***Research Networking Programme*-EURAPMON**

Research and monitoring *for* and *with* raptors in Europe

SAMPLING AND CONTAMINANT MONITORING PROTOCOL FOR RAPTORS

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Introduction and aim

There is much information available on the different types of matrices that are useful for contaminant monitoring. However, there are no detailed guides or protocols on appropriate sampling methods for contaminant monitoring schemes. Although some guidelines exist, they do not address specifically sampling for contaminant analysis (Friend and Franson, 1999; Fair et al., 2010). Different studies use different sampling and processing strategies, and different analytical methods, making it hard to compare and interpret results among studies. Consequently, there is a great need for developing a protocol on best practices. The proposed sampling protocol is based upon the experience of different research groups from different countries in Europe.

Research and Monitoring for and with Raptors in Europe (EURAPMON) is a European Science Foundation (ESF) Research Networking Programme (http://www.eurapmon.net). One objective of EURAPMON is to spread best practice and build capacitiy in Europe for harmonised monitoring with raptors (Movalli et al., 2008a). This includes making available guidelines and protocols for fieldwork, sampling, preparation, analyses, etc., that build on existing best practice.

From May 31st to June 2nd 2013, representatives from six countries gathered in Murcia, Spain, to attend the Workshop on “Setting best practices on raptor contaminant monitoring activities in Europe” funded by EURAPMON. The workshop developed a rough draft of the current protocol. The protocol was subsequently completed with the involvement of investigators from Belgium, Denmark, France, Germany, The Netherlands, Norway, Spain, Sweden and the United Kingdom. All contributors are experts in monitoring contaminants in raptors.

It is clear that sampling methods, and transport and storage conditions, may differ between sample matrices. The aim of this sampling protocol is to provide guidance on types of best practice that will facilitate harmonisation of procedures between existing and emerging schemes and so maximise the reliability, comparability and interoperability of data. The methods here do not require use of anaesthesia on birds. This protocol covers the sampling of blood and feathers from live birds, addled and deserted eggs, internal organs and tissues from dead specimens, and other samples such as faeces, preen oil and pellets.

1. General guidelines

1.1. Permission

- Approach and disturbance of nest sites and handling/sampling of protected species is regulated in most countries. Generally licences are required to undertake such activities and relate to animal welfare and use of animals for scientific purposes, conservation issues and hunting legislation. All necessary licences and permissions must be obtained from the appropriate national agencies before work is undertaken.
- Samples should be collected by trained and authorised personnel.

1.2. Identification

- Always note information systematically following a previously prepared scheme regarding the characteristics, specifications, and circumstances of the samples collected.
- Label the individual sample containers prior or immediately after the sample is collected.
- Each sample should be identifiable from a unique code.
- A short and self-explanatory identification system that is easy to implement in the field should be used to ensure traceability of samples and the relevant associated information (see 2).

1.3. Avoid contamination

- Provide appropriate material for sampling and storage conditions according to the aims of the study (take advice from the laboratory undertaking the chemical analysis).
- Pre-clean sampling and storage equipment before sampling. Choice of cleaning agent will depend on the specific contaminants of interest (take advice from the laboratory undertaking the chemical analysis).
- Handle the containers carefully to avoid situations of possible contamination.
- Keep some sampling material/containers for blank/control analyses.
- Do not smoke, drink or eat during sampling. Note if mosquito treatment has been used by the sampling personnel.

1.4. Personal safety and wildlife health

- Use appropriate personal protective equipment to avoid zoonotic diseases or zoonoses. According to the World Health Organization, zoonoses are those

diseases and infections that are naturally transmitted between vertebrate animals and humans. Chlamydiosis, Histoplasmosis, Newcastle Disease, Salmonellosis, Yersiniosis, Avian Influenza, and West Nile Virus, are some well-known zoonoses.

- The transmission routes of zoonotic diseases include: ingestion, inhalation, direct contact, penetration or absorption through mucous membranes and cornea, injection, bites and scratches (Cooper and Cooper, 2007; USGS, 2009).

- Put an effective barrier between you and the disease agent, such as masks, gloves, coveralls, boots, goggles, and respirators. The level of personal protection required depends on the situation (USGS, 2009).

- Prevent mechanical transmission of disease agents through disinfecting supplies and equipment between sites and/or animals. Bag and clean items before leaving site, clean more thoroughly as soon as possible (USGS, 2009).

- Apply safety requirements for climbing and hiking. Do not go into the field alone.

1.5. Animal welfare

- Avoid posing unnecessary stress to the birds:
    - Cover the head without contact between the eyes and cover. Avoid unnecessary noise and talking.
    - In case of sampling of living animals, avoid central hours of the day in order to limit heat stress.
    - Keep the handling time as short as possible. Handling duration is a trigger point for adverse effects of sampling on birds due to stress, and should ideally last less than 5 minutes. Since ringing, measurements and sampling usually require more time than this, handling time can be extended but should be kept short.
    - Avoid multiple handling of a bird. If possible, do sampling at same time as ringing to limit disturbance and stress to the bird.
    - Sampling of nestlings must be carefully timed such that chicks are old enough to be sampled, yet not so old that there is a risk that they jump from the nest (Movalli et al., 2008b).
    - Leave the nest site as you found it.

- Sample amount: check with the laboratory for minimal requirements, but in case of invasive sampling (blood, plucked feathers) take no more sample than safe for the bird (see details on the different sampling matrices below) and no more than is allowed under any licence requirement.

- Try to keep error to a minimum, e.g. follow a proscribed protocol and, if possible, a single person should collect and process all samples in a uniform manner.

2. Basic data and records

Any records, regardless of their type, should be secure and uniquely identifiable by the use of reference numbers, identification tags, scale (metric) and appropriate markings (Cooper and Cooper, 2007). The following information should be clearly described in the sampling report, which must always accompany the samples submitted for analysis:

- Date and time of sampling.
- Study area: country, province, and specific location such as map coordinates or site coding. When possible use geographic information systems. Note: there may be a need to keep specific location data confidential to avoid disturbance, illegal persecution or illegal egg and specimen collection.
- Type of samples and number of samples collected.
- Biological data:
    - Species: Scientific name and common name. Preferably use the nomenclature from “Handbook of birds of the world” (Del Hoyo et al., 1992-2013).
    - If possible, individual identification of the animals (e.g. ringing data). For information on Euring data and codes visit the webpage www.euring.org.
    - Age and gender (if can be determined) (Petersen and Thompson, 1977; Helander, 1981; Bijlsma, 1997; Martínez et al., 2002). Indicate how determined (e.g. morphometrics, colouration, dissection).
    - Morphometric measurements: e.g., weight, head and bill length, wing length, tarsus length, tarsus width (Figure 1). These measurements may be useful to calculate growth rate, and to determine age and sex in the field (Bavoux et al., 2006; Helander et al., 2007; Hernández et al., 2011), although the criteria used to separate sexes may have to be adjusted for each population (Helander et al., 2007).
    - Body condition index (Labocha and Hayes, 2011).
    - Nest information: e.g. numbers of eggs, live and dead nestlings, unhatched eggs, eggshells.
- Other general observations (e.g. prey remains in the nest).

PROTOCOL FOR EACH SAMPLE TYPE

1. BLOOD

1.1. Sampling: general requirement

- Prior to and after blood sampling, a clinical exploration should be done by a veterinarian experienced with wild birds in order to evaluate the health status of the bird. If signs of zoonotic disease are observed, the case should be reported immediately to the local health authority.

- This clinical exploration should include the evaluation of general body conformation, posture, attitude, stimulus response, character of respiration. Also it should include exploration of the feathers, skin, beak, eyes, ears, cere, nares, oral cavity, bones, muscles (especially breast muscle), wings, feces, abdomen and cloaca (Cooper, 2002; Espín et al., 2014a).

- Blood should be collected by a vet or properly trained and authorised personnel.

- Volume criteria:

    - A general rule is that the collection weight should not exceed 2% of the body weight of the animal in any 14-day period, or 1% at any one time (McGuill and Rowan, 1989; Voss et al., 2010).

