located in very dense sub-layer vegetation (bramble, sedge and reed beds), without possibility to see the animal but all the more disturbing it. The marten was not once found visible, e.g. resting on a bird's nest, although another pine marten was seen twice on a hawk's eyrie in the Heirnissee on the 4th and 12th of May 2011. In winter, dense spruce canopy was chosen as a day hide a few times, while in snow periods the marten invariably hid in the immense wood heaps, sometimes without leaving them for two or three days.

In the last ten days of March, the marten was repeatedly (but not permanently) recorded at one particular site, in an inaccessible private property at the Vette-meers sub-area. During the night of the 23rd to the 24th of March, the animal even never left the site, and on consecutive days the transmitter signal always indicated exactly the same site. The last signal was received on the 1st of April when the life time of the transmitter battery ended.

A once-only visit to the site on the 12th of April confirmed the assumption of a natal den at that location: an old nest cavity of a black woodpecker (*Dryocopus martius*) in a big gray poplar (*Populus x canescens*) with two entries and a massive latrine on a branch stump. According to Kleef (2000) and Kleef & Tydeman (2009), the lengthy and uninter-

Table 1. Pine marten reproduction at Sinaai between 2000 and 2011.

2000	Certainly	Traffic kill of young male on the 7 th of July
2001	No information	
2002	No information	
2003	Probably	Traffic kill of subadult female on the 5 th of June 2004 (about 14 months old)
2004	Certainly	Direct observation of at least two young on the 7 th of June
2005	Certainly	Direct observation of adult female with at least two young, on the 1 st of June
2006	No information	
2007	No information	
2008	Probably	Remarkable presence of field signs (scats, prey remnants)
2009	Certainly	Plenty of visual evidence from camera trapping of at least two young
2010	Probably not	Intensive camera trapping unsuccessful for young
2011	Certainly	Localisation of natal den with two young

rupted stay of the female at this site between the early evening of the 23rd and the morning of the 24th of March was indicative of her giving birth then.

The following weeks, a local naturalist with free entrance to the private domain observed the den tree during the day for many hours. The adult female was seen several times, leaving the cavity for a latrine visit or to go and search for prey. On the 20th of May, two kittens were noticed for the first time at the entries of the den, while on the 24th of May the adult female was seen encouraging her kittens to leave the den. On the 27th and 28th of May, no more martens were seen, in spite of about seven hours of observation.

What followed ...

From the 14th of June, the adult female was camera-trapped several times in the Heirnisse together with her two kittens (photo 3), indicating that those young martens had also swam successfully over the Moervaart channel. After some weeks, the size difference indicated that one young was a female and the other a male, with a much bigger stature than his mother.

Surprisingly, on the 19th of September the adult female (recognisable by her radio-collar) was camera-trapped in a remote corner in the Fondatie sub-area, about 1.6 km away from the

eastern border of her known home range. Since then, this animal could not be photographed any more in the Heirnisse, but was residing in the Fondatie as was proven by camera trapping, at least up until mid-November (the closing date for manuscripts for this issue).

On the 29th of September, the male young (recognisable thanks to his throat patch pattern) was live-trapped in the middle of the Heirnisse and also radio-collared. Recording this animal telemetrically (31 fixing days) showed its lasting presence in the natal home range at least up to the 22nd of November, being a late date not to have yet dispersed according to Broekhuizen & Müskens (2000a). At least two round trips over the Moervaart channel were recorded, but no fixing was made in the Fondatie. During this period, the animal was also regularly camera-trapped – all alone – on the usual feeding place in the Heirnisse.

Discussion and conclusions

The Sinaai location undoubtedly appears to be a hot spot for pine marten in Flanders, despite only having a small sized forest complex and being situated in a mainly open landscape. At least in 2000, 2004, 2005, 2009 and 2011 there was successful reproduction (table 1), and the species has probably been permanently present for more than a decade now.



Photo 3. Camera-trap photo (Reconyx HC600) recorded on the 11th of July 2011 in the Heirnisse at Sinaai: the radio-collared adult female (right) and male young (left) on a feeding table and the second young on the floor under the table. *Photograph: INBO.*

According to the overview of mean home range sizes of pine marten in Europe, given by Zalewski & Jędrzejewski (2006), the found (eight months) home range of the radio-collared breeding female appears to be very small ($<1 \text{ km}^2$), although this does lie within the range ($0.54\text{--}2.61 \text{ km}^2$) of the authors' own results from the pristine, continuous, forest of Białowieża (Poland) which has a very high marten population density. On the other hand, Mergey et al. (2011) found in the French Ardennes that home ranges in fragmented habitat, with an abundant food supply, were between two and three times smaller than in forest, probably due to the confinements imposed by the configuration of the landscape which contains only small suitable habitat fragments. As the home range found in our study was located in the most compact centre within the forest complex – leaving alone the integral sub-area of the Fondatie as well as a substantial part of the Vette-meers – the question remains how to explain the given situation. Zalewski & Jędrzejewski (2006) reported only very little inter- and intrasexual home range overlap, even in a dense population, while Mergey (2007) also found overlap to be very exceptional. Given the prolonged presence of the species and regular reproduction, at least the temporary and repeated presence of a

mature male in the (direct) surroundings must be guaranteed. However, as Mergey et al. (2011) found that male home ranges were 2.3 times larger than those of females, in our study area the possible configuration of the respective home ranges should cause spatial problems. So, as a social system of floating males and resident females seems unlikely in the given landscape, a cluster of small sized neighbouring and essentially overlapping home ranges can be supposed. As a fact, in Sinaai, the presence of a second adult pine marten beyond the mating season was noticed twice in the central breeding home range: in 2004 a false pregnant female was killed on the road bordering the forest core area, and in May 2011 a resting animal was observed in the very middle of the breeding home range.

