Universiteit Antwerpen	Evolutionary ecology group	Biodiv-Afreid project
	Biodiversity changes in African forests and Emerging Infectious Diseases: should we worry?	
	Protocol info: Evaluation of a protocol to link changes in biodiversity of the tropical rainforest to changes in small mammal communities and the pathogens they carry in the Democratic Republic of Congo	

Background

This document contains instructions to collect field data for the Biodiv-Afreid project (2020-2023) in Tshopo/BasUele province. More specifically, it will explain the selection of the field sites (i), set-up of the traps (ii) and the sampling/dissection protocols (iii) for small mammals.

Materials needed

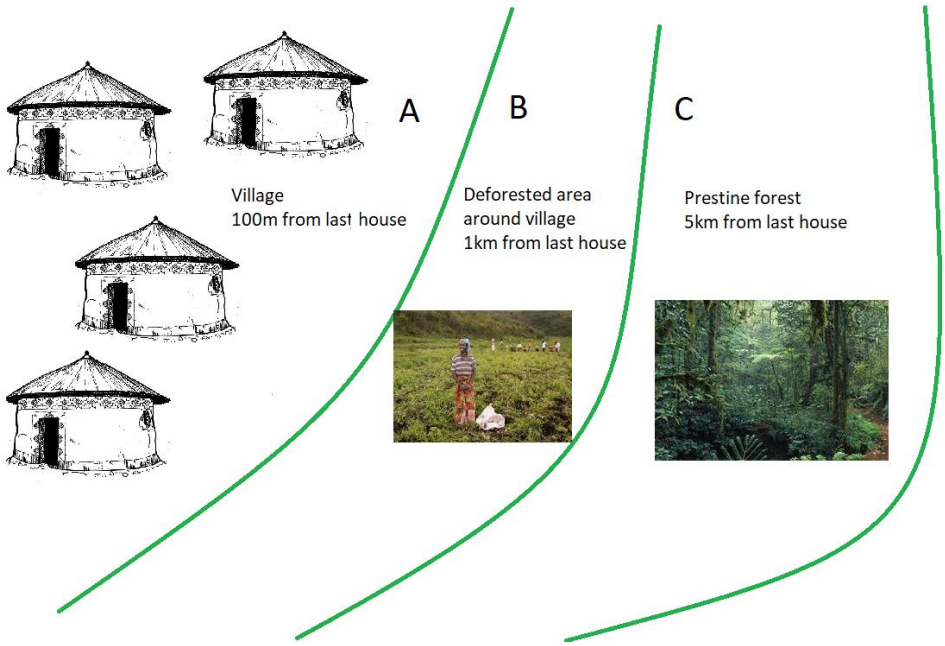
See attached excel list for all materials needed.

Procedures

Selection of the field sites

- The field sites will be setup in Aketi or Likati health zones (Bas-Uele, DRC) and Inkanamongo (Boende) where both Monkeypox and Ebola viral disease (two main targets of the project) have been noted the past 5 years. Another field study was performed in the same areas in 2017, which will facilitate the start of the field work (easier to convince the villagers) and allow comparison of biodiversity indices over years.

- We will assess the effect of habitat degradation on the small mammal community composition and on the pathogens they carry. We will therefore select 3 sites near one village (between 100-300 houses) where a variety of traps will be placed to collect small mammals. **Site A** is in the village (houses and fields) until 100m from the last house. **Site B** in a recently deforested area or secondary forest close to the village (1-5km), and **Site C** will be selected in pristine forest (>5km). The name of the field site is as follows Afreid_C, for the field site in pristine forest in area. This name will later be written and the general datafile.



- Before we start trapping, we will need to visit the local authorities to ask permission for entering the fields and forests. Make sure that the "ordre de mission" is already made in Kisangani.
- GPS coordinates will be taken in the centre of each Site (middle of a transect, see below) and four pictures (each wind direction to get an idea of the vegetation) are taken with a smartphone. All pictures are sent immediately on a dedicated whatsapp group including the name and GPS coordinates of the Site.

