

Protocols for field and laboratory rodent studies

Vincent Herbreteau, Sathaporn Jittapalapong, Worawut Rerkamnuaychoke, Yannick Chaval, Jean-François Cosson, Serge Morand

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Editors: Vincent Herbreteau, Sathaporn Jittapalapong, Worawut Rerkamnuaychoke, Yannick Chaval, Jean-François Cosson and Serge Morand

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Editors

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Front cover picture: *Bandicota indica*, Kalasin, Thailand, © Herbreteau V., 2006

Back cover picture: Tracks of *Bandicota indica*, Kalasin, Thailand, © Herbreteau V., 2006

Foreword

"Protocols for field and laboratory rodent studies" is a publication generated by the **CERoPath** project (**C**ommunity **E**cology of **Ro**dents and their **Path**ogens in a changing environment, www.ceropath.org) funded by the French Ministry of Research from 2008 to 2011 (French ANR Biodiversity ANR 07 BDIV 012). This book was written and compiled by the various partners directly involved either in the trapping of rodents or in the analyses of samples for rodent or macro- and micro-parasites identification.

These protocols summarize the techniques that we developed while studying wild rodents, during almost ten years. It aims to **providing accurate and practical knowledge and advice for future research on rodents**, keeping in mind that this information would have been precious to all of us when we started our studies.

Requirements for trapping and manipulating rodents differ regarding the objectives of the studies. Some observations can be made without capturing animals: by identification of foot tracks, feces, damage to crops or objects, burrowing activity, by using camera or video traps, by recording acoustic signatures with ultrasounds detectors, etc. These techniques have enabled identification of unknown species and allowed collection of information on the ecology of species (seasonal migrations, daily ranges, density). However trapping may be necessary in some cases. First to describe a new species, some invasive samples (such as hairs) can be taken for genetic analysis and some measurements can be performed on a live rodent lightly anaesthetized. Second, for a medical survey, a small volume of blood can be obtained using methods that minimize stress and pain for the animal. Third, longitudinal ecological studies may also require live trapping to mark (ear tag, toe clip, etc.) or follow (radio transmitter, GPS collar) animals. We placed emphasis on providing colorful illustrations for a better interpretation. Consequently, these protocols show some pictures of dead animals since sacrifice is necessary for some specific research purposes. Nevertheless we tried to present alternatives to sacrifice wherever possible.

We have learnt everyday in the field and these protocols have evolved with our different research projects and the experience we have gained from various collaborations. The list of co-authors and institutions is long but everyone has played a part in this publication.

Some South-East Asian rodents (Photos: Herbreteau V.)



Bandicota indica



Berylmys berdmorei



Chiropodomys gliroides



Leopoldamys neilli



Maxomys surifer



Mus cookii

Background: CERoPath project



CERoPath (**C**ommunity **E**cology of **Ro**dents and their **Path**ogens in a changing environment, www.ceropath.org) is a 4-year (2008-2011) project funded by the French Ministry of Research (French ANR Biodiversity ANR 07 BDIV 012). It involves scientists from France, Thailand, Laos, Cambodia and Finland.

The CERoPath project investigates the structure of murine rodent communities, their pathogens (microparasites) and their macroparasites (helminths and arthropods) in three South-East Asian countries, Cambodia, Lao P.D.R. and Thailand. South-East Asia is characterized by a high biodiversity (hotspot), threatened by rapid environmental changes under the pressure of economic development and insertion into the global economy. In South-East Asia, rodents are hosts and vectors of several agents causing diseases including leptospirosis, scrub typhus, viral hemorrhagic fevers and plague in humans and trypanosomiasis in livestock.

Seven sampling sites (Fig. 1) were selected along the Mekong River in:

- Thailand: Nan, Loei and Buriram provinces,
- Lao PDR: Luang Prabang and Champasak provinces (Pakse),
- Cambodia: Mondolkiri and Krong Preah Sihanouk provinces (Veal Renh).

These locations represent a gradient of deforestation and human pressure, from highly forested areas with low human density, to poorly forested areas with high human density.

Rodents were sampled twice in each site: during the dry season (November-May) and during the rainy season (June-October).

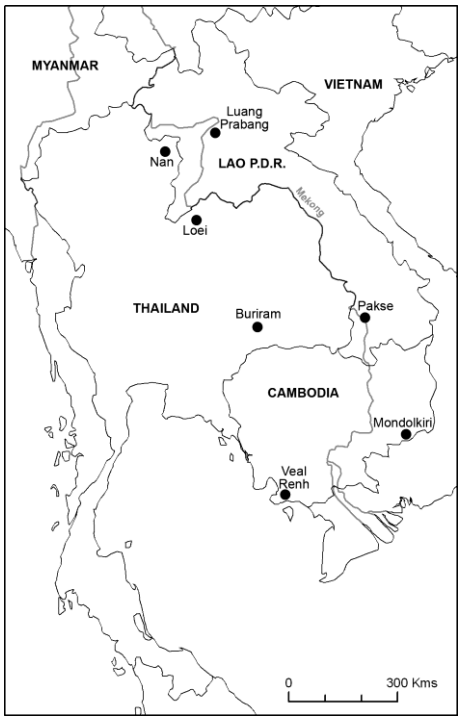


Figure 1: Location of the seven CERoPath sampling sites in South-East Asia

This project is based on the concepts and methods of evolutionary ecology and aims to:

- characterize the likely processes that contribute to the observed patterns of host-pathogen assemblages, taking into account the ecological environment of rodent communities (using remote sensing and geographic information systems) and the evolutionary history of the interacting species (using population genetics, molecular epidemiology and community phylogenetics);

- build projections of host and pathogen community change associated with ecological change, through the help of spatial analysis.

Consequently, **this book should be considered with the background of the CERoPath project**, in the geographical context of South-East Asia. Nevertheless, authors have tried to make information and recommendations as general as possible to help further studies of rodents and rodent-borne diseases in other contexts.

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1. Trapping rodents

(All authors)

1.1. Trapping techniques

Different methods have been used for generations for catching rodents showing the great ingenuity of rural populations and their knowledge of animal behavior. Traditional techniques are quite similar in different countries. Recently the commercialization of trapping devices tends to generalize the use of a few techniques to the detriment of ancestral practices.

A variety of traps have been created to catch rodents. The most common are:

- **Live-traps**: cage-traps (usually locally made, but also commercialized ones such as Ugglan traps), box-traps (like Sherman, Tomahawk, Longworth) and pitfall traps.
- **Kill-traps**: snap traps, snares.

These different traps vary in size and may consequently be intended to trap different sized animals. The smallest rodents and insectivores, such as *Mus* sp. and *Crocidura* sp., may escape from the cage-traps (depending on the size of the mesh fence). Box-traps have the advantage of protecting captured animals against rain and other animals, but they may repel some species.



Figure 2: Hunter with traditional trap in Phrae, Thailand (Photo: Herbreteau V.)

Locally-made traps, usually cage-traps, may be chosen because of their low cost (compared with commercial ones) and because of their availability in remote areas. Some live-traps can fold flat to be easily carried and stored. Others allow multiple captures (Fig. 3). Fences are sometime used to drive the animals into the trap and help to increase the trapping success.



Figure 3: A multiple-capture cage-trap in Cambodia (Photo: Herbreteau V.)

Live-trapping is required when animals must be released (demographic studies) and when specific tissue samplings or blood are required (medical studies). Also, kill-traps may damage animals and prevent certain analyses. For instance, snap traps usually destroy skulls and prevent any measurements for morphometrics.

Kill-traps are more effective in catching many species. They have the advantage of being cheap and small. They are often chosen by hunters who use large numbers of traps every night. There is a large variety of traps made by local people, using bamboo, wood and motorcycle tubes (Fig. 2 and 4). The trapping must be conducted cautiously. One should wear protective equipment (mask and gloves) to avoid contamination with blood or excretions from the animal. When required, animals should be killed rapidly after their catch.



Figure 4: Preparation of collars mounted on a wooden stick, in Kalasin province, Thailand (Photo: Herbreteau V.)

The traps should be placed so that the captured animals are protected from changing weather conditions and predators. Adding a shelter made with vegetation on the trap can help protecting captured animals.

The choice of baits to attract rodents into the trap usually generates great debates among scientists and local people. Different baits can be used in a single trap to be more appealing. Each hunter may recommend their own recipe but differences in trapping success are not so obvious. Nevertheless some bait is advantageous in that it is long lasting and easily cleaned. Bait attracting ants should be avoided.

There are other techniques for catching rodents yet some of them are more or less recommended. Digging burrows can be a solution when the species is rarely caught using traps. However animals may escape or they can bite easily. Glue traps and foothold traps are not recommended since they can hurt animals and because they can capture non-target species.

Recommendations:

- *When handling traps, **wear thick rubber gloves**. Keep in the car some gloves boxes, soap, disinfectant and water to wash hands whenever required.*
- *Transport the captured animals **in cars, outside of the passenger section.** A pickup car may be preferred so that the cages are placed at the rear of the car. This area should be ventilated by opening the windows if a hardtop is present.*
- ***Protect animals from the sun and the heat**. Do not pile cages up.*
- *Carefully wash and disinfect the car, once the traps have been unloaded.*

1.2. Trapping protocol

Trapping rodents for medical or ecological researches requires a trapping protocol to be defined. The description of this protocol is necessary to evaluate the success and the representativeness of the trapping. This protocol should be set up according to the objectives of the study. A certain minimum number of animals are generally required to assess the prevalence of pathogens or to investigate population genetics and morphometrics. However, it is very important to realize that rodent populations are seasonally heterogeneous in respect of age structure and breeding status, and consequently for various infection parameters that depend on the functional group of the host individual. Reliable comparisons can be made only between same functional groups. Multiannual differences in infection parameters may depend of differences in age structure, not on differences between comparable units, functional groups.

The trapping system and intensity should be similar at each sampling site in order to avoid biases when comparing the rodent communities between habitats and localities.

When targeting the diversity of rodent, the trapping protocol should take into account the landscape features. One simple way is to **categorize the landscape** into main categories (stratified sampling) and then to manage equal trapping pressures in each of them. In South-East Asia, we have distinguished four main landscape categories, further divided in sub-units, for the CERoPath trapping protocol. This may be adapted in other regions:

1- Built-up areas: villages and isolated houses;

2- Flood plains (humid or irrigated areas): alternating paddy cultivation and pasture in the rainfall farming systems or more complex rotations including legumes (soy bean, mung bean, peanut) and cash crops (paddy, vegetables, maize) in irrigated systems;

3- Dry lands: including annual (maize, dry rice), multi annual (cassava, grassland, pineapple), perennial (orchards and young bamboo, teak, young plantations of rubbers and eucalyptus), cultivated areas and fallow lands covered by grasses and shrubs;

4- Wooded areas: including forests (deciduous dipterocarp, mixed deciduous and evergreen forest), and mature plantations (bamboo, teak, rubber tree, eucalyptus…).

This typology is very simple from an ecological point of view but it allows a description of the major rodent habitats.

The organization of the trapping using several **lines of traps** is generally used to have a clear and reproducible protocol with a calculable trapping pressure. We recommend composing each line with 10 traps, placed every 5-10 meters. These lines should be retained for 3-5 nights at the same locality. We also recommend sampling each of the landscape categories using several trap-lines (10 to 20 lines). The duration of the setting of the trap-lines is a compromise between too much mobility (where the rodents do not have the opportunity to encounter the traps and to get used to them) and too much stability (where the majority of animals are removed from the area by the trapping, leading to a decrease in the trapping success over time). This trapping system will hopefully provide a good representation of the rodent diversity and community structures and take into account the variation within these landscape categories. However, we acknowledge that there are many other methods, and we recommend adopting this method according to the environment and the aim of the study.

To be accepted by local people (and in order to limit trap removal or displacement), the owner of the field should be contacted before setting the traps. In order to facilitate the daily control and the trap recovery, each trap should be marked by a piece of surveyor's flagging attached at proximity (i.e. 1-2 m) in the vegetation (being aware that it may increase the danger of traps to be stolen). A unique code is attributed to each trap line. Trap lines are localized using their centre with the geographical coordinates given by a GPS receiver.

Local hunters can help with the acquisition of additional animals. This has proven efficient in South-East Asia, where rats are often hunted for food. Hunters have a great knowledge of rat ecology and they can catch many animals each night. Farmers are also helpful because they notice the presence and the abundance of rats by observing their tracks and the state of their holes in the edge of fields. Usually farmers can inform if rodents are present or not in their field. However they may not know about small-size rodents. They also may provide a negative answer if they are against any intrusion in their fields.

Hunters should be informed of the objectives of the study. Animals are preferably needed alive, with a little information about the place of capture (land use, and location). The GPS location can be later taken near to the place of capture. Hunters should not put several animals together in a single cage or a container. They should store animals away from their living place. Hunters should not keep living animals too long and inform the surveyor as soon as they are caught.

If a species cannot be caught alive, killed animals should be processed rapidly or placed into a fridge, preferably in a plastic bag to avoid any contact with blood.

1.3. Recording trapping data

First, traps containing rodents should be carefully labeled to indicate the place of capture (with an identifying code), the date of capture (if the animal is kept alive) and landscape information. The label should be out of reach of the animal to prevent its destruction (so avoid putting it directly on the cage).

The place of capture should be precisely described and recorded for each individual and each trap-line. We provide in Appendix 1 a model of the data sheet to be filled during the fieldwork for this purpose. It should first indicate the identifying code of the sampling site and its **geographic coordinates**. This is required for all the analyses that are later done on animals: their description in a collection, the spatial analysis of their habitat, the study of their dynamics, but also in order to later come back to the same place for further samplings. A GPS receiver is the best instrument to use in the field to record the position of the capture place.

Definitions

*A **GPS** (Global Positioning System) is an accurate worldwide navigational and surveying facility based on the reception of signals from an array of orbiting satellites.*

A GPS receiver calculates its position by precisely timing the signals sent by these GPS satellites. Accuracy of a basic GPS receiver is about 10 meters. Some other systems, such as differential GPS have a higher accuracy (about 1 meter or less), by integrating external information into the calculation process. Some GPS receivers are integrated into a pocket computer, which allows bringing geographic maps, aerial pictures and satellite images in the field to better visualize the surroundings and the landscape. They can also include a GIS (Geographic Information System) software to directly record field observations into a spatial database (Fig. 5).



Figure 5: GPS receiver with integrated GIS software (Photo: Herbreteau V.)

Recommendations:

- *Always bring a GPS receiver to the field. Use the WGS84 datum (same as in Google Earth).*
- *Indicate, together with the GPS coordinates, the administrative location (country, province, district, sub-district, village) in order to help reading and sorting data.*

The accuracy of the positioning should be indicated together with the position on the trapping data sheet since it can vary widely with the system used. When a GPS receiver is not available, other but less accurate solutions may be used. For instance, the sampling point can be visually identified from Google Earth/Map images and then associated with geographic coordinates. If the precise origin of the animal is unknown (for example, animals brought by hunters, or found in a market), we recommend to record the geographic coordinates of the place where the animals were collected and to give the rough distance to the place of capture. One can use the following codification, based on decimal logarithm (base 10 logarithm):
Code = log10 (distance), i.e. “0” = 1 m, “1” = 10 m “2” = 100 m “3” = 1 km “4” = 10 km “5” = 100 km.

The surroundings of each sampling site should be precisely described. We propose in Appendix 1 a three-level classification: “low resolution” for the main landscape categories (for example: built-up / flood plains / dry lands / wooded areas), “medium resolution” for a more detailed category (for example: village, rice field, corn field, dry evergreen) and “high resolution” to give more precision (harvested, flooded, etc.). Some information about the distances to houses, roads, rivers, etc, as well as about the human presence can be recorded. Basic weather information should be added, especially if weather is exceptional and disturbing animal distribution and trapping.

The last table in the data sheet allows records of the animals captured at the given site every day to be made, and to keep track of the number of traps, used, lost and replaced, in order to calculate the trapping pressure.

We finally recommend taking four photographs in the direction of the cardinal’s points to help visualizing the landscape description recorded in the data sheet.

2. Dissecting rodents

2.1. Organization of the field laboratory

(All authors)

2.1.1. Processing site

The choice of a convenient processing site can be a difficult task, as there are several requirements. First of all, the laboratory should be set up in a secluded area, away from humans and domestic animals. Outdoor processing is an alternative if electricity and tap water are not needed (or available) and the weather is favorable. Water tanks can be used as well as an awning to cover the field laboratory from sun and rain. A restricted area can be created by placing a ribbon around the processing site to clearly limit the intrusion by non-authorized persons. Processing rodents indoors requires an isolated and ventilated space that can be easily and regularly cleaned.

Everybody entering the processing site should wear protective equipment, including gloves, mask and laboratory white coat.

Before processing the animals, traps containing animals should be stored in a ventilated area, preferably outside and downwind, to avoid the inhalation of aerosolized particles excreted by rodents. They should be protected from the sun and changing temperatures. Rodents should be adequately fed and provided with water (fruits and vegetable are perfect) if not processed immediately.

Garbage should be removed every day. Used needles should be kept separately in a disposal container and if possible treated by a regional hospital or laboratory in accordance with local regulations. Other waste can be burnt daily at the processing site.

2.1.2. Avoid handling live animals

Fieldworkers should avoid handling live animals due to the associated risks of disease transmission through bites, contact with contaminated urine, blood, etc. or inhalation of infected particles. However in some cases this may be inevitable, for example:

- morphological and taxonomic studies when the animal has to be released (when dissection is not required for medical studies). Measurements can be taken on a living animal: sizes and weight (the animal can be placed in a bag and weighted together). Also, a small piece of tissue (e.g. hairs, top of the ear) can be taken for genetic analysis.
- serological studies: it is possible to collect a small amount of blood from the tail arteries and veins or the orbital sinus (see chapter 2.5).
- population studies: animals can be tagged to estimate densities, demographic patterns, niche extent, etc.
- production of karyotypes: injections should be done on live animals before processing (see chapter 3.4).

For these purposes, live animals should be handled gently with care to minimize stress to the animal. Different techniques can be applied, making sure that the animal can breathe and does not suffer. Small-size rodents should be grasped by the scruff of the neck using the thumb and forefinger. The tail can be secured with fourth and fifth fingers or with the other hand. Larger rodents can be held by catching the neck between the thumb and index fingers, while the hand covers the abdomen and maintains the animal. Another method is to grasp the head between the index and middle fingers, while the thumb and ring fingers are placed under the elbows. Thus maintained, the animal is rolled over, resting on the back in the entire hand.