It is too early yet to be able to interpret the most recent findings on the presence and absence of individual animals. Although the camera trap technique has proved to be a very useful tool to detect or document pine marten presence and possible local reproduction, the other way round it is very tricky to postulate hard conclusions: absence can't be proven. Indeed, during preceding years, the technique was not always successful, sometimes resulting in periods without any marten pictures, while the animal(s) certainly were present. Although it is known that territorial individuals sometimes make short excursions outside their home ranges (see e.g. Müskens et al. 2000), our camera trapping results suggest that the adult breeding female has left her previous home range – in favour of the male young? – and moved into the Fondatie at least for a prolonged period of several weeks.

In the near future, we hope to recapture the adult radio-collared female and replace the silent transmitter by a new one for some extra months of tracking, and afterwards remove it definitively. We also plan to start genetic kinship research as a follow up step to gain more clarity about some of our findings. In the main time, our findings yet indicate that pine martens do have a future even in the small scale

fragmented forests of Flanders. Some lessons can be learned from the particular home range position on both sides of a middle sized channel, e.g. with respect to possible attempts of diminishing predation risks from martens (e.g. to rare breeding birds) by creating landscape 'barriers' such as broad ditches or even channels.

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Samenvatting

Hot spot voor boomarter (*Martes martes*) en eerste formele vaststelling van een nestboom in Vlaanderen (België)

Hoewel boommarters (*Martes martes*) reeds vele decennia bijzonder zeldzaam zijn in Vlaanderen en het zuiden van Nederland, duidt hun langdurige aanwezigheid hoe dan ook op lokale voortplanting. Voor Vlaanderen kon in 2000 daarvan voor het eerst bevestiging gevonden worden in een klein en geïsoleerd boscomplex te Sinaai (Oost-Vlaanderen). Sindsdien lijkt, op basis van een combinatie aan onderzoeksmethoden (waaronder de inzet van cameravallen), permanente aanwezigheid van boommarters in het gebied zeer aannemelijk en vond succesvolle voortplanting met zekerheid nog minstens vier keer (in 2004, 2005, 2009 en 2011) plaats. Tijdens twee voorjaren d.i. buiten de paartijd, werd de aanwezigheid van een tweede adult dier in het voortplantingsterritorium vastgesteld. In augustus 2010 werd een lokaal gevestigd wijfje met een halsbandzender uitgerust en gedurende acht maanden telemetrisch gevolgd, dit is

tot eind maart 2011. De home range van dit dier bleek minder dan 1 km² groot en beperkte zich tot het compacte, centrale deel van het boscomplex, evenwel gelegen aan weerszijden van een 20 meter breed kanaal dat enkel zwemmend kan worden overgestoken. Dit wijfje bracht op 24 maart 2011 twee jongen ter wereld – de eerste gedocumenteerde geboortedatum in Vlaanderen. Kort na hun vertrek uit de nestboom bleken ook deze jongen het kanaal reeds succesvol te kunnen oversteken. Voor een territoriale soort met grote individuele leefgebieden is de langdurige aanwezigheid en herhaaldelijke succesvolle voortplanting in een klein en geïsoleerd bosgebied opmerkelijk. De landschappelijke configuratie maakt het opbouwen van een klassieke sociale structuur, waarbij in principe ook de leefgebieden van mannetjes en wijfjes nauwelijks of niet overlappen, heel moeilijk. Voorlopig lijkt het er op, dat het moederdier in het najaar haar home range verplaatste naar een aanpalend deelgebied, terwijl haar mannelijk jong in het geboorteterritorium resideert.

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Estimating population differentiation between isolated root vole (*Microtus oeconomus*) populations in the Netherlands using geometric morphometrics

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Abstract: We investigated morphological differentiation in threatened populations of the root vole subspecies *Microtus oeconomus arenicola*, measured by using geometric morphometrics on skulls recovered from owl pellets. Using populations from Finland as a reference, we compared measures of morphological differentiation to levels of genetic differentiation reported in literature for the same populations. We found that the degree of morphometric population differentiation was generally lower than the degree of genetic differentiation, yet it revealed broadly similar patterns of geographic isolation. This suggests that skull shape is conserved in isolated root vole populations, and that geometric morphometric measurements from skeletal parts recovered from owl pellets may provide a cost-effective method to monitor population subdivision.

Keywords: conservation, population subdivision, habitat fragmentation, skull, root vole.

Introduction

Habitat loss and fragmentation are well known factors to threaten persistence of animal species through isolation of local populations (Hartl & Clark 1989). A high degree of isolation is very generally accompanied by a high degree of inbreeding, which decreases fitness through increased homozygosity and susceptibility to diseases (Frankham 1996). These factors slow the recovery of populations after major disturbances, rendering local populations more vulnerable to demographic fluctuations, and eventually (in combination with reduced recolonisation) more susceptible to extinction (Frankham et al. 2002).

Conservation efforts often study population genetic structure of endangered species solely

in order to measure the degree of isolation of local populations as a proxy for various other threats. A popular measure to quantify the genetic variability of populations is:

$$F_{ST} = \frac{V_b}{V_b + V_w}$$

(e.g. Weir & Cockerham 1984), where V is genetic variation between (V_b) and within (V_w) populations. As is easily understood from the above equation, $0 < F_{ST} < 1$. F_{ST} does not measure the total genetic variation in the population ($V_w + V_b$), but the variation that is found between populations, or rather between individuals within populations. More precisely, if F_{ST} is calculated from genetic markers that are