Biodiversity estimates by capture & sample collection for the study of pathogens

Set-up of traps: rodents/shrews

- A variety of traps will be placed in different transects to capture an as large as possible diversity of rodents and shrews. Transects will start 20-50 m from each other and not overlap. Mix traps between transects
    - **Sherman live traps** will be placed at 5m distances in four transects of 30 traps each.
    - **Snap traps** will be placed at 5m distances in 2 transects of 30 traps each
    - **Havahart traps** will be placed at 10m distances in 1 transects of 14 traps each
    - **Buckets for pitfalls** will be placed at 5m distances in 1 transects of 30 traps each
    - **Traditional traps** will be used to capture squirrels and other bigger rodents. These traps will not be placed in a fixed formation.
    - **Camera traps** (n=X) will be placed at 20m distance in 1 transect. They will be installed in the beginning of the sampling occasion and left undisturbed until the end, after which movies will be directly uploaded on a field computer.
- All traps are numbered (e.g. 1-120 for Sherman live traps) and used in transects lines according to number (e.g. line 1 will include the first 30 Sherman live traps). For labelling the traps, attach a piece of scotch to the traps and write down the number with a permanent marker.

- All traps are placed late afternoon, and collected in the morning, between 7AM (allowing "dawn-active" animals to get in the traps") and 9AM (after 9AM animals die of heat). They will be baited with a mixture of palm nut pulp, nuts and fish (depending on the availability).
- GPS coordinates are taken for all traps. If this is too labour intensive, take the beginning and end point of each transect line.
- Early morning next day: collect traps with animals inside using gloves, and take them to the place where you will dissect. Leave other traps on the field in case someone can guard them during the day. Close those traps, otherwise diurnal species will enter and die from the heat.
- If more than **40** rodents are captured that day, select 40 traps at random for dissections. The remaining animals will be marked and released after taking a blood, urine and saliva sample.
- Perform the dissections (see below)
- After dissections, clean the traps that had animals inside with 5% Dettol/Virkon/Bleach
- Place traps back in the grid, re-bait all traps (also the ones that no animal came in the previous night and that still contains bait: it doesn't smell strongly anymore).
- Traps will remain on the same location for **10**consecutive trapping nights and will be placed in the three sites at the same time to prevent temporal bias in field collections
- For each trapping occasion (i.e. three trapping nights), keep track of the number of traps used. Always try to work with the same number of traps to be as standardized as possible, but over time traps will disappear (stolen, broken, etc…).
- Traps will be replaced to three other locations surrounding the village so that we will have 3 replicates of each habitat.
- **<u>Set-up of traps: bats</u>**

Bags for bats
Handmade drawstring bags in water repellent fabric with one eyelet/metal grommet --> tested for 2 years, increase +++ the chance to get urine. Two different sizes.

Trap guidelines
Sampling will focus on catch and release methods with non-invasive sampling with harp traps and mist nets (9mx2.4mx2cm) set 1.5m from the ground to 7.5m high under the canopy. In open areas, we will use nets set 1m from the ground to 3.5m high. Traps will be installed around 4:30pm-5:00pm and opened at the beginning of the catch session. The catch survey will be carried out throughout the night (6:00 p.m. to 6:00 a.m.). Information on capture sites (GPS coordinates, ecological environment) will be recorded.

- Potential locations for mist-netting and harp-trapping will be surveyed during the day. Locations will be chosen based on their accessibility, the number of probable flight lines observed by the trees present and the level of clutter by vegetation. Potential travel corridors such as streams or logging trails typically are the most effective places to net. Place nets perpendicular across the corridor. Nets should fill the corridor from side to side and from stream (or ground) level up to the overhanging canopy.
- Time of trap opening and closing, capture and net location will be recorded. Weather conditions such as temperature and relative humidity will be recorded for each trap night.
- Depending on the number of people available/night, we will place 2 harp traps and 2 mist nets (minimum 4 people)/night.
- Immediately after mist-net opening at sunset, check if any birds have been caught.
- Each mist net should be checked every 10-15 minutes. Prompt removal from the net decreases stress on the bat and potential for the bat to escape (MacCarthy et al. 2006). Monitoring the net also allows the researcher to assess the effectiveness of their net placement (i.e., if bats are active near the nets but avoiding capture); this may allow for adjustments that will increase netting success on subsequent nights. There should be no disturbance near the nets, other than to check nets and remove bats.

