A protocol for the use of roadkill or stranded animals as material for research and teaching

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Abstract – Many aspects of the Brazilian fauna dynamics remain unknown. Often, researches on zoology depend on invasive protocols that require the death of specimens of rare or endangered species. This is the first time a combined protocol is proposed for the use of roadkill or stranded animals in research and teaching by different professionals. It turns such undesired deaths into a source of taxonomic material, tissue for histology, DNA analysis and parasitological tests and as ecological evidence. This is a non-invasive protocol that requires no sacrifice of any specimens. Skins and skeletons can be processed as taxonomic material and stomach contents provide information about feeding habits and ecology. In addition, regional zoological collections can be improved and these specimens can be used for practical classes in different areas, including environmental conservation. This article thus describes thirteen steps which can be taken to take the best advantage of these victims of human progress.
Additional key words: genetics, necropsy, parasitology, pathology, taxonomy, vertebrates.

Resumo (Protocolo de uso de animais atropelados ou encalhados em praias como material para pesquisa e ensino) **–** Muitos aspectos da dinâmica da fauna brasileira permanecem desconhecidos. As pesquisas em zoologia geralmente dependem de protocolos invasivos que exigem a morte de indivíduos de espécies raras ou em perigo. Assim, aqui é proposto pela primeira vez, por diferentes profissionais, um protocolo de ações conjuntas para o uso de animais atropelados ou encalhados para pesquisa e ensino. Ele torna essas mortes indesejadas numa fonte de material taxonômico, tecido para histologia, análises de DNA e exames parasitológicos, além de fornecer evidências ecológicas. A pele e o esqueleto podem ser utilizados como material taxonômico, enquanto o conteúdo gástrico fornece informações sobre hábitos alimentares e ecologia. Além disso, os acervos zoológicos regionais podem ser melhorados e estes animais podem ser utilizados também para aulas práticas em diferentes áreas, incluindo aquelas sobre conservação ambiental. Este artigo descreve então treze etapas que podem ser seguidas para tirar o melhor proveito dessas vítimas do progresso humano.
Palavras-chave adicionais: atropelamento, genética, necropsia, parasitologia, patologia, taxonomia, vertebrados.

Last century's economic and technological development have jeopardized the existence of many ecosystems and species on Earth, also compromising the health of the human populations. Therefore, the same development needs to create measures for nature conservation and human life quality maintenance. Habitat destruction, fragmentation, hunting, illegal trade, wildlife roadkill and contamination must be minimized or avoided through effective actions against each one of the mentioned problems. However, a lot of research is lacking to enable a better understanding of these problems and an effective action for mitigation of anthropogenic disturbance caused to ecosystems.

One of the consequences of the economic development is the running over of the fauna, which is constant in the Brazilian road network. Some studies, such as Turci & Bernarde (2009), provide a good information summary on this issue. Other works could be also accessed where the information in global scale is considered (Plowman & Imhoff 1972; Cândido Jr. et al. 2002). Sorensen (1995) emphasizes that in European countries, the deaths by run over have been identified as a major threat to wildlife. In Brazil, it is not different. In many highways in São Paulo State (e.g. Bandeirantes, Washington Luis, Anhanguera, Castelo Branco), which have a very large traffic flow, one could notice a huge decrease in the number of occurrences of roadkill carcasses if compared sightings ten or twenty years ago (pers. comm. Dr. Luciana Fernandes, Federal University of São Carlos and personal observations of the senior author). The same process should already be occurring in other Brazilian roads. Although these data are based on observations with no records, the impact that roads have on wildlife of any region is obvious.

Turci & Bernarde (2009) showed that in a stretch of 110 km of State Highway 383, in the State of Rondônia, there were 259 specimens belonging to 34 species of vertebrates, ran over in a period of one year, of a total of 3,300 km traveled. In this study, amphibians presented the largest number of samples victimized, with 68 individuals (two species), followed by 67 birds (12 species), 63 reptiles (13 species) and 61 mammals (seven species). These proportions should possibly be similar on other roads. Data from eight

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other studies (Vieira 1996; Cândido Jr. et al. 2002; Rodrigues et al. 2002; Rosa & Mauhs 2004; Pereira et al. 2006; Cherem, et al. 2007; Silva et al. 2007) showed an average of 0.0563 individuals hit by km (one animal per 17.762 km), with a minimum of 0.003 (or one animal per 333.34 kilometers) to a maximum of 0.145 animals per km (or one animal per 6.895 km).