2.1.3. Preparation of tubes

Recommendations:

- *Prepare tubes before starting dissection for quicker and safer processing (Fig. 6).*
- *If two tubes for one organ are needed, mark one of the two (with a cross for example) to differentiate the two series. It will help when sorting the tubes while transferring them from the liquid nitrogen to the -80°C freezer.*

Preparation:

***Tubes**:*

- *Use screw cap cryogenic tubes to store in a liquid nitrogen tank in the field (and in a -80°C freezer in the laboratory).*
- *Use color caps to rapidly sort and identify the organs collected. We use blue caps for lungs, white caps for spleen, yellow caps for kidneys and red caps for blood.*



- *Use Eppendorf® tubes to store in a fridge or a -20°C freezer.*
- *Prefer tubes with a safe lock to prevent any undesired opening.*



Labels:

- *Label tubes to be stored in liquid nitrogen with the rodent identification number. Use a permanent marker (beware or different qualities of markers; make a preliminary test to make sure that the label will last in liquid nitrogen).*
- *Prepare stickers with the rodent identification number for tubes stored in a fridge or a -20°C freezer.*

Tags:

- *Prepare two tags with the rodent identification number: one to be fixed to the body, and one to keep with the digestive tract in ethanol in a Falcon tube. We use the "Dymo®" stickers as they last a long time in ethanol. We use a nylon fishing line to attach this tag to the leg of each animal or the cheeks if the skull is separated for morphometrics.*

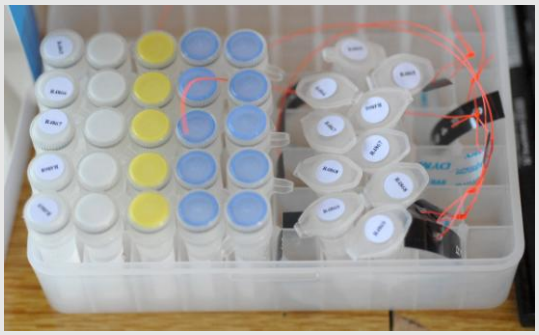


Figure 6: Tubes for dissection (Photo: Herbreteau V.)

Dissection table:

- *Prepare the processing table with all the equipment needed for each dissection.*
- *We recommend using a polystyrene board covered by a thinner plastic board to pin animals for a proper dissection. Such dissection boards can be easily disinfected and should be replaced regularly.*
- *Thoroughly clean the dissection board and tools with bleach then water then ethanol after each animal. Household bleach is a suitable disinfectant and can be easily found in remote areas. After cleaning with bleach, rinse with water to avoid the bleach corroding any metal surfaces (Fig. 7).*



Figure 7: Prepare Bleach, water and ethanol and replace regularly (Photo: Herbreteau V.)

2.2. Euthanasia

(Vincent Herbreteau, Yannick Chaval and Serge Morand)

Non-invasive techniques can be applied to several rodent studies (such as ecological surveys, morphological measurements, serology of pathogens, etc.). However, in certain cases, killing animals is necessary for research purposes: especially to keep voucher specimens for a reference collection and to isolate pathogens or parasites from internal organs. The necessity of sacrificing animals should be established under an approved research protocol guaranteeing the use of samples to answer the scientific objectives. When an animal is killed, a maximum of measurements, photographs, and samples should be taken for the purpose of the study but also for sharing with the scientific community if not required by the study.

Euthanasia is defined as the painless and stress-free killing of an animal. It is a sensitive question that recent guidelines have tried to clarify:

- AVMA (American Veterinary Medical Association Council on Research) Guidelines on Euthanasia (2007): http://www.avma.org/issues/animal_welfare/euthanasia.pdf
- CCAC (Canadian Council on Animal Care) guidelines on: euthanasia of animals used in science (2010): http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/PDFs/Euthanasia.pdf

We have tried to follow these guidelines for rodent studies and invite to read them carefully. We have summarized here the main recommendations.

Recommendations:

- *Euthanasia should be conducted following the reference guidelines* (American Veterinary Medical Association Council on Research, 2007; Canadian Council on Animal Care, 2010).
- *Euthanasia should be conducted by trained people.*
- *General anesthetics may be hazardous to workers and should be used with special care. Workers should wear the protective equipment adapted to each method.*

Different methods of euthanasia exist and those enabling a rapid death with reduced pain of the animal as well as safety for the field workers should be selected. Recommended physical methods are limited to cervical dislocation but only apply for small rodents:

- **Cervical dislocation** aims at quickly separating the cervical vertebrae from the skull (American Veterinary Medical Association Council on Research, 2007; Canadian Council on Animal Care, 2010)**:**
  - Training is required to ensure a rapid loss of consciousness and that dislocation is cervical and not lower in the vertebral column.
  - It can **only be applied for small-sized rodents** (mice and small rats). It should be applied if the number of animals is relatively low to prevent human error due to fatigue.
  - It consists of quickly pulling head from the body to dislocate the neck.
  - Method: one hand takes the head with the thumb and index finger on either side of the neck at the base of the skull; the other hand quickly pulls the base of the tail. It is also possible to simply press with thumb tip strongly into the neck immediately behind the skull.

After cervical dislocation, ectoparasites may remain alive. After death, we recommend to place the animal in a plastic bag containing anesthetics for a while to collect ectoparasites and to avoid biting during dissection.

Cervical dislocation is adapted to animals that can be manipulated easily. However, wild animals are generally more stressed by human contact and consequently are aggressive. Therefore we recommend methods without any handling of animals alive, for security reasons and to reduce their stress:

- **CO_2 inhalation** (American Veterinary Medical Association Council on Research, 2007; Canadian Council on Animal Care, 2010)**:**
  - The cage containing the animal is placed in a chamber where a flow of 100% CO_2 gas is initiated.
  - The flow of CO_2 gas should be monitored using a gas flow meter to reach 20-30% of chamber volume per minute (a higher flow rate would result in pain prior to loss of consciousness, whereas a lower rate would be too slow). (Ambrose et al., 2000; Canadian Council on Animal Care, 2010).

CO_2 is inexpensive, non-flammable, non-explosive, and poses minimal hazard to personnel when used with properly designed equipment.

After CO_2 inhalation, ectoparasites may remain alive. After death, we recommend here to place the animal in a plastic bag containing anesthetics for a while to collect ectoparasites and to avoid biting during dissection.

- **Overdose of inhalant anesthetics** (American Veterinary Medical Association Council on Research, 2007; Canadian Council on Animal Care, 2010)**:**
  - Euthanasia should be conducted in open-air places, far from the other animals captured.
  - Recommended agents are, in order of preference: enflurane, isoflurane, sevoflurane, methoxyflurane, desflurane with or without nitrous oxide. They are non-flammable and non-explosive under ordinary environmental conditions.
  - Chloroform and ether are not acceptable. Chloroform is very toxic and recognized as carcinogenic. Ether is irritating and flammable and requires a long wait before processing the animal.
  - Anesthetics should be used into an appropriate hermetic container resisting to the agents. We recommend the use of a hermetic box, which can fit a rodent cage with a limited volume (Fig. 8).



Figure 8: Material used for euthanasia (Photo: Herbreteau V.)

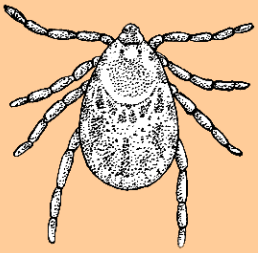
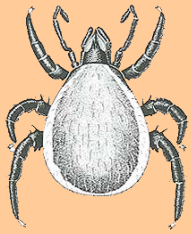
As the use of CO_2 is more difficult to set up in the field, inhalation of anesthetics is more commonly chosen. Animals should only be handled outside of the trap after been deeply anesthetized: as soon as respiration and circulation have ceased.

The collection of ectoparasites after euthanasia by inhalation of anesthetics is described in the following chapter.

2.3. Collection of ectoparasites

Definitions

Ectoparasites refer to the parasites that live on the body surface. There are 4 main groups on rodents:

Class Insecta		***Class Arachnida / subclass Acari***	
Lice *(Order Phthiraptera)*	***Fleas*** *(Order Siphonaptera)*	***Ticks***	**Mites** (all members of Acari except ticks)
- scavengers, generally feed on their host body, but some species feed on sebaceous secretions and blood, - wingless.	- haematophagous, - wingless, long legs.	- haematophagous, - usually, adult ticks are found attached to the skin (larvae and nymphs are difficult to see). They grow in size during several days before dropping off.	- various feeding, - microscopic size, - usually found moving on the body.
			
Figure 9: Sucking louse drawn By Pearson Scott Foresman (Public domain), not scaled.	*Figure 10: Flea drawn by Robert Hooke's in his Micrographia (Public domain), not scaled.*	*Figure 11: Tick drawn By Pearson Scott Foresman (Public domain), not scaled.*	*Figure 12: Mite drawing (from www.landcareresearch.co.nz), not scaled*

Protocol:

1- Stick a piece of kitchen paper on the face of the cage that will be below in the container for euthanasia (Fig. 8 and 13). Some ectoparasites will fall off the animal and will be directly collected on the paper with dissecting forceps (Fig. 14). Either prepare an ethanol (70% or absolute) tube for morphological or DNA study, or a cryogenic tube with phosphate buffered saline (PBS) solution (that will be placed in nitrogen tank) for pathogen isolation.

2- Inspect the body with a comb (Fig. 15) or dissecting forceps to find more ectoparasites:
- Ticks: usually on the head and underbody (Fig. 16);
- Fleas and lice: usually on the lower part of the abdomen and underbody;
- Chigger mites: in the ears.



Figure 13 : Tissue paper stuck under the cage to collect ectoparasites (Photo: Herbreteau V.)

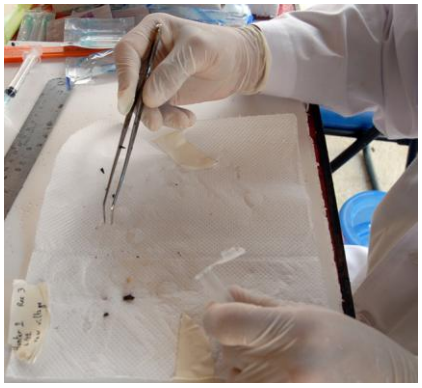


Figure 14 : Collection of from the euthanasia container before inspection of animal (Photo: Herbreteau P.)



Figure 15 : Use of a comb to collect more ectoparasites on the animal (Photo: Herbreteau P.)



Figure 16 : Ticks attached to the ear (Photo: Herbreteau V.)

We recommend collecting ectoparasites before dissection to avoid any contact with blood. Removing them before dissection may also reduce the risks of bites to workers.

2.4. Blood collection

(Vincent Herbreteau, Yannick Chaval and Serge Morand)

Collecting blood is probably the most delicate task but fundamental for the serological study of rodent-borne diseases. Blood collection requires training and practice: when manipulating animals alive to limit their stress, and generally to get a maximum of blood since this operation can be easily missed.

Different techniques can be used depending on the status of the animal, the amount of blood needed, the purpose of the study and the necessity of dissection for further sampling. These techniques can be separated into three groups (Hoff, 2000):

- Blood collection not requiring anesthesia: saphenous vein (on the caudal surface of the thigh), dorsal pedal vein (on the top of the hind foot);
- Blood collection with anesthesia recommended to minimize pain and stress: tail vein, orbital sinus (or retro-orbital), jugular vein (near the throat or neck);
- Terminal procedures: cardiac puncture, posterior vena cava, axillary vessels, orbital sinus.

The different techniques on living animals can be repeated frequently, except from the orbital sinus. Anesthesia aims at reducing pain but should also be carefully managed: a low dose of isoflurane can be used until the animal is lying on its side and breathes slowly.

We only develop below the techniques that we have adopted in the field and found suitable for the study of blood parasites or the identification of antibodies against pathogens.

Recommendations:

- ***Only trained people should collect blood****, to avoid accident when manipulating syringes, to minimize stress to living animals and to obtain a maximum volume of blood.*
- ***Used needles should be placed in a sharps bin*** *(needle containers are made of puncture-resistant and leakproof plastic, and can be incinerated or autoclaved).*
- *As soon as used,* ***replace the plastic cap*** *and then remove the needle from the syringe, to avoid any injuries. Under no circumstances should needles be left on the table without their plastic cap.*
- *Used syringes without needles can be disposed of in a different container and burnt everyday.*

2.4.1. Blood collection from the base of the tail

Blood can be collected from the base of the tail on living animals when small amounts are needed.

Protocol:

1- Warm the animal and the base of the tail to increase flood flow.
2- Carefully and gently grasp the animal (see chapter 2.2), eventually place the animal in a restraining tube covering the head and front legs.
3- Disinfect the base of the tail with ethanol. Puncture the vein with a 23-25 gauge needle or use collection tubes with capillary action to collect blood.
4- Afterwards, apply pressure or use a cauterizing agent (such as silver nitrate) until bleeding stops.

2.4.2. Blood collection from the orbital sinus (retro-orbital)

Retro-orbital blood collection is also possible for small amounts of blood. This technique requires practice.

Protocol (Hoff, 2000):

1- The animal should be anesthetized first.
2- Pull the skin away from the eyeball, making the eyeball protruding out of the socket as much as possible.
3- Insert the tip of a glass capillary tube or Pasteur pipette into the corner of the eye socket underneath the eyeball, with a 45-degree angle. Apply gentle downward pressure and then release until the vein is broken and blood is visualized entering the pipette.
4- Slightly withdraw the pipette and allow the pipette to fill.
5- Before removing the pipette, cover the open end with your finger to prevent blood from spilling out of the tube. Bleeding usually stops immediately and completely when the pipette is removed.

2.4.3. Blood collection by cardiac puncture

When the dissection of the animal is required by the research protocol, cardiac puncture appears **the best method to rapidly obtain large amount of blood in aseptic conditions**. It requires practice to maximize the volume of blood collected but has proved to be very effective.

Equipment:

A suitable needle and syringe should be chosen according to the animal size (Table 1):

Table 1: Recommended choice of syringe and needles for cardiac puncture according to rodent size

	Syringe	***Needle gauge (length)***
Large-size rat	*5 cc*	*18 – 21 (1 – 1.5 inch)*
Medium-size rat	*3 cc – 5cc*	*22 – 23 (1 inch)*
Small-size rat	*1 cc (or insulin syringe)*	*24 (1 inch)*

Protocol:

We recommend a direct cardiac puncture without opening the thorax for a quick and safe process. Nevertheless, it requires practice and some prefer directly taking the blood from the visible heart, which particularly suits for smaller species.

1- Blood should be taken as soon as possible after the death of the animal as blood can coagulate rapidly.

2- Fix the needle to the syringe and carefully loosen the needle cover. Expel the air from the syringe. Eventually coat the syringe with heparin by aspirating and expelling heparin, to prevent coagulation.

3- Place the animal on its back, in a horizontal position. Clean the thoracic fur with ethanol.

4- Locate the thoracic cage and xiphoid process (the lower part of the sternum) with one hand, making sure that the animal is well positioned (i.e. the xiphoid process is well centered).

5- With the other hand, insert the needle under the xiphoid process at about 30 degrees to the left (Fig. 17). While inserting the needle, gently pull the plunger to aspirate until the heart is perforated and blood begins to flow. As soon as blood is flowing, continue pulling the plunger without moving the needle to avoid any more perforations in the heart, which will stop the flow.

   If no blood appears in the needle, the action should be repeated. If the flow stops rapidly, the heart may have been perforated on the back: slowly withdraw and adjust the needle to restore the flow.



Figure 17: Cardiac puncture (Photo: Herbreteau P.)

   As soon as enough blood is collected, release pressure on the plunger and withdraw the needle.

6- To avoid damaging blood cells, we recommend disposing of the needle before expelling blood. Slowly expel the blood from the syringe onto a filter paper or into the appropriate tube.

2.4.4. Blood collection from a dead collected rodent

It is still possible to collect blood samples from rodents, that were collected dead (e.g. from snap trappings). Under these circumstances, cardiac puncture cannot be done and the body should be opened.

Protocol:

1- Dissect the heart and cut it partly using sterile scissors.

2- Press the heart against a filter paper. Immediately hang filter paper to let it dry and avoid any contact.

3- Or, aspirate blood from the thoracic cavity using a narrow syringe or a heparin-coated Pasteur pipette. If not enough blood is available, fold a filter paper and introduce it inside the cavity, in order that it can absorb some blood.

4- Or, insert the whole heart in a vial of suitable size and add 2-3 volume of phosphate buffered saline (PBS), and freeze. The extract can be used for seroscreening.

2.4.5. Serum separation and blood storage

We recommend to prepare serum the same day as the blood is collected using a microcentrifuge (Fig. 18), and to freeze separately serum and blood clot in liquid nitrogen. This can be easily done in the field and it ensures a better preparation of blood samples.

Serum separation:

1- Place the tube containing whole blood in a micro-centrifuge and spin at 4,000 rpm for 5 minutes.

2- Eventually remove any coagulated blood using a toothpick and place back in the micro-centrifuge for a further 5 minutes at 4,000 rpm.

3- Using a 200 µL pipette and sterile tips, remove the transparent serum and place in a new labeled cryovial. Also place the remaining blood clot in another labeled cryovial.

4- Place both cryovials in liquid nitrogen (Fig. 19).



Figure 18: Example of microcentrifuge suitable to realize serum in a field laboratory (Photo: Herbreteau V.)



Figure 19: Liquid nitrogen tank suitable for field use (Photo: Herbreteau V.)

If liquid nitrogen is not available, blood can be collected on different supports, such as test tubes (that help conditioning blood) or blotting papers (that have proven easy blood storage when equipment is limited).

- **Test tubes** (Fig. 20):

- **Coagulation tubes**: filled with sodium citrate (3.8%) and used for coagulation assays. A certain quantity of blood is required to ensure a correct dilution.
- **Serum separation tubes**: used for serology. They are coated with micronised silica particles, which activate clotting. Serum can be directly aspirated from the tube.
- **EDTA** (K2 or K3) **tubes**: used for hematology examination and complete blood count (CBC).
- **Heparin tubes**: used in blood collection and anticoagulation for clinical biochemical examinations.



Figure 20: Tubes used for blood collection (EDTA, serum and coagulation tubes) (Photo: Herbreteau V.)

After collecting blood in tubes, gently invert to 180 degrees several times to react the additive with the blood. Note that tube caps are coded: red for serum tubes, light blue for coagulation tubes, purple for EDTA tubes.

- **Filter or blotting papers:**

Filter papers can be used for DNA extraction by PCR. These present several advantages:

- They are **cost-effective**,
- They **can be stored easily** in zipper bags with desiccant (suitable for studies in remote areas),
- They **require a limited amount of blood**: suitable for the smallest mammals from which only a few drops of blood can be obtained, as well as freshly-dead animals (when puncture cannot be performed).

Recommendations:

- *Use small circular or rectangular blotting papers. One cm^2 of impregnated blood is enough.*
- *Carefully expel a few drops from the syringe (after disposing of the needle). Make sure that the blood is not coagulated; otherwise it may spurt if there is too much pressure. Try to work so that the syringe is not pointed at any of your co-workers.*
- *Hang blotting papers in an open-air space for drying (Fig. 21). Use a hairdryer for drying quickly but not heat the samples.*
- *Cut away any unnecessary paper.*
- *Pack into small individual zipper storage bags. Add some desiccant or preserve in a fridge.*



Figure 21 : Blotting papers drying (Photo: Herbreteau P.)