<u>Dissection protocol: Rodents/shrews/squirrels & bats</u>

Before you start make sure all your material is ready

- Select dissection place outside the sight of villagers, make sure that no one comes into a 20m perimeter from the dissection place
- Order all traps according to number, take a picture of all the traps collected that day
- Prepare dissection table, garbage bag
- Label tubes and filter paper (use the field ID, eg. BDA_x) Place them on tube holders already in the right order.
    1. Spleen (DNA/RNA shield, 700µl)
    2. Faeces + colon (DNA/RNA shield, only if present, 700µl)
    3. Kidney + liver (DNA/RNA shield,850µl)
    4. Lungs (DNA/RNA shield, 700µl)
    5. Testes and epididymis (700µl DNA/RNA shield)
    6. Tongue, nose (95-99%ethanol, for MPXV detection)
    7. ectoparasites (95-99%ethanol)
    8. Blood and/or Urine Whatmann filter paper

9. Eyes (10% formol)
10. carcasses (ID specimen) 10%formol
11. Aliquot of all organs in tube with 10%formol for histopathology in 10ml falcon tube

Info on DNA/RNA shield: https://www.zymoresearch.com/collections/dna-rna-shield

- Black rubber mat
- forceps and scissors
- Spraying bottle with 10% bleach for cleaning (put a hole in the lid)
- Beaker with 10% bleach (buy bleach Jik in any shop in town)
- Beaker with water
- Beaker with 70% ethanol (buy ethanol in lab shop in town)
- Big bucket with lid, with 10% formol
- Field lists + pencil (always write with pencil)
- Pesola (for weighing)
- Ruler
- Pins
- Tagging gun + plastics
- Capillaries + needles
- Lots of tissue paper for cleaning
- Waste bag

Before you start put on PPE

- 2x of gloves
- Surgical gown
- FFFP3 masks, goggles, face shield (or respiratory hood)

Start the process

- Clean your rubber mat with 10% Bleach (javel) and let it dry and take a tissue.
- Take a random trap with animal inside and write:
    - Identity number code (ID) according to pre-printed labels (BDA276500…)
    - Trap name (e.g. sh_1 sherman live trap 1)
    - Site name
    - Date
- Take animal from trap with a cotton bag, and place it in a zip lock bag with cotton drenched in chloroform or equivalent. Wait until it falls asleep and dies. Take the animal out of the bag put it on the tissue, and verify if it is dead.
- Weigh the animal with a pesola
- Measure the length of the animal (from the tip of its nose to its anus – stretch the animal a bit) with a ruler by placing pins into the rubber mat
- Measure the tail length of the animal with a ruler and pins.
- Determine the species, sex and reproductive activity (yes/no)

Dissection

- Perform the previous steps quickly so you start dissection as soon after death as possible (otherwise the blood might coagulate).

- Collect **ectoparasites** and store in 95-99% ethanol
- Spray ethanol on the abdomen, lift the skin + muscle layer with a forceps and cut a small hole. Then cut further longitudinally through the centre of the belly. Also cut through the rib cage. Cut laterally through the diaphragm.
- Take the following samples (in this order):
    - **Faeces** found are put in DNA/RNA shield
    - **Blood**. Use a syringe to suck out the blood from the heart (200ml can be obtained). Put the blood on the filter paper on fill at least 3 of the 5 pre-punched circle (make sure they are completely filled).
    - One **Kidney** and a small piece of the **liver** are stored in DNA/RNA shield
    - **Lungs** are stored in DNA/RNA later
    - **Spleen** is stored in DNA/RNA shield
    - **Colon** in DNA/RNA shield
- Take out the **eyes** with a forceps and store in 10%formol
- Cut piece of the **nose** and **tongue** and store in 95-99% ethanol

- For each locality: keep the carcass of 1 specimen of each species. Write on the paper label of the correct ID the locality, species, date and your name (coll) and attach this paper on the foot of the animal with a tagging gun. Put the carcass in a 10L bucket with 10% formaline. Throw away the rest of the carcasses and have them burned in a hole together with the rest of the waste.

