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TECHNICAL REPORT

Field sampling methods for mosquitoes, sandflies, biting midges and ticks

VectorNet project 2014–2018

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This report of the European Centre for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA) was produced by the VectorNet project (European network for sharing data on the geographic distribution of arthropod vectors transmitting human and animal disease agents).

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Abbreviations

CCHF	Crimean-Congo haemorrhagic fever
COI	Cytochrome oxidase subunit 1
COII	Cytochrome oxidase subunit 2
D2	D2 domain in 28SrDNA
EVS	Encephalitis vector survey
GPS	Geographic positioning system
HLC	Human landing collection
ITS2	Internal transcribed spacer 2
kdr	Kinase insert domain receptor
mAh	Miliampere hour
ND4	NADH dehydrogenase subunit 4
NUMTs	Nuclear mitochondrial DNA sequences
MALDI-TOF MS	Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
OVI	Onderstepoort Veterinary Institute trap
PCR	Polymerase chain reaction
SD	Standard deviation
UV	Ultraviolet

Glossary

Ascoid	A horn-like structure located on antennal segments of sandflies. It can be found between the 3rd and the 15th antennal segments.
Biopesticide	Certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals.
Cibarium	The space anterior to the true mouth cavity in which the food of the insect is chewed.
Diapause	Period of suspended development in an insect especially during unfavourable environmental conditions (e.g. winter).
Endophagic	An endophagic mosquito is a mosquito that feeds indoors.
Endophilic	An endophilic species is a species that tends to inhabit/rest indoors.
Exophagic	An exophagic mosquito is a mosquito that feeds outdoors.
Exophilic	An exophilic species is a species that tends to inhabit/rest outdoors.
Exochorion	Outer egg shell of a mosquito
Gonotrophic cycle	The average number of days that gravid females need to oviposit after taking a blood meal.
Haemorrhagic	Escape of blood from a ruptured blood vessel.
Hypereutrophic	Extremely rich in nutrients (e.g. a body of water)
Kairomones	A chemical substance emitted by an organism and detected by another of a different species which gains advantage from this.
Mammophilic	Biting insects feeding on mammal blood.
Multivoltine species	A species that has more than one generation per year.
Nuclear mitochondrial DNA sequences	The natural transfer of DNA from mitochondria to the nucleus generates nuclear copies of mitochondrial DNA
Ornithophilic	Biting insects feeding on blood of birds.
Oviposition	The process of laying eggs.
Pyrethroid	A pyrethroid is an organic compound similar to the natural pyrethrins produced by the flowers of pyrethrums. Pyrethroids constitute the majority of commercial household insecticides.
Status of a species	A rough estimate of a species' presence in a defined area (e.g. rare, patchy, ubiquitous).
Species complex	A group of related species where the exact demarcation between species is often unclear or cryptic; such a group can be denominated with the representative species name with the addition 'sensu lato' (s.l.)
Spermathecae	Organs of the female reproductive tract in insects
Surveillance	Systematic ongoing collection, analysis, interpretation and dissemination of highly structured information for action.
Univoltine species	A species that has only one generation per year.
Vectorial capacity	The rate (usually daily) at which a bloodsucking insect population generates new inoculations from a currently infectious case.
Vector competence	The ability of a vector (e.g. *Anopheles* mosquitoes) to acquire, maintain, and transmit microbial agents (e.g. malaria plasmodia).

Executive summary

ECDC has the mandate to strengthen the capacity of the EU for the prevention and control of infectious diseases. Vector-borne diseases, as a specific group of a (re-)emerging infections, pose a threat to European public health and require particular attention. One important aspect of preparedness for vector-borne diseases is the monitoring or surveillance of the introduction, establishment and spread of the main disease vectors.

An efficient surveillance or monitoring campaign starts with the development of a well-considered sampling strategy. Depending on the target species, a range of sampling methods is available. This implies that different teams use different approaches and as operational vector sampling methods often lack standardisation, quantitative comparisons across different settings are very hard to make. This also implies that teams starting a disease surveillance project can get overwhelmed by the sheer amount of options and may end up choosing a strategy that is less than ideal to meet their objectives.

Although some guidelines exist on the sampling of invasive and native mosquito species in Europe [1–3], there is no summary document on sampling strategies for the various vector species. In the framework of VectorNet, pan-European field campaigns were established to collect data on the presence/absence or seasonal distribution of mosquitoes, sandflies, biting midges and ticks. To standardise the efforts across different teams and to ensure comparable outcomes, a set of protocols were developed for every species group. These protocols describe a uniform way to sample specimens for the four above-mentioned groups. The protocols also describe the conservation of specimens, identification and quality control. Depending on the vector group, the protocols are subdivided according to activity (e.g. flying vs resting mosquitoes and sandflies), life stage (mosquitoes), or species (ticks). Each section describes in detail which traps should be used to meet specific study objectives, how to select a sampling site, when to sample and how long or how often, how to collect the samples, how to preserve them, how to identify them, and how to assess quality.

This document intends to provide support to professionals involved in the surveillance, monitoring or control of vector-borne diseases. It can also be of interest to decision-makers, policymakers and stakeholders in public health, as well as non-experts in mosquito surveillance, monitoring and control. The suggested methods can be applied to the entire geographic area of Europe, e.g. in all EU Member States and EEA/EFTA countries. The methods presented here can also be applied to few EU Outermost Regions and Overseas Countries and Territories, but for most of them, the methods presented in this document should be adapted to the local situation.

Background

VectorNet is a joint initiative of the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC). It was established in May 2014. The project supports the collection of data on vectors and pathogens in vectors, related to both animal and human health. It aims to build and maintain a common database on the presence and distribution of vectors in Europe and the Mediterranean basin through developing a network of experts and organisations from the medical and veterinary domains. The project provides ad-hoc scientific advice to support ECDC and EFSA concerning technical questions about vector surveillance and vector-borne diseases in humans and animals. VectorNet performs targeted entomological collections in specific habitats to fill knowledge gaps that have been identified through the now-defunct VBORNET project. This is done through analyses of the existing vector databases and EFSA scientific opinions.

This document presents vector collection methods and strategies used by VectorNet for targeted sampling. The main aim of the collection is to provide information on the distribution of the following vector groups: mosquitoes, sandflies, biting midges and ticks. The sampling protocols in this document also present methods (where possible) for data collection related to abundance and cover various aspects of the ecology, behaviour and vector status of the target species. The protocols were validated in the field in 2015.

This document provides entomologists involved in vector sampling with information on how to collect, store and identify disease vectors. It will also give public health professionals insight into the different collection strategies that can be applied to sample mosquitoes, sandflies, biting midges and ticks. The collection methods and strategies are addressed in four chapters, one for each vector group.

1 Sampling methods for mosquitoes

Introduction

Sampling methods for mosquitoes can be divided into four main categories: methods for collecting flying adults, resting adults, larvae and eggs. The combined use of these methods depends on the objective of the surveillance or monitoring campaign, the target species, the environmental conditions at the selected sampling sites and the availability of resources.

Adult mosquitoes spend less time flying than resting. Catching flying mosquitoes using an attractant is easier and less time consuming compared to other trapping methods. During their flight, they are in search of nectar and blood or suitable sites to lay eggs. Therefore, the search is shifted from the collector to the mosquito. The collection of flying mosquitoes captures almost only females at various stages of the gonotrophic cycle (unfed, fed, half gravid and gravid). Blood-engorged females are ideal for determining the preferred natural hosts. Mosquitoes caught in host-baited traps could be used to reveal the blood-feeding behaviour of a particular species. Sampling of blood-engorged females is also possible using host-baited traps provided ethical agreements on animal testing. Flying mosquitoes are most frequently sampled outdoors but miniature traps (with or without light) baited with CO_2 can also be used in semi-open places or indoors. For example, indoor trapping is used for collecting *Culex pipiens* and some *Anopheles* species. In general, however, most flying mosquito sampling techniques are designed to sample outdoor populations. Sampling techniques for flying adults are quite efficient for most mosquitoes of animal and human health.

Collections of resting mosquitoes yield females at various stages of the gonotrophic cycle. Additionally, such collections also yield male mosquitoes, which are rarely collected with traps that target biting or ovi-positioning females. The male mosquitoes can help to evaluate the vertical transmission of a pathogen, and to assess the overwintering of the pathogen in the vector at localities with high virus circulation. By design, the collection of mosquitoes at rest does not require bait and one rarely has to resort to traps. The collection of resting mosquitoes must differentiate between indoor and outdoor sites.

Surveillance of larvae is of key importance. All mosquitoes rely on water bodies for their development, which allows for a focused and rapid inspection of a site. Sampling and identification can be performed on the same day, which ensures a good cost–benefit ratio. In addition, larval sampling is particularly helpful when sampling mosquito species whose females are not, or only rarely, attracted to the commonly used trap systems. Depending on the species, there are large differences between the types and characteristics of colonised bodies of water (e.g. size, water quality, natural or man-made) and the ecological surroundings they are embedded in (e.g. rural, urban, nature). Also, larvae of univoltine species can only be detected at specific periods while those of multivoltine species often occur throughout the entire vegetation period. Larval surveys therefore require a detailed knowledge of mosquito ecology.

Container-breeding mosquitoes, such as the invasive species *Aedes aegypti*, *Aedes albopictus*, *Aedes atropalpus*, *Aedes japonicus* and *Aedes koreicus* are not necessarily attracted to commonly used adult mosquito traps. Instead of adult traps – or in addition to them – oviposition traps (ovitraps) can be used to check for the presence of these mosquito species by demonstrating the presence of mosquito eggs. By imitating breeding sites, ovitraps are meant to attract gravid female mosquitoes to lay their eggs in locations that are easily accessible to the survey team. Ovitraps can be used in situations where mosquitoes are suspected to occur (e.g. to check for introduction or spread) or to assess control efficiency. They can be particularly useful for surveying remote areas as they can be checked infrequently (intervals of several weeks to several months). In such situations, it is recommended that a long-lasting insecticide (biopesticide or insect growth regulator) is added to avoid the trap from becoming a breeding site. Efficiency is reduced when other breeding sites compete with the ovitraps, but their attractiveness can be improved by using dried oak leaves or hay infusion.

Objectives of field collection

All sampling methods mentioned in this report can be used to obtain information on the distribution (presence/absence) of the target species. Supplementary information can be collected, addressing different aspects of the mosquito ecology and behaviour, the vector status of the target species, and control options (Table 1). Information on the seasonality, ecology, behaviour, and vector status of a mosquito species are essential for the design of a successful campaign.

Table 1. Overview of sampling strategies and objectives

Objective	Flying adults	Resting adults	Larvae	Eggs
Distribution (presence/absence)	X	X	X	X
Seasonality and ecology of different mosquito	X	X (overwintering)		X*
Pathogen detection in natural population	X			
Host preferences	X			
Biting behaviour endophagic/exophagic	X			
Resting behaviour (endophily and exophily)		X		
Insecticide resistance	X	X	X	
Evaluation of control methods	X	X	X	X
Determination of breeding location			X	

** Egg density: association with adult density needs to be assessed*

Field sampling methods

Study design

The study design should meet the main objectives: a cross-sectional design is recommended primarily for distribution and abundance assessments; a longitudinal design is recommended for the collection of data on population dynamics.

Cross-sectional surveys collect data to assess the presence/absence or vectorial capacity (field components) of the target species at randomly selected or risk-based study sites and over a short time period. Cross-sectional surveys may be repeated periodically. Usually, in a repeated cross-sectional survey, sites sampled at one point in time are not intentionally sampled again, although a site of a previous survey could be randomly selected for a subsequent one. However, identical sites can be intentionally re-sampled: hotspots of invasive species, for example, are often sampled several times a year.

Longitudinal surveys are research studies that involve repeated sampling of the same sites. A limited number of sites selected by a predefined geographical range, biotope type/s and time frame are monitored over one or more mosquito seasons. These surveys could be used to assess vector presence/absence and population dynamics (e.g. seasonality, density peaks, number of generations) and vector abundance. Density peak assessments are essential to depict time windows for abundance sampling. If combined, both types of data can later be used risk assessment. Data on presence/absence can be collected by a number of appropriate sampling methods but the estimation of population dynamics and abundance should be carried out by a single sampling method in accordance with a standardised protocol.

When planning sampling activities, it is important to obtain land cover maps of the area of interest and its surroundings. Basic ecological and meteorological knowledge of the sampling area is important for the success of the study. However, this is not possible in all areas and should not be a limiting factor when planning sampling activities.

There are a number of tools available that facilitate the planning of sampling activities based on state-of-the art geographic information systems and integrated software components. They allow for the rapid and cost-efficient planning of surveys, which in turn increases the overall success rate.

Selection of sampling methods

Depending on the objectives (Table 1), the available funding and resources, and the size of the area to be sampled, a sampling method, trap type and the trapping frequency is determined.

The selected sampling method is chosen according target species, life stage and status. Sampling adult flying mosquitoes is the preferred method when all immatures have emerged (e.g. after emergence of a cohort of floodwater *Aedes/Ochlerotatus* species), during swarming (to collect adult males), when sampling larvae is more time consuming or when breading sites are scarce because of dry conditions.

Some species are difficult to collect with the available adult traps and should therefore be sampled in the larval stage. Larval sampling may also be required for vector control purposes: in case of mosquito nuisance, for example, it is essential to detect the source of the problem, so identification of the larval breeding sites of the species is key.

Sampling resting mosquitoes is the preferred method to collect adult females that are not attracted to traps (e.g. blood-fed or gravid females which ignore baited traps), to collect adult males (which can facilitate mosquito species identification), and to collect overwintering adult females.