    - Volume should be sufficient to ensure suitable analytical limits of detection (take advice from the laboratory undertaking the chemical analysis). When volumes are limiting, consider the potential of combining blood samples from different individuals (for example, nestlings from the same clutch). When separated fractions of blood (red blood cells and plasma/serum) are needed for analyses, consider the percentage in volume of plasma or serum and red blood cells that can be isolated from a volume of blood. Assuming a haematocrit (percentage of erythrocytes in blood) of 50%, you will obtain a 50% volume of plasma/serum from the total blood collected. Consider the possibility of collecting extra blood to repeat some analyses if needed (Ehret et al., 2002).

- Take blood samples using a hypodermic needle and a syringe. Change needles between birds. Avoid vacuum systems such as vacutainers as these may lead to collapsing veins (Jennings, 1996).

- Use the smallest needle possible:

    - For birds < 500 g body weight: 30 to 25-gauge (0.3 to 0.5 mm) hypodermic needle and a 1 or 2 ml syringe (Cooper, 2002).

    - For birds > 500 g body weight: 23-gauge (0.6 mm) hypodermic needle and a 5 or 10 ml syringe (Cooper, 2002).

- Veins of very small birds can be nicked with a scalpel blade and blood collected in a capillary tube (Dawson and Bortolotti, 1997). Butterfly catheters may lessen the effect of bird movements during sampling (Cooper, 2002).
- Smaller-gauge needles increase the risk of haemolysis. The use of larger needles increases the risk of haematomas (Fudge, 2000). Excessive negative pressure may collapse veins (Jennings, 1996).

- Use anticoagulants for whole blood/plasma. Take advice from the laboratory undertaking the chemical analysis as some anticoagulants may interfere with subsequent analyses. Take into account that EDTA may affect clinical chemistry analytes and enzyme measurements (Hochleithner, 1994), and promote a significant movement of Pb from the red blood cells to the plasma (deSilva, 1981). On the other hand, heparin interferes with white blood cells and sometimes with PCR analysis (Ehret et al., 2002), although heparinised plasma or serum is the recommended matrix for the vast majority of biochemical tests (Hochleithner, 1994). Sodium chloride can be used where samples are collected for organochlorine analysis (van den Brink et al., 1998).

1.2. Sampling: method

- Stimulate the local blood circulation, e.g. by allowing wing flapping before puncturing or putting pressure on vein.
- Use antiseptic at the phlebotomy site.
- Take blood samples puncturing brachial/tarsal/jugular vein (Figure 2). Brachial vein is the easiest one from which to obtain blood.
- Press the puncture site with sterile cloth or non-woven gauze before pulling the needle from the vein, and keep pressure on the cloth at the puncture site for some minutes to avoid bleeding and haematomas. Avoid cotton wool and woven gauze which fibres may hamper a proper healing.

1.3. Procedure after blood extraction

- Remove needle before placing the sample in tubes (otherwise the red blood cells may break).
- Tubes containing anticoagulants should be adequately filled in order to provide a proper blood-to-anticoagulant ratio. Under-filling tubes can cause inaccurate results.
- Use serum separator tubes for serum and centrifuge as soon as possible, ideally within 6 hours (maximum 12 - 24 h) after collection; the longer the elapsed time, the higher the risk of clotting and rupture of red blood cells). Record the time of sampling and spinning.

- Transport samples at 4-10 ºC. Avoid direct contact with ice bags and temperatures <4 ºC to avoid haemolysis.

- Centrifuge blood to separate plasma/serum from erythrocytes (10 minutes, 1600-3000 g). Plasma/serum/red blood cells separation is possible on fresh blood only and cannot be done on samples that have been frozen or where storage has led to rupturing of erythrocytes.

- Use different pipette tips for each sample during plasma/serum separation to avoid cross contamination.

- Keep all separated fractions (red blood cells and plasma/serum) in different labelled tubes. Note that, depending on the analyses planned on red blood cell samples, erythrocytes may need to be washed using saline solution. Take advice from the laboratory undertaking the chemical analysis.

- Record the rate of centrifugation [RCF (g) = 11.18 x 10^{-6} x *r* (cm) x N^2 (rpm); *r* = ratio of the rotor], the time of centrifugation and the capillary volume. Such information is important for example when reporting the volume of blood fractions, such as the haematocrit (percentage of erythrocytes in blood) and the buffy coat (fraction of white blood cells after centrifugation of the blood).

1.4. Storage

- Keep frozen at -20º/-80ºC/liquid N_2 (depending on the analyte or the studied biomarker) in darkness until analysis. Take advice from the laboratory undertaking the chemical analysis for further information about temperature and duration.

1.5. Characterisation of samples

- Consider the possibility of obtaining additional information. For example, haematological and plasma biochemistry values can be used to check the health status of the birds.

- Some haematological parameters can be determined in birds including haematocrit, erythrocyte count, haemoglobin concentration, and white blood cell count (Polo et al., 1992; Monks and Forbes, 2007). The mean corpuscular volume (MCV), mean cell haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC), can be calculated from the haematocrit, erythrocyte count and haemoglobin concentration (Polo et al., 1992; Monks and Forbes, 2007).

- To determine the haematocrit, blood is collected in heparinised micro-capillary tubes. These capillary tubes should be as full as possible to obtain the most accurate value. After centrifugation, the blood is separated into the component parts (red blood cells, buffy coat and plasma). A haematocrit tube reader or a digital caliper may be used to calculate the haematocrit. Haematocrit (%) is calculated as the percentage of the capillary tube occupied by red blood cells in

relation to the total length of the capillary tube with sample. For information on the measurement of other haematological parameters see for instance: Polo et al. (1992), Monks and Forbes (2007), Lobato et al. (2011).

- In order to obtain information about the health of the birds, some clinical biochemistry parameters should be analysed, such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), creatine kinase (CK), gamma-glutamyltransferase (g-GT), lactate dehydrogenase (LDH), albumin, total protein, cholesterol, glucose, triglycerides, uric acid, calcium and phosphorus. For further information about biochemistries see Hochleithner (1994).

- The plasma colour can give some indications of the state of health. The plasma should typically be clear or pale yellow. The yellowness of the plasma can be related to an increased in carotene levels in the diet. If the plasma has a reddish color, it is due to haemolysis or rupture of red blood cells. Haemolysis can occur due to a disease or lead poisoning, although difficulties in the blood collection are a frequent cause of haemolysis. Lipemia or presence of fat in the blood, is visualised by a cloudiness or white colour in plasma, caused by the suspension of fat cells. Lipemia can be found if the sample was collected after a high-fat meal or may be related with liver disorders (Sakas, 2002). Haemolysis and lipemia may alter some haematological and biochemical tests (Joseph, 1999; Cooper, 2002).

- Storage conditions have to be adapted according to the parameters to be analysed, e.g. haematocrit has to be done on fresh blood and not on frozen samples, enzymes measurements require samples to be stored at -80°C or liquid N_2, chemical residues can be measured in tissues stored at -20°C except for a few compounds. Take advice from the laboratory undertaking the chemical analysis.

2. FEATHERS

2.1. Sampling

- The number and type of feathers that need to be sampled, and from which birds (adults or nestlings), depends on the contaminant under investigation, the analytical technique available, and the objectives of the study. This should be carefully checked before starting sampling. Take advice from the laboratory undertaking the chemical analysis.

- Plucked (or cut at the skin) contour body feathers from nestlings are preferred. Plucked flight feathers will result in bleeding and may cause impairment of flight and physiological costs (e.g. McDonald and Griffith, 2011), but plucking a limited number of body feather is unlikely to have any substantial impact on the nestling bird (e.g. Movalli, 2000; Jaspers et al., 2011; Espín et al., 2014b). We therefore argue not to sample primary feathers which are essential for a proper flight, but to sample wing coverts or body feathers (not down feathers) instead.

- If you need to collect tail feathers, follow the suggestions of Odsjö et al. (2004) who collected the outgrown tip (15-20 mm long) of the 2 growing central tail feathers in nestlings, in such a way that further growth of the feather is not impaired.