Three methods for DNA extraction from filter papers were compared by Bereczky *et al.* in 2005. They show that the sensitivity of detection was dependent on the parasite density, the type of filter paper and the DNA extraction method. For 3MM® Whatman filter paper, the sensitivity was 100% with the Tris-EDTA buffer-based extraction, and lower with the standards methods: 73% with the methanol extraction, and 93% with Chelex® extraction (Bereczky et al., 2005).

Tris-EDTA buffer-based extraction is rapid, simple and inexpensive (Bereczky et al., 2005).

Protocol (adapted from: Bereczky *et al.*, 2005):

1- Excise the area of filter paper with the blood spot using a clean scalpel on a piece of glass. Transfer the piece of filter paper to a sterile 1.5 mL Eppendorf® tube using forceps.

2- Extraction (3 methods)

- Tris-EDTA buffer-based extraction:
  a. Prepare Tris-EDTA buffer: 10 mM Tris, pH 8.0 (Tris-base plus Tris-HCl) and 0.1 mM EDTA in distilled water, at room temperature.
  b. Place the filter paper punch in an Eppendorf® tube, and soak in 65 µL of TE buffer,
  c. Incubate at 50°C for 15 minutes.
  d. Mash punches, and change pipette tip for each punch.
  e. Heat at 97°C for 15 minutes to elute the DNA.
  f. Remove the liquid condensed on the lid and the wall of the tubes by centrifugation (2−3 seconds).
  g. Keep the DNA extract at 4°C for use within a few hours or store at −20°C.

- Methanol extraction:
  a. Soak the piece of filter paper in 125 µL of methanol
  b. Incubate at room temperature for 15 minutes.
  c. Remove the methanol and dry the samples
  d. Add 65 µL of distilled water.
  e. Mash punches, and change pipette tip for each punch.
  f. Heat at 97°C for 15 minutes to elute the DNA.

- Chelex extraction:
  a. Incubate overnight at 4°C in 1 mL of 0.5% saponin in phosphate buffered saline (PBS).
  b. Wash for 30 minutes in PBS at 4°C
  c. Transfer into new tubes containing 25 µL of stock solution (20% Chelex-100 and 75 µL of distilled water) and vortex for 30 seconds.
  d. Heat at 99°C for 15 minutes to elute the DNA.
  e. Vortex, and centrifuge at 10,000 × g for 2 minutes.
  f. Transfer the supernatants (65 µL) into new tubes.

3- Store the supernatant at -20°C (short term) or at -70°C (long term).

4- Use 1-5 µL of supernatant per 20 µL PCR reaction.

- **FTA cards** (adapted from Whatman website: http://www.whatman.com/FTAElute.aspx)

FTA® Elute Cards (Fig. 22) can be used for virtually any cell type (blood, cultured cells, bacteria, plasmids, microorganisms, solid tissue, viral particles, M13 Plaques, etc.) and a wide range of applications: multiplex PCR, sequencing after PCR Amplification, whole genome amplification quantitative PCR, genotyping, genetic identification, molecular diagnostics, etc.

The FTA® Elute matrix is chemically treated to lyse cells upon contact causing the release of nucleic acids.

FTA® cards are expensive but present several advantages:

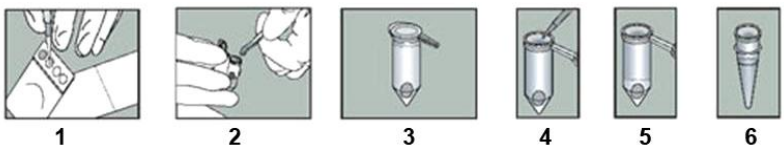
- Very small amount of blood can be used since the minimum volume required is 12-40 µL.
- Cards can be stored at room temperature and easily shipped.
- Security: organisms, including blood borne pathogens, are rapidly inactivated. It eliminates the risk of contamination and allows an easy transportation.
- Sample processing requires a simple hot water elution procedure to isolate DNA. Eliminates the cost of using a purification kit.



Source: www.whatman.com

Figure 22: Whatman FTA® Card

Protocol:



1- Apply sample to FTA® Elute matrix; dry thoroughly.

2- Punch a 3 mm disc and place in a microcentrifuge tube.

3- Rinse punch in 500 µL water, pulse vortex 3 times for 5 seconds.

4- Remove water and centrifuge for 5 seconds; pipette off excess water.

5- Add 30 µL sterile water, heat at 95°C for 30 min; pulse vortex 60 times; centrifuge.

6- Use 5-10 µL eluted DNA in a PCR mixture.

2.5. Dissection

2.5.1. Separation of organs

(Vincent Herbreteau, Yannick Chaval, Jean-François Cosson and Serge Morand)















Protocol:

1- **Pin the animal ventral side up**, on the **clean dissection board** (Fig. 23A). Avoid holding the animal in one hand, so as to keep your hands clean and to properly cut the organs.

2- **Clean the ventrum** with cotton wool soaked with ethanol to avoid introducing hairs in the body and infecting organs (Fig. 23B).

3- Pinch and raise the skin with dissecting forceps (Fig. 23C).

4- Cut through the body wall muscles just anterior to the genital opening and continue just to one side of the midline on the ventral side until the thoracic cavity (Fig. 23D). Use blunt-end scissors (either two blunt-end scissors or one blunt, one sharp point scissors) to avoid damaging the organs.

5- Pull the skin to keep the fur away from the body on each side of the thoracic cavity (Fig. 23E).

6- Cut large parts of **lungs** and place them quickly in the cryogenic tubes (Fig. 23F). We recommend keeping some more parts of lungs in another tube filled with RNA later.

7- Clean forceps and scissors (bleach + water + ethanol).

8- Move the stomach to the left to find the **spleen** beneath (the spleen can be identified by its triangular shape).

Figure 23: Dissection steps A to G (Photos: Herbreteau P.)

9- Pull the spleen with forceps and carefully remove the white tissue with sharp points scissors (Fig. 23G). Cut the whole spleen and place it on a filter paper to check its **weight** (in mg) with a precision balance (to get information for immuno-competence) (Fig. 24H). Then place it in the cryogenic tubes with and without RNA later.

10- Clean forceps and scissors (bleach + water + ethanol).

11- Move the intestines to find the kidneys beneath.

12- Separate the **kidneys** and place them entirely in two cryogenic tubes, one with and one without RNA later (if the kidneys are too big, cut it in pieces) (Fig. 24I).

13- Clean forceps and scissors (bleach + water + ethanol).

14- Cut the **diaphragm** and place it entirely in a cryogenic tube (Fig. 24J).

15- Place the **cryogenic tubes in the liquid nitrogen tank** (Only open the tank one time at the end of dissection of each animal to limit the evaporation of nitrogen).

16- Clean forceps and scissors (bleach + water + ethanol).

17- Cut a piece of **liver** and place it in an Eppendorf® tube prepared with absolute ethanol for DNA analysis (Fig. 24K).

18- Place the **Eppendorf® tubes in a fridge** or foam box with ice (if no fridge is available).

19- Clean forceps and scissors (bleach + water + ethanol).

20- Observe the **genital organs** to confirm the sex identification and eventually count embryos or measure the testes.

21- Cut the ears for studying Chigger mites (Fig. 24L) and place one in an Eppendorf® tube prepared with absolute ethanol for DNA analysis and the other one in a cryogenic tube for isolation of pathogens in Chigger mites.

22- Attach the identification tag to one leg. Use narrow point forceps to perforate the skin and introduce the string.

23- Verify that all measurements and samples were taken (Fig. 24M).

24- Place the animal into a watertight jar, filled with 70 degrees ethanol.

25- Clean the dissection board (bleach + water + ethanol).



Figure 24: Dissection steps H to M (Photo: Herbreteau P.)

Table 2: Rodent samples and possible analyses

	Samples	Storage*	Conservation**	Analysis***	Target
BLOOD	Blood	Filter Paper / FTA card	Dry in ZSB	Serology	Antibodies against the different pathogens listed below
		Filter Paper / FTA card	Dry in ZSB	PCR, RT-PCR	*Bartonella* spp. (infect, among other cells, erythrocytes, etc.), Protozoan (*Babesia* spp., *Hepatozoon* spp., *Plasmodium* spp., *Trypanosoma* spp., *Leishmania* spp.)
		CryoT	LN, -80°C F	PCR	
	Serum	SerST (to separate serum)	5°C R	Serology	Antibodies against the different pathogens listed below
		CryoT (after centrifugation)	LN, -80°C F		
	Blood cells	CoagT (to separate blood cells)	5°C R	PCR	*Bartonella* spp. (infect erythrocytes), Protozoan (*Babesia* spp., *Hepatozoon* spp., *Plasmodium* spp., *Trypanosoma* spp., *Leishmania* spp.)
		CryoT (after centrifugation)	LN, -80°C F	PCR	
INTERNAL SAMPLES	Different organs	CryoT	LN, -80°C F	PCR, RT-PCR	Orthopoxviruses (Cowpox, Monkeypox…), herpesviruses, hantaviruses, arenaviruses, *Babesia* spp.
		CryoT	LN, -80°C F	Culture	Hantaviruses, arenaviruses (cell culture and isolation in rodents)
		CryoT / RNA later	-20°C F	RT-PCR	RNA viruses (hantaviruses, arenaviruses)
	Heart	CryoT / PBS	LN, -80°C F	Serology	Antibodies against the different pathogens (see serum)
	Lung	CryoT	LN, -80°C F	PCR, RT-PCR	Hantaviruses, arenaviruses, orthopoxviruses (spleen or kidney samples are preferred)
		CryoT	LN, -80°C F	Culture	Hantaviruses, arenaviruses, orthopoxviruses (cell culture and isolation in rodents)
		CryoT / RNA later	-20°C F	PCR, RT-PCR	Hantaviruses, arenaviruses, orthopoxviruses
		CryoT / RNA later	-20°C F	PCR	Fungi (*Pneumocystis* spp.)
		EppT / 70% ethanol	5°C R	MicObs / PCR	Macroparasite (nematodes: *Angiostrongylus* sp.) morphological description, DNA identification
	Nematodes in lung cavity	EppT / 70% ethanol	5°C R	MicObs / PCR	Macroparasite (nematodes: Filaria) morphological description, DNA identification
	Spleen	CryoT	LN, -80°C F	PCR, RT-PCR	*Rickettsia* (scrub typhus, murine typhus), *Bartonella* spp., arenaviruses
		CryoT	LN, -80°C F	Culture	*Rickettsia* (scrub typhus – in cell culture and rodents)
		CryoT / RNA later	-20°C F	PCR	Genetic markers of immunity: major histocompatibility complex (MHC), Toll like receptors (TLR), integrins, Mx proteins
	Kidney	CryoT	LN, -80°C F	PCR	*Leptospira* sp., hantaviruses, arenaviruses
		EppT / Abs ethanol	5°C R	PCR	*Leptospira* sp.
		CryoT / PBS	LN, -80°C F	Culture	*Leptospira* sp., hantaviruses
	Liver	EppT / Abs ethanol	5°C R	PCR	Host DNA identification, macroparasites (cestodes larvae)
	Cysts on liver	EppT / Abs ethanol	5°C R	MicObs / PCR	Taenia (cestodes) morphological description, DNA identification
	Brain	CryoT	LN, -80°C F	PCR	*Toxoplasma gondii* (as bradizoites)
		CryoT	LN, -80°C F	PCR	Rabies virus
	Diaphragm	CryoT	LN, -80°C F	MicObs / PCR	Macroparasites (nematodes: *Trichinella* sp.) morphological description, DNA identification

	Gut	FalcT / 70° ethanol	Room temp.	MicObs / PCR	Macroparasites (trematodes, cestodes, nematodes, Acanthocephala and Pentastomida) morphological description, DNA identification Protozoan (*Cryptosporidium* spp., *Eimeria* spp., *Giardia* spp.) DNA identification
	Muscles	CryoT	LN, -80°C F	PCR	Protozoan (*Sarcocystis* spp., *Toxoplasma gondii*), nematodes (*Trichinella* sp.)
EXTERNAL SAMPLES	Tracheal mucus	Cotton swab	FT, Room temp.	Culture	Orthopoxviruses
	Urine	Filter Paper	Dry in ZSB	Culture	Orthopoxviruses
	Feces	EppT / 2.5% $K_2Cr_2O_7$	4°C R	MicObs / PCR	Protozoan (*Cryptosporidium* spp., *Giardia* spp., *Neospora* spp., *Sarcocystis* spp., *Toxoplasma gondii*), etc.
	Hairs or tissue (ex: toes)	EppT / Abs ethanol	5°C R	PCR	Host DNA identification
	Ears (to collect the ectoparasites attached to the ears)	EppT / Abs ethanol	5°C R	MicObs / PCR	Chigger mites morphological description, DNA identification
		CryoT	LN, -80°C F	PCR	*Rickettsia tsutsugamushi* (scrub typhus) in Chigger mites, *Borrelia* spp. in ticks and lice, *Anaplasma* spp. and *Babesia* spp. in ticks
	Ectoparasites	EppT / Abs ethanol	5°C R	MicObs / PCR	Ectoparasites (ticks, mites, fleas, lice…) morphological description, DNA identification
		CryoT	LN, -80°C F	PCR	Search of pathogens in ectoparasites

* SerST = Serum Separation Tube (coated with micronised silica particles), CoagT = Coagulation Tube (filled with sodium citrate, 3.8%), CryoT = Cryogenic Tube (2mL), EppT = Eppendorf® Tube (2 mL), FalcT = Falcon Tube (15mL or 50mL), FTA card = Whatman FTA® card
Abs = Absolute, PBS = Phosphate Buffered Saline;

** R = Refrigerator, F = Freezer, LN = Liquid Nitrogen tank, ZSB = Zipper Storage Bag

*** PCR = Polymerase Chain Reaction, RT-PCR = Reverse transcription PCR, MicObs = Microscope Observation

Equipment:

Several elements should be considered when organizing the field laboratory (Fig. 25):

- *Euthanasia should be conducted outside and away from the dissection place.*
- *Dissection should be conducted in a place protected from the rain, sun and wind.*
- *We recommend here to work in teams of two, one doing the dissection, and the other one taking notes, managing tubes and only touching the clean equipment (measuring scales, centrifuge). Another team can work on helminth isolation.*
- *For each person, the necessary equipment should remain close at hand. Security items are disposed on a different table.*



Figure 25: Example of organization of the field laboratory (CERoPath project, Nan province, 06/2008) (Photo: Morand S.)

2.5.2. Hemoparasites: detection, identification and cryopreservation

(Marc Desquesnes, Christina Milocco, Pornpan Pumhom and Sathaporn Jittapalapong)

Definitions

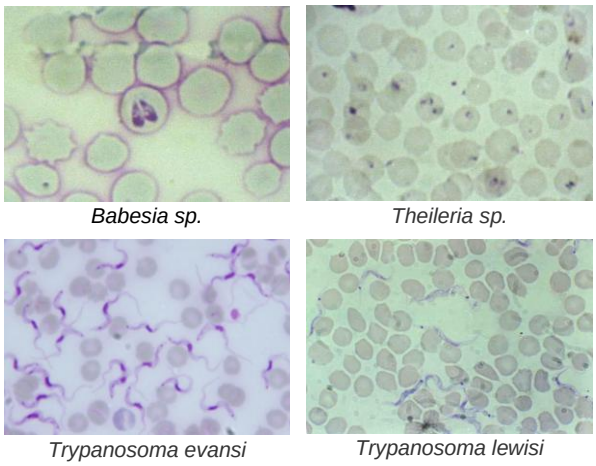
Hemoparasites *(or blood parasites, hematozoa) are bacterial or protozoan parasites that live in the blood of vertebrates. In humans, they are cause of major diseases (such as malaria, sleeping sickness, Chagas disease). Various genera may be found in the blood of rodents. They include: Bartonella, Haemobartonella, Babesia, Anaplasma, Theileria, Plasmodium, Eperythrozoon, Hepatozoon, Leishmania and Trypanosoma.*

Trypanosomes*, which are flagellate protozoa, can also be observed moving in fresh preparation, with or without previous enrichment of the sample by differential centrifugation.*

- **Preparation of stained blood smears:** must be done with fresh blood, preferably added with anticoagulant such as EDTA.

Protocol:

1. Place a small drop of blood (3-5 µL) at one end of a clean microscope slide and draw out a thin-film.
2. Air-dry briefly, fix in methyl alcohol for one minute and allow drying.
3. Stain the smears in Giemsa (one drop Giemsa + 1 mL PBS, pH 7.2) for 25 minutes. Nowadays, fast stains are increasingly used (Diff-Quick®, RAL 555® etc), allowing to fix and stain within a few seconds (for example RAL 555®: 5 deeps of 1 second in each of the 3 chemicals).
4. Rinse with tap water and dry the slide.
5. Examine with a high magnification (400-1,000 X) microscope with oil immersion.



Babesia sp. *Theileria sp.*

Trypanosoma evansi *Trypanosoma lewisi*

Figure 26: Examples of hemoparasites on Giemsa stained blood smear (Photos: Desquesnes M.)

This technique permits detailed morphological studies and identification of the main hemoparasites (Fig. 26). However, for trypanosomes, since the sensitivity is very low (can detect parasitaemia > 100,000 trypanosomes/mL of blood), it is only used for morphological studies.

- **Wet blood film:** examination is very fast and allows trypanosomes detection in case of high parasitaemia.

Protocol:

1. Place 2-3 µL of blood on a clean slide and a cover slip above to spread the blood as a monolayer of cells.
2. Examine by light microscopy (200 X) to detect any motile trypanosomes. Better observation can be obtained with dark ground or phase contrast (200-400 X). The sensitivity of this method is low, approximately 10^6 trypanosomes per mL, which is frequent in early or acute infections only.

- **Hematocrit Centrifugation Technique (HCT, or Woo technique):** to increase the sensitivity, heparinised (or EDTA) blood is centrifuged in a capillary tube and trypanosomes may be found above the buffy coat.

Protocol:

1. Fill capillary tubes (75 × 1.5 mm) with blood (70 µL).
2. Seal the wet end with plasticine and centrifuge tubes at 3,000g for 5 minutes (generally 12,000 rpm in hematocrit centrifuge machine) (Fig. 27). Value of the hematocrit (% of packed cells in the blood) can be recorded.
3. Place the capillary tube in a groove made with slide pieces glued to a slide (Fig. 27).

   Under microscope (100–200 X), observe trypanosomes, which concentrate at the junction between the buffy coat and plasma. Light conditions must be set to induce refringence of the cells and increase the visibility of the moving trypanosomes; this can be obtained when lowering the position of the light condenser or with intermediary positions of the turret light condenser.

Fresher is the sample, better is the sensitivity since strong parasitic movements make trypanosomes more visible. This technique can detect around 50-200 trypanosomes/mL of blood (Desquesnes and Tresse, 1996). The buffy coat can also be collected in a microtube and frozen; it can later be prepared for PCR.