not under selection (e.g. microsatellites) and if rates of mutation are similar in different populations (Nagylaki 1998) then F_{ST} is governed by the effects of migration and random genetic drift (Kimura 1983, Hartl & Clark 1989), and measures the degree of population differentiation resulting from drift and gene flow (Lande 1992). Hence, F_{ST} provides information about the degree of population isolation: if local populations are isolated and the genetic variation within populations is low, most genetic variation will exist between populations, resulting in a high value of F_{ST} . On the other hand, if there is extensive gene flow between local populations, much genetic variation is expected between individuals of the same local population, and little differentiation between populations, so that F_{ST} will be close to 0. From the above, it will be clear that population genetic analysis of endangered species can provide a wealth of information for conservation efforts, however, it should be kept in mind that the lack of genetic variation rarely threatens populations by itself, except through direct negative effects on survival and fecundity under extreme levels of inbreeding (Lande 1998, but see Spielman et al. 2004). The most general way in which decreased variation of local populations increases local extinction risk is probably a reduction in the capacity to adapt to changes in the environment: isolation decreases the genetic variation within a local population, and renders potentially useful genetic variants present in other populations inaccessible, thereby reducing the local population's capacity for adaptive evolution (Hartl & Clark 1989). Therefore, it is useful to infer not only the degree of genetic subdivision of a population, but also the degree of local adaptation.

The degree to which populations are adapted to their local environment can be inferred by comparing phenotypic (e.g. morphological) variation with genetic variation. In a similar fashion as explained for genetic variation above, phenotypic variation can be partitioned into within- and between-population compo-

nents. Specifically, we can calculate

$$P_{ST} = \frac{P_b}{2 * P_w + P_b}$$

where P_b is phenotypic variation between and P_w phenotypic variation within populations. Again, low levels of P_{ST} indicate that most phenotypic variation is found within populations, which implies little phenotypic differentiation of populations. If P_{ST} is high, on the other hand, populations are phenotypically distinct but foster little variation within them. It was shown (Spitze 1993) that when neglecting phenotypic differences due to different environments (for diploid species, assuming purely additive gene action and no linkage disequilibrium), P_{ST} is analogous to F_{ST} . That is, P_{ST} is the value of F_{ST} that would be obtained if F_{ST} were calculated from the genes that determine the phenotype (Wright 1951, Lynch & Spitze 1994, Latta 1998).

The analogy between P_{ST} and F_{ST} facilitates comparison of the variation in neutral (microsatellite) markers (F_{ST}) and that in metric traits (P_{ST}): A difference between the two values can tell us something about the direction of natural selection (McKay & Latta 2002). Typically, for divergent selection, where two populations become adapted to different environments, the degree of phenotypic differentiation between populations exceeds the degree of differentiation at neutral loci, so that $P_{ST} > F_{ST}$. Conversely, if the direction of selection is towards equal phenotypes in several populations (convergent selection), phenotypic variation between populations is smaller than genetic variation between the same populations, so that $P_{ST} < F_{ST}$. If $P_{ST} \approx F_{ST}$, the effects of genetic drift and selection are indistinguishable (Merilä & Crnokrak 2001).

Comparisons of the genetic and phenotypic structure of populations can be helpful to determine the risk for isolated populations to become vulnerable to stochastic events. This is of great interest for conservation biology, where

the goal is to preserve variation within populations and to ensure connectivity between sub-populations. An example of a population threatened by fragmentation due to human impact is the root vole (*Microtus oeconomus*). The Dutch subspecies, *M. oeconomus arenicola*, is the Netherlands' only endemic mammal subspecies and is endangered: its occurrence has been threatened by habitat fragmentation and loss during the last century. Recently, human activities have enabled other vole species (common vole, *M. arvalis* and field vole, *M. agrestis*) to colonise areas that were previously the exclusive domain of *M. oeconomus*. In these places, those invading species outcompete *M. oeconomus*, mainly in the drier parts of its habitat. This process further reduces the range of the root vole in the Netherlands (La Haye & Drees 2004). The population genetic structure of Dutch root vole populations has been studied using allozymes and microsatellite markers. While allozyme studies indicated low levels of genetic variation in local populations (Leijts et al. 1999), analyses using microsatellite markers showed that genetic differentiation is as large between regions within the Netherlands as it is between Dutch and Scandinavian populations (van de Zande et al. 2000). But, allozymes are variant forms of an enzyme that are coded by different alleles at the same locus, and may therefore reveal only genetic variation resulting from structural changes in enzymes. Thus, allozymes are more prone to selection bias than microsatellites and have a lower resolution in measuring genetic diversity. This suggests that populations of *M. oeconomus arenicola* experience substantial genetic isolation.

In this study we estimate morphological variation in root vole populations (P_{ST}) and compare it to literature reports of genetic variation (F_{ST}) from the same populations to infer selection regimes. We measure morphological variation from skulls found in regurgitated pellets of the barn owl (*Tyto alba*) and long-eared owl (*Asio otus*). Using this non-invasive sampling method we avoid removing individuals from the population. We quantify skull morphol-

ogy using geometric morphometrics, which is particularly sensitive to small morphological differences, and has earlier been applied successfully to show differences between root vole populations in Hungary (Ràcz et al. 2005).

Methods

Study species

The root vole has an almost circumpolar geographic range from northern Scandinavia eastward to Siberia, into Alaska and Canada. The main population stays above 50° north, but several isolated relict populations are remnants of a more southern postglacial distribution. In Europe, such relict populations can be found in Mid-Norway, Finland, Austria, Hungary, Slovakia and the Netherlands. Because of its endangered state, the Dutch root vole subspecies *M. o. arenicola* is included in the European Community Habitats Directive (97/62/EC) as a priority species; it is also classified as Critically Endangered (CR) by the IUCN (Gippoliti 2002 in: IUCN 2006) and it is on the Dutch 'Red List' for endangered mammals (Thissen et al. 2009).