- In adult birds, plucking tail or flight feathers does not generally result in bleeding but may cause stress to the bird (e.g. Katzner et al., 2012). Consider cutting the feathers at the base rather than plucking them. It is also preferable to collect contour breast feathers, as their limited removal does not affect thermo-regulation or flight (e.g. Movalli, 2000; Jaspers et al., 2011). Take advice from the laboratory undertaking the chemical analysis.

- Concentrations of contaminants in the first plumage of nestlings are generally unaffected by moulting and migration (e.g. Odsjö et al. 2004), and they may be relatively good indicators of local contamination (e.g. Movalli et al., 2008b). However, possible differences in diet between nestlings and adults have to be considered.

- Feathers sampling can be collected from museum specimens. Contour breast feathers are those most likely to be available for sampling from museum samples. Attention must be paid to potential contamination risks from preservatives and dust (eg. Hogstad et al. 2003; Lodenius and Solonen, 2013), but careful sampling can provide long temporal perspective (Bortolotti, 2010). Isolate and pluck individual feathers with clean, acid-rinsed, stainless-steel forceps. Use a pair of plastic gloves.

- Moulted feathers found in the nest or field can also be collected but may be less useful if it is unknown which individuals they are from.

- Down feathers are not recommended for contaminants monitoring. A large number is needed to provide sufficient sample for analysis and excessive collection may impair thermoregulation in chicks. In addition, downy feathers are more difficult to handle in the laboratory.

- Before they are stored, feathers that have been plucked from living birds or collected from carcasses should be cleaned of all fresh tissue (blood, muscle). Otherwise, if they are stored at ambient temperature in sealed bags, rotting will occur. Alternatively, freeze the uncleansed feathers in sealed plastic bags. Regarding the sample amount, normally 200-500 mg is required for organic compounds and 10-200 mg for metal analyses, but take advice from the laboratory undertaking the chemical analysis. Use the conventional numbering system for primary flight feathers from the inside out, i.e. nomenclature shown in Figure 3 (Hardey et al., 2009). This numbering system makes it easy for identification according to Cieslak and Dul (2006).

- Feathers can be transported at room temperature in aluminium foil and plastic sealed bags for the analysis of organic pollutants and just plastic bags for

metals. Dry feathers if they are wet to keep them stored at room temperature, or store them in the freezer.

2.2. Storage

- Feathers can be kept at room temperature if housed properly (eg. Rudnick et al., 2009) and if any soft tissue or blood residue is removed. Store feathers in aluminium foil and/or plastic sealed bags or envelopes, in darkness, and in a dry place (or use silica) if stored at room temperature. Alternatively you can freeze the feathers in sealed plastic bags. Volatile compounds are unsuited for feathers.

- Store moulted feathers in separate plastic sealed bags to clearly identify each bag with a different individual. Plastic bags protect feathers against contamination from the storage environment if stored for a long time.

- Don't put the label directly on the feather, but label the bags with a code referring to the individual bird along with an individual ID for the feathers (e.g. 001-TF1 is tail feather 1 from individual 1).

2.3. Characterization of samples

- Write down any remarks about parasites and state of the feather.

- Identify type and number of the feather (left or right) (Figure 3). In case of contour feathers, indicate the location on the body. Take a picture.

- Register date of sampling and estimate the time of the last moult.

- There has been significant debate about the best way to measure contaminants in feathers (for instance see Bortolotti 2010) and it is suggested that expression of concentration per unit length is most appropriate, although expression per mass has been used in many previous studies. Therefore, we suggest to measure both the mass and the length of the feather. The total length of the feather (expressed in mm) can be used to calculate the deposition rate of pollutants (Eq. 1) (García-Fernández et al., 2013; Figure 4).

  *Eq. 1: Deposition rate (ng contaminant/day) = mass contaminant (ng) / feather length (mm) * growth rate (mm feather/day)*

- Calamus (ie, the most proximal part of the shaft lacking vane; Figure 4) removal is probably a good standard practice because this may have a different growth rate as the rest of the feather (Bortolotti, 2010). Before taking feather length measurements, remove the calamus with a pair of sterilized stainless-steel scissors, just at the point where the first barbs of the vane join at the base of the feather (Figure 4). The calamus can be stored for genetic studies (using the blood in the quill).

- If the calamus is not required, it may be better to cut feathers instead of plucking them.

- Measure the length of each feather (in mm), using a caliper, from the cut end (the base of the vane) to the tip. Weigh (in mg) each feather. Make sure that the feather is not bent or broken. If bent, use steam to restore it to its original form. Work on a clean table and use an appropriately cleaned sheet of glass to flatten each feather for measurements.

- Other recommendations when using feathers may be found in García-Fernández et al. (2013).

2.4. Pre-treatment of samples

- Wash feathers prior to analysis using an ultrasonic bath if the analyte to measure is incorporated in the matrix of the feather. If the analyte is external (from preening or aerial deposition) or a combined measurement is wanted, we suggest washing feathers with deionised water to remove dirt (soil or faeces).

- Depending on the compound to be analysed different washing techniques should be employed. Most studies wash feathers with deionised water and acetone to remove external contamination with metals (Burger and Gochfeld, 2001; Dauwe et al., 2003; Jaspers et al., 2004; Espín et al., 2014b), or in case of POPs with distilled water only (Jaspers et al. 2007; Eulaers et al. 2011a). We recommend washing with deionised water, acetone, 2% nitric acid and again deionised water for lead (Cardiel et al., 2011); deionised water and acetone for metal and other elements; and washing twice with deionised water for perfluorinated compounds. Since more studies are needed to find the best washing technique to remove external contamination with organic compounds, for now we suggest washing feathers only with deionised water to remove dirt.

- Analytical grade acetone, nitric acid, etc. have to be used. Contact the laboratory for complementary information.

- Dry washed feathers at room temperature in a clean environment, and cut into small pieces of approximately 1 mm to expose the largest surface of the feather to the extraction process.

- For further discussion on the use of vane and shaft for some compounds see: Cardiel et al., 2011; Espín et al., 2012; García-Fernández et al., 2013; Lodenius and Solonen, 2013.

3. UNHATCHED EGGS

3.1. Sampling

- Collect only deserted eggs (collected during incubation period from abandoned clutches) or addled eggs (unhatched eggs collected after incubation period) from the nest. While it is recognised that use of deserted/addled eggs may

introduce sampling bias into monitoring datasets, collection of fresh viable eggs is unlikely to be acceptable for most species because of conservation and ethical concerns.

- Be careful about the time for egg collection to avoid nest abandonment.
- Use a graphite pencil to write information on both the eggshell and the container.
- Use suitable containers (e.g. chicken eggs boxes, padded containers, wraps) for transportation to avoid breaking.
- Collect pieces of the eggshells (remains after hatching or crushed eggs) found in the nest and keep them in sealed plastic bags. They may be useful for some contaminant analysis.
- Record the number of eggs found in the nest/ successful eggs and addled eggs.

3.2. Pre-treatment of samples

- Do not freeze the egg because it can crack. Keep cool and process egg as quickly as possible.
- Measure (length and width) and weight the egg (Figure 5). Open at the equator of the egg (Figure 6) and empty its contents into flasks, weigh and homogenise the content, and keep frozen until analysis.
- Homogenization tools should be cleaned depending on the contaminant of interest. For instance, tools used for metal analyses need to be metal free (ceramics) and acid washed, and for PFAS analyses wash them with Milli-Q water and methanol. Take advice from the laboratory undertaking the chemical analysis.
- Examine eggs for putrefaction, embryo development (according to the scale in Table 1) and deformities. If an embryo is present, keep frozen for future analyses. Homogenisation of embryo requires other tools which are able to homogenise bones and other tissues.
- Estimate the time between when the egg was laid and when collected. During this time the egg could be exposed to high temperature causing decomposition of the egg content and modifying the concentrations of some compounds (Herzke et al., 2002).
- Rinse eggshell carefully with tap water to remove all remains of egg contents from the inner surface. Such remains will influence the eggshell weight if not removed before drying the shell.
- Dry eggshells at room temperature to a constant weight, and record the constant eggshell weight.