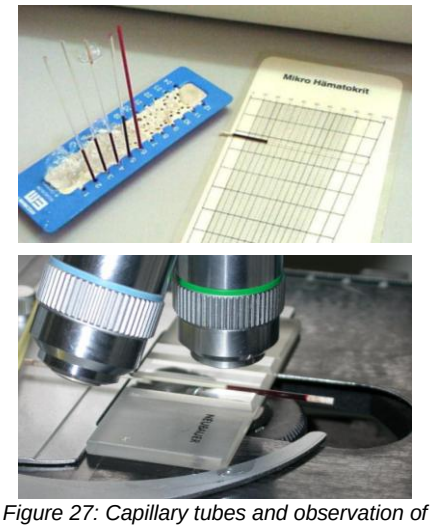


Figure 27: Capillary tubes and observation of the buffy coat area (Photo: Desquesnes M.)

- **Cryostabilates**

Blood parasites can be preserved alive in liquid nitrogen, providing the blood with anticoagulant (EDTA or heparin) is added 1:1 with cryopreservative made of 20% Glycerol and 80% of Phosphate Saline Glucose PSG (PBS with 1% glucose). They are gently mixed, put in cryotubes and frozen at -20°C (or in the vapour phase of the liquid nitrogen) for 2 hours before they are dropped in liquid nitrogen. These cryostabilates can be thawed to inoculate living parasites to laboratory rodents for further characterisation.

2.5.3. *Leptospira*: culture preparation from kidneys

(Kim Blasdell and Duangdao Waywa)

Leptospira, which are spirochaete bacteria causing leptospirosis in humans, concentrate in kidneys. Samples (kidneys) for *Leptospira* culture should be kept at the beginning of the dissection to avoid contamination.

Protocol:

1- Set up a lit Bunsen burner to flame the forceps and scalpel blade after touching any organ or surface.
2- Open up the animal trying to ensure that the intestines are not opened on incision. Locate the kidneys and avoid touching the other organs or the outside of the animal with the forceps.
3- Place the culture vial (EMJH medium) into a rack and label appropriately.
4- Remove gloves, which are inflammable, to avoid injuries. Flame the forceps for a few seconds. Using the forceps and a new sterile scalpel blade, collect the kidney, clean it with sterile normal saline solution and place it into a sterile Petri dish. Cut the kidney into smaller pieces.
5- Flame the forceps again. Open the culture vial using little finger and retain the lid. Flame the top of the vial briefly. Place a small quantity of kidney (not more than 10% of the volume of the vial) into the vial.
6- Flame the top of the vial again and then replace the lid, but unscrew slightly as *Leptospira* are aerobic and require oxygen.
7- If possible, collect a small quantity of urine directly from the bladder using a needle and syringe. Open a second culture vial, flame the top, remove the needle (to avoid contamination) and place the urine inside, flame the top and replace the lid (remembering to leave slightly unscrewed).
8- Place back into the rack and keep at 30ºC in a box in the dark (as *Leptospira* are light sensitive).

Culture vials can be kept in the fridge until required. Cultures should be examined weekly to detect growth (turbidity, haze or a ring of growth) and microscopically with dark-field illumination to observe spirochetes (6-20 µm long). If leptospires are present, subcultures should be realized in fresh medium.

2.5.4. Orthopoxviruses: culture preparation from urine and tracheal mucus.

(Nathalie Charbonnel and Anne Xuéreb)

Orthopoxvirus is a genus from the *Poxviridae* family, that includes many species of viruses causing diseases: *Cowpox virus*, *Monkeypox virus*, *Variola major virus*, *Variola minor virus*. This protocol aims at collecting orthopoxviruses alive in order to be cultivated. They can be isolated from urine and tracheal mucus.



Figure 28: Sampling urine (Photo: Herbreteau P.)

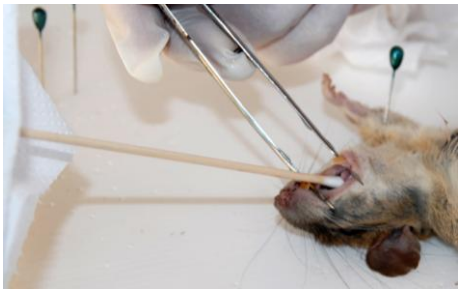


Figure 29: Sampling tracheal mucus (Photo: Herbreteau P.)

Recommendations:

- *Orthopoxviruses are sensitive to humidity and light. Thus it is very important that the urine and tracheal mucus samples are dried and stored in a dark room.*
- *These samples are complementary (detection in trachea for early infection, in urine for long lasting infection).*

Protocol:

1- Urine:

- Collect urine on small filter paper, from the bladder (dead animal) or directly on animals that urinate (Fig. 28). Note the animal identifying code.
- Let the paper dry for 24 hours, in a dark place. Store dry papers individually in zipper storage bags, then in a dry and dark room.

2- And/or tracheal mucus:

- Rub the trachea with a thin cotton swab (Fig. 29).
- Leave to dry during 24 hours, in a dark place. Store dry swabs individually in zipper storage bags with the individual code annotated, then in a dry and dark room.

3. Identifying rodents

The great diversity of rodents and their similarity in shape and size make their identification difficult in the fields. Rodentia is the largest order of mammals, with over 2,277 species (Wilson and Reeder, 2005). Some species can be morphologically almost identical while genetically they correspond to different taxa (such as sibling species). Facing this complexity, the taxonomy of rodents is still under revision, with new species regularly described. Nevertheless, rodent surveys, that usually investigate the implications of species in the transmission of diseases or the destruction of crops, rely on an accurate identification of each rodent at a specific level (Chaval et al., 2010). To reach this goal, different methods, which are described in this section, can be combined.

3.1. External identification

(Vincent Herbreteau, Yannick Chaval, Jean-François Cosson and Serge Morand)

Rigor in measuring animals is necessary for a correct identification of the species. Detailed information should be carefully recorded (See appendices 2 and 3 for examples of data sheets for rodent studies).

3.1.1. External measurements

Recommendations:

- *Be sure that everyone measuring rodents uses the same method, to limit discrepancies.*
- *Inexperienced workers should be trained and work as a duo during a few days to avoid such biases.*
- *Take time to read twice the measurements (especially while using a Vernier caliper) and verify what the assistant writes on the dissection sheet.*
- *Use a ruler with the extremity starting at "0". Measures (in mm) can be taken to half a millimeter.*
- *We recommend using a polystyrene board covered by a thinner plastic board to pin animals.*

Definitions

*The **Vernier caliper** is a precision instrument that can be used to measure internal and external distances with accuracy.*

***Example (f**rom V. Ryan, 2004: http://www.technologystudent.com/equip1/vernier3.htm):*

1- *The main metric scale is read first and this shows that there are 19 whole divisions before the 0 on the hundredths scale (Fig. 30). Therefore, the first number is 19.*

2- *The "hundredths of mm" scale is then read (Fig. 31). Only one division on the main metric scale lines up with a division on the hundredths scale below it. In this example, the 32nd division on the hundredths scales lines up exactly with a division on the metric scale.*

3- *This 32 is multiplied by 0.02 giving 0.64 as the answer (each division on the hundredths scale is equivalent to 0.02mm). The final measurement is 19.64mm.*

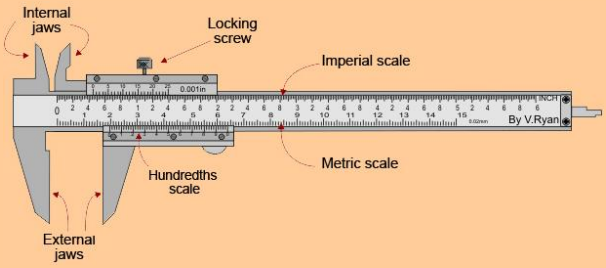


Figure 30: Drawing of a Vernier caliper

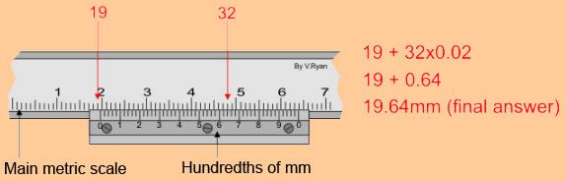


Figure 31: Example of measurement with a Vernier caliper

Protocol:

- **Head and body length:**

- The animal should be weighed (in grams) before blood collection.
- The animal should not be wet.
- Use a digital balance for accuracy to within one decigram (Fig. 32). Calibrate balance to zero before measurement.
- Supply a pesola scale in case no electricity or no battery is available, and for larger animals than accepted by the digital balance.



Figure 32: Balance used for small animals (0.1 g precision) (Photo: Herbreteau V.)

● **Head and body length:**

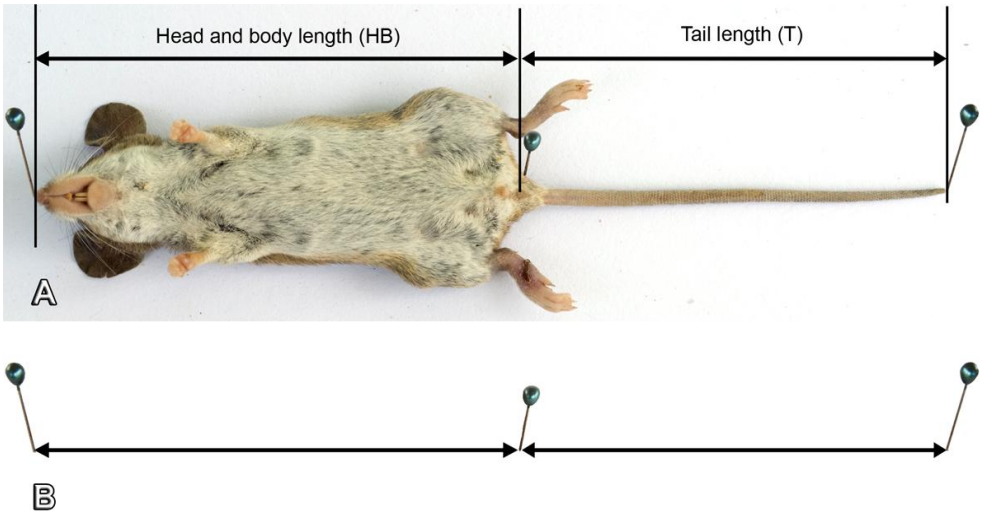


Figure 33: "Head and body", and tail measurements (Photo: Herbreteau V.)

- Place the animal lying on its back, in a straight position, without stretching the body (Fig. 33A).
- Use pins to mark the landmarks of the body on the dissection board. Place one pin on the tip of the nose and a second one on the side of the anus. For males, lift the scrotum up to see the anus before placing the pin.
- Then remove the animal and measure precisely the distances between the pins with a ruler (Fig. 33B).

● **Tail:**

- Place the animal lying on its back, with the tail in a straight position (Fig. 33A). Place one pin on the side of the anus and a second one on the tip of the tail. Do not include terminal hairs, if present, but measure their length separately (in the case of squirrels, etc.).
- Verify that the tail has not been cut at its extremity: in such case, the tail looks like intact but its extremity is square (the tail length would be a little shorter and it may result in a wrong identification). If the tail is cut or damaged, indicate, "cut" when recording data, and add in brackets the length of the cut tail (it can be useful for identifying the species of the animal).



Figure 34: Hind foot measurement not including claw (Photo: Herbreteau P.)

● **Hind-foot (or pes) length:**

- Position the ruler on the dissection board and flatten the hind-foot on it or directly flatten the ruler under the hind-foot.
- Measure the distance between the back of the heel and the tip of the central toe, without including the claw (Fig. 34).

● **Ear:**

- Introduce the ruler (with the extremity starting at "0") into the ear and measure the distance between the notch (at the base of the ear) and the extremity of the ear (Fig. 35).
- Verify that the ear is not cut or damaged at the extremity.



Figure 35: Ear measurement (Photo: Herbreteau P.)

● **Skull:**

- Use a Vernier caliper.
- Measure skull length between the back of the skull and the extremity of the nose (Fig. 36).
- Be sure to position one extremity at the back of the skull and not above or below (eventually locate the back with fingers).

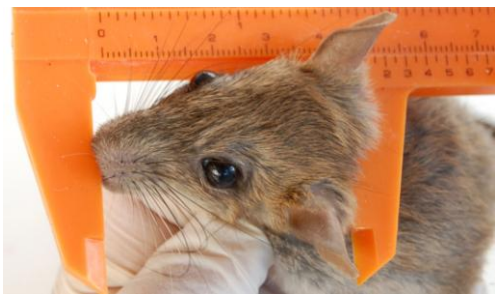


Figure 36: Skull measurement (Photo: Herbreteau P.)

3.1.2. Sex determination

Male and female can be differentiated by observing their external reproductive organs: presence of scrotum covering testicles in adult males and of the vagina in females (Fig. 37). Nevertheless, juvenile males have their testes inside the body and can be confused with juvenile females. Differentiation can be done by observing the distance between the anus and the genitals, which is greater in males than in females. If the identification cannot be done using external morphological features, the internal sexual organs should be observed at the end of the dissection.

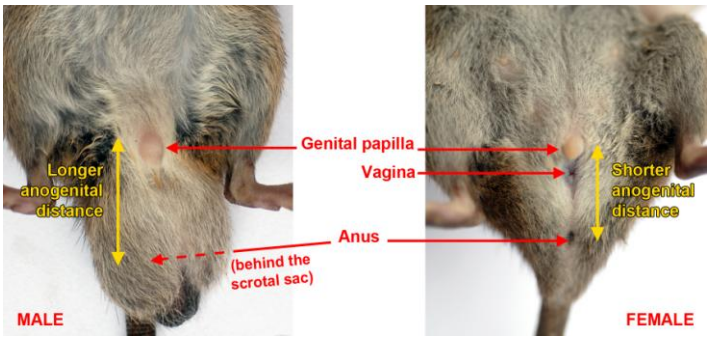


Figure 37: External comparison of male and female reproductive organs (Photo: Herbreteau V.)

- **Females:**

- **Vagina**: the vaginal opening is covered by a translucent skin in juvenile females. Record if the vagina is "not perforated" (Code = 1), or "perforated" (Code = 2).
- **Teats**: record if teats are "barely visible" (hardly distinguished and enumerated, Code = 1) or "prominent" (Code = 2).
- **Mammary formula** (Fig. 38): it corresponds to three parts of the body, from the top to the bottom: **pectoral + post-axillary + inguinal**

  This formula can help to distinguish different species (Aplin et al., 2003; Marshall, 1988).
- If the female is pregnant, mention the number of embryos on each side.

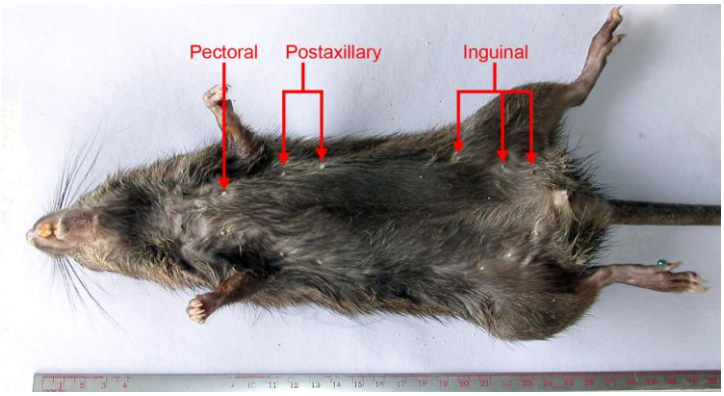


Figure 38: Position of teats, example of Bandicota indica (1+2+3) (Photo: Herbreteau V.)

- **Males:**

- Indicate if the **testicles** are "inside" (i.e. abdominal = juvenile, Code = 1), or outside (i.e. descended into the scrotal sac = adult, Code = 2).
- Measure the testicle length after the dissection (Fig. 39). If the testicles are in the scrotum, pull one out of it before measuring. The size of testicles differs between species and regarding the sexual activity.
- Indicate if the **seminal vesicles** are developed (visible) or undeveloped (Fig. 40). This informs on the sexual activity of males.



Figure 39: Measurement of testicle length (Photo: Herbreteau P.)



Figure 40: Developed seminal vesicles in an adult male (Photo: Herbreteau P.)

3.1.3. Maturity determination and development stage

The determination of sexual maturity (immature / mature) and that of the development stage (juvenile / sub-adult / adult) are both necessary to understand the ecological traits of an animal as well as to interpret epidemiological results. Also it can help to correctly identify an animal to the species level. In some case, juveniles could be misidentified with adults from another species. Figure 41 shows such difficulty with an adult *Rattus exulans* and a juvenile *Rattus tanezumi*, which look like similar in size and shape. However external observation can help to distinguish juveniles from adult individuals. Juveniles usually have a soft fur and their skull, ears and feet are larger (relative to their body size) than those of adults.

The observation of the sexual organs is highly informative to assess their sexual maturity:

- **Juveniles:**

- Juvenile females: the vagina is not perforated and teats are barely visible.
- Juvenile males: testes are abdominal (inside the body cavity), the scrotal sac is not apparent.

- **Sexually mature individuals:**
  - Females: the vagina is perforated and teats are easily visible. Verify the presence of embryos or scars in the uterus.
  - Males: testes are descended into the scrotal sac; seminal vesicles are well developed when the animal is sexually active. In rare cases, testicles can be inside the body even if the scrotum and the seminal vesicles are developed: note these observations in the data sheet.



Figure 41: Comparison between an adult female Rattus exulans (C0207, above) and a juvenile female Rattus tanezumi (C0206, below), from Veal Renh in South Cambodia (Photo: Herbreteau V.)

Finally, the close inspection of the dentition is also indicative of the development stage of a rodent. However it can only be performed on a skull perfectly cleaned up. In particular the growth of the third mandibular molar (M3) is a useful criteria. The M3 growth can be divided in at least three stages (Fig. 42). This information is interesting because in some cases rodents start to reproduce before the end of their morphological development. The stages subadult/adult are thus not fully congruent with sexual maturity (Aplin et al., 2003). The observation of the M3 growth gives a good idea of the age structure of a population and then of its demography. Another popular method to reach this goal is the use of the weight of the crystalline lens (Rowe et al., 1985). We then recommend estimating separately the sexual maturity and the development stage of the rodents (Fig. 43).



Figure 42: Different stages of the third molar (M3) growth (Photo: Chaval Y.)

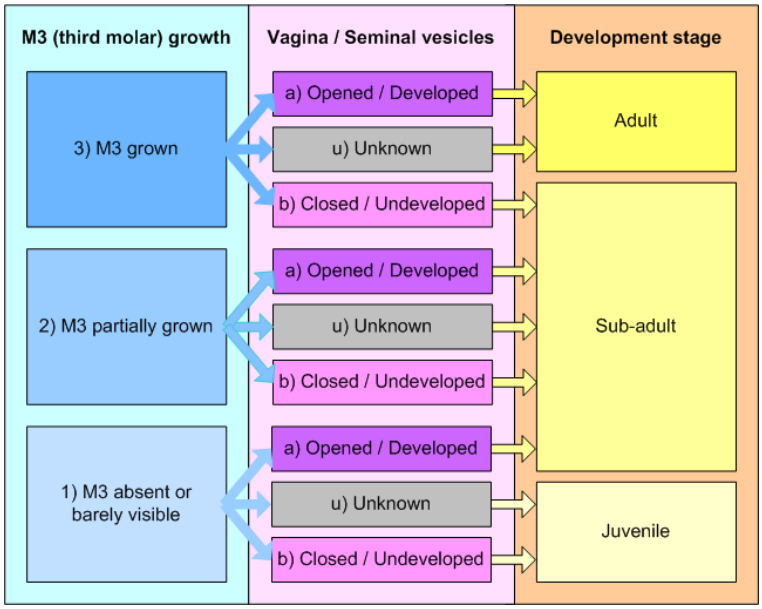


Figure 43: Decision tree to determine the maturity of a rodent, by considering the third molar growth and sexual organs externally

3.1.4. Data recording and species identification

External description of each animal should be recorded during the dissection work on a paper sheet. Then record the data in the computer as soon as possible. This will allow a safe storing of the data, on two different supports. Notice that recording the data directly on the computer may be risky as a power outage or a computer failure is not unlikely. In such case, one can get back the paper support and data are not lost.