Sampling

Root vole skulls from Dutch vole populations were obtained from barn owl and long-eared owl pellets. Home ranges of the owls are up to 5 km² in size (Arlettaz et al. 2010), so that the scattered occurrence of root vole populations renders it unlikely that pellets produced by an individual owl contain rodent samples from more than one region. For reference, we also used specimens from Finnish root vole populations, which were obtained from the zoological museum of the University of Oulu, Finland. These specimens had been collected by trapping at various locations.

The Dutch samples came from five regions; four of the five regional clusters described in the *Beschermingsplan Noordse Woelmuis* (Pro-

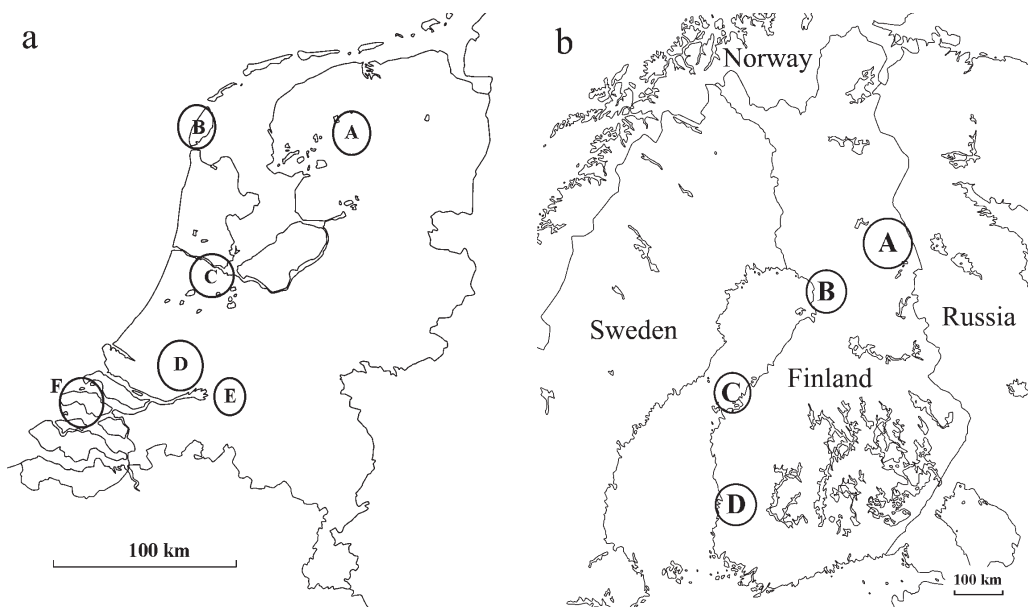


Figure 1. Maps showing sampling locations in the Netherlands (a) and Finland (b). Letters in the circles of figure 1a correspond with regional clusters of the Netherlands: A=Fryslân; B=Texel; C= Zeeland; D= Zuid-Holland; E= Biesbosch. Letters in the circles of figure 1b correspond with sampling locations in Finland: A=Kuusamo, B=Li, C=Tankari, D=Ahlainen.

tection plan Root Vole, La Haye & Drees 2004). The fifth region in this study is the Biesbosch area, a swamp which represents a habitat distinctly different from the neighbouring regions of Zeeland and Zuid-Holland (figure 1a). For Finland, populations were not combined into regions, since they are situated sufficiently far from each other to be all considered representative of separate regions (figure 1b). Sample sizes n were as follows: Fryslân: 60, Texel: 11, Zeeland: 181, Zuid-Holland: 56, Noord-Holland: 2, Biesbosch: 55, Kuusamo: 8, Li: 22, Tankari: 5, Ahlainen: 5. A table with exact locations and populations sampled is available from the authors upon request.

Geometric morphometrics

We used geometric morphometrics to quantify skull shape. Geometric morphometrics analyses the geometric configuration of a set of corresponding points on each speci-

men under study. These points, often placed at diagnostic features, such as the tip of the skull, or bone fissures, are termed landmarks, a term borrowed from craniometry and previously from topographic surveying. The analyses of this data use mathematical definitions of shape. The shape incorporates all features of the landmarks, except for *size*, *position* and *orientation*. A so-called Procrustes transformation can remove these factors from the landmark configuration, making the remaining descriptors suitable for standard multivariate analyses. The removal of size is achieved by scaling all samples to the same centroid size (the square root of the sum of landmark distances from the centroid point). Subsequently, centroids of all samples are superimposed. Finally, all samples are rotated for an optimal fit, in order to minimise distances between corresponding landmarks between individuals. (For statistical background of the process see Rohlf & Slice (1990) and Bookstein (1991, 1996)). The remaining variation in landmark

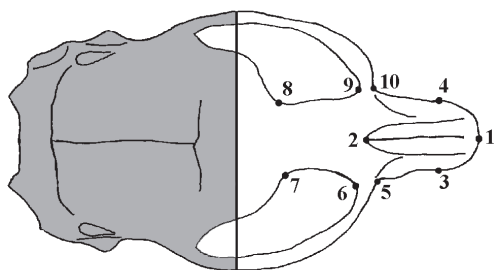


Figure 2. Landmarks used for morphometric analysis, on a skull of *M. oeconomus*. The grey area at the back half of the skull is usually broken off, and therefore no landmarks could be selected in that area (figure adapted from Rącz et al. 2005).

coordinates is variation in shape and can be used as input for standard multivariate statistics (Klingenberg & McIntyre 1998).

Geometric morphometrics possess two important advantages over traditional methods. The first is its ability to represent results graphically, which allows easy interpretation in relation to the object under study. Second is its remarkable statistical power, enabling detection of even very small phenotypic differences (Klingenberg et al. 2002).

Landmarks and selection of skulls

We used eight of the landmarks used by Rącz et al. (2005) in their study of the root vole, plus two extra, all located on the front half of the skull. Rącz et al.'s landmarks located on the braincase could not be used, since this part is usually fractured and missing in owl pellets. The complete representation of landmark locations is given in figure 2.