In 2010, the fully paved highways in Brazil covered 212,738 kilometers (http://www.brasil.gov.br/sobre/o-brasil/o-brasil-em-numeros-1/infraestrutura/print). If we extrapolate the data up to this highway extension, we can get a number of 3,778.65 vertebrates systematically slaughtered per year on paved roads only where most accidents happen. Even this number should be an underestimation, considering that not all roadkill were found. One should also take into account that some scavengers as vultures and raptors eventually eat these carcasses and can drag them from the road. Moreover, injured animals can move away and die elsewhere or even the impact can throw them away from the track. Teixeira (2010), in a review of differences between data collection, points that the numbers vary significantly depending on the taxa considered. There is an underestimation in some classes of vertebrates (birds, amphibians and reptiles), as scavengers more easily remove these animals, and the speed of the vehicle used by the researcher also does not allow an accurate observation of small animals on the roads.

Even data on midsize mammals can be underestimated. On September 22, 2010, the senior author of this article (PA) collected an adult raccoon around 17hs at kilometer 340 of the BR-135 highway in southern Piauí. There were only very few fresh remains on the yet articulated skeleton. Locals told that the animal had been hit in the early morning, and the vultures had completely stripped the skeleton during the same day. This example makes clear that the scavengers possibly cause an error of great influence on the numbers in these studies.

Monitoring studies on highways roadkills have been the subject of many articles (e.g. Vieira 1996; Cândido Jr. et al. 2002; Rodrigues et al. 2002; Rosa & Mauhs 2004; Pereira et al. 2006; Cherem, et al. 2007; Silva et al. 2007) in the last 30 years. Most of these studies aimed to quantify the number of lethal hits of a particular group of vertebrates over a period of time. Usually the sought data are identification of species, sex, weight and measures, as well as notes on the exact point where the animal was found, and these data are analyzed comparatively by statistics among the variables. These data are important for assessments of the effect of the construction of highways on wildlife surrounding ecosystem, which in turn are important for actions on protection for these species and other ecological data. However, in view of this undesirable scenario, it should be recognized the potential of these animals as a source of studies. Few studies emphasize that roadkill can have an advantage that goes beyond the statistics commonly applied to the data. Rocha (2009) reports that these carcasses may also be used for taxidermy and environmental education, for example.

The use of these animals can increase knowledge about the regional fauna in several aspects as well as enable the start or growth of universities' vertebrate collections, it can also provide material for studies on molecular biology, through the collection and storage of tissue samples. Still, each specimen may favor ecological studies with the analysis of stomach and intestine contents and, also, provide tissue samples of various organs for histology. Parasitological studies also enable epidemiological surveys, so little known areas about our wildlife. They can also be used for practical classes, in addition to the already mentioned use for environmental education. In short, the combined work of several professionals can maximize the use of this resource, with a short-term result in proposals for conservation or taxonomic/evolutionary biology.

It is important to note that any citizen may accomplish the procedure of collecting wild animals found stranded or dead without prior authorization from competent authorities (IBAMA in Brazil), since the collector takes the animal immediately to a research institution (IBAMA normative # 154, March 01 2007, in Brazil). For animals found on beaches, there is a procedure already established by several institutions studying Cetaceans and Pinnipeds, but that can be refurbished with the suggestions presented here.

The aim of this article is to suggest a new protocol to avail this roadkill and other dead animals (for example, stranded on beaches) as material for scientific studies or education. For the first time researchers from different fields of biology and veterinary medicine propose a multidisciplinary protocol to encourage the collection and use of this material, aiming at maximizing data retrieved on each animal. Other protocols deal only with specific problems as how to collect parasites and fixing them, or how to proceed taxidermy, and so on. This protocol suggests a combination of well known protocols mixed with new procedures that make this a new way to think about roadkill or stranded animals, to take the best advantage of these undesired deaths.

Protocol. This protocol seeks to optimize the usage possibilities of a carcass found in trampling conditions or other causes, such as stranding on beaches or simply found dead. For this, we used several biological and veterinary techniques already used in domestic animals that can be adapted to each local situation and its institutions. It is hoped that this protocol contributes to the conservation of biodiversity, with the opportunity to systematize possible research on wildlife fatalities, as a compensation for this environmental loss through scientific gain.