It should be ensured that traps do not interfere with each other: for example, the range of CO_2, chemical lures, and animal or human bait should be taken into account when placing traps.

Selection of study sites and exact location of sampling sites

Study sites can be selected based using the following criteria:

- Identified gaps in the knowledge of the distribution of the species
- The geographical area to be prospected is identified based on the following factors:
  - There are no field data or existing data are incomplete and/or outdated
  - Data collection through passive surveillance is impossible
  - Theoretical species distribution models highlighting areas with high probability of presence where model validation with field data is required.

Once the study sites are identified, the exact locations of all sampling sites have to be determined. This can be done using online satellite imagery (e.g. Google Earth). These maps can be used to identify urban, suburban and rural zones; floodplains and rice fields; urban 'green and blue islands'; large animal sheds and bunkers or caves. Maps also permit to identify resting vegetation (groves, forests) in or nearby flood lands.

Which locations are selected for sampling also depends on sampling methods:

- **Flying adults.** Identify places which meet the following criteria:
  - appropriate location and good accessibility
  - moist and protected from wind (mosquitoes prefer to fly through bushes and shrubs, and try to avoid open terrain like meadows; hedges are often used as flight corridors
  - out of public sight and not accessible by children.
- **Resting adults.** Identify all types of animal shelters and all natural and artificial hiding places.
- **Larvae.** In natural areas, larvae can be found in e.g. puddles, road tracks, swamp areas, drains, ditches, irrigated croplands, streams, riverbeds, ponds, and tree holes, but also in human-made containers such as drinking troughs, discarded waste containers, tyres, and tarpaulins. In urbanised areas, larval sampling should focus on the available human-made water bodies (both in private and public areas), below and above ground level (e.g. discarded containers, flower vases, flower pots in gardens and cemeteries, used tyres left outdoors, rain water barrels, road drains and catch basins, pits).
- **Eggs.** Identify places close to or under vegetation or near buildings. The position of all ovitraps should be marked on a map.

Sampling methods: flying adults

Malaise traps, truck traps and electrocution traps belong to a group of traps which are used to sample mosquitoes during dispersal and foraging flights. These traps do not rely on attractants and can be placed between breeding sites and those locations where mosquitoes obtain their blood meals. They could be particularly useful to determine mosquito flight periods and inform the timing of control measures against adult mosquitoes.

A second group of traps is based on the fact that the bait or host for a blood meal emits olfactory stimuli or kairomones which attract the host-seeking mosquitoes (foraging flight). Female mosquitoes respond to compounds such as carbon dioxide from exhaled breath and host odour components (e.g. lactic acid) deriving from sweat. They can also detect water vapour and body heat.

The most frequently used outdoor methods include:

- collection of females landing on a human (human landing collection, HLC) or inside a drop-net with aspirators (mouth or battery operated), provided that all ethical issues are fully taken into account
- animal bite catches (by removing mosquitoes directly from a tethered animal host with an aspirator or inside a drop-net); it is also possible to use a suction trap baited with small animals
- suction traps containing attractants like carbon dioxide (CO_2-baited traps), light (UV and incandescent light traps), or combination of both
- gravid and sticky ovitraps that attract ovipositing females.

The species/sex composition of the catch is not only determined by the sampling technique but also by factors such as mosquito activity patterns, host-seeking and resting behaviour, the physiological stages of the mosquito, and weather conditions.

In addition to the range of conventional mosquito traps used by mosquito specialists, a number of novel mosquito trapping or monitoring devices have been introduced over the last decades. Some incorporate novel chemical lures, others have alternative airflow systems, while yet others use novel technology to power the fan.

This document provides only sampling protocols for the techniques used by the VectorNet project. Silver [4] and Becker et al. [5] provide more information, as do two ECDC guideline documents [1,3].

Trapping methods[i]

- **Human landing collection (HLC).** HLC is probably the oldest and simplest method of collecting host-seeking mosquito females. Anthropophilic species can be efficiently collected when HLC is performed during daily activity peaks. Sampling for day-biting mosquitoes should occur in shaded environments. There are some disadvantages, such as labour costs and the risk of becoming infected, even though it is recommended to collect the females before they bite. For standard comparisons, a fixed sampling duration (e.g. 15 minutes) is recommended. It can be reduced to five minutes if mosquitoes are very numerous (≥5/5 min). Results depend on the collectors' skills and on the attraction a person exerts on mosquitoes, which needs to be taken into account when comparing numbers for an abundance estimation. However, this approach is not recommended in transmission areas and can only be used when approved by an ethics committee.
- **Animal-baited traps.** Mosquitoes are attracted by different cues from a number of hosts, and host preferences differ between species. For example, *Culex territans* feeds preferably on amphibians, *Culex pipiens* is known to feed on birds, other animals and also on humans; some *Anopheles* species feed on humans and animals. Animal-baited traps use a variety of animals such as cattle, horses, goats, pigs, rodents and various birds. Mosquitoes are directly removed from a tethered animal using an aspirator or are collected from the walls of the animal shed. Animal baits are often used in West Nile virus surveillance studies, but are less used in large-scale surveillance schemes.
- **CO_2-baited suction traps.** Suction traps baited with CO_2 are the most commonly used mosquito traps. They are operated with dry ice (producing carbon dioxide through sublimation) or a carbon dioxide tank and are a highly effective attractant for host-seeking females of many species. These traps are run on battery or have a power supply and can come with or without a light source. They have the advantage of being simple, robust, portable, often run on inexpensive 3 x 1.2 V batteries and are not significantly influenced by background light in urbanised areas. The main disadvantage is the need to have access to dry ice or gaseous carbon dioxide. When combined with light, they collect more male mosquitoes but this increases the sample pollution with other nocturnal insects, mainly Lepidoptera and non-hematophagous Dipterans. This make separation time consuming and often damages the sampled mosquitoes, slowing down identification. Incandescent light, which usually attracts mosquitoes, can have the opposite effect on some female mosquitoes and repel them. Carbon dioxide-baited traps also collect other hematophagous insects such as Phlebotominae (sandflies), Simuliidae (blackflies), and Ceratopogonidae (biting midges). The operational time of these traps is usually limited by the CO_2 supply (maximum of 24 hours) or battery capacity (12V, 10A, maximum 48 hours).
  CO_2-baited suction traps are an efficient sampling tool in mosquito-borne pathogen surveillance where live females are needed. An additional advantage is that dry ice left in insulated containers is an excellent medium for conservation on the way to laboratory. To set up and run the traps, experienced technicians are needed, and when a large number of traps are needed to monitor a large area, several teams might be necessary. A technician can cover a total distance of 450 km, setting up 40 dry ice-baited traps in nine hours.
- **Light traps.** Light traps are most often used in malaria surveillance, but have also been used in arboviral surveillance. Light traps operate at night and are most efficient when there is little light pollution. The advantages of the light trap are that they are portable and easily powered by a 6-volt battery (or 3 x 1.2V batteries in CO_2-baited light traps). The disadvantages are that they are only marginally or poorly attractive to diurnal and crepuscular active mosquitoes and they have a large by-catch of other insect species (see above). However, carbon dioxide can be added as main attractant, as described above. Live-trapped females can be tested for mosquito-borne arboviruses. As a light trap's batteries last only one night, a sufficient number of technicians is needed if light traps are to be used in large-scale surveys. A technician can cover a total distance of 450 km, setting up more than 50 traps in nine hours.
- **Gravid traps.** These traps consist of a black bucket filled with water, hay or an infusion of dead leaves and are designed to collect gravid females that fed at least once and need to oviposit. Gravid traps are used in arbovirus surveys, as the probability of detecting viral infections in a fed mosquito is higher than in samples obtained by CO_2-baited traps which collect host seeking females, most of which are unfed. Gravid traps are considered not to be particularly attractive to *Aedes* mosquitoes, but their attractiveness can be improved (for container-inhabiting species only) by using infusions of dead oak leaves or grass (bluegrass *Poa sp*.).
- **Sticky traps.** Like ovitraps (for egg sampling) and gravid traps, sticky traps attract gravid egg-laying females. The mosquitoes land on internal surfaces and stick to them. The samples can be used to identify the species and pathogen screening. Samples need to be collected daily.

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[i] This section was adapted from [3]

- **BG-Sentinel and Biogents Mosquitaire traps.** These traps were designed to attract *Aedes aegypti* and *Aedes albopictus* with a specific chemical lure (BG-Lure or Sweetscent). Their effectiveness can be increased by baiting the trap (e.g. a mouse in a cage) or by adding a carbon dioxide source (Biogents devices). Carbon dioxide makes the trap attractive to a wide range of mosquito species (e.g. *Culex pipiens* and *Anopheles plumbeus*). Traps can operate continuously when a power supply is available, but can also run on a single 12V-battery (which limits operating time). When CO_2 is added, these traps have also been reported to catch male mosquitoes, blood-fed females and gravid mosquitoes.
- **Mosquito Magnet traps.** Mosquito Magnet traps are CO_2-baited suction traps that produce carbon dioxide by burning butane. They can thus be used in remote locations without power supply and for up to three weeks. Chemical attractants such as Lurex (L-lactic acid) and octenol (1-octen-3-ol) can be added. Several studies confirmed the utility of traps such as the Mosquito Magnet Liberty Plus for broad non-specific sampling. Major disadvantages of these traps are that they are large, heavy and relatively expensive, which can be a major constraint to their use in large-scale studies.

Positioning of traps and sample collection

If a quantitative comparison is envisaged such as for longitudinal sampling to assess population dynamics and abundance, the same type of batteries and chargers should be used in all traps. As a minimum requirement, battery capacity in mAh should be the same. A detailed description of the placement of CO_2-baited traps is given below. Other trap types follow a similar procedure but details may differ.

Positioning of CO_2-baited traps

- Generally, traps should be placed with the insect entrance at about 1.5 m above ground level; if there is a light source built into the trap, it should be removed. Mammophilic mosquitoes tend to search for a blood source closer to the ground. If targeting *Aedes albopictus*, *Aedes aegypti* or *Aedes vexans*, the trap should be placed 50 cm above ground. Ornithophilic mosquitoes search above two metres, so traps should be placed higher than this. Different biotypes of *Culex pipiens* could express both patterns, depending on the preferences of the local mosquito population.
- If not already fixed to the motor+fan trap part, an insulated dry ice container should be placed above the trap. The container should be filled with at least 800 g of dry ice (for running from dusk to dawn), or more for a 24 h run.
- The net should be attached, and a label with site code, date of capture, and trap type should be placed inside the net.

Colleting CO_2-baited traps

- When removing the collection net, blow from above through the fan cylinder to move the mosquitoes down; close the net with the label inside and store it in a large insulated box if air temperature is above 22 °C. If the collection intended for virus detection, fill the box with dry ice and store the mosquitoes on top.
- Turn off the trap.
- Disconnect the battery by removing both cables; take the trap and the insulated container.
- Charge batteries immediately after returning to the laboratory.

Specific guidelines for sample collection

Where to search?

- In rural and peri-urban biotopes, place the trap at the border of forests, hedges, or, even better, hide it among vegetation to protect it from wind and public interference.
- Follow the above instructions when placing traps in urban green space. In gardens, choose the most humid and wind-protected places. If light is used as the only attractant in the trap, avoid intense background illumination.
- For each surveyed area, select a station in each urban, peri-urban, rural, and natural zone. Use either two or four traps for every zone. Temperatures should be recorded starting two days before the first day of sampling.

What time of year?

- Sampling should be performed during the mosquito season (April–October in temperate regions). Samples can be collected throughout the year as long as the mean daily temperature remains above 10 °C.
- To collect data on the initial population and their different overwintering locations, collect the first samples during the first week of the mosquito season when overwintering females start searching for blood meals. The first week of the mosquito season usually coincides with the short period when the outside temperature surpasses the temperature inside the overwintering shelters (10–12 °C). In addition to sampling at the beginning of the season, samples should be collected around the seasonal peak period (plus/minus one week) to capture peak abundance data.

What time of day?

- Samples can be collected all day and night, assuming that most battery-powered traps run for 24 hours; collection should start no later than in the early afternoon and continue into late morning the next day in order to catch diurnal biters; a more precise schedule should be applied for abundance monitoring (see below).
- If sample collection is targeted to a particular species in order to save time, make sure that the peak of biting activity of that species is covered.
- For each study area, the local literature (or data published for neighbouring countries or the same latitude) about seasonal activity of the targeted species (e.g. start of activity of hibernating females and seasonal peak) should be searched to find the best times for sample collection.

How many rounds of sampling?

- Preferably, sampling is repeated three times. Sampling should start at least two hours before sunset and end at least two hours after sunrise to include dusk and dawn peak activities.

What type of trap should be used?

- If possible, use the following trap types in parallel to test their performance:
  - CO_2-baited EVS trap
  - CO_2-baited CDC miniature light trap with light
  - CO_2-baited CDC miniature light trap, but with the light bulb removed
  - CO_2-baited BG-Sentinel trap with Sweetscent lure.

  This will allow for performance comparisons between different trap types and help evaluate cost effectiveness, resulting in the selection of the best trap type for presence/absence and abundance surveys. If such comparisons are not possible, use a CO_2-baited CDC miniature light trap with light and a CO_2-baited BG-Sentinel trap with Sweetscent lure. One of these traps should be used for longitudinal abundance sampling during the following sampling surveys of a project. See Table 2 for specific protocols for VectorNet field sampling.
- Select a standard trap after testing several trap types during the first field sampling year; decide on standard batteries, standard chargers, standard CO_2 release rate or standard dry ice pallet size and quantity.