For the selection of landmarks, a trade-off between as many landmarks as possible and as many samples as possible has to be made. Reduction in either of the two presents unwanted difficulties in concurrent statistical analyses, as discussed extensively by Adams et al. (2004). Thus, it was decided to concentrate on landmarks on the frontal part of the cranium, as most skulls, including the relatively

damaged ones, were intact in this part. Those skulls that were damaged in such a manner that not all landmarks were present, had to be removed from analysis, since for a Generalized Procrustes Analysis (GPA) it is necessary that all samples have equal numbers of landmarks. To date, there is no satisfactory solution to deal with this problem (Adams et al. 2004).

Preparation for analysis

Skulls from pellets were cleaned with a brush, hair and mud were removed with a pair of tweezers. Each skull was assigned a unique identification code. Each skull was photographed from a dorsal view with a tripod mounted Olympus E-500 digital camera. Included on each photograph was a fixed distance line as well as the unique identification code, to prevent accidental mixing-up of images. The digital images were then randomised using the program TpsUtil 1.34 (Rohlf 2005) before marking landmarks.

Ten landmarks were marked on each skull using tpsDig version 2.05 software (Rohlf 2006). To assess the accuracy of the measurements, VB measured all skulls twice in random order and from those measurements we calculated repeatabilities. For both series of measurements, all X and Y-coordinates of the ten landmarks were added up, to obtain one number per individual skull measured. Following Lessels & Boag (1987) repeatability was calculated based on a one-way ANOVA from this data with identity as factor and the two measures as response. Measurements proved to be very accurate with a repeatability of 0.9998 ($se = 4.3 \times 10^{-5}$, $F_{1,364} = 8030.5$, $P < 0.0001$). Further analyses were performed based on the averaged values from the two measurements.

Levels of comparison

The populations were compared at the level of the country, at the level of the region within

countries, and for the Dutch populations also at the population level. The data was entered into the statistical software PAST version 1.42 (Hammer et al. 2001), where the landmark data was transformed using Procrustes analysis. With this data a Shape Principal Components Analysis was performed, to identify the principal components (PC) that best described the variation in skull shape. From inspection of plots of magnitude, direction, and size of principle components, it was decided that only the first two principle components reflected systematic shape variation.

Subsequently, a MANOVA on the first two PCs was performed to identify differences in skull shape and finally, Hotelling's T^2 test was used to identify which pairs of populations were significantly differentiated in skull shape. In the computer program MATLAB a dendrogram based on the MANOVA was made, to visualise the differences of the different populations, using Mahalanobis distances between group means (A Mahalanobis distance tree is roughly equivalent to a phylogenetic tree, in that it expresses the amount of phenotypic variation between populations as distances between them. This is graphically displayed as a 'tree', with bifurcations depicting splits between populations).

Population variation

To investigate variation at the population level, P_{ST} values were calculated, and compared with F_{ST} values as found in the microsatellite analysis performed by van de Zande et al. (2000). Because we were not able to obtain F_{ST} -values directly in this study, we used estimates by van de Zande et al. (2000) instead, to give an indication. Those were obtained from populations from roughly the same regions as the samples in this study. The F_{ST} for their comparison between countries can only give an indication of the range in which the actual value for a comparison between Dutch and Finnish populations would be, since in their

article, the comparison also involved populations from Norway and Germany. The calculation was done for pairwise combinations of populations, which were then ordered to the level of comparison, to calculate average P_{ST} .

Results

Geometric morphometrics

For the comparison between Dutch and Finnish populations, the Procrustes transformed landmarks for all individuals reveals clear shape differences. The Shape PCA revealed that the first two components explained 52.9% of all variation. These two principle components were then selected to perform subsequent analyses. A shape deformation plot from mean skull shape also suggests a difference in shape between the Finnish and the Dutch populations (figure 3), which is confirmed by a Hotelling's T^2 test indicating significant differences in scores on principle components 1 and 2 between Dutch and Finnish populations ($P < 0.0001$).

Subsequently, regions within countries were compared, to identify possible differences on a regional scale. A plot of Procrustes transformed landmarks does not reveal any clear pattern, as there is considerable overlap between the shapes of skulls from different regions (plot not shown).

Nevertheless, MANOVA analysis of PC1 and PC2 indicated significant differences between regions ($F_{10,716} = 3.919$, $P < 0.0001$). To pinpoint the location of the differences in spe-

Table 1. Significant differences between Dutch regional clusters from Hotelling's T^2 test and sequential Bonferroni adjusted critical P -values (α). BB= Biesbosch, ZL = Zeeland, ZH = Zuid-Holland, TX = Texel.

Populations		P -value	Adjusted α
BB	ZL	0.000005	0.003414
BB	ZH	0.000109	0.003657
BB	TX	0.002972	0.003938

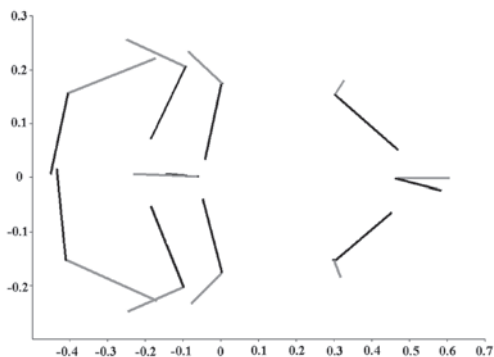


Figure 3. Plot of principal deformation from mean skull shape. Data for all skulls used in analysis. Lines indicate size and direction of the deviation for principal components 1 (grey) and 2 (black).

cific populations, a post-hoc Hotelling's T2 test was performed. This showed significant differences between regions BB-ZL, BB-ZH and BB-TX (table 1) after sequential Bonferroni correction for multiple testing. Thus, it appears that the Biesbosch region is significantly different in shape from the other Dutch regions. The Finnish populations were analysed in the same way as the Dutch for regional differentiation, but a MANOVA on the first two principle components indicated no significant differences between Finnish regions ($F_{6,70} = 1.135$, $P = 0.3514$).