- Prepare for the next dissection
    - Clean your tools in 10% bleach, water and 70% ethanol (in this order).
    - Clean your rubber mat with 10% bleach thoroughly.
    - Check if your gloves have remained clean. Otherwise change them

Capture-release (If more than 40 rodents are captured)

- Put animal in a bag and knot the bag
- Weight the animal in a ziplock bag
- Take out the animal carefully with cut proof gloves
- Note sex, species, and reproductive status
- Take samples
    - **Blood**. Use a microcapillary tube to draw the blood from retro orbital sinus. Put the blood on the filter paper on fill at least 3 of the 5 pre-punched circle (make sure they are completely filled).
    - Collect **oral swab** by forcing the animal to bite on whatman filter paper
    - **Anal swab** by gently insert one sterile swab tip at a time into the animal's rectum in DNA/RNA shield. Alternatively, collect fresh faeces
    - Cut part of first upper **toe** and store in 95-99% ethanol
- Put animal back into the trap and release at location from where it was captured
- Throw the bag away

After the dissection of rodents

- Throw away second pair of gloves
- Decontaminate the bench, chairs, tubes and other material on the table
- Spray surgical coat with 10% bleach and let it dry in the sun/or throw away if dirty (e.g. throw away the coat from the person who did the dissections)
- Remove face mask and goggles
- Remove first pair of gloves
- Leave the blood on filter paper to dry outside of the sun. After, transfer them to a small ziplock bag and put desiccant bag inside. Do not spray this bag with 10%bleach for decontamination (otherwise the filterpaper might become wet, if the bags are still wet).
- Keep the tubes with samples in boxes or if they are full in zip-lock bags. All DNA/RNA shield and RNA later samples are preferably stored in cooling boxes (even samples stored in DNA/RNA shield only remain good for 7 days at room temperature!). If cooling boxes are not available, store the samples in cool place (water, under ground...). Back in the lab samples are stored in –20 freezer, ethanol samples on room temperature.
- Take a picture of the field list and send with the pictures of the animals of that they to dedicated whatsapp group (if internet is available).

<u>Sampling protocol: bats</u>

All staff handling bats should be vaccinated for rabies.

Best to be 5 people minimum/night.

- A team-member is assigned to record and write data (writer)
- A team-member is assigned to handle and process sampling on each bat (manipulator)
- A team-member assist the manipulator for samples collection (assistant)
- One/two team—members is/are checking the traps every 15 min (animal collectors).

The organisation will depend on the catch rhythm. For example, if several bats (> 10-15) are trapped at the same time in a mist net, and/or if no other bats are waiting to be processed, everyone can help to remove the bats or checking the other traps.

COVID19- specific behaviour
Risk to transmit SARS-COV-2 from humans to bats. Make sure to always wear a mask and clean gloves when manipulating bats. If the FFP3 are with exhalation valve, it protects the person who wear the mask, but not the animals that are handled. Wear a surgical mask below the FFP3 with exhalation valve

Minimum PPE required for handling, capturing, or sampling live bats
The minimum PPE for handling bats during capture and sampling includes:

- Nitrile gloves x2 + leather/rubber gloves
- Disposable Tyvek gowns
- FFP3 masks
- Face shield

First aid protocol for a bite, scratch, or needlestick

1. The injured person must notify other research staff.
2. The bite, scratch, or needlestick site should be washed well with water and betadine (povidone-iodine) or benzalkonium chloride (this is known to kill rabies virus) for a full 15 minutes. It is recommended that benzalkonium chloride be kept readily available in a first aid kit for such purposes.
3. If the injury (bite or scratch) is from a bat, the post-exposure rabies vaccination should be obtained as soon as possible. It is recommended that the field team develop a postexposure vaccination plan with their physician prior to fieldwork if working in a remote location so that a booster dose can be administered soon after exposure. Otherwise, exposed personnel should immediately report to a medical clinic for administration of the booster doses.