We provide in appendices two models of data sheet that can be used during measurement and dissection of rodents. Appendix 2 aims at a detailed description of each animal, while Appendix 3 allows description of 10 individuals with less information. First, identification numbers should be attributed to the animal and the trapping place. The name of species should be indicated with a question mark if the identification is dubious. Different dates should be given to indicate the days of capture, of death and of dissection. A short description of the trapping place should be given, in addition to its detailed description in another data sheet (Appendix 1). Measurements and every sample collected (organs, helminths and ectoparasites) should also be indicated.

3.2. Morphometrics

(Julien Claude and Saksiri Soonchan)

Definitions

Morphometrics *concern the* ***study of the shape and size variation among organisms or objects****. Morphometrics can quantify morphological change occurring during evolution or ontogeny of organisms and can quantify differences among group of organisms (e.g., populations or species). There are several morphometric methods for extracting statistical data from organisms' morphologies; these include measurement of lengths and angles, landmark analysis and outline analysis.*

Applied to the study of rodents, morphometrics of the skull or other body features can help to identify unknown specimens and assign them to known groups, exclude dubious specimens (outliers), quantify and depict ontogenetic change, or discriminate different populations within a single species.



Figure 44: Skull cleaned for morphometrics (Photo: Soonchan S.)

Background: Much information can be gathered and analyzed via morphometrics from field and laboratory specimens. Basic linear measures can be directly collected from the field (head and body length, feet, ear, tail lengths, weight) with rulers and weighting machines and may provide a first step for taxonomic assignation (see Chapter 3.1.), Moreover, after preparation of a specimen a wide collection of measurements, especially on skulls can further help assignation to a given species. Voucher specimens are often associated with a dataset containing these measurements to be used as a reference.

Depending on authors, measurements can vary and users must adapt in order to collect a comparable data set on newly discovered specimens. To be reproducible by others, linear measurements should be defined between landmarks that are easily recognizable on the skull rather than maximum or minimum measure within skull region. Analyzing large sets of linear measurements requires the use of multivariate techniques, and visualizing shape differences is not always easy. Linear measurements can be and are often redundant (for example total length, head and body length, and tail length are three distinct measurements but they can be summarized by the last two variables). This redundancy may inflate the weight on some shape properties over others. Some techniques, such as geometric morphometrics allow this redundancy to be avoided.

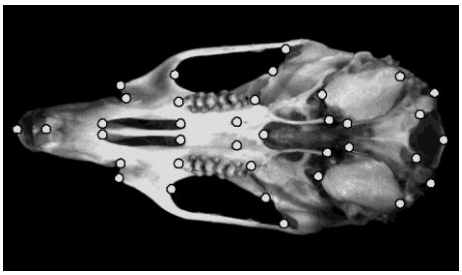


Figure 45: Example of landmarks on a palatine view of a Rattus sp. skull (Photo: Claude J.)

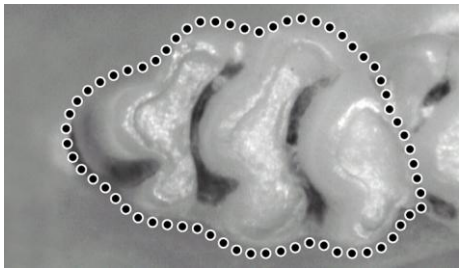


Figure 46: Pseudolandmark digitization on first upper molars of R. exulans (Photo: Claude J.).

Methods: Geometric morphometric methods developed during the last thirty years offer alternative opportunities in providing tools that allow shape difference, shape variation, and shape change to be visualized and quantified in an integrated way (Rohlf and Marcus, 1993). The strategy used by these methods is more to analyze the variation directly between configurations than inferred from measurements datasets. These methods can be split into two categories: some work on landmark positions (Fig. 45): Procrustes methods, euclidean distance matrix analysis, etc.; while others work on outline or surface shape (Fig. 46): Fourier analysis, semi landmark Procrustes analysis, etc.

Prior to morphometrical measurements, skulls should be correctly cleaned (Fig. 44) (See chapter 5.1.2). For both methods the first step is to record the position of a set of remarkable points (landmarks) on the specimen. Specimens are often 3D, and it would be better to record information in three dimensions. However 3D digitizers can be expensive and the user may prefer to directly analyze 2D variation that can be measured via pictures. In this later case, it is important to standardize the way to take pictures (standardization of the specimen position, magnification, focus for all specimens) to avoid inflating measurement errors. It is recommended to record a scale with the picture or to take a picture of the specimen directly on graph papers for further users to be aware of lens distortions.

Ideally, the same person should measure all the specimens of interest, by using the same camera during the digitization process to minimize errors (two different people will not manipulate the camera exactly in the same way, and different cameras generate different image distortion due to their lenses). Once the specimen is photographed, landmark (Fig. 45) or pseudolandmark coordinates (Fig. 46) can be gathered using a digitization program via the computer and a given user (here too: we recommend users to work with the same program and that all data must be acquired by the same user). Point coordinates are then submitted to statistical shape analysis using methods available in the literature (Claude, 2008; Dryden and Mardia, 1998) and a variety of freely available software (See http://life.bio.sunysb.edu/morph/).

3.3. Genetics

(Yannick Chaval, Marie Pagès, Maxime Galan, Johan Michaux and Jean-François Cosson)

Definitions

Genetics *aims at analysing the genetic variation within and among species and/or populations. Genetic analyses rely on the study of coding or non-coding genes, which are present in the nucleus or in the cytoplasmic organelles (mitochondria, chloroplast) of eukaryote cells. The use of genetic data for establishing evolutionary relatedness among various groups of organisms (for example, species or populations), group membership and group boundaries, is part of* ***molecular phylogenetics****. Genetics have also allowed the development of several molecular tools for a quick, cheap and unambiguous diagnosis of species when species are difficult to recognize using morphological criteria.*

Preparation:

- ***Tissue sampling for DNA analysis:***

  DNA can be extracted from a piece of liver or hind foot when animals have to be sacrificed for other analyses. If the animals are to be released after trapping, a small piece of ear can be used. When the tissue sampled is external (foot, ear), it is recommended to carefully clean the sampling area with bleach, ethanol and rinsing with water before cutting to avoid DNA contamination. Tissues conserved in 90° ethanol may be stored at room temperature for some days. For long-term conservation, the storage in refrigerator is recommended in order to slow down the evaporation of ethanol and degeneration of DNA.

- ***DNA extraction:***

  We mostly use the Qiagen protocol for purification of total DNA from animal tissues (Spin-Column Protocol and Quiagen DNeasy 96 Tissue kits for routine extraction). Purity of DNA extracted by this protocol allows long time conservation of the products. However, if the extracted DNA does not have to be kept for a long time, cheaper methods as Chelex 100 (Bio-Rad) can also be used.

3.3.1. Species-specific PCR-tests.

Over the past years, molecular tests were developed to allow a clear and rapid species diagnosis when external morphological characters are not fully discriminant. Most of these tests are based on the use of species-specific primers, which relies on the definition of PCR primer sets that amplify the DNA belonging to only one species.

Given the importance of the genus *Bandicota* as a vector for several human diseases, CERoPath project developed such a molecular test to unambiguously assign *Bandicota* specimens to a single species. This method which could be extended to most other species has the advantages of:

- being cheap and suitable for investigations of large series of specimens;
- being useful for field surveys, since it requires simple and easily accessible DNA samples, which are easy to conserve in field trip conditions (ethanol stored at room temperature);
- being also applicable on dry and/or osteological material, allowing a precise species assignment for specimens in collections and museums.

The test is conducted in two steps. The first step allows discrimination of *Bandicota* DNA samples from other samples belonging to other genera (**PCR1**). The specificity of the test was verified using the 27 different rodent species sampled during the CERoPath project. The second step allows the two different *Bandicota* species inhabiting the study area to be distinguised (**PCR2**). It is possible to combine these two steps as a **multiplex PCR**.

Table 3: Primers used to discriminate Bandicota indica from Bandicota savilei and from other rodent species.

Gene	Type of primer	Name	Primer
Cyt *b* gene	generalist	L14723	5'ACC-AAT-GAC-ATG-AAA-AAT-CAT-CGT-T 3'
	generalist	H15915	5' CT-CCA-TTT-CTG-GTT-TAC-AAG-AC 3'
	specific of *Bandicota* genera	FBan	5' TTA-ATC-TTA-GCC-TTC-ATA-CCT-CTA 3'
Dloop gene	generalist	F1043	5' GGC-CAA-CTA-GCA-TCC-ATC-AG 3'
	generalist	R1594	5' GAC-GGC-TAT-GTT-GAG-GAA-GG 3'
	specific of *Bandicota savilei*	FBsa	5' CAT-ACA-CCA-TAA-AGT-CCA-AAA-TCA 3'

Protocol: PCR-test for *Bandicota* species assignment

- **PCR1**: discrimination between *Bandicota* and other rodent genus (*Berylmys, Niviventer, Leopoldamys, Cannomys, Chiropodomys, Rattus)*.

1- Perform PCR amplifications in 10 µL reaction volumes with 2 µL of DNA matrix pre-diluted forty times after extraction (if using Qiagen Spin-Column Protocol).

2- Add 0.2 µL for the FBan primers and 0.2 µL for the other primer pairs L14723 and H15915 (10 µM) (Table 3), 0.4 µL of a Deoxynucleoside-triphosphate mixture (dNTP 2.5 mM), 1µl of reaction red Coral Load buffer (10*) (Qiagen), 0.5 µL of $MgCl_2$ (25 mM) and 0.1 µL of Taq DNA polymerase (Qiagen).

3- Use the following thermal cycling parameters: 2 min at 94°C, 30 cycles (30 s at 94°C, 30 s at 52°C, 60 s at 72°C), with a final extension of 10 min at 72°C

4- Visualize band products by migrating the DNA fragments in an agarose gel to 2.5% so as to clearly distinguish the specific PCR-fragments. Two PCR-fragments, one of 320 bp (FBan, H15915) and a second one of 1,150 bp (L14723, H15915) are obtained for *Bandicota* samples when only one PCR-fragment of 1,150 bp (L14723, H15915) is obtained for samples belonging to other genus (Fig. 47).

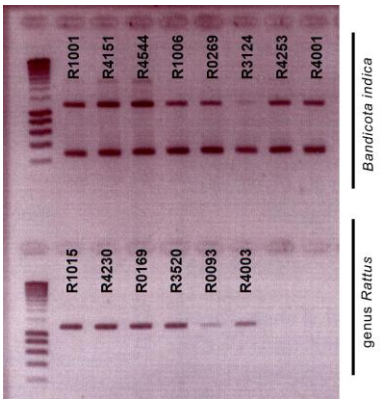


Figure 47: PCR1 agarose gel to differentiate genus Rattus and genus Bandicota (Photo: Chaval Y.)

- **PCR2**: discrimination between *Bandicota savilei and Bandicota indica*.

1- Perform PCR amplifications in 10µl reaction volumes with 2 µL of DNA matrix pre-diluted forty times after extraction (if using Qiagen Spin-Column Protocol).

2- Add 0.2 µL for the FBsa primers and 0,2µl for the other primer pairs F1043 and R15945 (10 µM) (Table 3), 3.2 µL of a Deoxynucleoside-triphosphate mixture (dNTP 2.5 mM), 1 µL of reaction red Coral Load buffer (10*) (Qiagen), 0.5 µL of $MgCl_2$ (25 mM) and 0.1 µL of Taq DNA polymerase (Qiagen).

3- Use the following thermal cycling parameters: 2 min at 94°C, 30 cycles (30 s at 94°C, 30 s at 52°C, 60 s at 72°C), with a final extension of 10 min at 72°C.

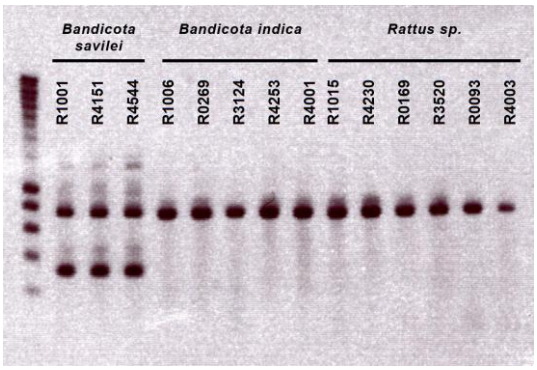


Figure 48: PCR2 agarose gel to differentiate species of the genus Bandicota. (Photo: Chaval Y.)

4- Visualize band products by migrating the DNA fragments in an agarose gel to 2.5%. Two PCR-fragments, one of 350 bp (FBSav, R1594) and a second one of 750 bp (F1043, R1594) are obtained for *B. savilei* while only one of 750 bp (F1043, R1594) is obtained for *B. indica* and samples of *Rattus* genus (Fig. 48).

- **Multiplex PCR**: discrimination between *Bandicota savilei*, *B. indica* and the genus *Rattus*.

Further tests revealed that mixing these primers (FBan, L14723, H15915, FSav, F1043, R1594) within a single PCR produces reliable results.

1- Perform PCR amplifications in 10 µL reaction volumes with 2 µL of DNA matrix pre-diluted forty times after extraction.

2- After adjusting the PCR reaction's conditions, each specific reaction includes 0.2 µL for all the six primers (10 µM) (Table 3), 0,4µl of a Deoxynucleoside-triphosphate mixture (dNTP 2.5 mM), 1µl of reaction red Coral Load buffer (10*) (Qiagen), 0.2 µL of MgCl2 (25 mM) and 0.1 µL of Taq DNA polymerase (Qiagen).

3- Use the following thermal cycling parameters: 2 min at 94°C, 30 cycles (30 s at 94°C, 30 s at 52°C, 60 s at 72°C), with a final extension of 10 min at 72°C.

4- Visualize band products by migrating the DNA fragments in an agarose gel to 2.5%. Five PCR-fragments (320 bp (FBan, H15915), 350 bp (FBSav, R1594), 750 bp (F1043, R1594), 950 bp (FBan, R1594) and 1,150 bp (L14723 ,H15915)) are obtained for *B. savilei.* Four PCR-fragments (320 bp (FBan, H15915), 750 bp (F1043, R1594), 950 bp (FBan, R1594), 1,150 bp (L14723, H15915)) are obtained for *B. indica.* Two PCR-fragments (750 bp (F1043, R1594) and 1150 (L14723, H15915)) are obtained for *Rattus* genus (Fig. 49).

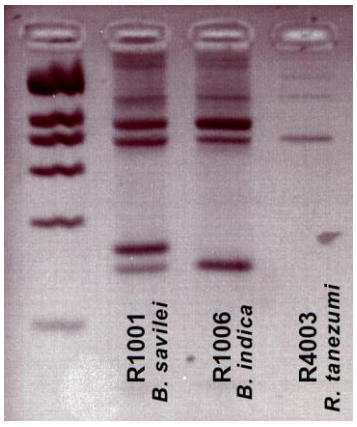


Figure 49: Multiplex PCR agarose gel to differentiate the genus Bandicota and R. tanezumi. (Photo: Chaval Y.)

3.3.2. DNA barcoding

Definition

DNA barcoding *is a recent tool increasingly used to identify species in large-scale studies (the Barcode of Life Data (BOLD): http://www.barcodinglife.org/views/ login.php).*

It uses a very short genetic sequence from a standard part of the genome. For almost all animal groups this sequence is a 648 base-pair region in the mitochondrial ***cytochrome c oxidase 1 gene*** *("CO1").*

Example of the CERoPath barcoding tool "RodentSEA":

In order to facilitate the work of field mammalogists in South East Asia, CERoPath developed a reference Barcoding database ("RDBSEA"), which is available online and allows species assignation by reference of a DNA database "RodentSEA" (http://www.ceropath.org/barcoding_tool/rodentsea).

RodentSEA accepts DNA sequences from the barcode region and returns a taxonomic assignment to the species level when possible. It offers a reliable expert system for systematic assessment between an unknown sequence submitted by the user and a collection of reference sequences. This comparison is based on K2P (Kimura 2 Parameter) distances matrix computation and tree representation using Neighbour-Joining clustering method (Fig. 50).

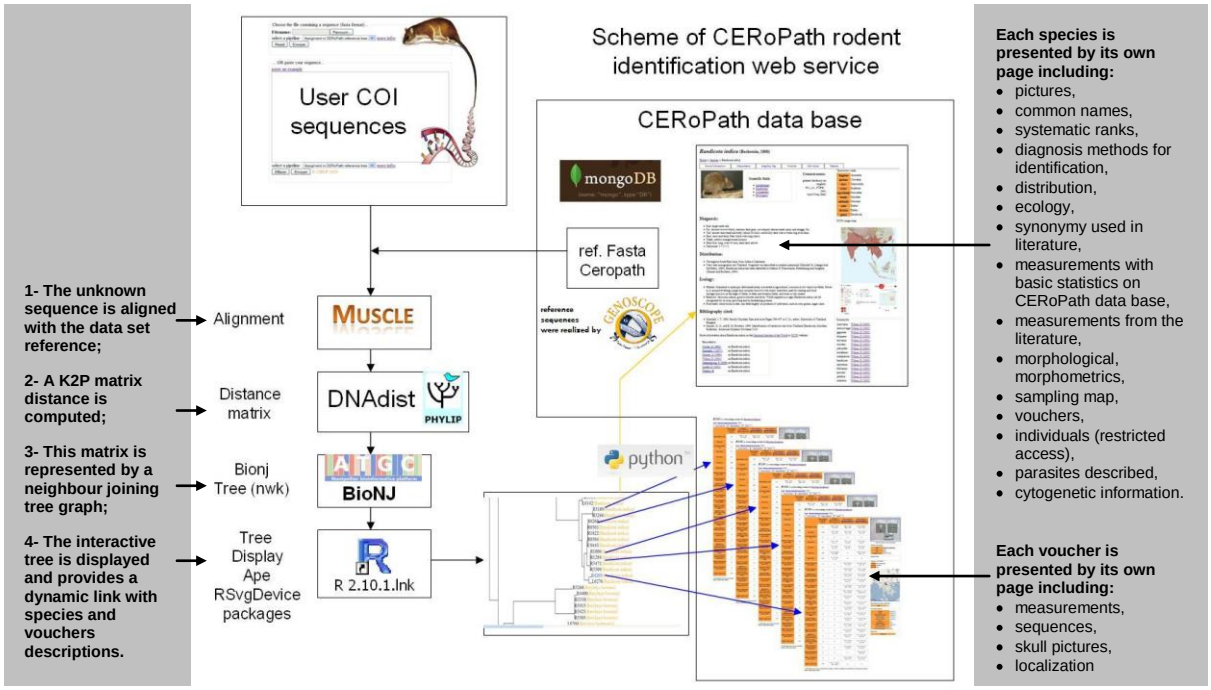


Figure 50: Structure of the CERoPath identification tool and the links with the virtual museum to present vouchers and species information (http://www.ceropath.org/barcoding_tool/rodentsea).