Table 2. Specific protocols used for the VectorNet field sampling surveys

Sampling	Objectives	Trapping methods
Cross-section sampling	Presence/absence	• Follow the general procedure: use the four types of traps listed above; if not possible, use a CO_2-baited CDC miniature light trap with light and a CO_2-baited BG-Sentinel trap with Sweetscent lure.
	Vectorial capacity data (field component)	• Follow the general procedures and use the two traps mentioned in the bullet point above or gravid traps when *Culex* spp. are targeted. • Samples should be collected every day in order to have a high number of live mosquitoes; this avoids having to separate live from dead mosquitoes.
Longitudinal sampling	Presence/absence	• Follow the general procedures: preferably use two different types of traps (see: What type of trap should be used?)
	Population dynamics (e.g. seasonality, density peaks, number of generations)	• Follow the general procedures: preferably use the four types of traps listed under: What type of trap should be used? Use the same type of trap/s at all localities and for all sampling dates in order to make results more comparable.
	Abundance data	• This sampling protocol is designed for sampling of adults from a group of *Culex* species (*Cx. pipiens complex, Cx. torrentium, Cx. perexiguus, Cx. tritaeniorhynchus*). • In case of comparing abundance data; a trap type must be selected optimally combining attractiveness, reliability/robustness and cost efficacy (see more below). • This 'sustainable' trap type will be chosen after analysis of the data gathered during the first sampling season.

Sampling methods: resting adults

Indoors

Only a few mosquito species rest in man-made shelters such as houses or cattle sheds. However, the dominant vector species of malaria parasites and filarial worms (more rarely arboviruses) are mostly endophilic and therefore should be collected indoors. Collections in the morning are usually more efficient than in the afternoon or evening.

The most frequently used methods for indoor sampling include: 1) collection with tubes; 2) collection with aspirators (mouth or battery operated) after locating the mosquito (with the help of an electric torch) on walls, ceilings, clothing, furniture, under mosquito nets; and 3) knock-down pyrethroid spray catches. Searching indoors

for resting adults is particularly efficient for *Anopheles* spp., members of the *Culex pipiens* complex and, to a certain extent, for *Aedes aegypti*.

Outdoors

Most mosquito species rest outdoors or in natural shelters (e.g. in vegetation, tree holes, crab holes, animal burrows, caves, rock crevices, and artificial shelters). The most frequently used methods for outdoor sampling include: 1) collection with hand-nets, 2) collection with motorised aspirators (large mechanical suction sweepers); 3) drop-net cages in the vegetation; 4) resting boxes of various types and sizes; and 5) an artificial Muirhead-Thomson pit. Searches for mosquitoes resting outdoors are usually very time-consuming and not very effective. In order to collect a large and representative number of mosquitoes, a major effort must be made.

Catching methods

- **Net sweeping.** Rustle the vegetation with a stick in one hand; with the other hand, sweep the space above the vegetation with a hand-net; quickly remove all caught mosquitoes from the net, using a mouth or mechanical aspirator to avoid the loss of scales from the integument of the mosquito.
- **Aspirating in shelters.** Illuminate the wall with an electric torch and catch adults resting on/hovering above the walls (usually in shaded and humid places) with a small mechanical aspirator (or directly with a mouth aspirator).
- **Aspirating in tree holes, rock crevices, small hiding places.** Catch hovering adults (usually in shaded and humid places) with a small mechanical aspirator, or, if available, a backpack mechanical aspirator.

Specific guidelines for sample collection

Where to search?

Bedrooms, bunkers, animal sheds, caves, (semi-)underground cellars (including wine cellars, mushroom-rowing cellars, etc.), vegetation (grass at breeding sites, tree holes and piled-up branches in the surrounding); at favourable places.

What time of year?

Collections can be performed during the entire year; during the cold period of the year, mosquitos can be sampled in overwintering places (e.g. caves, bunkers, animal shelters, (semi-)underground cellars, tree holes); during mosquito season, all places listed above can be surveyed.

What time of day?

Collection should preferably take place early in the morning during the mosquito season; any time of day is fine for winter sampling.

For how long?

- Duration: 30 minutes at each site.
- Abundance data: quantitative data are related to the intensity of the sampling effort (total number of mosquitoes collected in 30 minutes per collector per site) or to the number of sites examined.

Sampling methods: larvae

Trapping methods

Six main classes of larval habitats can be distinguished:

- Stagnant temporary water bodies (ditches, ponds, forest ditches, fens, flooded meadows or forests)
- Running waters (rivers, streams, ditches, drains)
- (Semi-)permanent water bodies with vegetation (ponds with vegetation, marshes, canals)
- Semi-natural water bodies without vegetation (e.g. puddles, road tracks, new ditches)
- Natural containers (e.g. tree holes, rock pools)
- Man-made (artificial) containers (e.g. pots, catch basins, pits)

Depending on the size and type of their breeding sites, mosquito larvae can be collected by netting, dipping or aspirating. Larger water bodies can be sampled with classic dippers or plastic trays (e.g. a frisbee) or a fine-meshed (≤ 0.5 mm) aquatic net (aquarium net) and sieve. Smaller water bodies (e.g. tree holes) can be checked for the presence of larvae by dipping with a ladle or by aspirating water with a tube or a pipette. Collected water can be inspected better for the presence of juveniles when decanted in a white plastic tray/bowl.

Dippers can be used as a survey tool to determine the abundance of larvae by taking several samples from designated sites in the habitat of interest and then counting the larvae of each dip. Netting, which allows to sample larger parts of the habitat, is more appropriate to determine presence/absence. The netting/dipping method will

vary with water depth, presence of aquatic vegetation or debris, and water clarity. Five to 20 sweeps/dips are recommended, depending on the site size and diversity of micro habitats (e.g. with or without vegetation, floating or erect vegetation, different water depths, shaded or sun-exposed).

Several dipping techniques exist, with different efficiency to collect the various mosquito genera (Table 3). Complete or partial submersion (depending on the water level) is recommended for routine immature monitoring of all species (general, non-genus-specific approach) in order to make comparisons across a range of aquatic habitats.

Larvae should first be transferred with a pipette to a small cup or bowl with fresh clean water as a washing procedure. If much debris or sediment is still present, additional serial transfers should be made until all suspended particles are eliminated. Using a pipette, as much water as possible should be removed from the cup or bowl. In the laboratory, water should be heated to about 60 °C and poured into the cup or bowl. In the field, the larvae should first be transferred to a vial with a pipette and then pure ethanol should be added. As soon as the larvae float up to the surface, the liquid is removed with the pipette and replaced with 70–80% ethanol. After five minutes, the larvae are transferred with the pipette (do not use forceps) to a vial with 70–80% ethanol. After filling the vial completely with ethanol to remove all air, it should be capped tightly. No more than 20 larvae should be placed in a 50 ml single vial because the water contained in the bodies of the larvae will significantly dilute the concentration of ethanol and jeopardise preservation.

Table 3. Various dipping techniques

Dipping method	Targeted mosquito genera	Method details	Notes
Shallow skim	*Anopheles*	The leading edge of the dipper is submerged at approximately 45° and about 2.5 cm below the water surface. The dipper is drawn along the water surface and filled at the end of the stroke.	The method works better for *Anopheles* larvae that remain at the water surface longer than other mosquito larvae after the dipper enters the water. This is a good sampling technique when submergent macrophytes (aquatic plants) have leaves just below the water surface.
Complete submersion	*Aedes, Ochlerotatus*, (*Culex, Culiseta*)	The dipper is submerged quickly in open water, usually in floodwater habitats. The dipper is brought up to the water surface through the submerging larvae that have reacted to the disturbance created by submerging the dipper.	This method is used primarily to sample mosquitoes whose larvae react rapidly to the dipper entering the water, but are visible. It is also appropriate for sampling larvae adjacent to vegetation. The dipper is brought to the water surface while brushing against the emergent vegetation.
Partial submersion	*Anopheles, Culex, Culiseta*	The dipper is submerged at approx. 45° along the emergent vegetation. Water flows rapidly into the dipper. The dipper should not be moved horizontally but vertically to scrape along the edge of the emergent vegetation.	The method works well when sampling in robust emergent vegetation such as cattail and bulrush. The suction created by water flow into the dipper and scraping also collects small insect predators and herbivores associated with mosquito larvae on or near the vegetation.
Flow-in	*Aedes, Ochlerotatus*, (*Culex*)	This technique is used in shallow water that has a depth < height of the ladle of the dipper. The bottom of the dipper is pushed into the substrate and the water with associated larvae and debris flow into the dipper.	This method works well in shallow habitats, root masses and other habitats that are shallower than the dipper's profile.
Scraping	*Coquillettidia*	The dipper is scraped against the underside of floating vegetation to dislodge attached larvae. The scraping action is usually a vigorous back-and-forth motion.	Used to sample larvae that reside under and usually attached to floating vegetation or the roots of floating plants. Because a vigorous back-and-forth motion is used with the dipper completely submerged, this technique works best with dippers having a screened bottom.
Simple ladle	*Culex*	A quick flip of the wrist is used to completely submerge the dipper just below the water surface. The technique is similar to taking water to drink.	Not a preferred method, especially if the sample is not taken adjacent to a mosquito microhabitat. This technique would be adequate in hypereutrophic situations where the abundance of larvae often approaches 1000 larvae per dip.
Background	*Aedes, Ochlerotatus*	The dipper is used to provide a light background against which darker coloured immature mosquitoes are more easily seen. When mosquitoes are found, they are collected by quickly pulling the dipper through the water surface.	A technique used primarily to identify mosquitoes inhabiting woodland ponds and pools.

Source: [6]

Sampling methods: eggs

Trapping methods

Ovitraps can be any kind of black plastic bowls (0.3 to 2 l volume) filled with water (ca. 2/3) and supplemented with an oviposition support (e.g. a wooden stick or a piece of polystyrene). The size of the ovitrap (i.e. the volume of water it can contain) has to be adjusted to the trap-checking frequency as well as to the local rainfall frequency and intensity in order to prevent the trap from drying out.

Specific guidelines for sample collection

Recommendations for trap density, frequency of trapping and trapping period are listed in Table 4.

Table 4. Recommendation for trap density and period of trapping

Surveillance aim and sites	Density of traps	Frequency of trapping	Period of trapping
Introductions at point of entry			
Main parking lots at country borders[1], highways and road axes that originate in colonised areas, storage platforms of imported tyres	1/2500 m^2	biweekly	Apr – Nov
Ports	1/5000 m^2	biweekly	Apr – Nov
Airports	1/ha	monthly	Apr – Nov
Persistence in colonised area			
Inspection of colonised area	1/5 ha	biweekly	Apr – Nov
Abundance and seasonal dynamics	6/site	biweekly	Jan – Dec[2]
Spread into areas surrounding colonised area			
Inspection around colonised areas	1/15 ha	monthly	Apr – Nov

Source: Adapted from [1]

[1] Includes parking lots at commercial centres close to country borders.

[2] Required during the first year; can later be limited to the period of development in the local climate.

Field data and parameters to be recorded

Table 5. Recommendation for field parameters to be recorded

Field data and parameters	Flying adult collection	Resting adult collection	Larval sampling	Ovitraps
Type of trap	X	X		
Location (geo-referenced)	X	X	X	X
Meteorological data (temperature, humidity, wind speed, precipitation – if possible recorded with data loggers)	X	X		X
Date and time of trap positioning and pick up	X	X		
Environment/land use	X	X		X
Type of site inspected	X	X	X	X
Composition of the entomological team	X	X	X	X
Duration of sampling	X	X	X	
Results (absence/presence, trap performance at the end of sampling)	X	X	X	X
Information provided by locals	X	X	X	

Conservation of specimens

Adults

- Tightly closed sampling nets with mosquitoes should be transported to the laboratory for further processing (if possible in dry-ice containers)
- For morphological identification (females, males): males in vials with 70–80% ethanol (for genitalia); females pinned as soon as possible in insect boxes (if not possible, keep frozen; then pin in the lab).
- For blood meal analysis (freshly blood-fed females): abdomen squashed on filter paper (ELISA and/or PCR) or in vials with 70–80% ethanol (PCR detection + gene sequencing).
- For pathogen search (females): frozen or in vials with 70–80% ethanol (depending of pathogen and subsequent techniques, do not use ethanol if virus detection is foreseen), collected every day.
- For detection of insecticide resistance gene (e.g. knockdown resistance) (females and males): in vials with 70–80% ethanol.

Larvae

- For immediate morphological or genetic identification: collection in vials with 70–80% ethanol.
- For morphological identification only after further development: collection in vials/small containers together with water from breeding place for rearing L1-L3 larvae to L4 larvae (which can be identified with higher reliability) or for keeping the larvae until adult emergence.

Eggs

Oviposition supports can be stored in a closed plastic bag, at room temperature or in a fridge (5–15 °C). The bag should not contain free water, but should still be humid (around 55% relative humidity).

- For genetic identification (PCR, DNA sequencing), the eggs can be put in 70–80% ethanol.
- For MALDI-TOF MS, eggs are best kept on the oviposition support (but should not dry out) and transferred to (dry) microtubes before sending them out for analysis.

Identification methods

Adults and larvae

- Different techniques can be used for the identifications of the different developmental stages of mosquitoes. Adults can be identified based on morphological characteristics or processed in a non-morphological manner (e.g. PCR and sequencing or MALDI-TOF MS). It is sometimes very difficult to distinguish species morphologically, especially if specimens are damaged or the species is complex. For those samples, it is recommended to use molecular tools (especially COI or ITS2 sequences) or MALDI-TOF MS.
- Morphological keys that can be used all over Europe are: Becker et al. [5], Schaffner et al. [7] and Gunay et al. [8].