- Measure eggshell thickness at equator after drying at room temperature. Use a calliper (digital if it is possible) to take at least five measurements by the same investigator from the dry shell (Figure 6). Often the outer shell membrane is firmly attached to the mammillary layer and to remove it might affect the shell thickness. It should be recorded if the shell thickness includes the membranes.

- Eggshell index should be calculated according to Eq. 2.

  *Eq. 2: Eggshell index= Weight of the eggshell (mg dry weight)/(Lenght in mm*Width in mm)*

- Desiccation index should be calculated according to Eq. 3.

  Eq. 3: Desiccation index= (W - S) / V, where W = weight (g) of the egg after sampling, S = dry shell weight (g) and V = inside volume (ml) of the egg (Helander et al. 2002).

- Correct all eggs for desiccation/moisture loss (Best et al., 2010; Hoyt, 1979; Stickel et al., 1973).

3.3. Storage

- Use a glass container with Teflon sealed cup if considering determination of industrial pollutants (e.g. PCBs, bisphenol-A, but not perfluorinated compounds). Plastic containers may be used for inorganic contaminants and PFAS determination.

- Store in darkness.

- Freeze homogenised content at -20ºC until sample preparation.

4. INTERNAL ORGANS AND TISSUES

4.1. General considerations

- Keep carcasses in sealed plastic bags to avoid desiccation and put a label inside the bag (written with pencil or waterproof marker) and on the bag with a waterproof marker.

- All the information about the carcass should be recorded: date, location coordinates, circumstances in which the carcass was found, contact information of the finder, species, age, sex, body weight, measurements - length of wing (wing cord), status of decomposition, etc. See section 2. Basic data and records.

- General recommendations for handling and necropsy may be found in chapter 2 of Friend and Franson (1999).

4.2. Necropsy

- Necropsies should be carried out using protocols that avoids both potential exposure of the researcher to zoonotic diseases and chemical contamination of the sample. Check with the laboratory on equipment to avoid contamination.
- Necropsies should be done on fresh carcasses where possible or the carcass should be kept frozen (-20º) until necropsy.
- If the carcass is frozen, thaw it overnight. For larger species additional time may be required.
- If possible a radiograph should be taken before starting the necropsy.
- External examination of the carcass is necessary to find possible signs of trauma or evidence of clinical symptoms previous to the death, such as haemorrhages, diarrhoea, salivation, etc.
- The cause of death may introduce a bias when interpreting contaminant concentration and should be determined if possible with the help of an experienced pathologist.
- Body condition can affect contaminant levels in internal tissues (Wienburg and Shore, 1994; Crosse et al., 2013). Body condition should be recorded using one or more of the methods outlined below, although some methods may not be practical when carcasses are not intact.
- Body condition can be estimated as a relative score using the criteria in figure 7. Methods that estimate condition as a continuous variable include residual variation in body mass or pectoral muscle mass after correcting for variation in size measured by wing and sternum length (Gosler et al., 1998; Gosler and Harper, 2000) and measurement of pectoral muscle mass as a proportion of body mass [pectoral muscle index; PMI = 100*total pectoral muscle mass / (body mass – (mass of crop contents + gizzard contents))]. Body condition can also be measured by the thickness of subcutaneous, body cavity and coronary fat tissue together with the shape of the breast muscle and the weight of the bird (Krone et al., 2003); the thickness of fat tissue should always be measured on the same side (e.g. caudal edge of sternum).
- During necropsy, record organ weight, lesions/alterations, sex and status of the gonads (developmental stage) (Figure 8). Take pictures if possible.
- If it is believed that the bird has died due to disease seek help of an experienced avian pathologist to record diseases, organ alterations and causative agent (parasite, bacteria, virus).

4.3. Sampling

- Take advice from the laboratory undertaking the chemical analysis as to selection of tissues for analysis (choice will partly depend on the endpoint and the kinetics of the compounds to be analysed).
- Use suitable dissection material depending on the contaminants of interest and clean the material between the organ sampling and between individuals.
- Regarding sampling of liver, kidney and other internal organs, the whole organ must be taken if possible. In case of muscle sampling, the pectoral muscle is often used and is the preferred choice.
- Different bones may have different Pb concentrations (Ethier et al., 2007), and homogenised whole bone should be used. A special mixer mill may be used to homogenise bones. Choose the same bone from different individuals.
- Remove bone marrow if present from bones mechanically, when cleaning and opening the bone (Zong et al., 1996; Pain et al., 2005). Erythrocytes may accumulate lead in acute intoxications, while the measurement of bones should be independent from erythrocytes lead levels. The homogenised bone should appear as a whitish powder.
- If fat is to be analysed for lipophilic compounds, use abdominal fat instead of subcutaneous fat (e.g. Espín et al., 2010). Abdominal fat is more stable in its concentrations than subcutaneous fat because the latter is metabolised first and lipophilic compounds are mobilised and distributed throughout the bloodstream (Hela et al., 2006), so concentrations are more variable.
- If sampling tissues are dispersed through parts of the body, such as abdominal fat, it is recommended that the tissue is sampled consistently from the same part of the body to avoid differences in contaminant distribution leading to increased variability within your samples.

4.4. Storage

- Keep organs in separate containers/plastic bags and label the containers. Consider whether the materials of the containers may interfere or affect analysis of the contaminants of interest. Take advice from the laboratory undertaking the chemical analysis. Wrap in aluminium foil (washed with water and methanol) when uncertainties regarding plastic containers for perfluorinated compounds or use special containers. Use aluminium foil between samples and screw tap. Never use aluminium foil in case of trace metals analyses.
- Store the organs at -20 ºC or -80 ºC. PCBs and most chlorinated pesticides in tissue samples frozen at -20 ºC has been found to be stable for at least one year, and tissues stored at this temperature are protected indefinitely against trace metal loss (McFarland et al., 1995). However, tissues may desiccate to some extent with time and this should be taken into account when analysing

compounds. Furthermore, freezing at -20 ºC does not prevent degradation for some compounds, see for instance: O'Brien et al. (1981), Vanderkop et al. (1989), Kaneene and Miller (1997), MacLachlan and Lunn (2003).

4.5. Other considerations

- Collect and transport carcasses according to national and international (CITES) regulations (obtain necessary regulatory licences).
- A standardised necropsy protocol should be followed, preferably by a qualified pathologist. We provide a necropsy form and figures of anatomy of birds to facilitate the sampling (Table 2, Figure 9). This form is provided as an example but it is not a format that should be followed.
- Dispose of waste (papers, gloves, etc.) according to national regulations relating to biological waste.
- Where possible and resources permit, retain key organs, feathers, and bones in a long-term archive.
- Rests of carcasses: retain frozen if possible or investigate potential to donate to museums. Otherwise, dispose carcasses according to national regulations.

5. OTHER SAMPLES

- Although less frequently used, other interesting samples can be collected such as excrements, preen oil, and regurgitated pellets.
- Fresh faeces samples may be collected by inducing individuals to defecate on handling. Collect samples in sterile plastic bags/tubes. Excrements have different applications such as diet composition studies, hormones analyses, genetic studies, pollutant monitoring, etc. Check with the laboratory on amount required, and transport/storage conditions (room temperature/-20º/-80ºC).
- The preen oil are waxes from the uropygial gland, a sebaceous gland located at the base of the tail feathers that excretes an oily secretion (Figure 9). Preen oil is a specially interesting matrix when collecting feathers, since lipophilic compounds excreted in this oil may be the main source of external contamination on the surface of the feather (García-Fernández et al., 2013). When sampling from carcasses, the whole preen gland may be removed. In living birds, press the gland softly and collect the expelled oil in a sterile tube. Two stainless steel spoons (one with a hole) can also be used for oil collection (van den Brink and Pigott, 1996; van den Brink et al., 1998). Transport preen oil under cold conditions. Normally, 0.01-0.05g are required for organic compounds analysis. A sufficient amount of preen oil may be difficult to obtain from nestlings and may be hard to sample from juvenile or adult individuals (Eulaers et al., 2011b). Check with the laboratory on storage conditions. Normally, preen oil/gland can be stored at -20º for lipophilic pollutants determination.