Protocol for COI amplification under standard PCR conditions:

Table 4: Generalist primers used to obtain amplicons of COI genes and produce references sequences (See the list of taxa assessed by the CERoPath barcoding tool below)

Gene	Type of primer	Name	Primer
COI gene	generalist	BatL5310	5' ACTTCTGGGTGTCCAAAGAATCA 3'
	generalist	R6036R	5' CCTACTCRGCCATTTTACCTATG 3'

1. Perform PCR amplifications in 35 µL reaction volumes with 3.5 µL of DNA matrix pre-diluted forty times after extraction (if using Qiagen Spin-Column Protocol).
2. Add 0.7 µL for other primer pairs BatL5310 and R6036R (10 µM) (Table 4), 4.2 µL of a Deoxynucleoside-triphosphate mixture (dNTP 2.5 mM), 3.5 µL of reaction red Coral Load buffer (10*) (Qiagen), 0.7 µL of $MgCl_2$ (25 mM) and 0.28 µL of Taq DNA polymerase (Qiagen).
3. Use the following thermal cycling parameters: 4 min at 94°C, 40 cycles (30 s at 94°C, 30 s at 48°C, 60 s at 72°C), with a final extension of 10 min at 72°C.

List of taxa assessed by the CERoPath barcoding tool:

Bandicota indica (Bechstein, 1800), *Bandicota savilei* (Thomas, 1916), *Berylmys berdmorei* (Blyth, 1851), *Berylmys bowersi* (Anderson, 1879), *Cannomys badius* (Hodgson, 1841), *Chiropodomys gliroides* (Blyth, 1856), *Hapalomys delacouri* (Thomas, 1927), *Leopoldamys edwardsi* (Thomas, 1882), *Leopoldamys neilli* (Marshall, 1976), *Leopoldamys sabanus* (Thomas, 1887), *Maxomys surifer* (Miller, 1900), *Mus caroli* (Bonhote, 1902), *Mus cervicolor* (Hodgson, 1845), *Mus cookii* (Ryley, 1914), *Mus fragilicauda* (Auffray et al., 2003), *Niviventer fulvescens* (Gray, 1847), *Niviventer hinpoon* (Marshall, 1976), *Niviventer langbianis* (Robinson and Kloss, 1922), *Niviventer tenaster* (Thomas, 1916), *Rattus andamanensis* (Blyth, 1860), *Rattus argentiventer* (Robinson and Kloss,1916), *Rattus exulans* (Peale, 1848), *Rattus losea* (Swinhoe, 1870), *Rattus nitidus* (Hodgson, 1845), *Rattus norvegicus* (Berkenhout, 1769), *Rattus* phylogenetic r3 (taxonomic statute unclear), *Rattus tanezumi* (Temminck, 1844), *Rattus tiomanicus* (Miller, 1900), *Rhizomys pruinosus* (Blyth, 1851).

Protocol for using the CERoPath barcoding tool "RodentSEA":

Perform COI sequences for the samples to be assigned at the species level.

1- Connect RodentSEA: (Fig 51, nb 1) http://www.ceropath.org/barcoding_tool/rodentsea

2- Either paste the COI sequence on the dedicated window (Fig 51, nb 2) or upload a file containing COI sequences in fasta format (Fig 51, nb 2bis).

3- Select the reference dataset (Fig 51, nb 3), either the CERoPath dataset, or the CERoPath dataset with additional data from the literature made available online.

4- Send the query (Fig 51, nb 4).

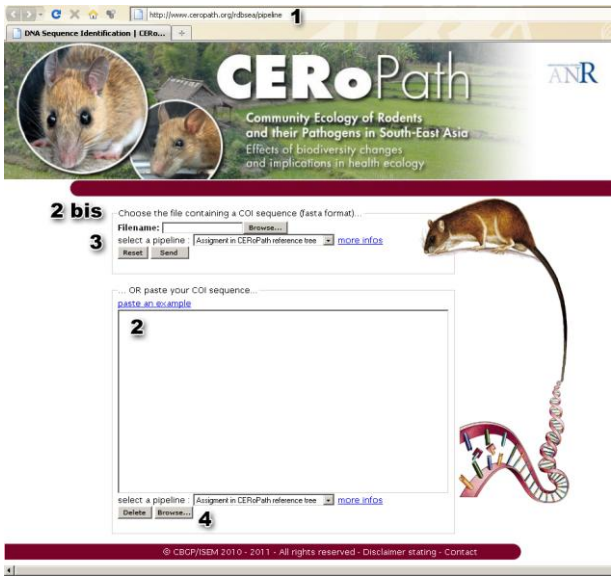


Figure 51: CERoPath identification tool based on COI comparison between a user sequence and the CERoPath data set reference (http://www.ceropath.org/barcoding_tool/rodentsea)

5- The analysis returns a phylogenetic tree, which places the unknown data sequence among the reference samples of the database (Fig. 52).

Detailed information about species and voucher specimens in the reference database can be accessed from the website. In 2010, the reference database of CERoPath was constituted by 170 individuals belonging to 27 species sampled in Cambodia, Laos and Thailand. 156 of these individuals were used in two taxonomic issues of the CERoPath project (Badenhorst et al., 2009; Pagès et al., 2010). The number of species and specimens of the database might increase after 2010.

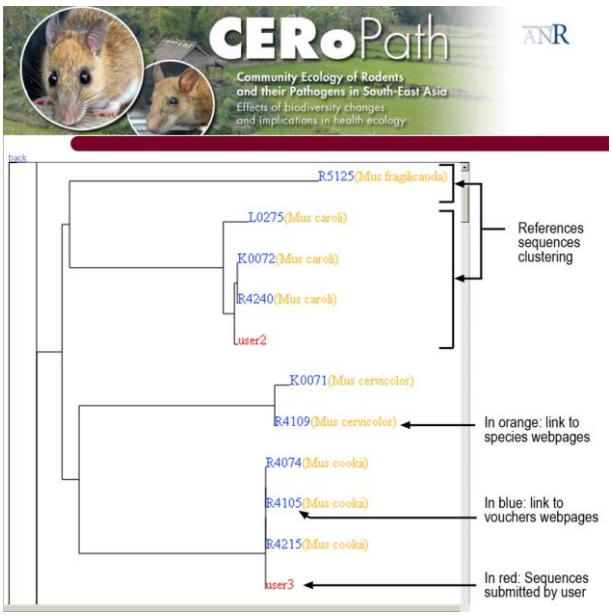


Figure 52: Interactive tree generated by the CERoPath identification tool after alignment of unknown sequences

The CERoPath barcoding tool is connected to a large rodent database that can be consulted online and presents molecular, morphometrical, museological, parasitological, cytogenetical, phylogenetical, ecological and spatial information collected by the project and ensures that these data are clearly linked to voucher specimens.

3.4. Cytogenetics: karyotyping and tissue sampling for fibroblast cell culture

(Gauthier Dobigny and Anne Xuéreb)

3.4.1. Karyotypes: mitotic chromosome preparation from bone marrow

Definitions

*A **karyotype** corresponds to the arranged chromosomes of one eukaryote cell. It allows one to describe the number and appearance of chromosomes. Special attention is paid to their length, the position of the centromeres, identification of the sex chromosomes, among other characteristics. The preparation and study of karyotypes is part of **cytogenetics**.*

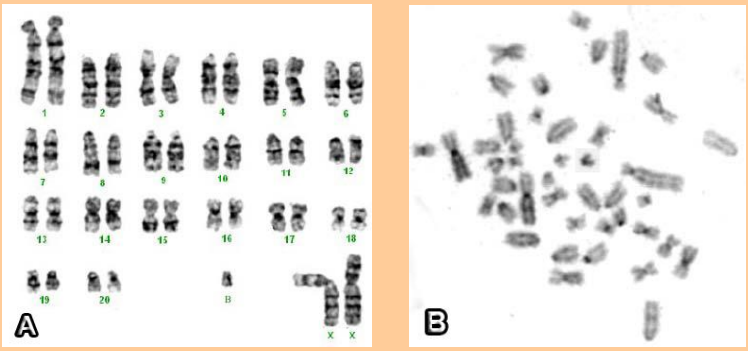


Figure 53: G-banded karyotype (Fig A) and C-banded metaphase (Fig B) of a female Bandicota savilei *(R4408) from Thailand (Badenhorst et al., 2009).*

Preparation:

- ***Yeast solution:***
  0.5 g of yeast (baking powder) + 1.1 g of glucose + 5 mL of water
  Leave fermenting from 10 to 20 minutes at room temperature
  Keep in fridge at 4°C

- ***Hypotonic solution:***
  KCl 0.075 M (equivalent to 0.56 g of KCl in 100 mL distilled water)
  Can be kept in fridge at 4°C for 1-3 days, but should preferably be prepared just before use

- ***Vinblastine sulfate solution:***
  (Preparation depends of the packaging of the Vinblastine sulfate solution)
  40 mL NaCl (Sodium chloride) 8.5 ‰ (1g NaCl in 118 mL distilled water)
  10 mg Vinblastine sulphate (an anti-mitotic drug used to treat some types of cancer)
  Aliquot in 1.5-2 mL Eppendorf® tubes
  Keep in freezer at -20°C (the solution is not stable)

- ***Fixative solution:***
  Methanol / Acetic acid (3 / 1 v:v)
  For 10 animals, prepare around 120 mL of fixative: 90 mL Methanol + 30 mL Acetic acid
  Can be kept in freezer at -20°C for 1-4 days, but should preferably be prepared before use.
  To be used cold (i.e. kept stored at -20°C all along the experiment)

Correspondence tables between solutions and animal weight:

Table 5: Volume of yeast to inject according to the animal weight

Animal weight (g)	*10*	*50*	*100*	*200*	*300*	*400*	*500*
Yeast volume (mL)	*0.1*	*0.5*	*1*	*2*	*3*	*4*	*5*

Table 6: Volume of Vinblastine solution to inject according to the animal weight

Animal weight (g)	*10*	*50*	*100*	*200*	*300*	*400*	*500*
Vinblastine solution volume (mL)	*0.1*	*0.25*	*0.5*	*1*	*1.33*	*1.66*	*2*

Karyotyping from bone marrow:

Chromosomes are observable only during mitosis (i.e. in dividing cells), especially at metaphase. Therefore, bone marrow is the most appropriate tissue to target since it shelters developing (so actively dividing) immune cells, hence providing numerous mitoses.

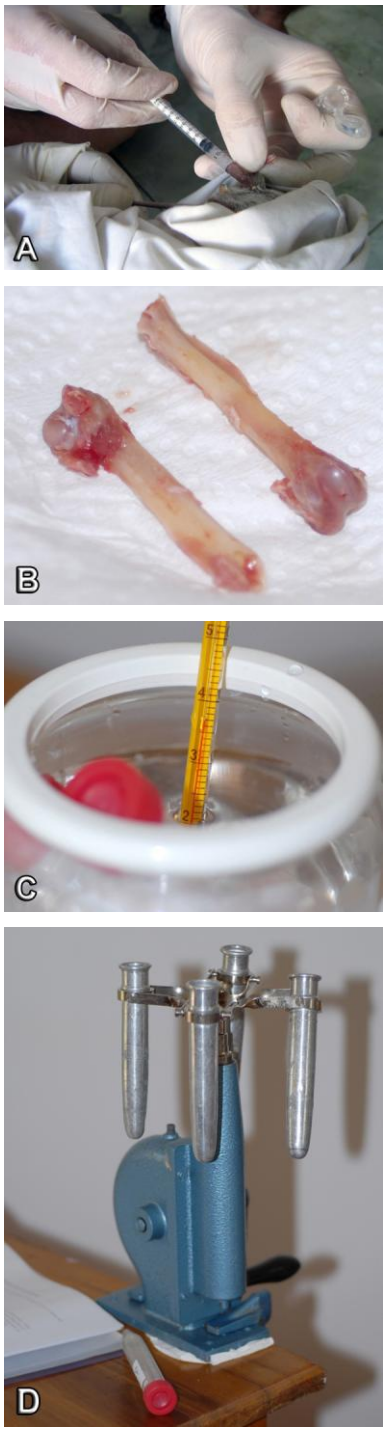


Figure 54: Karyotyping from bone marrow (Photo: Herbreteau V.)

Recommendation:

Inject a yeast solution one day before processing, in order to increase mitotic divisions, thus allowing for a better mitotic index (ratio between the number of dividing cells vs. the number of non-dividing cells).

Protocol:

1- Inject the yeast solution in the animal's back (in order to avoid lethal internal damages) 12-24 hours before the next step. See Table 3 for quantities in regards to the specimen weight.

2- Inject the rat with the Vinblastine sulfate solution (see Table 4 for proportions) intra-peritoneally (into the peritoneum = body cavity). Wait 45-60 minutes before sacrifice (Fig. 54A).

3- Sacrifice the rat and remove one femur or tibia carefully, with the bone head if feasible.

4- Clean and dig the extremities of the bone heads in order to insert a 23 gauge needle into the marrow cavity (Fig. 54B).

5- Flush out cells into a 15 mL conical centrifuge tube using a 1 cc syringe filled with the KCl hypotonic solution.

6- Incubate the tubes at 37° C for 20 minutes, with smooth agitation every 5 minutes (Fig. 54C).

7- Add 1-2 mL of cold fixative and centrifuge at 400 g (or 1,200 t/min) for 5 minutes (Fig. 54D).

8- Remove the supernatant without disturbing the cellular pellet, add 1-2 mL of cold fixative and resuspend. Complete with 6-8 mL of cold fixative.

9- Allow the tubes to sit at 4-20° C for at least 20 minutes.
The procedure can be interrupted at this point and resumed later. Always refrigerate cells if they are to be left standing in fixative longer than 30 minutes.

10- Centrifuge at 400 g, remove the supernatant, and resuspend the cellular pellet in cold fresh fixative (Fig. 54D).

11- Note that step 9 can be performed once more.
The last addition of fixative should be just enough to make a thin cell suspension (the solution in tube should look slightly opaque due to the cells in suspension. However, if cells are present but rare, the solution will look clear).

12- Metaphasic preparations are usually conserved in 1.5-2.5 mL (depending on the cell concentration) at -20°C. However, they may support 1-2 days at room temperature, provided the fixative is then replaced by fresh fixative before long-term storage at -20°C.

Slide preparation for karyotype observation:

1- Immerse pre-cleaned slides in 100% ethanol prior to use, then wipe them dry using either a Kimwipe (or other lint-free tissue) or a flame.

2- Drop 20-100 µl of cell suspension onto the slide surface with a Pasteur pipette and allow it to spread. Spreading of chromosomes (which guarantees a clear observation of the metaphases) may be improved by adding a drop of cold fixative onto the just-dropped cell suspension.

3- Leave it to dry at room temperature or close to a flame. Note that rapid drying is usually an indication of well spread metaphases. Keep the slide in a flat position while adding the fixative and until the slide is completely dry.

4- Control for cell concentration and metaphases spreading under a phase contrast microscope.

5- Slides can be stained using a buffered (pH=6.8) 4% Giemsa solution (conventional staining) or kept at -20°C as long as necessary, waiting for further treatment (banding or molecular cytogenetics).

3.4.2. Tissue sampling for cell culture

Definitions

Cell culture *is a complex process by which cells are grown under controlled conditions.*

Cells can be isolated from tissues for ex-vivo culture in several ways. A simple way, i.e. ***explant culture****, consists to place pieces of flesh that are rich in conjonctive tissues in growth media, in order to allow fibroblast cells to adhere and proliferate.*

Cells are grown at an appropriate temperature and gas mixture-regulated pH (typically, 37°C, 5% CO_2 so pH 7 for mammalian cells) in a growth medium (that includes glucose concentration, growth factors, and all required nutrients) and maintained in a ***cell incubator*** *(Fig. 55). In some rare cases, culture conditions vary widely for each cell type, and slight variation in growth medium composition, pH and/or temperature conditions for a particular species.*



Figure 55: Cell incubator at CBGP, France (Photo: Xuéreb A.)

Recommendations:

- *Tubes containing culture medium must be kept in a 4°C fridge until use. Tubes may not be used if the color of growth medium has turned either to dark pink (basic pH) or yellow (acid pH). Trouble medium means bacterial and/or fungal contaminations, and should not be used.*
- *Ideally only place a single sample (even from the same animal) per tube, in order to limit the contamination risks as well as sample cross-contamination.*
- *Since cell cultures require living tissues, sampling should be performed at the very beginning of the dissection process, as soon as the body is opened. In the same manner, processing must occur within 1-4 days.*

Protocol:

1- Thoroughly disinfect (using ethanol and/or an antiseptic solution) the working bench and the dissection tools. Limit any draught if working in an open-air space.

2- Prepare two sets of scissors and forceps, one for “external dissection” (opening of skin, or cutting of tail), and another one for the tissue sampling per se. Keep those for sampling in ethanol 100% (for over 20 minutes). If possible, use a Bunsen burner (or ethanol burner) to disinfect tools again a couple of seconds before use. Beware of transporting the flame with wet tools to an ethanol-soaked processing board or ethanol-soaked animal (guaranteed fire).

3- Tissue sampling:
   - For live animals: the simplest way is to cut a small piece of ear.
     - Clean the ear with ethanol as well as possible (at least one minute);
     - Use the disinfected forceps and scissors to cut a small piece of ear and place it as soon as possible in the culture tube.
   - An alternative for live animals: cut the extremity of the tail.
     - Cut the extremity of the tail.
     - Pull out the skin off the tail (at its tip) with clean forceps.
     - With other forceps hold the tailbone to cut its extremity with clean scissors.
     - Place it as soon as possible in the culture tube.
   - For animals to be dissected: cut a small piece of intercostal tissue.
     - Clean the body with absolute ethanol (at least 2 minutes) or, even better, antiseptic solution (e.g. Chlorhexidine).
     - Pull the skin out of the thoracic cage with forceps and scissors without touching the thorax.
     - Use the disinfected instruments to cut a small piece of intercostal tissue and place it as soon as possible in the culture tube.

4- Quickly close the tube and secure the culture tube with Parafilm® if necessary.

5- Send the tube to the laboratory as quick as possible (after a few days at room temperature, culture might be spoiled). If delay cannot be avoided, preferably keep the sample in culture medium in a fridge at 4°C.