Remove bats from traps

- Remove the bat from the mist-net by removing the animal starting from the head to the wings. If the removal is complicated and takes too long (>10 min) or if the bat is strangled by the net, one could cut one mesh with small scissors.
- When bats are removed from the trap, hydrate them with a syringe without needle (without touching the mouth to not contaminate the syringe). It will increase the chance to get urine.
- Place one animal /bag. If possible, for a same capture session, bags will not be re-used to prevent cross-contamination between bat individuals. The next day, bags will be washed by hands with 10% bleach.
- People in charge of removing the bats will be each equipped with one collar with 2-3 carabiners on each to hang the bats around their neck.
- On arrival at the processing table, hang the bat bags with the carabiners on a rope stretched between 2 trees.
- Wait about 20min before processing the bats to increase the chance to get urine.

Before you start make sure all your material is ready

- Select a processing place at least 30 m away from each trap. Outside the sight of villagers, make sure that no one comes into a 20m perimeter from the dissection place.
- Eppendorf tubes for oral swabs and wing punch will be pre-labelled with the type of sample and the animal ID (ex: “Oral Swab- AFREID-XX”). For others (feces, urine, ectoparasites), tubes will be only labelled with the sample type (“Feces”,”Urine”) and the animal ID will be added during the sample collection (ex: “Feces- AFREID-**XX**”).
- Prepare the vials to collect samples

-Eppendorf tubes with 300 µL of DNA/RNA shield for urine
-Eppendorf tubes with 300 µL of DNA/RNA shield for oral swabs
-Eppendorf tubes with 300 µL of DNA/RNA shield tube for feces
-Eppendorf tubes with 1ml of ethanol 95% for wing punches
-Eppendorf tubes with 1ml of ethanol 95% for ectoparasites

→ Prepare tube for oral swabs and wing punch on a 96-microtube 1.5mL rack.

→ Display the tubes for feces, urine and ectoparasites in a box compartmented with carboard pieces.
→ Add these other tubes on the rack with the other type of samples when processing the bat.

- Prepare the processing table

-Field lists + pencil
-2 garbage bags (one for trash, one for dirty bat bags)
-Headlights with batteries
-Squirt bottle with 70% ethanol for cleaning
-Eppendorf with 70% ethanol (to clean tweezers)
-Small battery-operated scale/tabletop scale (and Pesola) (+batteries)
-Small plastic box to use as a container to weight bats
-pipette 20µL
-20µL filtered tips.
- 2-3 tweezers
-swabs
-calliper
-small cutting board
- 3-4 mm puncher
-A small cutting plier
-Heparinized haematocrit capillaries
-27 G and 30 G needles
- White Posca Water Based, Non-Toxic Paint Pen Marker
-Tissue papers for cleaning

The bags containing bats will be processed in the order of capture. An exception will be given to bats which appeared weak or were not extracted from the nets in a short amount of time due to entanglement in the net and for mothers with babies.

Animals that do not survive capture will be sampled for organs as described in the dissection protocol above (cf Rodents, squirrels, and shrews). Their carcasses will be preserved in formalin (10%) and stored at Kisangani University. In dead animals, eventual foetuses will be collected as separate specimens to test for vertical transmission of pathogens.