- Pellets are masses of undigested matter from food such as bones or feathers that some birds can regurgitate. Regurgitate pellets may be used for diet characterization, presence of Pb shot determined by radiograpy, and chemical determinations (Eadsforth et al., 1996; Mateo et al., 2001). Pellets encountered near roosting sites and nests can be collected in plastic bags to identify food items and Pb shot. To collect pellets for chemical analysis, follow the strategy described by Eadsforth et al. (1996). Briefly, a first visit to each site is needed in order to remove existing pellets so that subsequent pellets collected will be of a known age. Those that are fresh (having a coating of mucous) can be reserved for prey identification and possible residue analysis. In the second visit, fresh pellets can be collected and place into individual plastic bags clearly labelled. Transport under cold conditions and stored at -20º until chemical analysis. Both old and fresh pellets can be used to identify prey content (see Eadsforth et al., 1996 for detailed descriptions).

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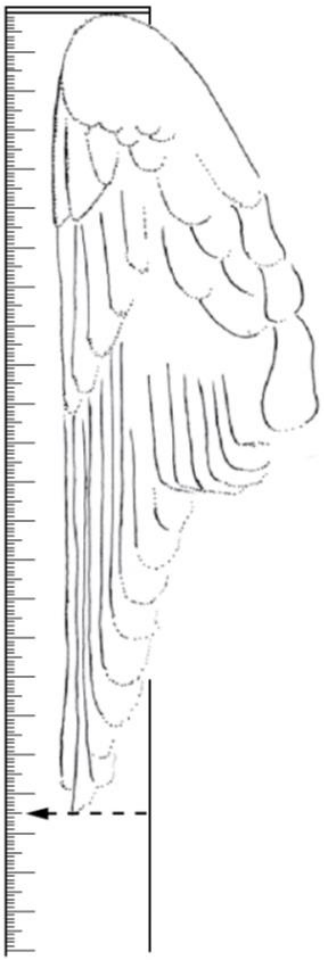
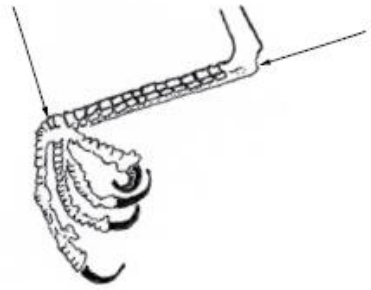
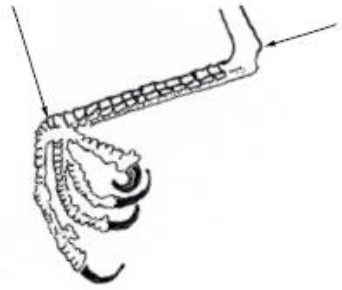
Figure 1. Morphometric measurements: “maximum chord” wing length measurement, tarsus length and head and bill lengths (from Hardey et al., 2009).

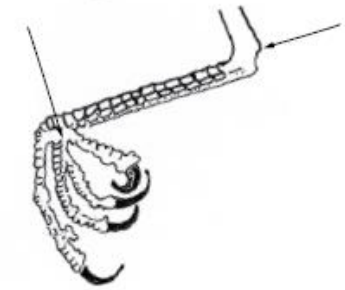




(i). Tarsus measurement.



(ii). Tarsus and heel measurement.



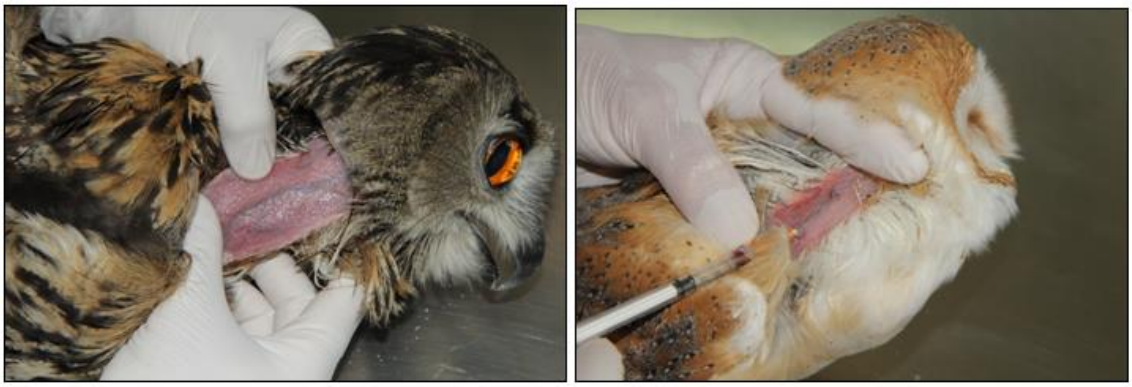
(iii). 'Foot' and heel (from 'ball' of foot to heel) measurement.

(i). Measurement of bill (culmen) length.

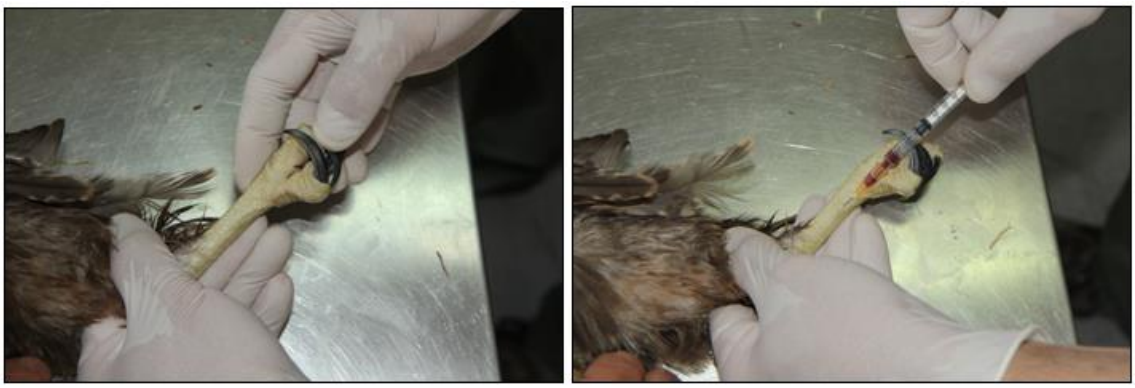
(ii). Measurement of head and bill length.

Figure 2. Brachial/jugular/tarsal veins (Photos: Pedro María-Mojica).

Brachial vein.



Jugular vein.



Tarsal vein.

Figure 3. Location and nomenclature of wing and tail feathers (Photo: Mark Hamblin) (from Hardey et al., 2009).