Step 1: Collecting the specimen. Dead animals are not always found in suitable conditions to be collected;

sometimes they were crushed by other cars, partially eaten by scavengers or are in an advanced state of decomposition, etc. For this reason, it is necessary to counterbalance both the rarity as much as the carcass state. It is up to the collector whether to house it or just gather data and collect a tissue sample for molecular analysis and a blood sample via cardiac puncture for serological analysis. If there is a fresh clot in the specimen, it is important to collect it for studies on disease occurrence. If you adopt one of the last two options, follow the 5th and 6th step of this protocol. If it is not possible an immediate dispatch to a research institution and it is not possible to perform a necropsy and sample collection immediately, the animal should be frozen. Be aware the necropsy of a fresh animal allows us to collect more information than after freezing.

When collecting specimens, one should take as much information as possible about the location. Besides the date and time of collection, record the geographic coordinates. This will be the best way to indicate the origin (including altitude), although not always a GPS is available. In addition, the annotation of the mileage of highway signposts provided by the lateral route is the easiest way for indicating the location. Moreover, you should check what the municipality is, the environmental conditions of the area (pasture, outskirts of town, forest) and the type of biome. Take pictures of the specimen and surroundings. Whenever possible take some pictures of the animal before removing it from the place where it was found. Use objects such as scale in the photos (e.g. a ruler or a coin). Knowing the position in which the animal was found, as the flank position or another one, assists in identifying cadaveric changes (hypostasis mortis and hemoglobin impregnation, for example) and reduces possible mistakes in necropsy (reference pathology). Ideally, this piece must be packed in a Styrofoam box with ice until arrival at the research institution. To do so, always keep in the car, a Styrofoam box with ice packs. If this is not possible, collect the animal in a plastic bag and take it, as soon as possible, to a research institution. The collection of specimens should be made wearing disposable gloves or, when it is not available, collectors must use a plastic bag to protect their hands.

Step 2: Identification and packaging. If it is impossible to immediately identify the species, adopt a numeric ID. A number or a field number must be given to every specimen, taking into account they will be later receive an official number in the collection. Every piece taken from the specimen should receive the same number, preferably the number of zoological collection where it will be deposited to avoid possible misunderstandings.

The labels must be made with special paper that avoids humidity. They must be written using pencil instead of pen since the information remains intact even when the paper gets wet. Labels must be bound directly to the animal's body. These labels should contain at least the date of collection, the species (if known), number of registration, place where it was found and the collector's name.

Each specimen of different zoological class must be packed in a different way (Auricchio & Salomão 2002). Birds have very delicate bodies and feathers and should receive a wad of cotton or other absorbent material inside the beak and anus. It avoids any liquid from the esophagus and intestines to spill over and get onto the feathers. Bloodied areas as fractures and/or exposed cavities parts must be protected and wrapped with tissue paper or newspaper. For amphibians, reptiles and mammals simply repackage them in plastic bags. Prioritize performing these procedures immediately after the arrival of the animals in the research institution, however, if this is not possible, store the samples in a freezer for later procedure. It can also be useful in order to fix and conserve parasites, to avoid bleeding since the blood coagulates, etc. and avoid diseases (see Auricchio & Salomão 2002). Do not rely on memory. Never fail to identify the specimen with its number and place of collection. Annotations should also be made in a lab book that will be supplemented later with the procedures of biometry, taxidermy, necropsy, etc.

Step 3: Collecting ectoparasites. Remove the specimen from the freezer a while before starting the procedures, allowing it to thaw. This time varies depending on the animal's body volume. It should be considered that the animal could be in any stage of decomposition, so that the defrosting time must be carefully monitored to avoid losses. Then you must make a thorough search for ectoparasites among feathers, hairs and scales on the eyelids, on the edge of the anus or cloaca, beak and mouth looking for helminth species. All ectoparasites found must be collected and fixed in 70° GL ethanol. Microtubes of 1.0 mL or other vial can be used for storage. Each vial must be strictly identified with the number of the specimen in the collection catalog, along with the part of the body where the parasite was found for future identification. This information should also appear in the necropsy form.

Step 4: Necropsy. In most cases, the cause of death of animals found on roads is obviously car hit and, as the specimen will be used for several purposes, necropsy cannot follow its usual protocol, and veterinarians who carry out necropsies should pay attention not to damage skin and bones. It is suggested that the necropsy is accompanied by biologists who master the preparation techniques to guide the cuts in the skin. Avoid long incisions like the usual paramedian cutaneous incision from the mandibular symphysis to the ventral base of the tail, or even cut the bones like those that usually occur in domestic animals necropsies. Aside of these precautions, a necropsy can be performed according to Hendrix (2005), with some modifications. Briefly, for mammals