- All individual bats will be processed to record basic data (i.e., sex, age, reproductive state, forearm length, and mass) and released at the site of capture. If newborns (naked skin, eyes closed, sucking reflex) are found attached to bats, the pup will not be processed for biological samples. For fruit bats mothers, right after processing, they will be fed with either small pieces of fruit (banana) with tweezers or at least with sugar water before release.
- Tare the weight scale with the bat + bag. Individual bats will be weighed using a small, battery operated scale. Tare the weight scale with a plastic box on it. Place the bag with the bat on the plastic box on the weighing scale. After removing the bat and bags, record the mass. *NB: For big bats, the use of a pesola may be necessary.*

- Carefully remove the bats from the bag, handle it gently and try to take it out of the bag to collect **urine** with a pipette and a 20uL tip. In cases where urine cannot be collected with a pipette, urogenital swab could be used.
- Collect **feces** directly on the bat and check in the bag with clean tweezers and placed into an Eppendorf tube containing DNA/RNA shield.
- Species identification will be done using the taxonomic key in "The bats of Congo and of Rwanda and Burundi Revisited (*Mammalia: Chiroptera*), 2017; European Journal of Taxonomy, "Bats of Southern and Central Africa: A biogeographic and taxonomic synthesis, second edition" by Monadjem and al, 2020 (or first edition from 2010) and "Keys to the Bats (Mammalia: Chiroptera) of East Africa" by Patterson & Webala, 2012 which are reliable sources to identify bats in DRC.
- The sex of the bat will be identified by assessing its genitals. The reproductive status was recorded by assessing the presence of the penis, testes, and nipples of the bat. Female bats with hairless or 'chewed' nipples were recorded as bred before since it indicates that the bat had offspring. Reproductive status: female= NP: non-pregnant, P: pregnant, L: lactating/ Male= testis, epididymis
- The dental wear will be an additional indicator to check the age of the bat. U0: translucent teeth, U1: normal teeth, U2: used teeth.
- **Salivary swab** samples will be collected by using cotton buds which will be swabbed in the mouth of the bat (salivary glands, left and right sides). The salivary swab will be then cut in half and placed into an Eppendorf tube containing DNA/RNA shield.
- The age of the bat (i.e., juvenile or adult), will be assessed by checking the ossification of the finger joints (bone fusion) with a light. If the joints were transparent and elongated after stretching the wing out and shining a light upon it, the bat would be considered as a juvenile. NB: newborn, JUV: juvenile, AD: adult.
- Length of the forearm (maximum length measured when the wing is folded, between the elbow and the wrist) will be measured with a calliper (in cm).
- A **biopsy wing punch** of 3mm in size will be collected by spreading out the bat wing until the skin is stretched out. The biopsy punch is pressed between the veins found in the lower half of the wing between the third and fourth finger, while taking care that the biopsy punch does not cross any large veins. The wing punch collected is then stored in an Eppendorf tube with 70% ethanol. Two wing punches will be collected in a same tube. If a bat wing is damaged with holes in it prior the wingpunch collection, only one wingpunch will be collected.
- **Ectoparasites** may be occasionally collected on bats with a tweezers and place in an Eppendorf tube with 300 µL of ethanol 70%.
- **Whole blood** will be collected by venipuncture of the propatagial or brachial vein and blood drops were directly transferred onto Whatman ® filter paper. The blood volume of bats comprises ~10% of the total body weight. As in other species, it is considered safe to remove a blood sample equivalent to 1% of the total body weight. The maximum volume of blood that can be collected on 10g-100g bats is 60µL. Manually restrain bats during blood collection. For larger bats, two or preferably three people are required for these manipulations: one person to safely restrain the bat, one to take samples, and a third to manage the tubes and record samples. Smaller insectivorous bats may be restrained and sampled by a single person. The person restraining the bat is responsible for monitoring respiration and communicating respiratory status appropriately.

Recommended venipuncture sites include the propatagial (cephalic) vein, the uropatagial (saphenous) vein, or the brachial vein. The selected area will be cleaned with ethanol 70% before puncture. Use a non-heparinized syringe with a 30-gauge needle to puncture the vessel in small bats (between 10 to 30g; 27G for bats >30 g like Pteropodidae and Hipposideridae). Whole blood will be collected into a microhematocrit capillary tube directly from the puncture site with a 10° angle without touching the animal skin. Compress the puncture area with a dry cotton for 1 min. Blood will be immediately transferred on a Whatman® filter paper. Let it dry. After, transfer them to a small ziplock bag and put desiccant bag inside. Do not spray this bag with 10%bleach for decontamination (otherwise the filterpaper might become wet, if the bags are still wet).