4. Isolation of helminths from rodents

(Alexis Ribas Salvador, Kittipong Chaisiri, Serge Morand, Jean-Pierre Hugot, Voitto Haukisalmi and Heikki Henttonen)

Definitions

***Helminths**, also called parasitic worms, constitute a division of eukaryotic macroparasites that live inside their host. They can be found in different locations, known as microhabitats (body cavity, lungs, stomach, small intestine, caecum, blood…). They derive nourishment by disrupting their hosts' nutrient absorption and circulation, sometimes causing disease.*

Rodents are definitive or intermediate hosts for adult or larval stages of helminths, respectively. Helminths are categorized into four groups (Cestoda, Nematoda, Trematoda and Acanthocephalans). Pentastomids are also sometimes studied by helminthologists, as they are recovered during dissections. In rodents, helminth size varies in length from few millimeters to tens of centimeters (the longest are found within the Cestoda).

Cestoda (tapeworms)

- *Segmented plane, covered with tegument.*
- *Presence of a scolex with 4 suckers and sometimes with an armed rostrellum in adults.*
- *Hermaphrodites.*



Figure 56: Scolex of Raillietina sp. with 4 suckers and armed rostellum (20x) (Photo: Ribas A. and Chaisiri K.)

Nematoda (roundworms)

- *Cylindrical shape, covered with collagenous cuticle.*
- *Various styles of head, with / without lips and teeth.*
- *Dioecious.*



Figure 57: Filarid worm (Photo: Ribas A. and Chaisiri K.)

Trematoda (flukes)

- *Unsegmented plane, covered with tegument.*
- *Presence of two suckers (oral and ventral or posterior sucker), rarely one.*
- *Hermaphrodites.*



Figure 58: Notocotylus loeiensis (Photo: Ribas A. and Chaisiri K.)

Acanthocephala (thorny-headed worms)

- *Externally segmented and round, covered with fine cuticle.*
- *Presence of a protrusible proboscis, covered with hooks, to pierce the gut of its host. No mouth.*
- *Dioecious.*

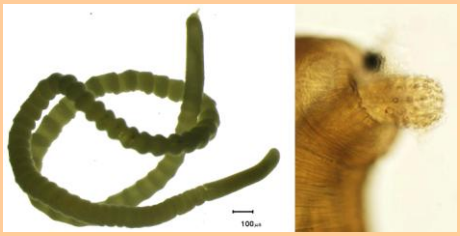


Figure 59: Moniliformis moniliformis with pseudo-segmented body (Photo: Ribas A. and Chaisiri K.)

4.1. Gastrointestinal (GI) tract and helminths isolation

Recommendations:

- *Helminths should be separated by organs, cestodes relaxed in water, and preserved in 70% ethanol in a tube. Tubes should be carefully labeled with the animal identification number and name of organ.*
- *Do not use formalin to store specimens that would prevent genetic analysis.*
- *Isolate the gastrointestinal tract, as soon as possible.*



Figure 60: GI dissection in Kalasin province, Thailand (Photo: Herbreteau V.)

Protocol:

1- **During the dissection**, check each organ for the presence of helminths. Especially check:

- Liver where *Taenia* and other tapeworm cysts may be present in some species (Fig. 61A). Cysts should be separated and preserved in ethanol (Fig. 61B). If a cyst is opened, slowly and carefully pull the worm out of the cyst, avoiding any damage. Larval tapeworm cysts can also occur freely in body cavities
- Lungs. Although lungs are collected to screen for other parasites and pathogens, they should be observed for the eventual presence of *Angiostrongylus* sp.
- Thoracic cavity, where *Filaridae* can be found (Fig. 62).

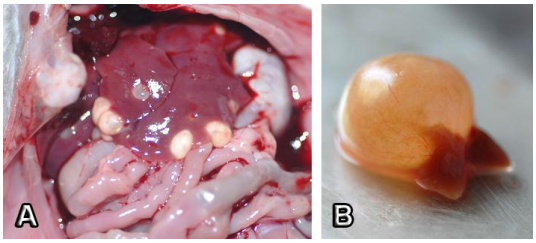


Figure 61: Cysts of cestode (Taenia taeniaeformis) isolated from liver (Photo: Herbreteau V.)

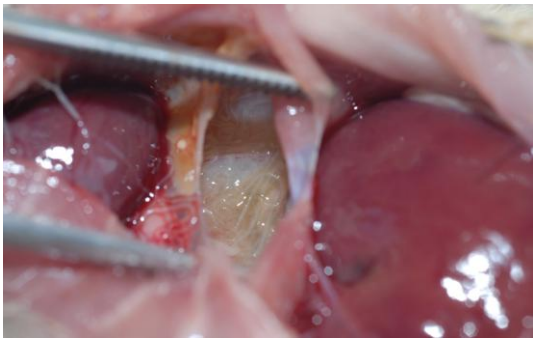


Figure 62: Filaria from the lung cavity (Photo: Herbreteau V.)

2- **Separate the gastrointestinal tract:** cut the end of rectum before the anus and lift the GI tract (Fig. 63). Carefully cut ligaments connecting the GI tract to the dorsal side of the body cavity. Separate it from the liver and finally cut it from the esophageal sphincter of stomach.

3- **If the gastrointestinal tract cannot be examined the same day,**

- but within one or two days: GI tract can be kept in a refrigerator and the material remains good enough for microscopical and genetic studies. GI tract should be placed in a small zipper bag, air pressed out.

- or not in a few days: place the GI in a zipper bag, press air out and freeze (normal freezer is OK). Frozen and later thawed helminth material is perfect for microscopical and genetic work.

- otherwise (this is the least preferred alternative): place the GI tract in a hermetic plastic box, Falcon tube (Fig. 64 and 65) or even a plastic bag, filled with 80% ethanol as a fixative agent. If possible, ethanol should be changed the next day because released water from GI tissue will dilute the ethanol. Cestode material fixed this way is contracted, not relaxed, and can seldom be used for detailed microscopic morphology (species descriptions).



Figure 63: Separation of the gastrointestinal tract (Photo: Herbreteau P.)

For a better conservation, a minimum of 4 volumes of ethanol should be added to one volume of viscera. Do not forget to put an identification tag with the GI. As tags tend to be attached to viscera and become dirty with blood, use plastic tags (such as Dymo®) instead of normal paper. Be sure the container is completely hermetic to avoid evaporation as dry viscera are not suitable for helminthological study. In case containers used in the field are not big enough, renew ethanol once back in the laboratory; this will allow a better conservation of helminths.



Figure 64: Preservation of GI in Falcon tubes (Photo: Chaisiri K.)

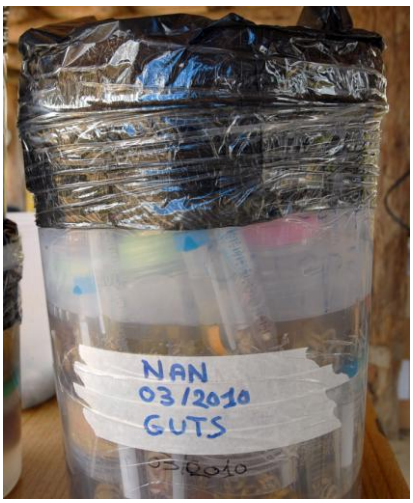


Figure 65: Preservation of Falcon tubes in a container filled with 70% ethanol to avoid evaporation (Photo: Herbreteau V.)

4.2. Helminths collection in the gastrointestinal tract

Table 7: Possible organs with helminths in rodents

Organs	Cestoda	Nematoda	Trematoda	Acanthocephalans
Stomach	No	Yes	Yes (ex: *Riberoia* spp.)	No
Small intestines	Yes	Yes	Yes	Yes
Caecum	Detached gravid segments	Yes	Yes	No
Body cavity	Larval cestode cysts	Yes	No	Yes
Other organs	Larval cestode cysts (*Taenia taeniaformis*) in liver	In lungs (*Angiostrongylus* sp.), thoracic cavity (filarids), diaphragm and muscles (*Trichinella* sp.), reproductive organs, mesenteric veins, urinary bladder	Lung (*Paragonimus* sp.), liver, mesenteric veins, (*Schistosoma* spp.)	No

Protocol: (Henttonen and Haukisalmi, 2008)

1- Bring a gut sample out from the container and put it in a Petri dish (Fig. 66). Do not forget to record the identification number of each animal.

2- Pour some saline solution or tap water (not too much) into the Petri dish to facilitate the dissection and examine under the stereomicroscope (In case viscera are not fresh and have been previously stored in ethanol, use water instead of saline solution). First uncoil the small intestine by cutting mesenteries that attach intestine and stomach together.

3- Separate into 3 parts: stomach, small intestine and caecum. Put each part in individual Petri dishes and start dissection one by one, however, if you see from outside that there is a cestode in, do not cut it:



Figure 66: GI tract in a Petri dish (Photo: Chaisiri K.)



Figure 67: Observation of nematodes (Spiruridae) from stomach (Photo: Herbreteau V.)



Figure 68: Dissection of intestines under binocular (Photo: Herbreteau V.)

- **Stomach**: cut it along the surface layer in a separate Petri dish with some saline solution, and spread the contents. Use more water to separate helminths. Use a pipette to remove the excess of liquid. Large spirurid nematodes are the normal findings (Fig. 67).

- **Small intestine (SI)**: cut the mesenteries to straighten SI. Open lengthwise starting from the anterior part by carefully sliding blunt tipped (iris) scissors to cut open and expose the lumen (Fig. 68). Alternatively, dissecting can be started from the posterior end, allowing the dissector to encounter the posterior end of the worm first and thus more easily to save the scolex (recommended for newcomers). Try to slide the scissors along the intestine surface layer not to cut the potential worms inside. The SI can be cut into 2 or 3 segments to make the dissecting easier to avoid parasites to be cut.

  Stir opened SI in water in a Petri dish to separate the helminths. Remove tapeworms (Cestoda) to a separate dish in about 1 cm of water to make them relax (extended to natural shape) and allow the observation of internal organs by microscopy.
  If a cestode scolex remains attached to the SI wall, let it relax for a while in water before trying again. Stir carefully the relaxing tapeworm a couple of times so that possible intestinal debris will be detached from the surface. Change the water if there is too much “mud” and stir again. Too much debris on the surface of the tapeworm prevents the good microscopical visibility to the in internal organs. Record the location of the worm in the small intestine (first, second, or third part – this is important because various species/species complexes have specific locations in the intestine). If the relaxing tapeworm has no scolex, look carefully for the scolex in the water of the dissecting Petri dish -- this will be helped by using a dissecting scope or magnifying loop. Pay attention to the presence of small nematodes and trematodes before throwing the content of the Petri dish, since they are very difficult to see (few millimeters in length for the adult form). Nematodes, such as trichostrongylids and syphaciids, can be found in great numbers.

- **Caecum**: nematodes are the normal findings in the caecum. Use of a stereomicroscope or magnifying glass is recommended since some nematodes are relatively small and hard to observe. The genus *Trichuris* shows the thin oesophagus strongly attached to the mucosa of the caecum. Gently use tweezers to detach oesophagus from mucosa. Trematodes might be covered with "greenish mud" and are difficult to separate from plant materials. It may be useful for a newcomer to run material through a small sieve to first discard some fine particulates.

4- Collect helminths:
   - **Cestodes** should be held and washed (stirred) in water at least 2 hours, depending on their size, until totally relaxed, i.e. flat and straightened. This is necessary to enhance their observation, especially to examine the internal structure of the proglottids (segments). Following relaxation and death in water, all tapeworms will be preserved in 70% ethanol. Fixation should be done flat without pressure, and let the cestode stay (preferably) overnight on covered Petri dish. Use fresh ethanol when preserving cestodes in vials. Ethanol should be changed once after 24 hours, especially for the large-sized species. It is recommended that ethanol-preserved cestode materials used later for genetic studies will primarily be stored in refrigerator.
   - **Trematodes** can be relaxed and washed in water, which often allows specimens to expel eggs that might otherwise obscure some organs. Preserve flukes in 70% ethanol.
   - **Nematodes** should be held in saline as osmotic pressure will cause the specimen to burst if kept in normal water. Specimens should be washed in saline and then soon preserved in 70% ethanol. Keep parasites from different organ systems separated.
5- Separate parasites by group, based on morphology, for each individual animal, to help further identification. Count helminths under the stereomicroscope. Label (by pencil) the details of collection on drawing paper and put it into the tube (at least: identifying number of parasite and host, group or genus of parasite, date, location in the GI tract). The absence of helminths should also be recorded for further analyses.
6- Keep vials in a cool place and clean instruments between the individuals to avoid contamination of samples.

4.3. Method for karyological analysis of helminths

First of all, a subsample of the helminth to study must be preserved for morphological study (see above), as this method will destroy the sample.

Protocol:

1- Treat living worms with 0,025 % colchicine in normal saline for 30-60 min at 37°C in order to increase the number of observed metaphases.
2- Subject the whole worms to hypotonic treatment (0.6 sodium citrate or 75 mM KCl, 20-60 min at 37°C).
3- Dissect testes or ovary (or small tissue pieces containing the organs), put them into the hypotonic solution for 15 min.
4- Put the tissue pieces into the fixative (cold, freshly mixed Carnoy: methanol: acetic acid 3:1 - if methanol is problem, you can provisionally use ethanol) for 30-60 min., then change the fixative one or two times.
5- Put the samples within the new fixative to refrigerator and let them frozen till next use for chromosome preparations.
6- Then, dissociate one small tissue piece in a drop of 60% acetic acid on the slide, put the slide onto a heating plate at 45°C and spread the drop of acetic acid with the cells slowly on the slide. Let the slide dry and use it for staining (Giemsa, C- banding, fluorescent dyes etc.). Alternatively, slides can be dehydrated in an ethanol series (70%, 80%, and 100%, 1 min each). Store slides at -20ºC until use.

4.4. Helminths fixation for scanning electron microscopy

Even if using hot ethanol is correct, a higher quality of images can be obtained by following this protocol:

Protocol:

1- Different reagents must be mixed the day of use for an optimal conservation:
   1 mL of gluteraldeide + 4 mL of distilled water, then add 5 mL of cacodilate.
   This fixative must be refrigerated before and after use.
2- Once helminths are as clean as possible (in physiologic solution), place them in a microtube with the fixative. Fill microtubes completely with fixative to ensure that specimens remain immersed during transportation. Very small individuals can be stored together in the same microtube to be mounted (this will allow a better chance to get them mounted in a perfect position in the support).
3- Samples have to be kept in a fridge until complete dehydration process is achieved.

5. Preparing rodent specimens for collections

(Yannick Chaval, Surachit Waengsothorn, Julien Claude, Saksiri Soonchan and Vincent Herbreteau)

Keeping the whole specimens in a collection may have different purposes. First, these specimens may complete an institutional collection established for the sake of taxonomical and biological conservation studies. Second, they may constitute an educative collection for teaching exercises. Third, when trapped for a research project, it might be useful to keep the whole bodies till the end of the project in order to be able to control identifications or even solve any mistakes made during data recording.

Rodents can be prepared as dry or wet specimens, depending on these purposes, on the available time in the field, on the animals' state of conservation and on the experience of the workers.

Recommendations:

Before preparing specimens to enter a collection, it is necessary to:

- ***Record all relevant information*** *relative to the animal and the circumstances of its capture. This information should be precisely kept into a database and summarized on a label that must be tied to the animal.*
- ***Keep a piece of tissue for DNA analysis****.*
- ***Take pictures*** *of the animals before preparation.*

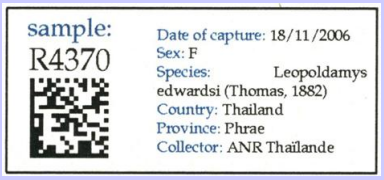


Figure 69: Label of the skull of R4370 with barcode (Photo: Chaval Y.)

Labels can now integrate a ***barcode*** *to link with a detailed database (Fig. 69).*

Labels *traditionally give details of (Fig. 70):*

- *Collection: name, collector's name, animal's identification number;*
- *Sampling: date, location (administrative details + GPS coordinates), habitat;*
- *Animal: species, sex, morphology.*

Labels should be durable, with high quality ink and paper, and securely tied to the specimen.

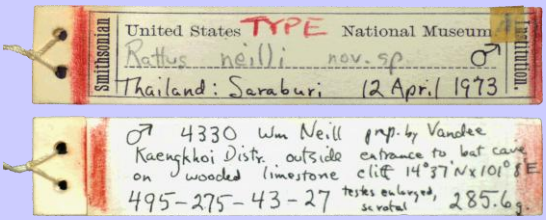


Figure 70: Label of Leopoldamys neilli *type specimen (Photo: Herbreteau V.)*

5.1. Dry collections

Traditionally, dry collections include the skin and skull of a few specimens for each collected species (Fig. 71 and 72). Well-prepared, these dry specimens are easily observables and can be conserved durably in suitable dry conditions. These collections are generally hosted by national museums or universities.

New species, as well as typical specimens of every species (adults, males and females, body not damaged) should be selected, prepared and kept into a dry collection. These animals should be preserved shortly after death; otherwise the skin will start degrading and loose hairs. Both skin and skull are prepared and carefully hung and labeled together. DNA sample should also be preserved; such specimens can potentially be used as voucher specimens.



Figure 71: Dry specimens of Bandicota savilei from the Thai national collection, at TISTR (Photo: Herbreteau V.)

5.1.1. Skin preparation

Protocol:

Use a scalpel and small scissors. Use cotton to absorb liquids and avoid tainting the pelage, while skinning.

1- Opening: lay the animal on its back and start the dissection around the genital opening and then to the leg halfway between knee and foot. Then cut between the anus and genital opening, and down the other leg.

2- Feet: keep the feet attached to the skin: cut the wrists or ankles (the feet will dry without treatment).

3- Tail: pull out the tail from the skin by gripping the tailbone close to the body with two fingers, and hold back the skin on the tail with two fingernails.

4- Back: skin up the back of the animal. Cut front legs loose inside the scapula (shoulder blade).

5- Head: peel skin down over neck to ears. Cut ears free from head, with sharp scissors close to skull. Peel to eyes. When cutting the eyelids free with a scalpel, work close to the eyeballs so that lids are not injured. Peel to mouth and cut close to jaw bone when severing lips and nose from skull.

6- Clean the skin from dirt, blood and tissue.

7- Preservation: skins can be covered with salt to enhance drying or preserved in ethanol. It must be later stored with desiccant, and be protected from parasites and fungi with chemicals.



Figure 72: Dry specimens at the London Natural History Museum (Photo: Herbreteau V.)

Dry skins can later be mounted with wires and filled with cotton (Fig. 71-73)



Figure 73: Rattus tiomanicus from the Thai national collection, at the TISTR (Photo: Herbreteau V.)

5.1.2. Skull preparation

Preparing skull is needed for:

- **Dry collection,** to keep a cleaned skull together with the dry skin.
- **Morphometrics**: in this case, the skull can be separated from the body just after dissection and conserved in ethanol. A label containing the identification number of the specimen should be tied to the skull (we recommend to keep a nylon string in the cheeks) before preparation in the laboratory.