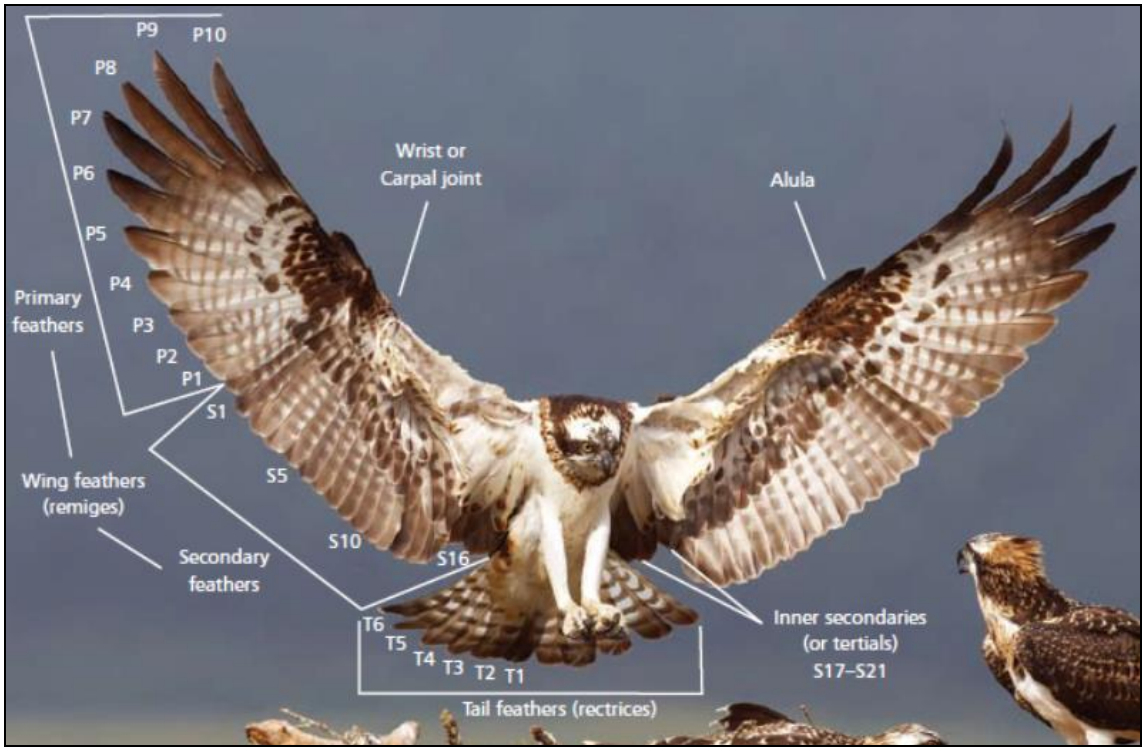


Figure 4. Structure of a primary feather (from García-Fernández et al., 2013).

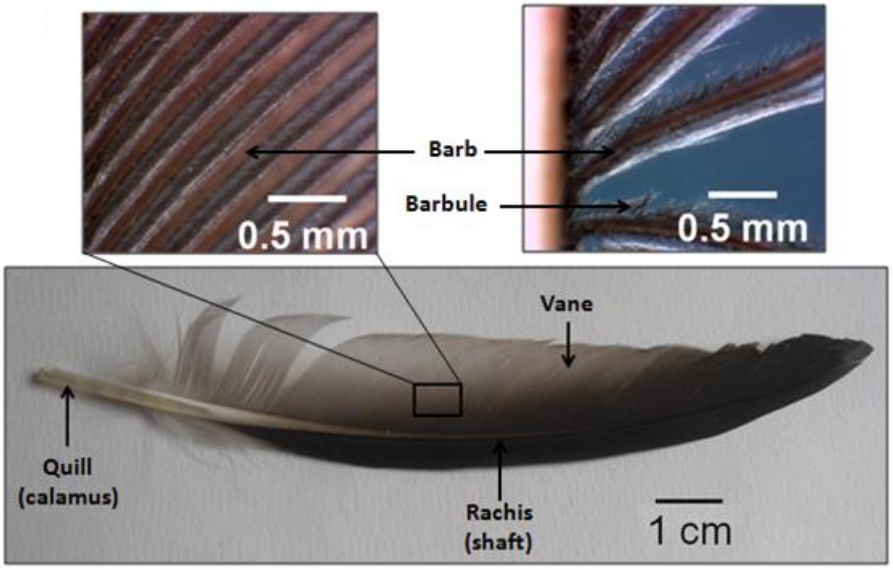


Figure 5. Measurement of length and width of the egg (Photo: Toxicology and Forensic Veterinary Medicine group, University of Murcia, Spain. http://www.sertoxmur.com/).

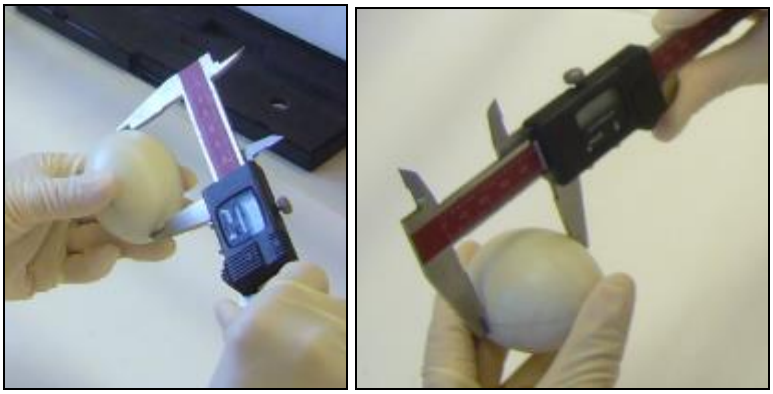


Figure 6. Measurement of eggshell thickness at equator (Photo: Toxicology and Forensic Veterinary Medicine group, University of Murcia, Spain. http://www.sertoxmur.com/).

Figure 7. Body condition index. Scored according to a four-point scale (from van Franeker, 1983, 2004).

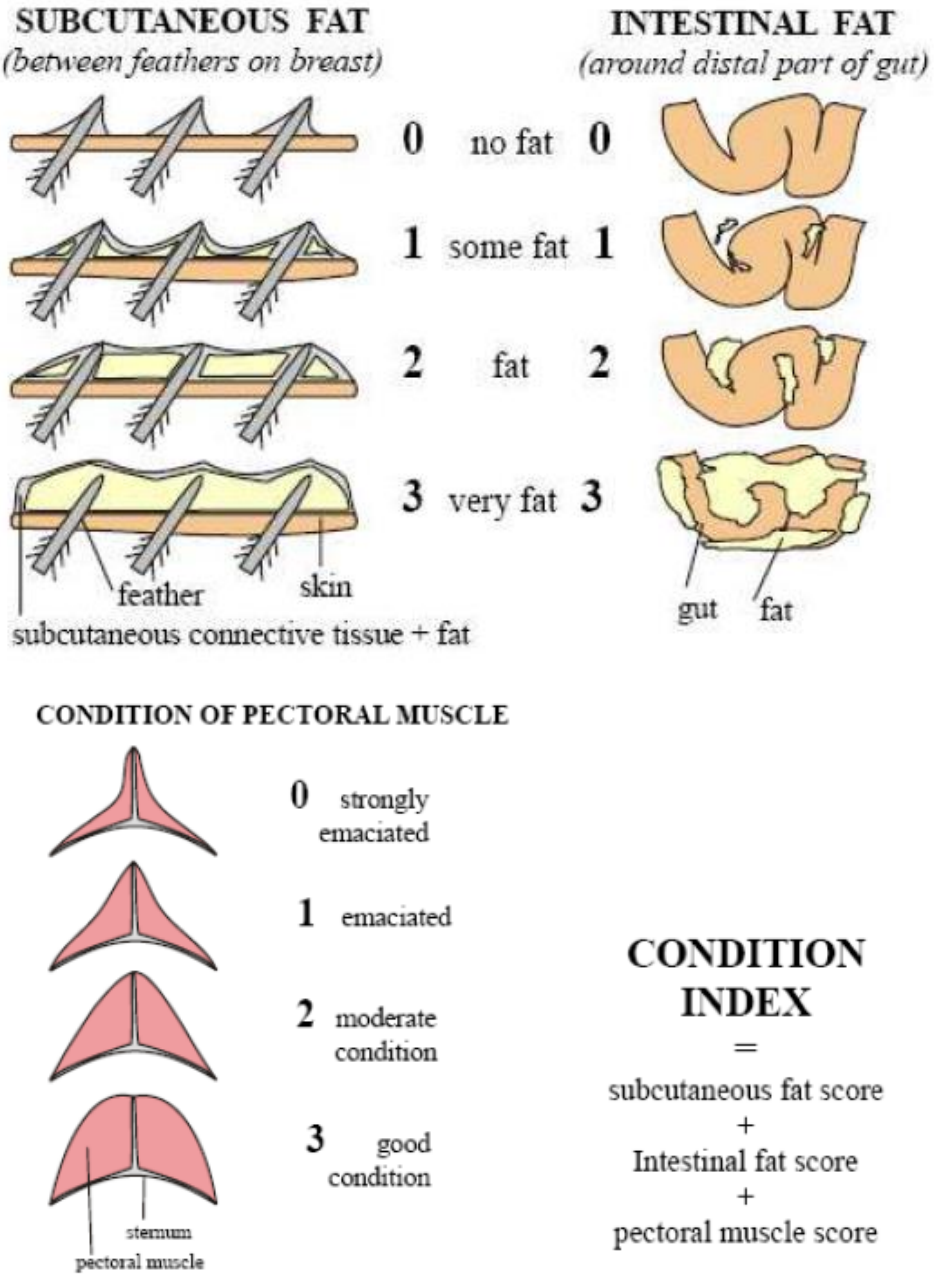


Figure 8. Gonads (ovary and testis) of Eurasian Eagle owl (Photos: Pedro María-Mojica).