- Finally, photographs will be taken of the bat including frontal view and side view which showed the details of the ears and any facial features (example: nose of Rhinolophidae and Rhinonycteridae bats; tails) and a full body view. After processing and identification, the bat will be temporary marked with a white dot on the head (Posca marker) and released.

For specimens not identified *in situ* using taxonomic keys, one male and one female of the species were sacrificed for subsequent determination based on craniometry and DNA barcoding. All procedures for handling live Chiroptera were in accordance with the American Society of Mammalogists guidelines (Gannon, et al., 2007; Sikes, et al., 2011).

After each animal, tweezers will be cleaned with ethanol 70%. Before handling another bat for sampling collection, the bat manipulator will clean his/her gloves with ethanol 70% before processing another animal.

After the night

- Throw away second pair of gloves
- Decontaminate the bench, chairs, tubes and other material on the table
- Spray surgical coat with 10% bleach and let it dry in the sun/or throw away if dirty (e.g. throw away the coat from the person who did the dissections)
- Remove face mask and goggles
- Remove first pair of gloves
- Keep the tubes with samples in boxes or if they are full in zip-lock bags. All DNA/RNA shield and RNA later samples are preferably stored in cooling boxes (even samples stored in DNA/RNA shield only remain good for 7 days at room temperature!). If cooling boxes are not available, store the samples in cool place (water, under ground...). Back in the lab samples are stored in –20 freezer, ethanol samples on room temperature.
- Take a picture of the field list and send with the pictures of the animals of that they to dedicated whatsapp group (if internet is available).

Additional bat sampling

If bat roosting sites are easily accessible during the day, we will collect fresh droppings under roosting sites. The droppings will be collected on plastic film/trash bags using a pipette or a swab. Samples will be conserved in Eppendorf tubes with 300 µL of DNA/RNA shield.

Indirect biodiversity estimates (summary of literature survey by Daan Lemmens 2020-21)

1. eDNA in the soil

To reliably infer the animal biodiversity, we will sample along a grid following rule of thumb: 'the distance between the sampling points in a grid or transect should be smaller than the distance required to represent the variability in the study area'.

We will take soil samples of 10-15g of wet soil for a consistent and representative result, especially when researching the diversity of mammals. Because the adsorption capacity of the soil is influenced by the pH value, the pH of soil adjacent to the individual sample point will be measured.

We will use a dedicated table to determine how many samples are required to achieve a predetermined relative error at a selected confidence level.

The depth at which we will take samples from the soil surface layer (0-15cm depth). Since the more DNA retaining clay layers will be sampled, we will avoid sandy places will be avoided, sampling the soil top layer is the most efficient to collect as much eDNA as possible.

We will combine the individual soil samples to assemble eDNA sampled. We will grind the individual samples to a grain size of <0.5 mm in subsample of <1 g. This has to be done while avoiding possible contamination of the soil by the grinding material.

We will no dry the samples and store them at a temperature of -20°C for a maximum of one month (this is tricky, perhaps we should fix the DNA fy fixing it in the soils samples with ethanol?)

2. Camera trap deployment

As we are only concerned with the biodiversity inventory, and not with the estimations of population sizes/densities, we will place most (number remains to be defined) available camera-traps at different locations 30-60cm above the ground, at places where we see animals' tracks. A smaller set of camera-traps will be placed on tree trunks (between 8-13m high) to document the diversity of arboreal species (e.g., squirrels and primates for our preliminary faunal inventory.

3. Acoustic sound estimates

We will use six AudioMoth sensors, a customizable open acoustic monitoring device (58 x 48 x 18 mm^3 with a single 4.72 x 3.76 mm^2 inbuilt microphone and a weight of 80 g including batteries) with a sample resolution of 16-bit.