Eggs

- Eggs can be recognised by their general shape, their size and the structure of the egg shell (exochorion). However, the morphological identification of eggs can be challenging, and identification keys only exist for a few species.
- Eggs can be flooded in the laboratory to let the larvae hatch and identify the resulting 3rd or 4th larvae instars or adults, although this can be inefficient for some species, in particular in case of diapausing eggs.
- MALDI-TOF MS or molecular methods are useful methods for accurate identification.

Quality control

To ascertain the quality of the identifications, 10% of the collected samples can be verified by an external expert, either by using morphological or non-morphological/molecular identification techniques.

Checklist

Table 6. Checklist for mosquito field specimen collections

Field data and parameters	Flying adult collection	Resting adult collection	Larval sampling	Ovitraps
Traps				
Checked and functioning traps	X			
Charged batteries	X			
Collecting nets	X			
Dry ice containers (part of the trap) or CO_2 gas bottles and release system	X			
If dry ice is used: dry ice in an insulated box	X			
Ladle/scoop for dry ice	X			
Insulated box for transport of collected nets	X			
Mechanical aspirator (BioQuip insect vaccuum, Hausherr’s handheld aspirator, or own construction) or mouth aspirator	X	X		
Hand net		X		
Back pack aspirator (only if available on site)		X		
Dipper			X	
Fine-mesh aquatic net			X	
Fine-mesh sieve			X	
Kitchen ladle			X	
Pipettes			X	
Ziplock plastic bags (10x15 cm)				X
Ovitraps: black plastic bowls (diameter 11 cm, height 9 cm, volume 0.62 l), piece of polystyrene (5 x 5 x 2.5 cm)				X
General				
Rope	X			
Swiss army knife	X			
Permanent marker	X			
Heavy-duty adhesive tape	X			

Field data and parameters	Flying adult collection	Resting adult collection	Larval sampling	Ovitraps
String	X			
Meteorological data logger	X	X		
Electric torch	X	X		
Labels	X	X	X	X[2]
Pencil	X	X	X	X
GPS or smartphone with geolocation function	X	X	X	X
Vials		X	X	X[1]
Ethanol, 70%[1]		X	X	X[2]
Magnifying glass		X	X	X
Entomological forceps		X		X[2]
Ethyl acetate		X		
Insect boxes		X		
Insect pins (n°2), micro pins, and small polyethylene foam or cardboard pieces		X		
Tubes			X	
Tissue paper				X
Outline map				X

2 Sampling methods for sandflies

Introduction

Phlebotomine sandflies, mostly known as vectors of leishmaniasis but also acting as vectors of arboviruses, belong to the family Psychodidae which contains some of the most primitive Diptera. The family is characterised by the presence of a dense clothing of hairs on the wings that are pointed to form a V-shape. Phlebotomines are small (body size of 2–4 mm in length), delicate, hairy flies with distinctive long, slender legs [9–13]. Of the approximately 900 species estimated to exist, less than a hundred (including 42 *Phlebotomus* species in the Old World [14]), belonging to *Phlebotomus* and *Lutzomyia* genera are proven or suspected vectors of human disease in the Old and New Worlds, respectively [15–18]. It is a rather difficult group to study due to the cryptic life of adults during the daytime and difficulties to find larval stages. Sandflies are found mainly in tropics and subtropics, with a few species penetrating into temperate regions in both the northern (up to 50°N) and southern hemispheres (to about 40 °S) [19]. Unlike in the New World, where sandflies are principally found in the tropics, in the Old World man-biting species are mostly confined to the subtropics, especially in more arid regions, including the entire Mediterranean basin.

Sandflies are typically crepuscular or nocturnal, biting at different times of the night according to the species but, they will bite during the day when disturbed (e.g. in dense forest, caves or buildings) [19]. Adult sandflies spend much time at rest, far more than flying (in search of hosts or oviposition sites).

Trapping host-seeking adults is based on light traps, sticky traps or CO_2 traps. Diurnal collections from sandfly resting sites in the vicinity of nocturnal trapping sites can reveal where these insects rest during the day. Although these collections can be standardised to some extent by determining the number of insects collected per unit time (usually per man-hour), the unique physical characteristics of each microhabitat, together with differences in the aptitude of collectors, make direct comparisons difficult. Regular resting site collections can be used to measure population changes. Collections of resting sandflies also provide females at various stages of the gonotrophic cycle (unfed, fed, half gravid, and gravid). Blood-engorged females collected at rest are of high interest to determine the natural host preferences. Additionally, such collections can also assist in evaluating the transmission of a pathogen, e.g. the detection of phlebovirus and/or parasites in females in the vicinity of the last season's hotspots (locations where a high circulation of viruses was recorded during the summer). By design, the collection of sandflies at rest does not require bait and rarely relies on traps.

Objectives of field collection

In the context of VectorNet, the primary objective of flying adult sampling is to collect qualitative and quantitative presence/absence and abundance data. General objectives of adult flying collection are (see also Table 7):

- To study the relative prevalence and density of sandfly species.
- To study sandfly behaviour, including endophily and exophily.
- To study the host preferences of sandflies and human/animal biting rates in a given area.
- To obtain additional information on sandflies, which can then be used in risk analyses and models.

The primary objective for the sampling of resting adults is to collect presence/absence data. However, with little additional effort in the field, supplementary data can be collected such as temperature activity threshold, natural host preferences, endophilic/exophilic behaviour, detection of insecticide resistance, given that an associated laboratory is willing to analyse the samples for that purpose.

Table 7. Overview of the objectives for sandfly sampling

Objective	Flying adults	Resting adults
Distribution (presence/absence)	X	X
Seasonality of different sandflies	X	X
Pathogen detection in natural population	X	X
Host preferences	X	X
Biting behaviour: endophagic/exophagic	X	
Resting behaviour		X
Insecticide resistance	X	X
Evaluation of control methods	X	X

Field sampling methods

Study design

The collecting methods are employed for two types of studies [20]:

- Qualitative studies, when there is a need to study the presence/absence, distribution, type of behaviour of different sandfly species in different macro- and micro-environmental conditions; and
- Quantitative studies, when collections are made to measure relative density, seasonality and abundance in different situations.

As it is impossible to examine entire populations of sandflies in different habitats spread over large areas, sampling is carried out using an intensive methodology. A sandfly sample is a representative part of a sandfly population collected over a fixed period of time in a given situation within its habitat. A population of sandflies is composed of individuals that possess the same basic characteristics, with some variation within known normal limits due to the physiological status and environmental factors. Therefore, a sample of a certain number of individuals of a given population will be satisfactory for the study of the characteristics common to the whole sandfly population in that area, e.g. presence/absence data, abundance, or variability of the density.

Many factors should be taken into account when conducting a sampling campaign. Of those factors, three are essential:

- Efficacy and limitations (feasibility) of the sampling method, taking into account the information mentioned above.
- Applied sampling method and techniques should be accurate and of satisfactory duration. In longitudinal studies, the intervals between repeated collections of samples in the same location should allow for the recording of changes in a given population of sandflies.
- Investigated sites and numbers of individuals sampled should be representative for relatively homogeneous areas [21].

Observations of house-resting sandflies versus studies on outside populations will give indications of endophily/exophily. While some species of sandflies show some degree of endophily, this behaviour can be limited to certain periods when houses are visited to obtain a blood meal (endophagy) [22].

The outside activities of most sandfly species fall into two categories; they either take place in sylvatic or peridomestic areas (including artificial shelters). Direct searches in sylvatic resting sites are difficult. In peridomestic areas, direct searches tend to be productive and can yield useful qualitative data. For example, artificial shelters, such as box shelters or pits, are often very productive and can provide quantitative data, but they also compete with natural breeding sites, the availability of which varies with the seasons.

There are three recommended locations for artificial shelters: a) close to hosts, i.e. in a village or near animal sheds, b) in open areas surrounding villages, and c) near expected breeding sites. When deciding on where to place artificial microhabitats in order to collect host-seeking sand flies, researchers should keep in mind interception and attraction methods.

Selection of sampling methods

The selection and application of methods for sampling flying and resting adult sandflies are determined by: the objectives of the study for which the collection is made; the biology and behaviour of vectors (if known); housing conditions and human behaviours; and the environmental conditions (natural or modified by man) in which the collection is made, e.g. temperature, relative humidity, winds, rains, absence of breeding sites, position of breeding sites, insecticide spraying, presence of domestic animals. It should be stressed that the significance of collected data will vary, depending on how the above-mentioned factors are taken into account at the time of collection.

Vector surveillance aims to detect the presence of a vector or a given population. It also measures vector abundance in order to estimate the risk of disease transmission. Insect traps should be as sensitive as possible in order to detect vector presence and vector abundance of a given species. Traps for the assessment of abundance traps must reflect the diversity and quantity of the insects. The most frequently used trapping methods are light traps; sticky traps are preferred for host-seeking females; while aspirator collection is the preferred method for resting sandflies.

Selection of study sites and exact location of sampling sites

Once the type of sampling site has been defined, the exact location of the outdoor sampling sites has to be identified:

- On online maps or using satellite imagery (e.g. Google Earth): identification of large animal shelters or sheds, farms, old quarries, bunkers or caves; identification of vegetation (groves, forests).

- On site: identification of all possible habitat types, identification of all natural and artificial sites, identification of all human settlements.

It is important to note that all data are associated with geo-referenced points and reported to a central database. Data are shared with the VectorNet project, with real-time updates of web-based distribution maps of bloodsucking insects. Ideally, data recording is supported through information technology, especially in these four areas: 1) definition of sampling sites (i.e. identification with geo-referenced sampling points/areas in land use units), 2) data reporting via a smart phone-to-web system, 3) analysis of the distribution data, and 4) modelling the potential distribution of the most common species and mapping species richness in relation to earth observation and climate data.

After a first fieldwork season and a comprehensive data analysis, the design could be adjusted, e.g. to include further sampling sites.

Collection methods: flying adults

The trap types listed below are used in the VectorNet project.

Light traps and CO_2 traps

CDC miniature light traps are the most widely used insect traps for collecting outdoor flying sandflies. A battery-operated light-suction trap (CDC miniature light trap) is used for field studies. The main advantage of this type of trap is that it can operate overnight to collect sandflies. Miniature light traps are highly efficient if they are located close to resting or breeding places and if the right light source is used. The catch of sandflies can be augmented by adding a carbon dioxide source such as dry ice [23]. Sandfly response to CO_2 becomes saturated at levels close to that of human exhaled breath (4.5%), and gas released from a trap would therefore remain above the background atmospheric level (0.03%) until dispersed downwind and diluted by a factor of 100. CO_2 is usually a long-range attractant for sandflies whereas light is probably perceived by sandflies at much closer range [24]. The addition of a source of CO_2 can therefore improve catches by increasing the effective sampling area of a light trap [25,26]). The productivity of a light trap or CO_2 baited-trap depends on several factors such as: the position; type of light used; species of sandflies to be collected; physiological conditions of sandfly adults; environmental conditions during the collection; the amount of contrast between the light source and surroundings: the greater the contrast, the greater the 'catchment area'; sandflies have a tendency to withdraw from the high light intensity immediately adjacent to lamp.

In general 0.1 cm size netting is sufficient for use in sandfly traps.

General considerations for the use of light traps

- Traps should be placed before dusk and collected in the morning after dawn. Sandflies should be rapidly removed from the trap to limit damage from heat and/or desiccation. Traps should be placed close to the ground or adjacent to vertical surfaces (walls, tree trunks), preferably far from an external light source. Background light intensity adversely affects trapping capacity. It is therefore better to avoid areas near other sources of artificial light. Other locations to avoid include sites exposed to strong winds and areas exposed to industrial fumes and smoke.
- A single trap usually reflects sandfly flight activity within a few metres of its location. Sometimes a shift of only a few metres makes a considerable difference in the number of sandflies attracted. A sufficient number of traps must be utilized to ensure a representative sample [23].
- Usually traps operate from dusk to dawn. The insulated bottle filled with dry-ice up to 2/3 (800 gr) (for CO_2–baited traps), is hung above the trap (or use insulated box fixed trap). The net has a label inside on which the site code and date of capture is noted. The battery should be protected from rain. A data logger can be attached to a trap for measuring temperature (°C) and relative humidity (RH%).
- Traps are generally collected within two hours after sunrise. First, the net should be tied, then removed from the trap, and only then the battery should be disconnected. Nets should be stored in conditions preventing the desiccation of sandflies. Specimens are collected from the nets with mouth aspirators and should be transferred to an appropriate carrying box or similar.
- Specific procedure for the transfer of specimens: care should be taken not to collect too many sandflies by single aspiration (reservoir type of aspirator is essential); sandflies should be transferred immediately to a paper cup, cage or larval pots with a layer of plaster of Paris. Particular care should be taken not to damage the sandflies when they are collected for different experimental studies.
- Where to search: inside houses, outside houses, in/around animal sheds, caves, all suitable shaded open areas (including sylvatic habitats), several microhabitats such as sheep/goat barns, poultry houses, pigpens or -sties, and areas near hosts, i.e. in villages or near animal sheds, in open areas near villages, near potential breeding sites.
- Time of year (trapping density and frequency): the trapping density and frequency depends on several factors such as study objectives, capacity of the team, and finances. It is difficult to decide on the exact

trapping density in a study area, particularly for ecological studies. First, researchers have to make a decision on the number of samples per habitat unit. One aspects to consider is whether different areas of the habitat unit have to be sampled separately (with regard to altitude, vegetation, or microhabitats). Another aspect is the number of samples to be collected within each unit/subunit for maximum efficiency. If the distribution of the population in the habitat is biased towards certain subdivisions and the samples are taken randomly, 'systematic errors' will occur. This can be overcome either by sampling so that the differential number of samples from each subdivision reproduces the gradient in the habitat, or by regarding each part separately, with corrections made at a later stage.
The objectives of a study also determine the sampling pattern. If the aim is to obtain estimates of the mean density, then variance should be minimised. It is preferable that both the trapping density and trapping frequency are high. Trapping biweekly with a sufficient number of traps is required to keep variance errors at a minimum level. If the aim is to obtain presence/absence data and relative abundance of sandfly species in given area (e.g. aim of the project), trapping once (three to four consecutive nights) in one or two months with a sufficient number of traps during sandfly season should be sufficient.
- Abundance data: quantitative data are usually related to the sampling effort (total number of sandflies collected per night at a given site).