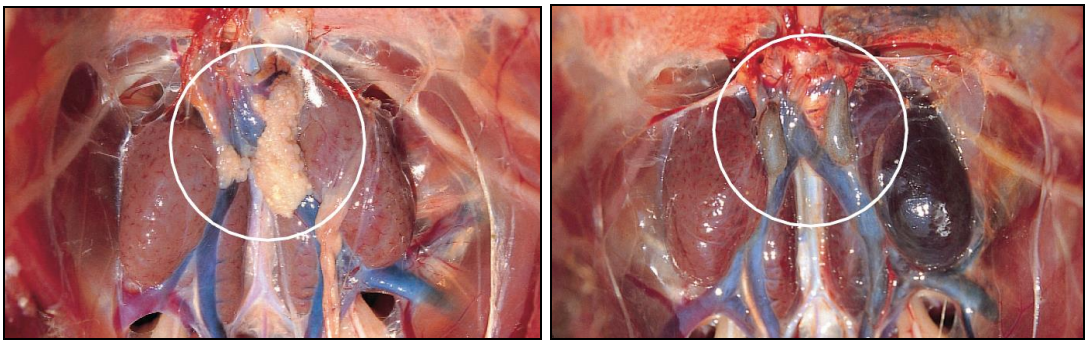
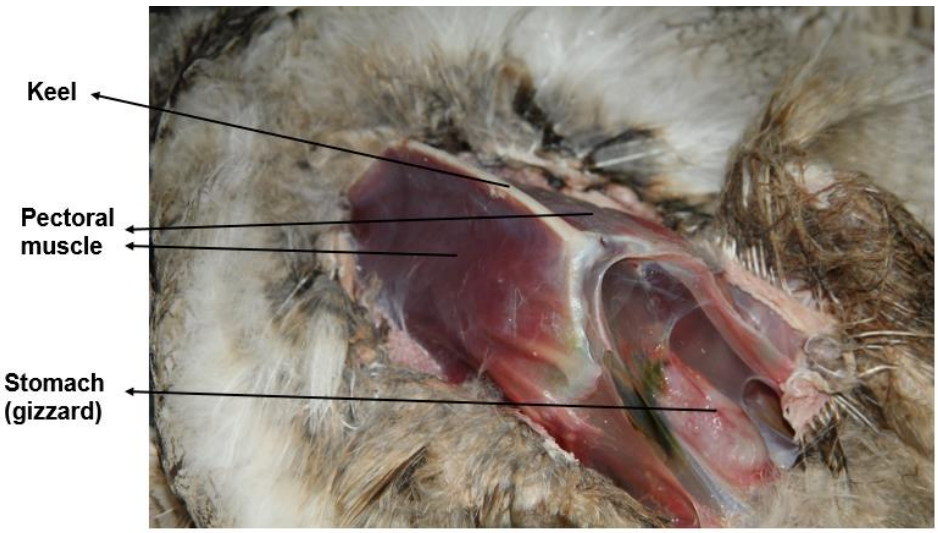
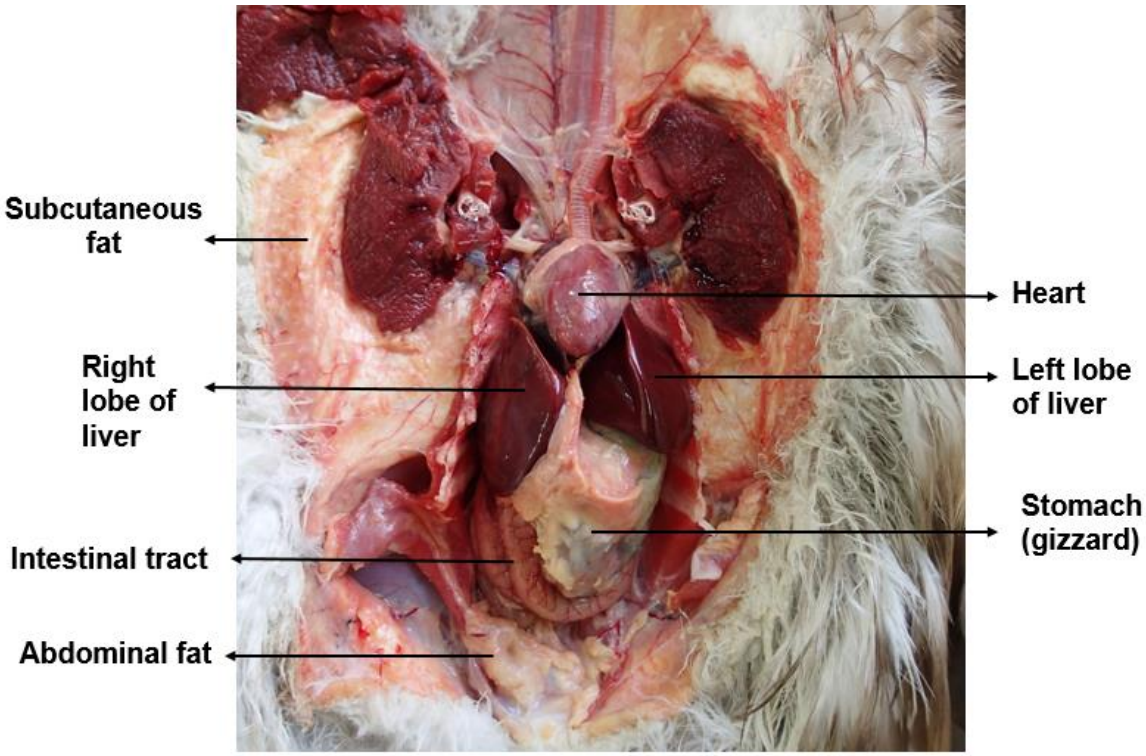


Figure 9. Anatomy of raptors (Photos: Pedro María-Mojica).