and reptiles: 1. The animal is positioned in its left lateral position; 2. Bounce off the legs dorso-lateraly after cutting the muscle insertions close to the chest and abdomen, disarticulate the hip joints and scapular-humeral without cutting the bones; 3. The cut must be long sufficient for observation of internal organs or, preferably, proceed the skin removal before the necropsy (as shown in Vanzolini & Papavero 1967; Auricchio & Salomão 2002); 4. Remove the jaw without damaging it or the skin to access the tongue. The hard palate should not be cut and a cut around the pharynx to the level of the hyoid bone should be done. After they are dismantled, move away the tongue, pharynx, esophagus and trachea through the entrance of the thoracic cavity; 5. Open up the chest with an exact cut in the fibrocartilage or remove the sternum without damaging any bone; 6. Pull the tongue, pharynx, esophagus and trachea, together with the thoracic organs from the chest cavity; 7. Tie the caudal end of the esophagus to separate these organs from the thorax, together with the rest of the gastrointestinal tract. This set of organs is the first part of internal organs; 8. Open the abdomen by cutting the *linea alba* to the brim of the pelvis; 9. A tie must be done in the cranial portion of the duodenum, in the pyloric region, in order to detach the second group of organs which comprises of stomach, liver, pancreas and spleen; 10. The third piece of the body, composed by the intestines, is removed by cutting mesenteric ligation in the rectal region. The fourth portion consists of the kidneys, ureter, bladder and reproductive organs.

For birds, the following steps must be tracked: preferably, remove the skin before the necropsy to avoid dirtying the feathers (Auricchio & Salomão 2002). 1. Place bird in a supine position; 2. The skin is incised in ventral midline avoiding cutting through the neck to the beak; 3. Incise up the abdomen and expose the abdominal cavity, everything with the utmost care not to dirty or cut the feathers; 4. Disarticulate up the ribs and shoulder blades to reveal the chest cavity, exposing all the internal organs.

Step 5: Collection and storage of fresh blood or clot. Serum samples should be collected for research on occurrence and prevalence of diseases. In the case of roadkill sent to the laboratory, it is recommended to collect fresh clot immediately at the beginning of the necropsy, doing a cardiac puncture for removal of clot from this organ. You can perform ligation of major blood vessels and remove them along with the heart, opening it and collecting all the blood inside a glass tube without anticoagulant. Centrifuge to extract the serum and the concentrated red blood cells. Both serum and concentrated red blood cells should be stored frozen, preferably at -80ºC freezer. It should be noted that several pathogens may degraded after the death of the animal, such as viral agents. Therefore, the laboratory that performs the isolation and serological tests should be aware of the serum collection and extraction method of so that the results are reliable.

Step 6: Collection and storage of Genetic Material. Liver tissue fragments (~ 1 cm^3) and animal muscle are stored for further molecular analysis (DNA). Samples must be packed in individual microtubes or cryogenic tubes of 1.5 or 2.0 ml, containing ethanol (PA). In the case of absence of PA ethanol, commercial ethanol may be used 92º or 96º GL (or INPM). The samples are preferably kept at -20°C, to reduce the degradation of nucleic acids. However, they can be kept at room temperature for a couple of days if it is impossible to store in freezer immediately. The sample tubes must always be identified with the specimen collection number, species and location.

Step 7: Collection of gastrointestinal parasites. The parts of the digestive system should be directed to the careful collection of gastrointestinal parasites. In the stomach and intestines: an incision is made from cranial to caudal portion of these organs, search and collect with a brush all parasites visible by naked eye, placing them in microtubes or other bottle with 70% ethanol. Scrape the wall of the organ with the edge of a glass slide by adding water. Place the contents in a Petri dish and then put small portions of the collected content into another Petri dish with a little water or saline solution. Make stereomicroscopy observations, placing the parasites in microtubes or another bottle with 70% ethanol, properly identified.

Step 8: Preparation of gastrointestinal parasites. Most of the parasites will preferably be stored in other solutions than ethanol. However, a few specimens should be fixed only in ethanol (70% or PA) for further genetic analysis (Innis et al. 1990). The use of other substances may difficult PCR (Polymerase in Chain Reaction) procedures. To Helminthes: They must be collected from all organs, particularly the gastrointestinal tract, lungs, liver and kidneys. They should be carefully collected, cleaned and processed following the classical methodologies (Amato et al. 1991). Trematoda: They should be set after compression between the slide and the cover slip and fixed in AFA (Amato et al. 1991) for further species identification. Samples must be analyzed thoroughly *in toto* mounts, stained with hydrochloric carmine and then prepared into permanent slides for analysis of systematic value structures (Andrade 2000; Rey 2001). For Nematodes: They should also be submitted to fixation in AFA (Amato et al. 1991) for later identification. The specimens must be submitted to the lactophenol Aman clarification (Andrade 2000), to enable further analysis and observation of systematic value structures. Cestodes: they should be set after compression between slides and cover slips and fixed in AFA for identification. After this, cestodes be stained with hydrochloric carmine and then prepared on glass slides for systematic analysis (Andrade 2000; Rey 2001). This procedure allows drawings in *camera lucida* adapted to an optical microscope and morphometric data and photomicrographs can be obtained from computerized image analysis (e.g. QWin Lite 3.1, adapted DMLB microscope Leica).