Sticky traps

Sticky traps constitute another method of sampling sandflies by interception rather than attraction. Standardised pieces of unwaxed paper or thin cardboard (e.g. 20 x 20 cm or 25 x 20 cm or A4 plain paper) are soaked in castor oil and placed in sandfly resting places or in places where sandflies are likely to be active (for at least one night, and up to between three and five consecutive nights). In order to obtain quantitative results, either one or both sides of the paper must be completely exposed. The paper can be placed in the proximity of resting places and should be secured, e.g. with a piece of wood or bamboo, a wire, hook, staple or paper clip, depending on the local conditions. The number of oil-impregnated papers used at each sampling event is normally constant (minimum 20/village) [23]. These traps are generally inexpensive and easy to manufacture in large numbers. They can be prepared in bulk before field studies and stored until required. Castor oil-impregnated sticky traps can be used productively, even in dry areas if protected from the wind [10,23].

General considerations for the use of sticky traps

- Sandflies are removed from the oiled paper (e.g. with a small brush), washed in saline solution or a drop of alcohol (70%) and then stored. Only dead specimens can be collected with these traps [23].
- Where to search: sticky traps can be located around resting places in large numbers. Possible resting sites include animal barns (inside/outside), houses (inside/outside), poultry houses (inside/outside), caves, tree holes, leaf litter, spaces between (or under) rocks, animal burrows, rock crevices, holes in stone walls, and among vegetation.
- Time of year: in Europe, sampling can be performed during the high season between end of May and middle of October. The best collecting period for most Balkan locations is from the middle of June until the end of August, while in Turkey and most of southern Europe (e.g. Crete or Cyprus), April until the end of September (sometimes until the end of November) are good collection times.
- Trap positioning: On average, 20–30 traps set up in each sampling site. Rows of traps are hung 20–50 cm above the ground. In animal barns or houses, the ceiling level can be used. Animal burrows and rock crevices are used as diurnal resting or breeding sites by many species, and sticky traps can be hung across the entrances [27].
- Trap collection: traps are collected after a minimum of one night and no later than after five consecutive nights. Traps must be collected one by one and stored in ziplock plastic bags, with a small label indicating the sampling site and date. The number of sticky traps placed at each site should be recorded on every plastic bag.
- Abundance data: catch results are expressed as number of sandfly species per m^2 of sticky paper per night.

Collection methods: resting adults

Sampling from resting sites can be carried out with several aspirator types (mouth aspirator, reservoir type aspirator, tube aspirator, hand-held version powered by flashlight batteries). The collection of resting sandflies must differentiate indoor versus outdoor sites.

Indoors

Collections in the morning are strongly suggested. If not possible, early afternoon collections are usually more efficient than those in the evening. Most frequently used methods for indoor sampling: 1) collection with tubes; 2) collection with aspirators (mouth or hand-held, battery operated), after locating the sandfly on walls, ceilings, clothing, furniture, under mosquito nets; and 3) collection by knock-down pyrethroid spray catches.

Outdoors

Some sandfly species rest outdoors or in natural shelters (among vegetation, in tree holes, animal burrows, caves, rock crevices, artificial shelters). Most frequently used methods for outdoor sampling: 1) collection with different types of mouth aspirators, 2) collection with large mechanical suction sweepers. The searches for outdoor resting sandflies are typically time consuming and not very effective.

Diurnal resting places are comparatively cool and humid and include houses; latrines; cellars; stables; caves; fissures in walls; holes in stone walls, rocks or soil; dense vegetation; tree holes and buttresses; burrows of rodents and other mammals, bird's nests and termitaria. Females of most species are predominantly exophagic and exophilic. By contrast, endophilic species rest indoors during the maturation of eggs.

Trapping methods

Aspirating in shelters and houses. Illuminate the wall with an electric torch and aspirating adults resting/hovering on the walls (preferably on shaded and humid places) with a small mechanical aspirator (or directly with a mouth aspirator).

Aspirating from tree holes, stone wall holes, rock crevices, and small hiding places. Catch hovering adults (usually in shaded and humid places) with a small mechanical aspirator. Duration: 10 to 15 minutes at each site.

Specific guidelines for sample collection

Where to search?

Bedrooms (behind photo frames, TV and other furniture), latrines, bunkers, animal sheds, caves, any (semi-) underground cellar, vegetation (grass on breeding site, tree holes, wall holes); at favourable places.

What time of year?

In Europe, sampling can be performed in the mosquito season (between the end of May and the middle of October). The best collecting period for most Balkan locations is from the middle of June until the end of August, while in Turkey and most of southern Europe (e.g. Crete or Cyprus), April until the end of September (sometimes until the end of November) are good collection times.

What time of day?

During sandfly season, the preferred time of day is early in the morning.

General procedures for the sampling of resting sandflies

- Care should be taken not to collect too many sandflies by single aspiration (reservoir type of aspirator is essential); sandflies should be transferred immediately to a paper cup, cage or larval pots with a layer of plaster of Paris.
- In general, three different types of mouth aspirators can be used. The most commonly used type is composed of a glass or plastic tube (15 mm in diameter), a rubber or plastic tube, and a mouthpiece with a fine-mesh net plus a cotton piece (which prevents the user from swallowing dust and sandflies). VectorNet field studies are usually conducted with a 'reservoir type' mouth aspirator which uses a glass tube (30–40 mm in diameter). One potential problem is that sandflies shed large numbers of hairs which are inhaled by the collector and can cause respiratory problems. Also, resting sites are often in dark and humid microhabitats which poses a potential danger of fungal and bacterial infections, such as histoplasmosis or Q-fever caused by *Coxiella burnetii*. Therefore, some researchers use specific filters to prevent the inhalation of dust, hair and microorganisms.

Field data and parameters to be recorded

Table 8. Checklist for field data and parameters recorded during field work

Field data and parameters	Flying adult collection	Resting adult collection
Type of trap	X	X
Light intensity for CDC miniature light traps (Lux)	X	
Location (georeferenced)	X	X
Altitude	X	X
Meteorological data (temperature, humidity, wind speed, precipitation – if possible recorded with data loggers)	X	X
Date and time of trap positioning and pick up	X	X
Environment/land use	X	X
Type of site inspected*	X	X
Distance from nearest house	X	
Composition of the entomological team	X	X

Field data and parameters	Flying adult collection	Resting adult collection
Duration of sampling	X	X
Results (absence/presence; for resting adults, include physiological status of the collected insects; trap performance at the end of sampling)	X	X
Information provided by locals	X	X

** Buildings: construction material, condition of inner walls, ceiling material, presence of a separate kitchen, presence of domestic animals, producing dried dung, toilettes, distance from barn, insecticide usage, type of insecticide, date of last residual spraying.*
Shelters: construction material, condition of inner walls, ceiling material, distance from other shelters, insecticide usage, type of insecticide, date of last residual spraying.
For open areas: description of habitat, aspect/exposure/direction, sunniness/shadow, description of vegetation, distance from urban sites, insecticide usage, type of insecticide, date of last residual spraying.

Conservation of specimens

In principal, phlebotomine sandflies are stored in 70% (non-denaturated) ethanol at room temperature until laboratory processing and identification.

- For morphological identification: male and female sandflies are stored in vials with 70% ethanol until laboratory processing and identification.
- The best way to transport adult specimens to the laboratory for identification or establishment of a colony is to keep them alive in larval pots lined with plaster of Paris and closed with a snap-cap top and fine gauze. Pots are put into a plastic ice container or stored on ice bags to keep them cold.
- For MALDI-TOF MS studies: sandflies may be kept alive until processed in the laboratory. Alternatively, they are stored at temperatures below -20 °C or in liquid nitrogen.
- For DNA analysis: dried, fresh, frozen or at least alcohol-preserved (70 or 96%) specimens can be used.
- For blood meal analysis (freshly blood-fed females): abdomen squashed on filter paper (ELISA and/or PCR) or in vials with 70 or 96% ethanol (PCR detection + DNA/RNA gene sequencing).
- For pathogen search (females): frozen or in vials with 70 or 96% ethanol (depending on pathogen and subsequent techniques).
- For virology studies: collected sandfly adults must be transported to the laboratory on dry ice, or alive (see section on larval pots above). If adults are transported alive to the laboratory, they should be placed and killed in a freezer (-80 ºC) This process will preserve the virus RNA [23].

Identification methods

In general, species identification is based on morphological characteristics but not all closely related species can be adequately identified in this manner. The morphological identification process requires a high-level of expertise. Morphological keys are available, but often limited to some genera and/or limited to a region, and are sometimes not accurate enough. There are restrictions which complicate the morphological identification of sandfly specimens. For example, since it is almost impossible to find sandfly eggs and larvae in the field, only keys for the adult stage are available. Also, since adult sandflies are quite small and fragile, trapped specimens are often damaged and essential morphological features are missing.

Alternative identification methods have been developed, initially to allow for the identification of sibling species. These methods were gradually extended to cover a wide range of species and have improved the identification process, especially for routine operations. Molecular techniques allow for the rapid identification of species and can be carried out by non-taxonomists by analysing some gene sequences or peptide profiles. These methods offer the advantage of identifying damaged specimens and can also process samples simultaneously. A vast range of molecular techniques and markers is used to study sandfly taxonomy, systematics, population structure and dynamics.

The use of molecular techniques requires a well-equipped laboratory and trained personnel, which makes it a rather costly and laborious method for large-scale studies. If only a small number of species-specific genetic markers are available, or if they are completely missing, identification can be difficult. Polymerase chain reaction (PCR), combined with sequencing of the amplicon, can identify a specimen, provided that corresponding sequence data are stored in online molecular databases such as GenBank or BOLD Systems. For some groups of species (e.g. species complexes) specific conventional real-time PCR assays have been developed for rapid molecular identification. As an alternative, protein profiling by using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) was recently described for the characterisation of some sandfly species such as *Phlebotomus perniciosus* and *Phlebotomus tobbi,* and this approach was extended to include other sandfly species. MALDI-TOF MS, which is a rapid, simple and reliable method, was demonstrated to be suitable for the routine species identification of sandflies [28].

Egg and larval characters play only a secondary role in the identification of phlebotomines. By contrast, adult sandflies provide numerous reference points, most notably the male genitalia and the female spermathecae.

Morphology of the ascoids of the antennae and numbers of cibarial teeth are frequently used to differentiate species group. Length ratios are used in combinations with wing veins, antennal and leg segments, or mouthparts. Approximately 90 characters have been demonstrated as effective species descriptors and were recommended for inclusion in every new species description. Recognition and measurement of all character elements require mounting individual species on microscope slides and observation magnifications of 100-400X. The taxonomic nomenclature of phlebotomines is undergoing substantial change at the genus and subgenus levels. Efforts are underway to revise the nomenclature, generally consisting of the elevation of currently recognised subgenera or groups to the genus level.

Morphological identification

Based on the morphological characters, Artemiev [29] produced a classification scheme of 24 genera and 40 subgenera. Morphological identification keys used by VectorNet project mainly include:

- Theodor O [30]
- Artemiev MM [31]
- Lewis DJ [32]
- Lane RP [19]
- Niang AA, Hervy J-P, Depaquit J, Bousses P, Davidson I, Geoffroy B, et al. [33]
- Killick-Kendrick R., Tang Y, Killick-Kendrick M, Sang DK, Sirdar MK, Ke L, et al. [34]
- Perfiliew PP [35].

Most of the specimens will be identified morphologically using classical identification keys and the expertise of members of the project. Briefly, specimens are cleared in boiling Marc-André solution and mounted in a drop of Berlese or CMP-9 (or equivalent mounting medium). The head is mounted dorsally. Male genitalia are mounted laterally and female spermathecae are dissected before mounting. Spermathecae could be mounted also directly in Marc-André solution (especially those belonging to the subgenus *Larroussius*, the vectors of *Leishmania infantum)*.

For some complexes of species (*Phlebotomus sergenti/Phlebotomus similis*; *Phlebotomus perfiliewi* s. l., the major group of Larroussius, *Phlebotomus kandelakii*, *Phlebotomus burneyi*; the subgenus Transphlebotomus; *Phlebotomus perniciosus/Phlebotomus longicuspis* in North Africa if applicable), an additional molecular identification is also necessary on a representative proportion of the specimens sampled. Specimens selected for molecular identification are dissected: head and genitalia are mounted as previously indicated whereas the thorax is stored in 70% ethanol. A mitochondrial marker (e.g. cytochrome b, COI) and a ribosomal marker (ITS2 or partial 28S) are sequenced; part of the samples will be assayed with MALDI-TOF MS.