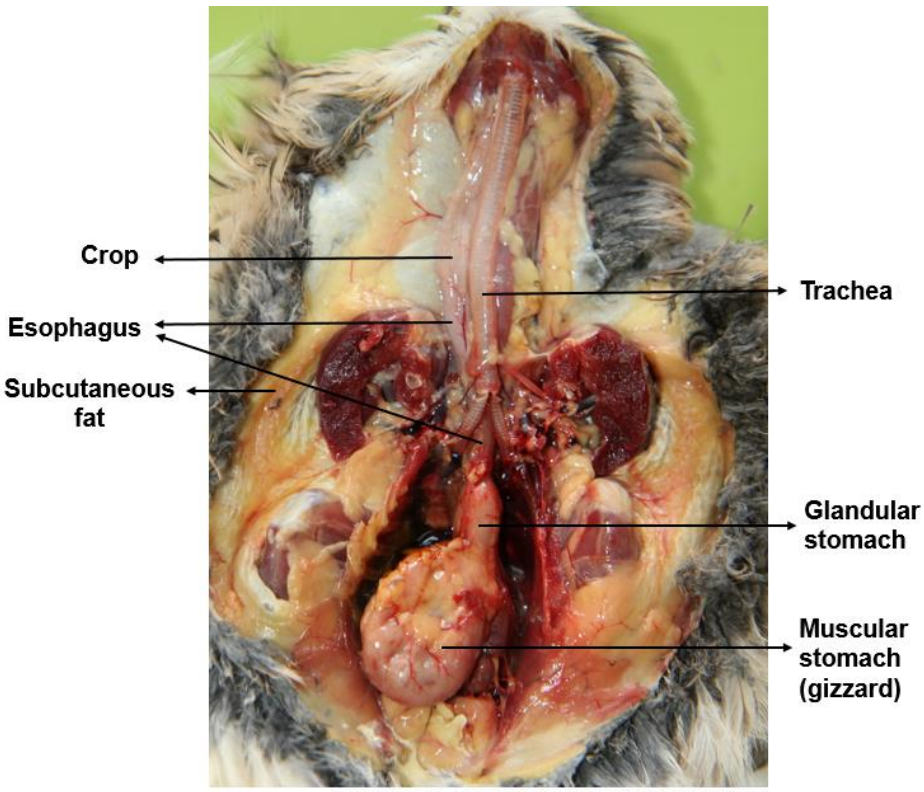


Eagle owl (*Bubo bubo*)
Photo: Pedro María-Mojica

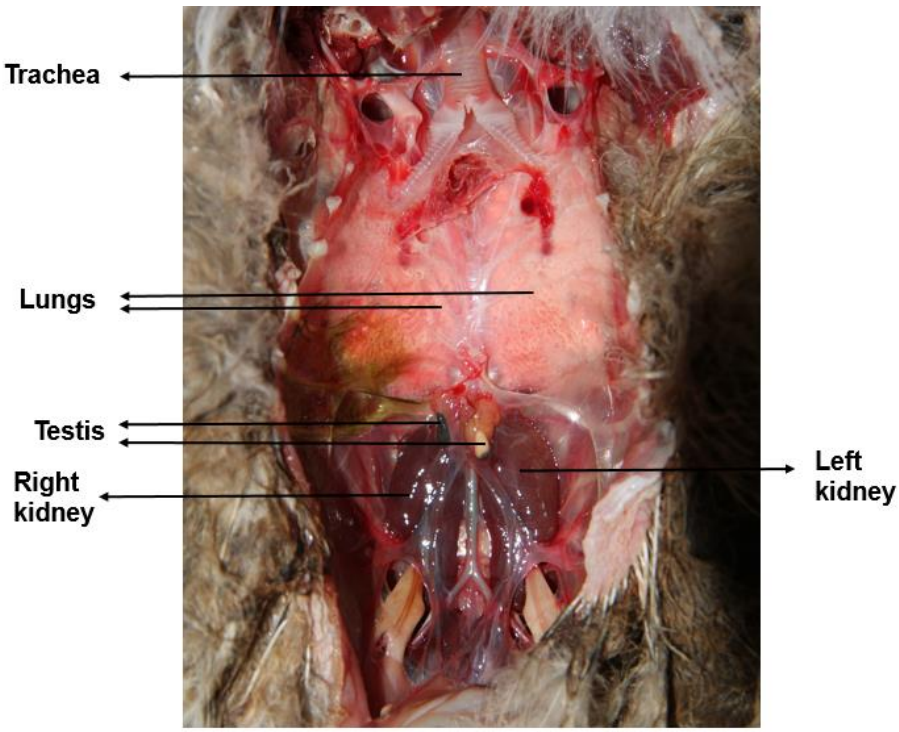


Booted eagle (*Hieraaetus pennatus*)
Photo: Pedro María-Mojica

Figure 9 (continued). Anatomy of raptors (Photos: Pedro María-Mojica).



Common kestrel (*Falco tinnunculus*)
Photo: Pedro María-Mojica



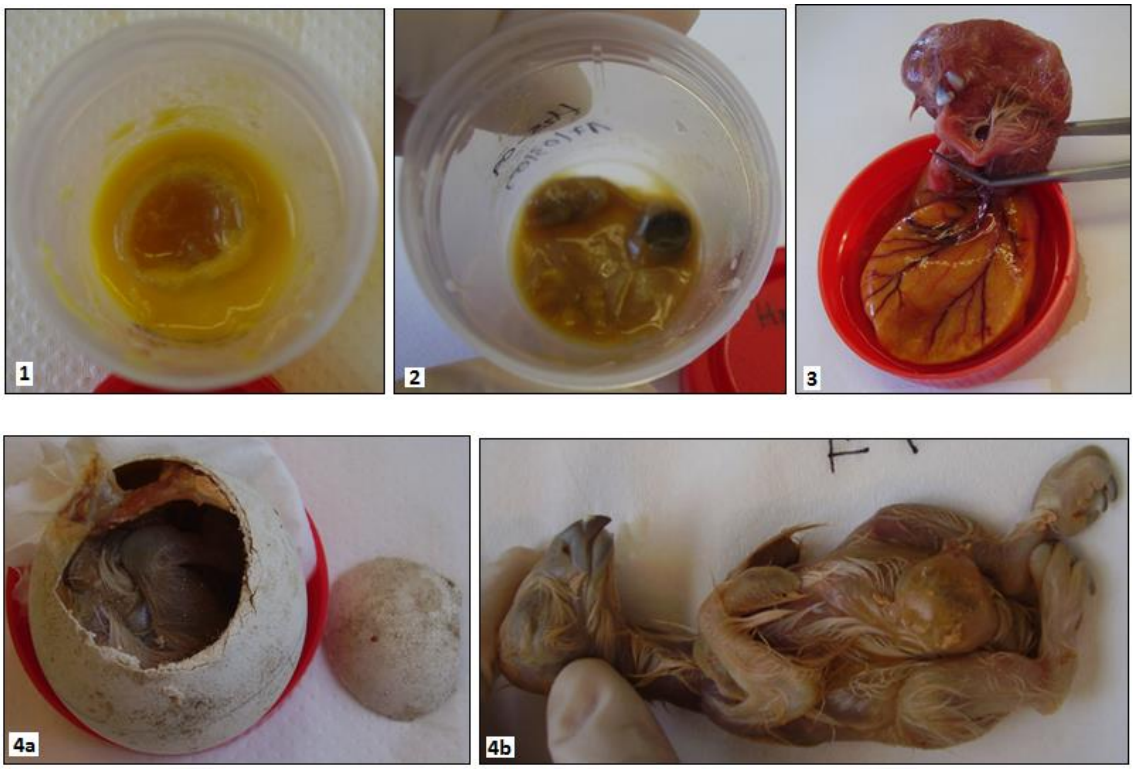
Eagle owl (*Bubo bubo*)
Photo: Pedro María-Mojica

Figure 9 (continued). Anatomy of raptors (Photos: Pilar Gómez-Ramírez).

Eagle owl (*Bubo bubo*) **Photo: Pilar Gómez-Ramírez**

Table 1. Embryo development (from Gómez-Ramírez et al., 2012; photos: Laura Ramón-Vaquero).

Classification	Development
1	Vascular formation
2	Morula or blastocyst
3	Embryo in first development stages
4	Very advanced embryo development



1. Vascular formation, 2.Morula or blastocyst, 3.Embryo in first development stages, 4a,b.Very advanced embryo development.

Table 2. Example of necropsy form.

NECROPSY FORM					
Carcass information					
Species			**Identification code**		
Age			**Sex**		
Wing length		**Tarsus length**		**Tarsus width**	
Head length		**Bill length**		**Body weight**	
Location of the carcass					
Date collected					
Circumstances in which the carcass was found					
Collector/Finder (contact information)					
Necropsy information					
Thawing	**Date and time**				
Necropsy	**Date and time**		**Person in charge**		
DESCRIPTION OF ALTERATIONS					
Status of decomposition					
External examination					
Cause of death					
Body condition					
Head and neck	**Eyes**		**Ears**		
	Oral cavity		**Nares**		
	Brain (weight)				
	Observations (lesions/alterations)				
Thoracic cavity	**Pectoral muscle**		**Subcutaneous fat**		
	Heart (weight)				
	Lungs (weight)		**Trachea**		
	Observations (lesions/alterations)				
Abdominal cavity	**Liver (weight)**			**Abdominal fat**	
	Kidney (weight)				
	Esophagus			**Crop**	
	Intestine			**Cloaca**	
	Observations (lesions/alterations)				
Gonads	**Developmental stage**				
	Observations (lesions/alterations)				
SAMPLES COLLECTED FOR CONTAMINANT MONITORING					
Liver	Kidney	Bone	Feathers	Subcutaneous fat	
Abdominal fat	Brain	Pectoral muscle	Preen oil	Crop content	
Other					