Method: Each recorder will be deployed on a tree in a grip-sealed bag using a cable tie, and positioned at a height of 10 m to reduce acoustic divergences between the understory and the canopy layers. We will record one minute every ten minutes, day and night. The recording parameters for each site will be homogenized. This will generate (2 sites × 3 recorders × 6 recordings/h × 24 h × 21 days). According to the Nyquist ratio, the sampling rate should be at least twice the highest frequency that expects to be recorded to ensure a proper recording of the signal. We will obtain all the recordings at a sampling rate of 48 kHz with low audio gain to tone down the background noise. This should result in a full-spectrum sound stored as a waveform audio file.

https://www.sciencedirect.com/science/article/pii/S2468067219300306

Fly sampling protocol (RKI)

1. Why do we collect flies?

Flies are perfect field assistants, because they feed on carrion and feces, and they find every single carcass and every pile of feces. In flies we can detect the species they have been feeding on and also the pathogens present on a carcass or shed in feces. Flies can therefore be used for biomonitoring and outbreak monitoring.

2. How to collect flies

Materials

- Nets
- Prepared synthetic attractant ("bait") or chicken or fish meat that gets nice and stinky.
- Plastic container for bait and piece of mesh with rubber band to stop flies from getting to bait
- Cover for plastic containers
- Ether/Isofluran or 90% alcohol to kill flies
- Cotton
- 50 ml falcon tube to kill flies.
- 50ml Falcons with silica gel and cotton to store flies
- Bleach to sterilize nets

Preparation

Flies are caught with self-made pyramide fly nets and artificial attractant or piece of meat ("bait").

To make your trap, you can use mosquito netting or other fabric that is fine enough to trap flies, but make sure that the material is not coated in insecticides or insect repellant. Cut three triangles of netting approximately 50cm long on each side and sew along the edges to make a pyramid that is open on the bottom. At each of the corners, it is nice to sew on a small loop of sturdier fabric that can be used to stake the corners down to the ground and to hang the net from a tree.

The synthetic bait needs to be prepared in advance:

- Add one bag of bait powder to 1L of water
- Put the bottle in the sun for at least 3 days (better 1 week)
- The sun and warmth activate the bait until it develops a strong smell which is irresistible for flies
- After using the nets or tubes to kill the flies, please sterilize them in a bleach/water mix by soaking overnight
-

Procedure

- Fix the top of the net to a branch of a smaller tree or bush
- Fix the other lashes to sticks in the ground
- Leave about 5cm space between the ground and the net, for flies to enter
- Place a plastic container under the net and fill in with ca. 100 ml bait
- Cover the plastic container with a small piece of netting and fix with a rubber band
- Wait for 30 minutes or until at least 20 flies have entered the net.
- Put a bit of ether/isofluran or alcohol on a piece of cotton and put it in a 50ml Falcon
- Wearing gloves, chase the flies to the top of the net, so that they can't escape
- Take the open Falcon with ether/isoflurane-cotton and move it around under the net to collect the flies (the flies will fall into the tubes once unconscious from the vapor)
- Randomly select 20-30 per sampling point and fill into a 15ml Falcon tube

FIELDWORK- BAT COLLECTION FORM example

Place: Trap type : Opening time: T° (°C) :

Date: Habitat: Closure time: Humidity (%):

					Sex		Age			Samples collection						
Sample ID	Species	Capture time	Start time process	Weight (g)	M/F	Reprod. status	Dental wear	Bone fusion	Forearm length (cm)	Urine	Feces	Oral swab	Wing punch	Blood	End time process	Remarks
DRC-A-1																
DRC-A-2																
DRC-A-3																
DRC-A-4																
DRC-A-5																
DRC-A-6																
DRC-A-7																
DRC-A-8																
DRC-A-9																
DRC-A-10																
DRC-A-11																
DRC-A-12																
DRC-A-13																
DRC-A-14																
DRC-A-15																
DRC-A-16																
DRC-A-17																