All specimens are kept at least for one year after the end of the study; an additional number of specimens (at least two specimens of each species from each location) are kept in long-term storage, i.e. parts of the body mounted on slide, an entire body in alcohol (to be mounted later), or the male genitalia mounted on slide.

Molecular identification methods

Molecular identification is performed in specially equipped laboratories. DNA-based protocols can use dried/field-collected, fresh, frozen or at least alcohol-preserved (70% or 96%) specimens. Preferably, each specimen is stored in a separate vial to avoid contamination. Although DNA can be extracted from dried sandflies, results are better if frozen specimens are used.

PCR exponentially amplifies the target DNA; the process can also be applied to RNA after reverse transcription. This occurs by a cyclic reaction of different steps with changing temperatures. The temperatures are specific for each step,and for each PCR reaction, mainly depending on the nucleotide sequence of the primers. The PCR reaction also demands the use of correct buffer conditions, presence of target DNA, the dNTPs and an enzyme (mostly Taq polymerase), and a thermostable enzyme originating from *Thermus aquaticus*. A simple visual detection of the amplicons can be done by electrophoresis on an agarose gel followed by an ethidium bromide staining (or alternative method). Amplified products can afterwards be sequenced or they can be processed further by adding restriction enzymes (PCR-RFLP) to the amplified product. The latter technique is often used to rapidly distinguish between closely related species (e.g. *Phlebotomus major* complex).

DNA barcoding

DNA barcoding is a technique based upon the use of a standardised short DNA sequence (ca. 500 bp) that can be universally used to identify species. DNA barcodes provide a unique 'tag' for each of the studied species. For this purpose, cytochrome b and/or a fragment of the cytochrome oxidase subunit 1 (COI) in the mitochondrial genome should be selected due to several advantages such as maternal lineage, lack of recombination, lack of 'index' and higher mutation rates. The COI region is also the most conserved gene among other amino acid coding genes, which aids resolution of deeper taxonomic affinities and primer design. One of the main advantages for worldwide research is the comparability of DNA barcodes. The complete or partial COI gene is increasingly used in phylogenetic, systematic and population genetic studies. However, so far this region has rarely been targeted in 'basic' sandfly barcoding research in Europe. Other mitochondrial regions that are often used for sandfly species

identifications are the ND4, COII and D2. Several primers have been developed for these regions and can be found in the literature. In addition, a frequently used protocol was developed to amplify the extracted DNA. Obtained sequence data are aligned (several freely available alignment tools can be found) and compared with data available in BOLD (www.barcodinglife.org) or GenBank (BLAST, www.ncbi.nlm.nih.gov/guide/sequence-analysis/).

The method used by VectorNet. DNA is extracted using the QIAgen BioSprint DNA Tissue Kit (QIAgen, Crawley, England, UK) on the QIAgen automated DNA extraction platform, with all solutions at half the manufacturers recommended volumes. The universal LCO and HCO barcoding primers of Folmer et al. [36] are used to amplify the barcode region of the mtDNA COI gene (658-bp after primer removal). The PCR reactions comprise 1 µl template DNA, 1 µl 10 × NH4 buffer, 129 0.5 µl dNTPs at 2.5 mM, 0.3 µl each primer at 10 µM, 0.4 µl MgCl2 at 50 mM and 0.2 µl of Taq polymerase (BioLine, London, England) made up to 10 µl with ddH2 O. Reactions comprise initial denaturation at 95 °C for 5 min, then 34 cycles of 95 °C for 30s, 48 °C for 30 min and 72 °C for 45s, followed by a 5-min extension at 72 °C and a 10 °C hold. PCR products are visualised on 2% agarose gels stained with ethidium bromide. Products are purified using the Millipore vacuum manifold system, following the manufacturer's instructions. Bidirectional DNA sequences are generated using the Big Dye Terminator Kit (PE Applied BioSystems, Warrington, England) and run on an ABI 3730 automated sequencer (PE Applied BioSystems). Sequences are edited using Sequenchera version 4.8 (Genes Codes Corporation, Ann Arbor, MI) and alignments verified in CLUSTAL X [37]. Nucleotide sequences are translated to amino acid sequences using the invertebrate mitochondrial code [38]. The second base of the 658-bp barcode sequence is equal to the first position of the amino acid codon. Alignment of the COI fragments usually are unambiguous and no evidence of pseudogenes is expected. Sequences generated during this study will be directly compared with those publicly available in GenBank using Blast (http://blast.ncbi.nlm.nih.gov/) and as yet unreleased sequence data held in the BOLD database. Sequence statistics, calculation of pairwise distance parameters using Kimura's 2-parameter algorithm [39] and the bootstrapped neighbour-joining tree [40] are constructed in MEGA v. 5.2.2 [41].

MALDI-TOF MS

A newly developed technique in sandfly identification is protein profiling with MALDI-TOF MS. It has been widely applied for routine identification of microorganisms in clinical microbiology laboratories, but it seems also suited for robust, low-cost and high throughput identification of sandfly vectors. This technique is especially useful in routine operations to screen large samples of mixed species and at various stages (i.e. eggs, larvae, adults).

Species identification of sandflies with MALDI-TOF MS is feasible and represents a novel and promising tool to improve biological and epidemiological studies on these medically important insects.

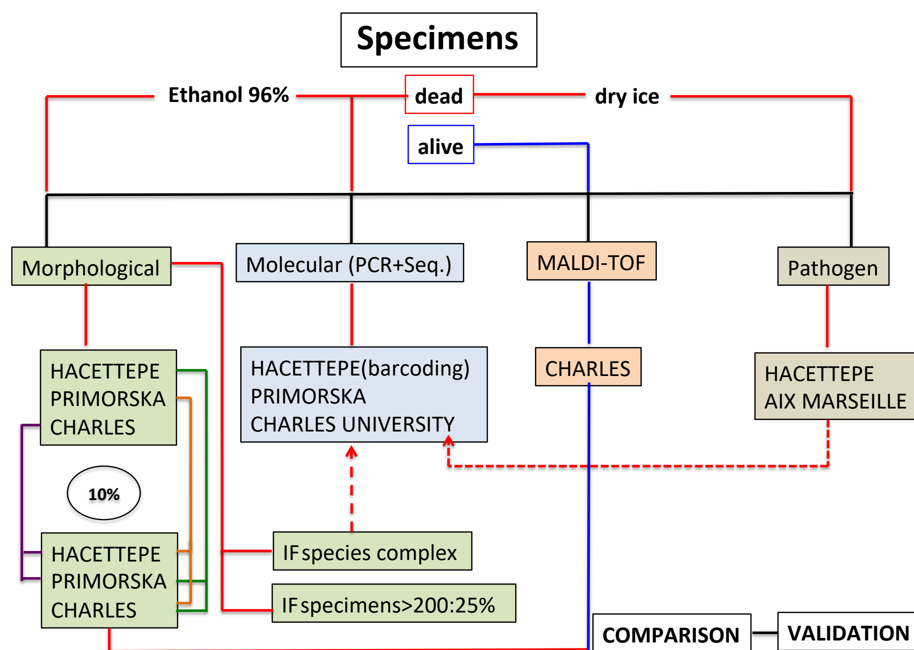
Method used by VectorNet. Insect bodies, stored as described above, are dissected, cutting off the head and abdomen so that body parts bearing decisive characters can be mounted on slides for morphological analysis; the rest of the abdomen are spared for DNA isolation. Remaining thoraxes will be manually ground in 1.5-mL microtubes with 10 µl of homogenisation solution using disposable pellets and pestles [28]. Two homogenisation solutions are tested: sterile distilled water and 25% formic acid. Two µl of the ethanol or the water protein extract are mixed with 2 µl of a MALDI matrix in a tube. One µl of the resulting mixture are deposited on the MALDI target and allowed to air-dry. The MALDI matrix are prepared daily as an aqueous 60% acetonitrile/0.3% TFA solution of sinapinic acid (30 mg/ml; Sigma). Positive-ion mass spectra are measured in linear mode on an Ultraflex III MALDI-TOF spectrometer (Bruker Daltonics, Bremen, Germany) within a mass range of 2–25 kDa and calibrated externally using the Bruker Protein Calibration Standard I. Each acquired spectrum corresponds to an accumulation of 1000 laser shots (5×200 laser shots from different positions of the target spot). The spectra are exported to the MALDI Biotyper 3.1 software for data processing (normalisation, smoothing, baseline subtraction, peak picking) and evaluation by cluster analysis. Only a maximum of 100 peaks with signal-to-noise ratio of >3 and relative intensity of at least 0.1% of the most intense peak from the spectra are considered for choosing peaks. For MSP dendrogram creation, an individual main spectrum are generated from each of the acquired spectra [28].

Quality control

All three VectorNet-affiliated laboratories in Turkey (Hacettepe), the Czech Republic (Charles University) and Slovenia (Primorska) are responsible for morphological and molecular identification. The collected specimens are divided in two groups and distributed to two groups, Turkey and Slovenia, for morphological identification. Alive specimens are assayed by MALDI-TOF in the Czech Republic. The Czech lab also performs morphological identification using the rest of the body parts (genitalia and head of each specimen) after MALDI-TOF. The laboratories in Turkey and Slovenia carry out DNA sequencing if necessary. When performed by each team, a proportion (10 %) of the samples is identified by other consortium laboratories for quality check and validation (see Figure 1).

If the number of specimens is above 200 in a trap, 25% of specimens are usually identified morphologically in a first step. The rest of the specimens is stored in vials with 70% ethanol in -80°C freezer. Identification of these specimens continues step by step to generate a species profile for each locality. In addition, detection of pathogens in specimens can be conducted by the laboratories in Turkey (Hacettepe) and France (Aix-Marseille University).

Figure 1. Species identification pathway



Checklist

Table 9. Checklist for sandfly field specimen collections

Field data and parameters	Flying adult collection	Resting adult collection
Field		
Checked and functioning traps	X	
Charged batteries	X	
Dry ice containers (parts of the traps) or CO2 gas bottles and releasing system	X	
In case dry ice is used: dry ice in an insulated box	X	
Ladle/scoop for dry ice	X	
Insulated box for transport of nets collected	X	X
Mechanical aspirator (BioQuip insect Vac, Hausherrs handheld aspirator, or own construction)	X	X
Mouth aspirator	X	X
Hand net		X
Castor oil		X
Papers or cards		X
General		
Data logger	X	
Rope	X	X
Knife	X	X
Heavy-duty adhesive tape	X	X
String	X	X
Meteorological data logger	X	X
Electric torch	X	X
Labels	X	X
Pencil	X	X
GPS or smartphone with geo-location function	X	X
Vials	X	X
70% and 96% ethanol	X	X
Field magnifying glass	X	X
Entomological forceps	X	X
Fine-mesh gauze	X	X
Insect boxes	X	X
Tubes	X	X
Tissue paper	X	X
Outline map	X	X

Field data and parameters	Flying adult collection	Resting adult collection
Cotton	X	X
Small cages	X	X
Larval pots	X	X
Paper cups	X	X
Ziplock plastic bags	X	X
Nitrogen tank (if possible)	X	X
Plastic ice container	X	X

3 Sampling methods for biting midges

Introduction

Culicoides are among the smallest haematophagous insects. Their development cycle is quite similar to mosquitoes; females lay eggs, which will develop into four larval stages, followed by a nymph stage. Emerging adults will mate, and both sexes feed on nectar, whereas only females take a blood meal on vertebrates for egg maturation. The main difference to mosquitoes is the nature of their larval habitats. The larvae of biting midges are not aquatic and can be found in humid/mud substrates enriched with organic matter: wet soil ecotone between aquatic and terrestrial habitats (coastal salt mud flats, freshwater vegetated swamps, etc.), dung pats or moist decaying vegetative material (decaying fruits and flower heads, forest leaf litter, etc.). Techniques to collect larvae (substrate sampling or emergence traps) exist, but they are time-consuming and laborious. Thus, the sampling of populations is mostly carried out using adult collections.

Most adult collections focus on flying adults, using passive aspiration traps such as Rothamsted suction traps or active traps such as truck traps [42]. However, UV light trap collections remain the most time efficient and widespread method to assess *Culicoides* presence and abundance at a wide scale. UV light traps collect a fraction of the *Culicoides* populations, mostly host-seeking females. The assessed abundance depends to a large degree on the trap location, including distance to animals, height above ground level, etc. [43,44].

Due to the emergence of bluetongue in Europe in 1998 [45], large-scale trapping was carried out using UV light traps in many European countries. During 'vector-free periods', EU regulations now allow the easing of emergency measures such as restrictions on the movement of animals. A massive amount of information exists on the distribution and dynamics of biting midges; there are also abundance data for *Culicoides* across Europe based on sampling with UV light traps.

Several publications have highlighted the discrepancy between *Culicoides* abundance data recorded with UV light traps and those data collected with host-baited traps [46–48], indicating that abundance data based on UV light traps could not used as a direct estimation of biting rate. However, Viennet et al. [49,50] showed that UV light trap collections were linearly correlated to attack rates on animals for several *Culicoides* species. This showed that abundance assessed by UV light trap collections may still be useful for risk assessment: abundance data based on UV light trap collections are likely to be broadly representative of biting rates (in identifying order-of-magnitude differences in population size according to spatial or temporal variation) but may be misleading at a smaller scale. It should be highlighted that abundance data collected with UV light traps have been used for more than a decade to manage animal movements under EU regulations, and that this system has demonstrated its utility.