The dendrogram based on Mahalanobis distances reflects the significant differentiation between Finland and the Netherlands, and the significant differentiation of the Biesbosch region within the Netherlands. Differentiation of populations within Finland is also considerable, but not significant, most likely due to low sample size.

P_{ST} Values

Values of P_{ST} for comparisons between pairs of populations show that the P_{ST} between the Netherlands and Finland is larger (0.0471) than that between Finnish populations and that between Dutch populations separately (0.0224 and 0.0152 respectively). This suggests

that the Finnish and Dutch populations are morphologically further apart than the populations in both countries are from each other. Furthermore, the average P_{ST} for the Finnish populations are higher, which would concur with the fact that the populations are separated by greater distances, and perhaps have been separated for longer periods of time, than those in the Netherlands.

Comparison of genetic and morphological divergence

F_{ST} values reported by van de Zande et al. (2000) are higher than the P_{ST} values found in this study. For differences between regions in the Netherlands they found an average F_{ST} of 0.1582 (95% confidence interval 0.1323-0.1840). Between countries, the average F_{ST} they found was 0.1708 (95% C. I.: 0.1415 to 0.2001). The difference between P_{ST} and F_{ST} ranges from three- (Dutch regions) to ten-fold (between countries). This suggests that the populations are under (strong) stabilising selection for skull shape.

Discussion

P_{ST} - F_{ST}

The calculated values for P_{ST} can be slightly inflated because phenotypic plasticity can have an influence on the variation measured: populations from different regions will experience different environmental conditions. This may affect phenotypic variance so that the between-population component increases. In other words, differences found between populations will not only represent the underlying genetic variation, but also environmental variation. This would increase variation between populations (V_B), which would thus increase the value of P_{ST} . On the other hand, our estimate of the within-population phenotypic variability V_w includes environmental variation and measurement error. The total

difference between the true value of P_{ST} and our estimate is determined by the, unknown, strength of these biases. Ideally, a multi-generation common garden experiment should be set up with animals from the different habitats in similar conditions to study the magnitude of the environmental influence on the phenotype, but such is difficult to achieve in practice. Despite these uncertainties, the difference between the P_{ST} values found and the F_{ST} values is three to ten-fold, making it unlikely that the conclusion that there is stabilising selection acting on the phenotype, would be altered. Apparently there is selection on an optimal phenotype for the separate habitats, making phenotype variation smaller than the neutral genetic variation.

Sampling bias

We do not know if and how the barn owls and long-eared owls selectively choose their prey in a way that is related to skull shape. This means that we are not entirely sure whether or not the sampling of skull shape was random: in theory, differences in skull shape between geographic regions that we reported could be due to differences in prey choice between owls from different regions. However, as Finnish populations (sampled by trapping) showed differentiation in skull shape to be comparable to variation in Dutch populations (sampled by owls), we believe that skull samples from owl pellets reliably describe differences between vole, not owl, populations. Second, we cannot be strictly sure whether one owl only sampled from only one population. However, for all analyses we grouped local populations in regions far exceeding the home range size of an owl, so that sampling from more than one population is not likely to be an issue.

Another problem could be the unknown age distribution of the root vole populations sampled. Since adult animals are larger than juveniles this could influence the analysis. However, geometric morphometrics studies

mainly shape, and not size. And even though the shape of a skull or other skeletal features will change during ontogenesis, it is still possible to assess shape differences, even between adult and juvenile specimens (Marcus et al. 2000).

Similarly, it was not possible to discriminate between males and females based on the skulls alone, so possible sexual dimorphism could interfere with test results. Several other studies on morphometric analysis in rodents (Reutter et al. 1999, Barčiova & Macholán 2006) and also one other on *M. oeconomus* (Rącz et al. 2005) found no sexual dimorphism, suggesting sexual dimorphism is low relative to total phenotypic variance. However, looking at specific age classes Markowski (1980) and Markowski & Østbye (1992) claimed evidence of sexual dimorphism in certain phenotypic characters of root voles, though without correcting statistically for testing a large number of characters. Thus, it was not possible to account for potential effects of age and sex on skull shape, but if such effects exist they are unlikely to have much effect on population comparisons using geometric morphometrics of skull shape.

Phylogeography

In the glacial periods up to the last glacial maximum (21,000-17,000 years ago), the root vole had a large habitat range in Europe, expanding its range further south than the current distribution. It is believed that there were several glacial refugia in central Europe (Chaline 1987). As the climate warmed, the population withdrew, leaving some populations isolated. The now isolated populations in the Netherlands, Slovakia and Hungary are very probably remnants of this larger range: analyses of mitochondrial DNA confirm this historical model, as these populations are part of the same mtDNA group (Brunhoff et al. 2003).

As temperatures rose after the ice-age, Scandinavia was released from its ice cover,

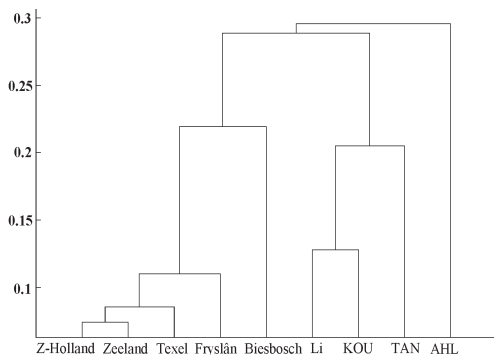


Figure 4. Dendrogram based on Mahalanobis distances between populations in the Netherlands and Finland. LI= Li, KOU = Kuusamo/Oulanka, TAN= Tankari, AHL = Ahlainen.

which made it possible for the root vole to recolonise Scandinavia (Brunhoff et al. 2003). Similar patterns have been observed in other mammals (Jaarola et al. 1999), and in particular a similar pattern has been found for field voles (Jaarola & Searle 2002), which are ecologically very similar to root voles. For the Finnish populations this means that the populations in the south may have become isolated from those in the north when the main population of root voles withdrew with the receding ice. If that scenario is correct, it is precisely reflected by the phenotypic distance tree (figure 4) which also shows an increasing phenotypic distance between populations with increasing latitudinal separation. The population in Ahlainen in southern Finland would have become isolated first, followed by Tankari in mid-Finland, and so on (see figure 1b).