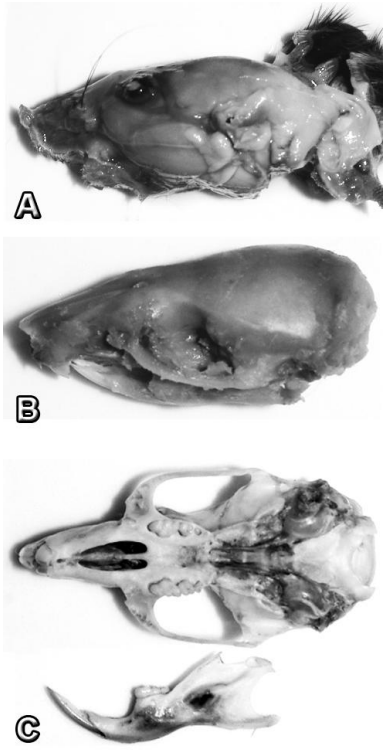


Figure 74: Skull preparation (Photo: Claude J.)

Protocol:

1- Use a scalpel or scissors to cut the skull free from the body. Beware of cutting the hind part of the skull: it is better to cut a few vertebrae down the neck to be sure not to damage the skull.

2- Put the skull and its label in a plastic vial with screw cap and fill it with water.

3- Then, leave the plastic vial into a boiler for 15 to 30 minutes depending on the skull size and fragility (beware that the plastic vial does not melt down). Boiling does little to remove material inside the nasal and other cavities. Slow cooking is preferable to make meat tender. Add some herbs if smells bother the neighbourhood…

4- Take the skull out of the vial and put it in cool water.

5- Peel the skin off the skull, scrape away most of the meat and pull out the eye balls (Beware of breaking the zygomatic arch) (Fig. 74 A and B).

6- Remove the brain by using a syringe or long-end dropper filled with water: inject water through the spinal hole (foramen magnum) with pressure.

7- Delicately remove the jaw from the skull in water. Make sure to save all teeth that may have come loose, since jaws are delicate.

8- Clean the skull and jaws from all tissue. Scrub the skull with liquid detergent (washing liquid) and a toothbrush, making sure all tissue is removed.

9- Skull should be whitened with very dilute hydrogen peroxide. If the skull is badly stained, it cannot be completely whitened (Fig. 74C).

10- Carefully label with a permanent pen or with a pencil. Always label both the container and the specimen for avoiding mix-up in the collection.

11- Store skulls in a plastic or glass vial, with desiccant.

5.2. Wet collections

Preparing dry specimens is time-consuming and might be difficult to prepare in the field. Therefore, we recommend keeping the whole bodies in a wet collection. It will allow one to study them later and eventually recover missing information. However, if skulls are cleaned for a morphometric study, they can directly be separated from the body, which will not be preserved in order to limit the storage.



Figure 75 : Wet collection in individual jars (Photo: Herbreteau V.)

For a small wet collection, animals can be kept individually in hermetic tanks. Such collection is easily sorted out and observable (Fig. 75).

When sampling lot of animals, they can be kept together in large watertight jars. Animals can be grouped according to the field survey or species, in order that they can be recovered quickly. Labels should be tied to each animal with care to avoid any mixing.

We recommend the use of 70 degrees ethanol, which is suitable for a good conservation of animals and DNA analysis. Ethanol is also easily obtained and not toxic. 10% formalin is recommended for anatomical and histological studies, but will preclude any DNA analysis.

Animals, especially when they are large and not dissected, should be opened to absorb the ethanol. Otherwise ethanol could be introduced in the body with a syringe.

Once back from the field to the laboratory, ethanol needs to be renewed because animal bodies have rejected water, which has decreased the concentration of ethanol. Each jar should be carefully closed to avoid evaporation, and labeled (animal identification number, locality, date of capture, collector).

Animals can be kept several years in such containers. Every jar should be placed in dark rooms, free from any animals (especially other rats).

5.3. Data sharing with online collections

Beyond preserving specimens, the aim of collections is to **allow anybody to consult the samples, learn and share information about species**. Collections should be accessible for remote users through the Internet and, in return, benefit from new technologies to integrate more information and access possibilities.

We have tried to reach this goal by providing an online access to the CERoPath osteological collection.

Labels of skull samples integrate a barcode (Fig. 69) that can be read by any camera device to directly link with the CERoPath web page of the virtual museum (Fig. 76).

This innovation provides wide and updatable information that could not be contained or managed on usual labels in collections.

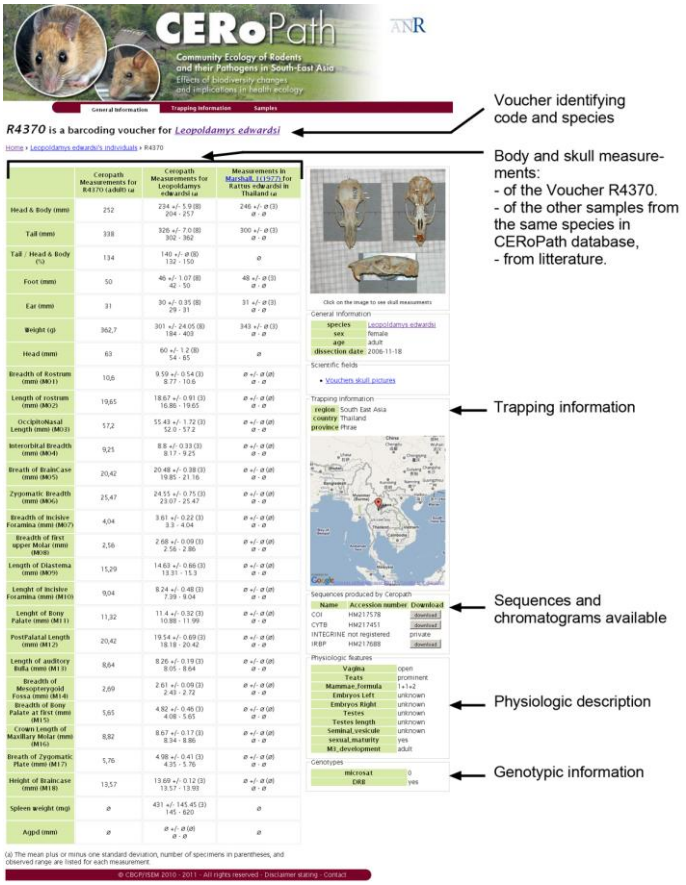


Figure 76 : Example of information accessed from the CERoPath virtual museum after reading the barcode of sample R4370.

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Glossary

allopatric: occurring in separate geographic areas

anesthesia: loss of sensation with or without loss of consciousness, by the administration of gases or the injection of drugs before surgical operations

ankle: the joint connecting the foot with the leg

arboreal: living in trees

axillary: of or relating to the armpit

biopsy: the surgical removal of tissue for diagnostic purposes

cardiac puncture: penetration of the heart for removal of a blood sample

carrier: a person or an animal carrying an infectious agent without showing any symptoms of the disease and capable to transmit it to other humans or animals

caudal: at or near the tail or the posterior part of the body

cell culture: complex process by which cells are grown under controlled conditions.

commensal: (from the Latin *com mensa*, meaning *sharing a table*) living on or within another organism and deriving benefits without harming or benefiting the other. Some rodents are considered as commensal because of their intimate relationships with humans.

cosmopolitan: qualifying the distribution of an animal found around the world in suitable habitats

cytogenetics: a discipline of genetics, concerned with the study of the structure and function of the cell, especially the chromosomes. It includes routine analysis of G-Banded chromosomes, other cytogenetic banding techniques, as well as molecular cytogenetics such as fluorescent in situ hybridization (FISH) and comparative genomic hybridization (CGH).

diastema: (from the Latin *diastēma*, meaning *interval*) vacant space in the toothrow between the incisors and the premolars and molars

dimorphism: occurring in or representing two distinct forms in a single population

dioecious: having two distinct sexes (antonym: hermaphroditic or monoecious)

diurnal: active in the daytime

dorsal: relating to the upper side or back of an animal or plant, opposite to ventral

dorsum: the back of an animal, opposite to ventrum

ectoparasite: parasite (see parasite) that lives on its host (examples: ticks, mites, fleas)

endemic: native or confined to a specific area

endoparasite: parasite (see parasite) that lives inside its host (examples: worms, protozoans)

estrus: (or oestrus) (from the Latin *oestrus*, meaning *frenzy*) a recurring period of sexual receptivity and fertility in many female mammals

euthanasia (good practices): painless and stress-free killing of an animal

feral: a population of domesticated animal that lives in the wild

fossorial: adapted for digging

gestation: length of time between conception and birth

GPS (Global Positioning System): a worldwide navigational and surveying facility based on the reception of signals from an array of orbiting satellites.

gravid: pregnant

groin: the area between the abdomen and the thigh on either side of the body

habitat: the area or environment where an organism or ecological community normally lives or occurs

haematophagous: feeding on blood (especially ectoparasites such as ticks and mites)

hallux: the innermost digit of the hind foot of vertebrates

hemoparasite: an animal parasite living in the blood of a vertebrate

home range: an area over which an individual or group of animals regularly exploits for feeding, resting or mating, and which may overlap with those of neighboring animals or groups

host: an animal or plant on which or in which another organism lives

incisor: a narrow-edged tooth at the front of the mouth, adapted for cutting

indigenous: originating or occurring naturally in a particular area (= native)

inguinal: of or relating to the region of the groin

introduced species: an animal or plant that has been introduced to an area where it normally does not occur

jugular: relating to, or situated near the throat or neck (ex: the jugular vein).

karyotype: describes the number and appearance of chromosomes in the nucleus of an eukaryote cell. The term is also used for the complete set of chromosomes in a species, or an individual organism.

lamellae: (singular: lamella) (from the Latin, diminutive of *lamina*, meaning *thin plate*) a thin layer, membrane, scale, or plate-like tissue or part

lamellated: composed of, or arranged in lamellae

mammae: (singular: mamma) organ of female mammals that contains milk-producing glands

mammal: belong to the class of tetrapodes: constant temperature, have lungs and a developed central nervous system. Female have breasts to feed their young.

mandible: lower jawbone in mammals

manus: (from the Latin, meaning *hand*) distal part of the forelimb of a vertebrate, including the wrist and hand or the carpus and forefoot

maxilla: the upper jaw holding the molar and premolar teeth in mammals

morphology: the form and structure of an organism

morphometrics: concern the study of the shape and shape changes of organisms or objects. It is assumed that morphometrics can quantify a trait of evolutionary significance, and by detecting changes in the shape of organisms, deduce something of their ontogeny or evolutionary relationships.

murid: belonging to the family Muridae

Muridae: the largest family within the class Mammalia: it contains over 1300 species, 281 genera and 17 subfamilies. The origins of the Muridae are believed to be in South-East Asia

murine: 1- relating to a rodent of the subfamily Murinae; 2- caused, transmitted, or affected by such a rodent: a murine plague

Murinae: a subfamily of the family Muridae, comprising of at least 529 species in 122 genera: the Old World true mice and rats, and relatives.

nasal: (from the Latin *nasus*, meaning *nose*) of, or relating to the nose

niche: (from the Latin *nīdus*, meaning *nest*) the relational position of a species or population in its ecosystem

nocturnal: active at night

omnivorous: feeding on a variety of food of both plant and animal origin

opposable: capable of facing and touching the other digits on the same hand (opposable thumb or opposable hallux)

pad: fleshy underside of feet

parasite: an organism that lives on or in a different organism, a host, by benefiting at the expense

parturition: the action of giving birth

pectoral: of or relating to the breast or chest

pelage: the fur, hair, or wool of a mammal

pes: (from the Latin *pēs*, meaning *hand*) foot or footlike part, used for the foot of a four-footed vertebrate, generally refers to hind foot

phylogeny: the evolutionary history of a species or higher taxonomic grouping of organisms , often visualized using tree representation (i.e. phylogenetic tree)

protocol (in science): a written description of a study to ensure its quality and integrity

protrusible : capable of being protruded or extended

reservoir: an organism or a population, in which an infectious agent is maintained, and that directly or indirectly transmits a pathogen while being often not affected by

rodent: (from the Latin *rōdēns, rōdent-*, present participle of rōdere, meaning *to gnaw*) member of the mammalian order Rodentia, such as a mouse, rat, squirrel or beaver, characterized by large constantly growing incisors adapted for gnawing or nibbling

sexual dimorphism: differences in the morphology of males and females from a same species

sympatric: inhabiting the same habitat, or living in the same area

taxonomy: the science of identifying and classifying organisms into groups

terrestrial: living on land

territorial: defending a territory

territory: a geographic area defended by an individual or group of animals against other individuals from the same species

vector: an organism, such as a mosquito, tick or rodent, that carries micro-organisms causing disease from one host to another

vector-borne transmission: indirect transmission of an infectious agent that occurs when a vector bites or touches a person

ventral: relating to the underside of an animal or plant, opposite to dorsal

ventrum: the belly or underside of an animal, opposite to dorsum

vibrissae: (singular: vibrissa) or whiskers: coarse hairs richly supplied with nerves and growing around the nostrils, specialized for tactile sensation

voucher: an animal specimen or a part of an animal showing specific features associated to its DNA sequence and permanently maintained in a collection and made accessible for scientific study

wrist: the joint connecting the hand with the forearm

zoonoses: (singular: zoonosis) infectious diseases naturally transmitted between vertebrate animals and humans

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Appendixes

Appendix 1: Trap location data sheet.

Appendix 2: Rodent observation data sheet for individuals.

Appendix 3: Rodent observation data sheet with limited information for 10 specimens.

Codes used for the Appendix 2: "Rodent observation data sheet for individuals"		
Measurements	***HB*** *= Head + Body length* ***Tail*** *= Length from the middle of the anus to the tip of the tail* ***HF*** *= Hind foot (= Pes) length, not including the claw*	
Vagina	***1*** *= Not open (juvenile)*	***2*** *= Open (adult)*
Teats	***1*** *= Barely visible (juvenile)*	***2*** *= Prominent (adult)*
Mammae formula	*Indicate the number of pairs of teats in the following order: pectoral + postaxillary + inguinal*	
Embryos	*Indicate the number of embryos on each side:* ***L*** *= left, and* ***R*** *= right*	
Testes	***1*** *= Inside (abdominal testes)*	***2*** *= Outside (descended testes = adult)*
Seminal vesicles	***1*** *= Undeveloped (juvenile)*	***2*** *= Developed (adult)*
Parasites	*Indicate the number and type of ectoparasites (ticks, fleas, mites, chiggers…)*	
Pictures	*Yes / No*	

SAMPLING SITE	Written by:
	Date:

Id_Site:	**Method:**	☐ Trapped by project	☐ Line ☐ Isolated trap
		☐ Collected from locals	Who: ……

Location	**Administrative:**	Province:		District:
		Sub-district:		Village:
	GPS coordinates **WGS 1984**	Decimal degrees	Lat: ……	Long: ……..
		UTM/UPS	Easting: ……	Northing: ……
		Elevation:	…… meters	

Sampling place / Typology	**Low**	**Medium**	**High**
	Comment:		

Environment		
	At the sampling point:	Site “nickname”: Other comments:
	Surrounding landscape:	**Type:** **Distance:** • Closest village • …… • …… For distances, use the following codification, based on decimal logarithm (base 10 logarithm): Code = $\log_{10}$ (distance): 0 = 1 m 1 = 10 m 2 = 100 m 3 = 1 km 4 = 10 km 5 = 100 km

Human presence			
	Houses:	☐ Yes	☐ No
		Number:	
		Distance from sampling point:	

Traps / Captures	**Date**	**Traps**			**Captures / remarks**
		lost	replaced	remaining	

RODENT SAMPLING	Dissected by: Written by:

Id_individual:	**Species:**
Id_Site:	

Dates	**Capture**	**Death**	**Dissection**
	…… / …… / ……	…… / …… / ……	…… / …… / ……

Origin	☐ Trapped by CERoPath ☐ Collected from: ……	☐ Alive ☐ Dead	**Location accuracy:** (1 High – 5 Low)

Sampling place / Typology	**Low**	**Medium**	**High**
	Comment:		

Measures	**Weight (g)**	**Head & Body**	**Tail**	**Hind Foot**	**Ear**	**Skull**	**Spleen (mg)**
			Brush:	Claw:			

☐ Male	**Testes**
	☐ Inside ☐ Outside Testes length (mm) : ……
	Seminal vesicles
	☐ Undeveloped ☐ Developed

☐ Female	**Vagina**	**Teats**
	☐ Not open ☐ Open	☐ Barely visible ☐ Prominent
	Mammae formula	**Embryos**
	…… + …… + ……	Left: …… Right: ……

Blood	**Blotting Paper**	**Whole blood**	**Serum**	**Toxoplasmosis / Trypanosomiasis**
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes, Nb: … ☐ No	☐ Citrate (blue) for PCR ☐ (red) for serum ☐ (purple) for general morphology

Organs		**Lung** *(blue)*	**Spleen** *(white)*	**Kidney** *(yellow)*
	Frozen:	☐ Nb:…	☐ Nb:…	☐ Nb:…
	RNA later:	☐ Nb:…	☐ Nb:…	☐ Nb:…

In ethanol	**Collection**	**DNA**	**Diaphragm**	**Gut**
	☐ Body ☐ Skull	☐ Liver ☐ Foot ☐ Other: ……	☐ Yes ☐ No	☐ Yes ☐ No

Ectoparasites	**Chigger mites**	**Ticks**	**Mites**	**Fleas**	**Lice**	**Others**
	☐ Frozen ☐ In ethanol	☐ Frozen ☐ In ethanol	☐ Frozen ☐ In ethanol	☐ Frozen ☐ In ethanol	☐ Frozen ☐ In ethanol	

Pictures:	**Comments:**

RODENT SAMPLING	Date:	Author:
Province:	District:	Sub-district:	Village:

Lab-Nb										
Place (Obs-Nb)										
Biotope										
Dates	Capture									
	Death									
	Dissection									
Species										
Sex (M/F)										
Measurements	HB									
	Tail									
	Pes									
	Ear									
	Weight (in g)									
	Skull									
Teats										
Mammae formula										
Vagina										
Embryos (L/R)										
Testes										
Samples	Blood									
	Lung									
	Spleen									
	Kidney									
	DNA									
	Gut									
	Body									
Parasites										
Pictures										
Comments										



Abstract:

"Protocols for field and laboratory rodent studies" present the best practices for the studies of rodents and rodent-borne pathogens and parasites from the field to the laboratory. It aims at covering the different steps of rodent studies: 1) Trapping, 2) Dissection and preparation of tissue samples for pathogens screening, 3) Identification of species, 4) Isolation of helminths, 5) Preparing rodent specimens for collections.

This book gathers accurate recommendations and techniques, based on those generally accepted in the literature and adapted from the experience of the different authors during rodent surveys and laboratory work. Its presentation is designed to work in the fields with a clear and colorful organization of each chapter, with: inserts providing definitions and recommendations, protocols detailed step by step, and an emphasis on illustrations with several photographs.

This book is a publication generated by the CERoPath project (Community Ecology of Rodents and their Pathogens in a changing environment, www.ceropath.org) funded by the French Ministry of Research from 2008 to 2011.

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