The VectorNet *Culicoides* group gathers existing information from national surveillance systems and collects *Culicoides* data across Europe in a standardised way so this information can be used in analyses for risk assessments.

Objectives of field collection

The objectives of field collections by the *Culicoides* group are:

- to gather existing information from national surveillance systems
- to study the geographic distribution of *Culicoides* vectors, focusing on regions where data from national surveillance systems do not exist or are unavailable, including regions bordering Europe (Northern Africa and the Middle East)
- to understand factors that influence the distribution of *Culicoides* and may affect the ability to extrapolate data across geographic areas (e.g. climate, land cover, altitude, host availability and species) using altitudinal transects and by exploring northern/southern limits
- to ensure that collected data, mostly from cattle farms, can be used for risk assessments on African horse sickness; methodologically, this is accomplished by comparing the species composition in cattle/horse farms.

The sampling protocol for biting midges was validated during the first VectorNet field season. It is based on experience gained from past network projects, e.g. the surveillance network for reovirus, bluetongue and African horse sickness in the Mediterranean basin (see MedReoNet, medreonet.cirad.fr) and national surveillance studies.

Field sampling methods

Study design

Surveys conducted by the VectorNet *Culicoides* group usually follow a longitudinal study design (collection of population dynamics data and assessment of peak abundance), but cross-sectional study designs for other purposes are also employed occasionally.

National surveillance datasets are typically longitudinal surveys, with repeated sampling at the same sites, as their purpose is to follow the *Culicoides* population dynamics and to determine the vector-free period. From these data, VectorNet extracts the yearly maximum abundance (the largest single night trap catch in the collecting period) as an index of species abundance. To obtain data that are comparable, the VectorNet *Culicoides* group has mostly carried out studies based on a longitudinal design. Climate, livestock density and land cover have been demonstrated as drivers of *Culicoides* seasonality and species composition [51,52]. An efficient strategy to assess abundance with a relatively small number of collection sites is to use latitudinal and altitudinal transects to sample different classes of livestock and land cover. The number of collections over time depends on the duration of the activity period. The number of collections necessary to estimate maximum abundance can also be reduced in areas where *Culicoides* dynamics have already been characterised with targeted trapping.

Cross-sectional surveys could be used to rapidly update the list of *Culicoides* species from a country (or region). This approach allows for the collection of *Culicoides* in many more sites than an approach based on a longitudinal design. Selection of sites could be based on agro-ecological classifications, on climate, land cover and livestock density. In places where detailed information on population dynamics is known, the cross-sectional surveys could be used to assess abundance at a large number of sites. Sampling should take place during a period when abundance peaks are expected.

Selection of sampling methods

To collect adult *Culicoides*, VectorNet uses only UV light traps in the field. During national surveillance activities conducted in several European countries, a variety of traps was used. In France and Italy, the Onderstepoort Veterinary Institute traps (OVI traps) were used; Spain used CDC miniature UV light traps (mini CDC trap); and in Germany, adapted BG Sentinel traps with UV light attachments were used (BG trap). The EU-funded concerted action MedReoNet compared five suction UV light traps: the OVI, the Rieb trap developed in France in the 1970s, the BG trap, the mini CDC trap, and the Pirbright trap were used for the collection of *Culicoides* midges under field conditions (e.g. different climatic conditions, different midge densities, different locations in South Africa, Germany, France and Spain [43,53,54]. The project concluded that the OVI traps usually capture more *Culicoides* compared with other traps, but no major differences were noted between traps with regard to the *Culicoides* composition. As the OVI trap is the most widespread trap, because it is the most sensitive due to its mesh system which excludes large insects and other bycatch, the OVI trap is also the reference trap for VectorNet *Culicoides* activities.

Selection of study sites and exact location of sampling sites

The species targeted for distribution mapping at the NUTS-3 level are: *Culicoides imicola*, *Culicoides obsoletus* s.l./*Culicoides scoticus*, *Culicoides dewulfi*, *Culicoides chiopterus*, *Culicoides pulicaris* s.l./*Culicoides lupicaris*, *Culicoides newsteadi* s.l., and *Culicoides punctatus* s.l. *Culicoides obsoletus* s.l., *Culicoides pulicaris* s.l., *Culicoides newsteadi* s.l. and *Culicoides punctatus* s.l.; taxa may include cryptic species.

All trapping should be carried out in farm, barn or stable environments, which are the primary focus of disease transmission in Europe. Sampling sites have to be stratified according to agro-ecological units (climate, soil, vegetation, agricultural practices) and land cover. Trapping sites should be selected according to a review of published and unpublished datasets to complement known distributions. A comparison between cattle/horse farms should be conducted. Horse farms are currently poorly surveyed. Instead of comprehensively mapping all horse farms, VectorNet experts propose to develop a conversion factor for vector abundance which can be derived from data collected on cattle farms: as a first step, researchers have to conduct a parallel trapping exercise (horse farm and cattle farm) in neighbouring locations. Vector abundance data from cattle farms can then be converted to horse farms, which allows researchers to model abundance on horse farms.

When combined with filters for known determinants of *Culicoides* distribution, this should highlight any gaps in our current knowledge of distribution. This approach should include the existing spatial distribution modelling outputs over a larger geographic space for presence/absence of *Culicoides* species where appropriate. A cut-off for probability of presence/absence should be used.

Within selected areas, farms have to be chosen according to a number of criteria:

- How representative are the farm's husbandry practices for the wider environment?
- How representative is the surrounding ecology for the wider environment?

Trapping should be performed next to specific hosts (cattle, sheep, goats, horses). It is important that farms where the traps are placed do not use unusually high levels of insecticides, either directly on the farms or in surrounding areas. Longitudinal studies should take place over a maximum of 30 weeks between April and October, but duration can be reduced if the *Culicoides* dynamics have already been characterised earlier; duration can also be shortened in high latitudes or altitudes.

Sampling methods: trapping

The most time-efficient and widespread method for the biting midges capture are UV light traps. VectorNet's trap of choice is the OVI trap (Figure 2):

- OVI light/suction traps are manufactured by the Onderstepoort Veterinary Institute (South Africa). The trap operates on a 12 volt car battery or a 220 volt connection and uses an 8W UV light to attract *Culicoides*.
- A mosquito net should be installed around the light to avoid larger insect entering the trap
- A beaker containing 100–200 mL water with one to two drops of detergent, added to reduce surface tension, is attached to the net funnel of the trap to collect the *Culicoides*.

At least one trap should be hung per farm.

Figure 2. OVI light/suction trap, manufactured by Onderstepoort Veterinary Institute, South Africa

Source: Venter et al. [43]

Specific guidelines for sample collection

- GPS locations must be recorded accurately for all farms. Placement of traps on farms: the location of traps in the area could follow climatic and/or latitude and/or altitude gradients, with a trade-off between practical issues and the sampling of different classes of livestock density and land cover.
- From April to October, collections should take place at least once every two weeks to determine the maximum abundance in absence of information on the *Culicoides* dynamics at the collection site. Frequency and interval can be adjusted, based on previous information on *Culicoides* dynamics.
- Traps should be installed at a standardised height: the UV tube should be 1.60 m above ground. Traps can run on a 220V mains power supply (preferred) or connected to a car battery. Traps should not be placed near contaminating lights on the farms as this can affect the attraction of biting midges and their capture. Most importantly, traps should be placed as close as possible to animals while at the same time remaining out of the animals' reach.
- 48-hour or 72-hour collections are recommended to avoid adverse meteorological conditions except where specific experimental procedures require otherwise.
- Sample collections should be carried out as soon as possible to prevent the degradation of the samples. To collect the samples, the content of the plastic jars should be filtered on a mesh of <1 mm. The mesh with the insects should be placed in a jar with ethanol 70% and labelled with pencil. Capture site and collection date should be recorded on the label.
- Both male and female insects should be counted.
- Presence/absence data obtained with other UV light/suction traps may be considered without any correction because these traps collect the same diversity as OVI traps although abundance could be reduced. However, data from Venter et al. [55] are available to evaluate the impact of the various trap types and models on data quality.

Field data and parameters to be recorded

- Location (georeferenced)
- Environment/land use
- Host species present
- Date, start and end dates of collection
- Weather parameters (temperature, rainfall) during the period of trap activity
- Person collecting traps
- Photograph of collection site (panoramic if possible)

Conservation of specimens

The samples of identified *Culicoides* must be labelled with the species name. Identified samples should be placed in a labelled vial filled to the top with ethanol 70%. Vials should be labelled with site code, date of capture, and an identification code.

Identification methods

Culicoides are initially identified under a stereomicroscope at species-group level according to wing pattern morphology, using main taxonomic keys for palaearctic *Culicoides* [56–60]. Specimens will then be identified down to species level. The number of individuals of each species will be counted, and males and females will be tabulated separately. In the case of females, their status (nulliparous, parous) may be identified.

Culicoides pulicaris s.l. and *Culicoides lupicaris* may be grouped into *Culicoides pulicaris* s.l./*Culicoides lupicaris*. *Culicoides obsoletus* s.l. and *Culicoides scoticus* include cryptic females, and they are classified as:

- *Culicoides obsoletus* s.l./*Culicoides scoticus* for females;
- As species for males, as they differ between *Culicoides obsoletus* s.l. and *Culicoides scoticus*.

Molecular assays should be used to separate *Culicoides obsoletus* s.s. and *Culicoides scoticus* females. Depending on available funding and identification interest, VectorNet performs molecular assays for some collections. Many methods exist to identify single specimens of midges (including multiplex PCR, MALDI-TOF MS or microarray assay). VectorNet uses the methodology described by Nolan et al. [61].

For larger collections, Mathieu et al. [60] have developed a molecular assay to simultaneous quantify the relative abundance of *Culicoides obsoletus s.s.* and *Culicoides scoticus*. However, due to the discovery of abundant cryptic diversity within the *Culicoides obsoletus* taxon in some regions (northern Europe), a DNA barcoding strategy on 30 individuals per collection date and site would be preferred to assess the relative abundance of both species in samples. This will also allow for an assessment of the relative abundance of cryptic species.

Quality control

Due to the huge number of *Culicoides* collected during each survey, it is not possible to carry out identification quality checks on a certain percentage of the samples. Thus, an external expert will be asked to evaluate the quality of morphological identification. Particular attention has to be given to unexpected records, or new records, for a given country. To ascertain the quality of the molecular identification, all molecular assays have to be centralised in a single laboratory.

Checklist

Table 10. Checklist for biting midges field specimen collections

Field data and parameters	*Culicoides* collection
Field	
Checked and functioning traps, including UV tubes	X
Charged batteries or extension cable	X
Plastic pot which fits into conical net	X
Site form and collection form	
Sample pot (one sample pot per trapping night)	X
Wash bottle with ethanol (> 70%)	X
Filter to separate insects from soapy water	X
Cardboard for labels	X
Pencil (do not use ink pen, it will be washed out by alcohol)	X
Plastic pipette	X
Meteorological data logger if required	
Rope	X

Field data and parameters	*Culicoides* collection
Swiss knife	X
Permanent marker	X
Heavy-duty adhesive tape	X
GPS or smartphone with geolocation function	X

4 Sampling methods for ticks

Introduction

Many European countries lack spatial and longitudinal data on vector distribution and activity, which makes continental-scale mapping difficult due to data gaps. There is a clear need for better surveillance of ticks across Europe to enable improved public and veterinary risk assessments and ensure better preparedness for tick-borne diseases. This requires standardised protocols for tick collection, tick identification and tick-borne pathogen detection. The Terrestrial Animal Health Code [62] of the World Organisation for Animal Health (OIE) lays down the general principles that should be followed in the surveillance of arthropod vectors of animal diseases. The most commonly used tick collection methods are based on manual collection from hosts or suitable habitats with dragging or flagging methods. This document outlines sampling protocols for the standardisation of tick collections.

The sampling protocols are presented by species groups 1) *Ixodes ricinus, Ixodes persulcatus* and *Dermacentor reticulatus*, 2) *Rhipicephalus* species and 3) *Hyalomma* species because the biology, hence sampling methods, are very different between these genera of tick species.

Objectives of field collection

The primary objective for sampling ticks is to collect presence/absence data with a geographical focus on the current known distribution limits of the targeted tick species. Data can be used to complete existing maps, feed models, and to inform risk assessments.

An established tick population in any given area is usually defined by the annually recurring presence of all active stages of the tick (larvae, nymphs, adults) during the months when ticks are usually active.

Field sampling methods

Study design

Ixodes and Dermacentor

These species are sampled using a cross-sectional study design and can easily be sampled both from hosts and vegetation. However, one of the problems with sampling from hosts is that it does not define the exact location where the tick originated from, but still provides important information. Owing to the fact that ticks of these genera can be easily collected from vegetation, it is better to conduct surveys of vegetation to better define the habitats. Any data collected on other species during host surveys should be recorded.

Rhipicephalus species

The best procedure is to survey the kennels (or the walls of homes where dogs are kept). To plan a survey, it is essential to collect information on the location of kennels (or homes with dogs) in all areas where *Rhipicephalus* is known or anticipated to be present. If *Rhipicephalus* is present, gap analysis techniques can be used to identify presence areas. Based on this information, a cross-sectional survey can be developed.

Hyalomma species

The best strategy for detecting and collecting *Hyalomma* is to survey large ungulates (both domestic and wild). Collections from the ground are difficult as there is no reproducible way to collect *Hyalomma marginatum* with a standard flagging method. One potentially effective collection method is to stamp heavily on the ground in areas where this tick species can be found and inspect the terrain. This procedure causes hidden ticks to emerge and search for hosts. This sampling method can be very time-consuming and is less efficient than using large animals as sentinels. To plan a survey, it is thus necessary to gather information on the location of host animals in areas where *Hyalomma* is known or anticipated to be present. If *Hyalomma* is present, gap analysis techniques can be used to identify presence areas. Based on this information, a cross-sectional survey can be developed.