The P_{ST} values, which are comparable with the Mahalanobis distance-based tree, support this. Also here, the further apart geographically the Finnish populations are, the larger the pairwise P_{ST} values are. For the Dutch populations, where the Biesbosch population differs significantly from Zeeland, Zuid-Holland and Texel, also the distance tree shows a split between the Biesbosch population and the others. This would mean that the Biesbosch population has been isolated from the others for a longer period of time.

Conclusions

Geometric morphometrics has proven to be a very powerful tool since it was possible to detect even small differences between populations, based on a limited number of landmarks from incomplete skulls. A dendrogram of population morphological differences (figure 4) is consistent with molecular phylogenies based on allozymes, and microsatellites (Leijds et al. 1999, van de Zande et al. 2000). On the small geographical scale of the Netherlands, morphological differences between populations exist. What was slightly unexpected is that the divergence between Dutch and Finnish populations based on morphological characters was smaller than the average F_{ST} from between-country comparisons by van de Zande et al. (2000). This suggests stabilising selection on skull shape for all populations, which keeps morphological variation low. Overall, our findings suggest that geometric morphometric analyses of skulls fragments obtained from owl pellets may provide a cost-effective, non-invasive tool to monitor subdivision of small mammal populations in fragmented habitats.

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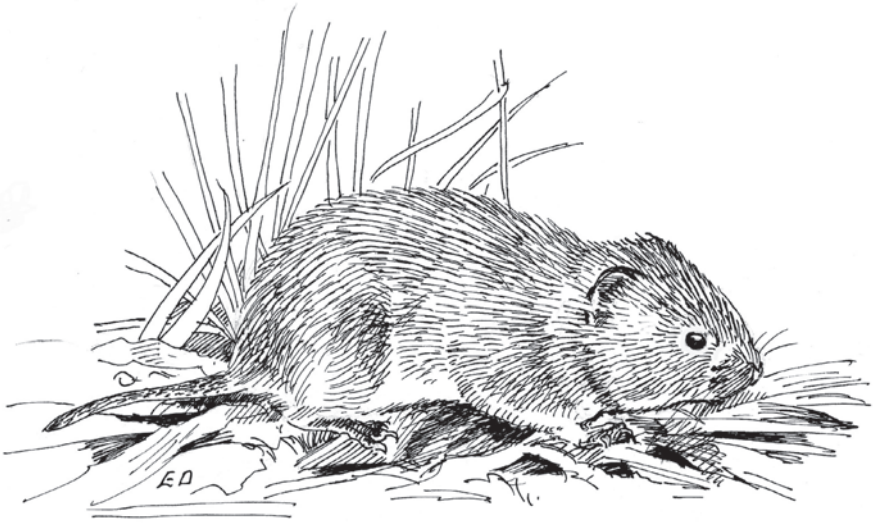
Samenvatting

Verschilt de schedelvorm tussen geïsoleerde populaties van de noordse woelmuis (*Microtus oeconomus*) in Nederland?

We hebben verschillen in morfologie tussen verschillende populaties van de Nederlandse ondersoort van de noordse woelmuis (*Microtus oeconomus arenicola*) onderzocht. We hebben hierbij gebruik gemaakt van geometrische morfometrie-metingen aan woelmuizenschedels afkomstig uit braakballen van uilen. Daarnaast hebben we de gevonden morfologische differentiatie vergeleken met waarden van genetische differentiatie voor dezelfde populaties afkomstig uit de literatuur. Hierbij zijn de populaties uit Finland als referentie gebruikt. We vonden dat de morfometrische populatiedifferentiatie in het algemeen lager was dan de genetische, maar dat deze wel dezelfde patronen van geografische isolatie vertoonde. Dit suggereert dat de vorm van de schedel geconserveerd is in geïsoleerde woelmuizenpopulaties en dat geometrische morfometrische metingen van onderdelen van het skelet afkomstig uit uilenbraakballen een goedkoop alternatief kunnen zijn om subpopulaties van dezelfde soort te vergelijken.

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Maternal care, calf-training and site fidelity in a wild harbour porpoise in the North Sea

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Direct, undisturbed observations of interactions of mother and offspring in harbour porpoises (*Phocoena phocoena*) in the wild are extremely rare. Because harbour porpoises are seldom held and hardly breed in captivity (Blanchet et al. 2008), even observations of mother-calf interactions of porpoises under human care are uncommon (Oleksenko & Lyamin 1996, Borowska 2009, Delgado & Wahlberg 2009). The second author, by chance, encountered a unique opportunity to observe wild porpoises (mother and calf as it turned out) associated with an offshore production platform during several weeks in September 2011. Interested in the animals as the second author was, but untrained as a biologist, the observations are only descriptive in nature. However, what could be seen repeatedly, were fully undisturbed interactions between a mother and a calf in the wild. This provided unique insight in the maternal care of porpoises and other aspects of the behaviour of this species, which would be extremely difficult to obtain in 'normal' (e.g., survey) conditions.