Selection of study sites and exact location of sampling sites

Ixodes and Dermacentor species

Gap analysis techniques can be used to delineate suitable areas on a larger scale while habitat suitability models generate information on a local scale which can be helpful to plan and guide the survey. Once the sites have been selected, planning should focus on the activity period of the species.

The beginning and end of the tick activity season varies depending upon the latitude and the actual weather (temperature) situation. In Europe, *Ixodes ricinus* can, in general, be collected from ground vegetation throughout

most parts of the year, except in northern Europe. It should be noted, however, that this species tends to show reduced activity during the hot and dry months of the summer.

Further to the north in Europe – for instance in southern Sweden – this tick species is usually active from April or May until October or November. Variations in the duration of the activity period are due to the local weather situation. Further to the north in Sweden, in the Stockholm area (58–60 °N), host-seeking *Ixodes ricinus* larvae and nymphs are most active in May–June and August–September. In coastal northern Sweden, the tick season is much shorter so that active ticks may only be present from early July until mid-August.

Surveying should take place when the vegetation is relatively dry, not shortly after or during rainfall nor when rain is expected within the next two hours. If the humidity is relatively high at ground level, the activity of ticks increases with temperatures in the range of +5 °C to about +25 °C. *Ixodes ricinus* nymphs and adults are very rarely collected by blanket-dragging/flagging if the temperature at ground level is ≤5 °C. Particularly wet or very hot days will also drastically reduce the activity of the ticks and should be avoided (if possible) during surveys.

The most appropriate months for collecting *Ixodes ricinus* and *I. persulcatus* depends on the latitude and the actual temperature (weather) situation. The shortest collection period in Europe is in the north of Sweden, where sampling efforts may begin in late May and continue through August. Further to the south in Sweden, sampling may start already in April and continue throughout October.

Activity periods for adult *Dermacentor reticulatus* ticks are different and show a peak between March and May and again in October. It is very unlikely that immature stages of this tick species will be collected.

Rhipicephalus species

Sampling sites should be identified based on the presence of kennels, or any other places where dogs (i.e. hunting dogs) are kept. Examples of potentially productive sites are old buildings made up of adobe or clay in which cracks have formed that serve as shelters for tick. Pastures and vegetation along river courses are ideal for the collection of ticks of the *Rhipicephalus sanguineus* complex. Areas that are too dry are most probably not adequate habitats for these ticks. Areas with dense vegetation such as forests should be avoided, but this tick species may be present in coniferous forests, where its density is usually very low.

The optimal season for collections is spring (March–April). Collections can be complemented with surveys on dogs living in kennels. It is also necessary to complement indoor collections with the outdoor collection of ticks attached to shepherd and hunting dogs. This is done by examining potential tick hosts. In many parts of the Mediterranean region, adult ticks are active between late March and late May. This, however, depends on how long the winter has been and how fast spring is coming. For example, after the short winter of 2013–2014, tick activity in Spain started up to three weeks earlier than usual. The complete month of April and the first half of May are usually for the collection of adult *Rhipicephalus sanguineus* ticks.

Hyalomma species

Sampling sites should be identified based on the presence of large ungulates. Access to sample livestock species should be arranged by local veterinarians and farmers.

Sampling methods: *Ixodes* and *Dermacentor* species

Ixodes ricinus, *Ixodes persulcatus* and *Dermacentor reticulatus* will be sampled directly from the vegetation via dragging or flagging (depending on the height of the ground vegetation) using a 1 m^2 white cotton cloth. Using this material will ensure that any ticks found are detected and thus removed, and not accidentally transferred to further sampling sites. Depending on the size of the sampling area, a predetermined number of 5–10 m transects (spaced at least 10 m apart) will be surveyed at each site. After each transect, the cloth will be inspected for all active tick stages, which will be collected (except for larvae, which will be noted on the recording form but brushed off).

A standard approach involves the following techniques:

- During a number of site visits in the selected region, the availability of suitable habitats is assessed, logistic needs are determined, and the size and number of the municipalities to be surveyed are established.
- At each location, tick dragging/flagging is carried out; the choice of technique varies depending upon the species (*Ixodes ricinus*, *Ixodes persulcatus* and *Dermacentor reticulatus:* flagging or dragging also depends on the height of the ground vegetation).
- Usually, the cloth is dragged for between 5–25 meters. If abundance is to be measured, five metres is preferable in order to avoid tick drop-off.
- Tick flagging/dragging is repeated at each location at least 30 times (30 x 5 metres, with a 5-metre gap between drags/flags; if at least 50 nymphs or adults are collected, this should be sufficient to provide data on the abundance of questing ticks (± standard deviation - SD). If fewer than 50 ticks are present after 30 5-metre drags, an additional 30 drags are conducted. After that, no further surveying will be done. This information can be used to estimate the mean number of ticks per m^2 (and standard deviation).

- If no ticks are found after 60 drags, ticks are presumed absent at this site.
- There may be a temptation to conduct exhaustive surveying at one location. However, the aim of a study should be to survey a number of different locations, rather than investing all efforts at one, or just a few, locations.
- Ideally, each site will be visited at least once during spring or early summer. If ticks remain active, they should be surveyed again during summer and, if possible, also in autumn.

If a tick population has become established in a location or region just recently, one will presumably only encounter individual ticks in areas that are ideal tick habitats. During the first few years of their establishment, ticks are usually not encountered outside these ideal habitats. If we limit our reasoning to *Ixodes ricinus* and *Ixodes persulcatus* in northern Europe, the ideal vegetation type to search when looking for these species can be found on islands and in sheltered coastline locations with a relatively warm climate and a recently established deciduous broad-leaved woodland or in a mixed coniferous and deciduous broad-leaved woodland.

Of course, habitats searched for ticks should harbour (or should have harboured) at least one maintenance host (tick reproduction host). Such hosts may be roe deer, red deer, moose and other cervids, hares, cattle, or sheep, and in some regions even squirrels, brown rats or large ground-dwelling birds such as pheasants.

By contrast, *Dermacentor reticulatus,* whose range extends throughout most of central Europe, can be found in its favoured habitats: meadows with occasional shrubs and ecotones between lowland forest and meadows in lowland river valleys (often periodically inundated). Flagging or dragging through low vegetation and low shrubs is most desirable, and this does not necessarily mean forest/woodland edges as this tick is found also in grassland.

Sampling methods: *Rhipicephalus* species

It is not necessary to individually examine every single crack in the wall of kennels or private dog housings. Using a hairdryer works very well: the hot air activates the ticks makes them come out from cracks and other resting sites.

A standard approach involves the following techniques:

- During a number of site visits in the selected region, the availability of suitable habitats is assessed, logistic needs are determined, and the size and number of the municipalities to be surveyed are established.
- At each location, tick dragging/flagging is carried out; the choice of technique varies depending upon the species (flagging or dragging also depends on the height of the ground vegetation).
- Usually, the cloth is dragged for between 5–25 meters. If abundance is to be measured, five metres is preferable in order to avoid tick drop-off.
- Tick flagging/dragging is repeated at each location at least 30 times (30 x 5 metres, with a 5-metre gap between drags/flags; if at least 50 nymphs or adults are collected, this should be sufficient to provide data on the abundance of questing ticks (±SD). If fewer than 50 ticks are present after 30 5-metre drags, an additional 30 drags are conducted. After that, no further surveying will be done. This information can be used to estimate the mean number of ticks per m^2 (and standard deviation).
- If no ticks are found after 60 drags, ticks are presumed absent at this site.
- There may be a temptation to conduct exhaustive surveying at one location. However, the aim of a study should be to survey a number of different locations, rather than investing all efforts at one, or just a few, locations.
- Ideally, each site will be visited at least once during spring or early summer. If ticks remain active, they should be surveyed again during summer and, if possible, also in autumn.

Sampling methods: *Hyalomma* species

Tick specimens are frequently obtained from their hosts. However, it is seldom feasible to examine all livestock, companion animals or wild animals for ticks. Instead, it is often more efficient to examine a specific area on the host's body; this is particularly relevant for ticks because they are known to have sites where they prefer to feed (predilection sites). Ticks can be collected directly from livestock or companion animals but also from wild animals such as small mammals and migratory birds.

In order to survey for adult ticks, grazing livestock (used as sentinels) will be targeted. Ticks on the animals can be removed with tweezers or by hand. The activity period of adults of *Hyalomma marginatum* usually lasts from March to November. Adults feed mostly on cattle, horses, sheep, goats and wild ungulates.

Adult ticks prefer sites with thin skin like the dewlap, axillae, udder, groin and perineum. Cattle can be used as sentinel for monitoring the seasonal activity of ticks. Although biweekly sampling would be the best choice, monthly samplings may also accurately describe the seasonal activity of adult ticks. Ticks can be detected on host animals by palpation of predilection sites, including those covered by hair. Ticks can be removed by hand (field personnel should wear gloves if there is a risk to contract Crimean-Congo haemorrhagic fever virus (CCHFV) or with tweezers. However, special attention should be taken to remove the ticks intact. Immatures can be collected

directly from hunted wildlife such as hares, crows, and partridges. Hunted wildlife collection is usually not used by VectorNet, although hunters can be asked and encouraged to submit ticks.

A standard approach involves the following techniques to collect *Hyalomma marginatum* ticks:

- Sites should be selected based on proximity to suitable habitats and presence of free-grazing livestock.
- The best way of detecting the presence of *Hyalomma marginatum* is through the collection of adult ticks from cattle or horses.
- 10% of all livestock at a location (e.g. a farm or village) will be inspected for ticks.
- All detected specimens will be collected, regardless of tick species.
- In view of the risk of CCHFV infection, all ticks should be removed by a specialist, e.g. a veterinarian.
- The ticks will be stored in vials for later identification.

Field data and parameters to be recorded

The following field parameters should be recorded on site:

- Location (georeferenced)
- Environment/land use. Keeping records of land use should be compulsorily, while the type of vegetation can be obtained through georeferencing; standard denominations, probably GlobalCov, should be used.
- Habitat/vegetation (general height of the ground vegetation where dragging/flagging takes place)
- Type of site/host inspected
- Presence of any tick maintenance hosts; species and approximate numbers should be recorded
- Date, start and end times of sampling
- Temperature and relative humidity (%) at ground level and in the shade. Record climatic parameters at the start and end of the sampling period.
- Brief description of weather situation (calm, windy, clear sky, cloudy, etc.)
- Composition of the entomological team
- Duration of sampling and distance covered (e.g. number of drags/flags completed, etc.)
- Results: species, numbers of each tick stage (nymphs, adult females, adult males, approximate number of larvae)
- Observational data on presence of humans in the survey areas (for public health exposure measure) and information provided by locals
- Photograph of survey site (panoramic if possible)

Conservation of specimens

Any ticks collected should be stored in collection tubes in the field and labelled with a pencil: information should be written on a piece of card, which is placed inside the tube. Alternatively, heat-stable permanent pen can be used to write on the outside of the tube.

Long-term collections of ticks are stored wet in laboratory alcohol (= 95% ethyl alcohol) diluted with water and glycerol to reach the final concentrations of 75% ethanol and 5% glycerol. Even if the ethanol accidentally evaporates, glycerol will still keep the tick specimens moist. Also, the glycerol prevents the specimens from drying out when examined in the open. Colours of arthropods will fade in ethanol. This problem can be reduced if 1% percent of chloroform is added to the alcohol: the chloroform will prevent colours from fading; this is particularly useful if the specimens are photographed.

The labelling should be performed in accordance with information given in Walker [63]. The text on the label to be put inside the vial with the collected ticks should be written in pencil because pencil is resistant to the preservative fluid. The text should include name of the species, time and date of collection, the collector's name, method of collection (e.g. drag sampling or from live/dead host), scientific genus and species name of host, locality and country of collection. The site (location) should be given as the distance from a permanent reference point, and compass points should be added, e.g. '1.5 km WNW of Morup church, province of Halland, Sweden'. Latitude and longitude should also be recorded. The use of a village name is discouraged; instead VectorNet recommends that a building (e.g. church, castle) or a natural feature is used as a reference point. The universally accepted system is to use latitude and longitude to at least the nearest minute. These are read from a map of the area or from a GPS-enabled instrument (e.g. a smartphone).

Identification methods

Ticks will be stored and morphologically identified using appropriate keys.

- For *Ixodes* and *Dermacentor* species, the following key is recommended: Hillyard [64]
- For *Rhipicephalus* species, the following key is recommended: Walker et al. [65]
- For *Hyalomma* species, the following key is recommended: Apanaskevich and Horak [66]

Quality control

To ascertain the quality of the identifications, 10% of the collected samples will be verified by an external expert (either using morphological or non-morphological/molecular techniques).

Checklist

Table 11. Checklist for tick field specimen collections

Field data and parameters	All tick genera
Field	
GPS device or smartphone with geolocation function	X
1m² cotton cloth (plus spare for replacing flags + material to replace flags)	X
Fine-tip tweezers/forceps/fine-pointed brushes	X
Thermo-hygrometers	X
Screw-cap tubes	X
Pencil/pen	X
Card labels	X
Camera	X
Plastic bags for transporting used tick drags	
PPE	
Notebook	
Large plastic bags for tick-infested clothes and tick-infested tick drags/flags	

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