During at least three (possibly four) weeks in September 2011, a female and calf harbour porpoise were seen in close association with the offshore gas production platform Rijn Charlie (30 km west of Scheveningen, the Netherlands) in the southern North Sea. In

this area, the seafloor is sandy and the water depth is approximately 18 m. From photos provided, the calf could be estimated to be circa 70-75% of the total length of the female (hence, circa 100 cm if the female would have been 140 cm total length, which is about the minimum size for sexually mature females) (photo 1). Peak calving in the southern North Sea is centred around June/July, with a fairly large number of births occurring in May and August (Addink et al. 1995) and an estimated age of circa 3-4 months for the calf would be in accordance with that approximate body size. Even though there is no proof, the observers claim that there were no reasons to believe that more than this couple of porpoises were involved, visible as the animals were during virtually every watch and given the highly consistent behaviour and interactions of the animals during their stay near the platform.

The calf was seen suckling frequently (snout 'attached' to the vaginal region, mother more or less stationary and slightly turned to one side). Suckling was positively observed in the morning and the evening, but dedicated observations by the platform crew during mid-day were less frequent. There were very many sightings of the mother disappearing under water for a long dive, to return with a fish that was still alive and that was released just in front of the quickly approaching calf,



Photo 1. Harbour porpoise mother (left) and calf. *Photograph: A. Krop.*

apparently in an attempt to encourage the calf to capture the fish. Flatfish and roundfish were offered to the calf, all alive, but fish species were not recognised by the crew. During the deeper and more prolonged dives of the adult female, the calf was left alone near the surface (cf. Amundin & Amundin 1973). The calf would perform quick spurts (fast swimming) around the platform (interpreted by the crew as 'playful behaviour'), or remain stationary in one place. The crew remarked specifically that the calf would suddenly spurt towards a spot where the mother was later indeed seen to surface and where the fish was offered. No sounds were heard, but the observations strongly suggested that a vocal signal by the approaching adult from under water was released, in response to which the calf sprinted to the area where the adult would surface and offer her prey. Deliveries of live fish were frequent (up to 3x per half hour at times), and some fish were successfully captured and swallowed by the calf (no data were kept on frequencies and success rates). When the female was at the surface, mother and calf

were usually close together, even though short 'playful absences' by the calf occurred (e.g., making short spurts towards or around the superstructure of the platform).

A number of interesting aspects were provided by these observations. First, a mother-calf 'couple' of harbour porpoises were apparently 'residents' near an offshore platform for several weeks. Perhaps the platform provided shelter or safety for marine predators, perhaps the structure served as a beacon where mother and calf could easily rejoin in an otherwise more or less homogeneous sea area. Secondly, the calf was apparently trained to capture fish, even though the animal must have been very young and unlikely to be weaned for several more months. Instead of feeding the calf fish, she would release them to have the fish captured (or missed!) by her calf. This behaviour agrees with what is known of harbour porpoises: lactation lasts up to ten months with a marked reduction after 5-6 months, but calves start taking small fish when already 2-3 months old (Yasui & Gaskin 1986, Evans et al. 2008). Thirdly, there were

indications for playful behaviour of the young animal, but here lurks the risk of over-interpretation of the data. Some fish offered by the adult were not swallowed but played with and subsequently left to sink when the calf's interest faded. To the observers, the rapid spurts seemed playful behaviour, but it may simply have been excitement of the calf in anticipation of the return of the mother. Finally, there seemed to be vocal communication between the two animals, as a result of which the calf and the adult could rejoin exactly at the point where the mother would surface with her fish (North Sea waters near the Dutch coast are very turbid and the visibility under water is very low).

Strong social bonds, prolonged periods of maternal care (a long phase of immaturity), and even apparent training to hunt are all known for several species of cetaceans (Whitehead 1996, Addink & Smeenk 2001, Miles & Herzing 2003, Bender et al. 2008), but are less well known for elusive species such as the harbour porpoise. These small cetaceans are notoriously difficult to study in the wild, and the sightings reported here are unique as far as we could deduce. The temporary association of small cetaceans with offshore structures (platforms or buoys) has been observed in several species of dolphins (Camphuysen 2011), but no observations are known to us for harbour porpoises. Such associations may have implications for acoustic monitoring studies, where listening devices are attached to objects. Marine mammals may deliberately approach and investigate such structures (for example because the availability of food may be enhanced near offshore constructions or fixed objects), or stay close with them during certain periods of time. Both tendencies would result in overestimates of the presence of porpoises based on the recorded clicks with TPods or CPods.

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Samenvatting

Moederzorg, training van het kalf en plaatstrouw bij wilde bruinvissen in de Noordzee

Bij en onder het gasproductieplatform Rijn Charlie, 30 km westelijk van Scheveningen (zuidelijke Noordzee), werden gedurende ten minste drie weken in september 2011 twee bruinvissen (*Phocoena phocoena*) waargenomen. Het bleek om een volwassen wijfje met een kalf te gaan. De bemanning van het plat-

form was getuige van wat kennelijk trainingen van het jong door de moeder waren. Herhaaldelijk bracht het diep duikende, adulte dier nog levende vis naar de oppervlakte, die vlak voor het toesnellende jong werd losgelaten. Soms werd het jong ook nog gezoogd. De beschreven waarnemingen zijn uniek, omdat bruinvissen in het wild bijzonder moeilijk te bestuderen zijn. Behalve de training werd ook speels gedrag van het jong beschreven. Het toesnellen van het jong naar een plaats waar het volwassen dier pas later bovenkwam, suggereerde dat het jong een geluidssignaal kreeg van de opduikende moeder. Langdurig verblijf van bruinvissen in de buurt van offshore-installaties (zoals dat ook wel bij dolfijnen wordt gezien) heeft implicaties voor de interpretatie van gegevens die met akoestische monitoring worden vergaard, aangezien er dan overschatting van de presentie kan optreden waarvoor gecorrigeerd moet worden.

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