Step 9: Analysis of gastric contents. After investigation of endoparasites in the stomach, you should proceed with a thorough analysis of stomach contents to separate and identify food items. To do this, place the gastric contents into a Petri dish. Instill distilled water on the content and with the aid of a double sieve or two pieces of cheesecloth, filter them as often as it deems necessary to facilitate analyzing the material. Transfer the sieved material to another Petri dish and proceed the separation with the aid of a magnifying glass or a microscope. Separate them into vegetable, animal or mineral, let them dry and store them in plastic bags with proper identification. Plastic bags should be packed in aluminum cans with silica to control moisture until they are forwarded to professionals who can identify the food pieces.

Step 10: Preparing the skin. The skin should be managed according to information found in Vanzolini & Papavero (1967) or Auricchio & Salomão (2002), in preparation for zoological collection or as didactic piece. Each vertebrate class has specific details and a revision in the bibliography is essential for the success.

Step 11: Preparing the skeleton. After necropsy and skin removal, proceed with the manual stripping of the skeleton. Care must be taken so that no bone is damaged or lost, since there are now a lot of blood and debris mixed with the carcass. After the removal of soft tissues, bones must be placed in cold maceration to obtain a fully disarticulated skeleton, or they can be boiled and manually cleaned which would result in a semi-articulated skeleton. Another way to clean the skeleton would be drying it off (never under the sun or in an oven) and leave it to dermestid beetles as indicated Auricchio & Salomão (2002). Use India ink to mark the already dried bones with the same collection number of the corresponding skin received. Disclaimer: all pieces in this preparation (skin, tissue, skeleton, stomach contents, etc.) should receive the same number to facilitate future correlation among the specimens.

Step 12: Assembling other organs and tissues. After being removed, the reproductive organs (ovaries, testicles and uterus) and other organs of histological interest must be washed in physiological saline or distilled water, packed in previously identified vessels with the same number and name of the specimen from what it came, specimen number, age and approximate date of collection. Add to the containers fixing solutions of 10% formalin or 4% paraformaldehyde. Preferably, the volume of fixative should be approximately 20 times greater than the volume of stored tissue. When possible, the fixing solution should be prepared in phosphate buffer (0.1 M, pH 7.4). Fixing time will depend on the volume of tissue to be secured. Consequently this material will be able to perform the majority of histological techniques that use resin or paraffin embedding and can be stained with different methodologies (for details, see Junqueira & Junqueira 1983).

Step 13: Report the roadkill finding to the competent organs. Whenever you encounter a hit wild animal, the researcher who receives the specimen must inform the agency and / or institution responsible for the maintenance of the road or highway. The animal species as well as the geographic coordinates, mileage and photos of the specimens can be informed. This will help the development of a statistics data bank of roadkill in the region and will subsidize these organs to build up concrete measures, such as the addition of signposts and speed control at strategic spots to prevent more animal deaths.

Obviously it is necessary to be careful and use personal protective equipment such as glasses and rubber gloves for all this procedures, avoiding all direct contact with the carcass. It is not unusual that fluids go beyond the preparation and squish or contaminate the skin.

This protocol has been standardized for recently dead animals or those dead a few hours. However, on several occasions the found carcass should be in decomposition state, not allowing its full use. Sometimes the skeleton is crushed; the skin may be dropping fur or feathers, etc. Even in these cases it is worth to emphasize that it is always possible to remove a sample of tissue for molecular analysis, as described in Step 5, even if it is in an advanced decomposition because this ensures minimal sample and record of the case.

Animals *in situ* are valuable sources of information, several times regionally unknown to science. Many are considered environmental quality indicators when they are in reduced population, facing environmental imbalances where they are exposed or, even, when they are ecologically healthy (Aguirre et al. 2002). Considering the conservation purposes, it is necessary to expand our knowledge about fauna in relation to normal physiological and morphological parameters, eating habits, pathology, among others. However, such studies are often impractical due to the difficulty of capturing and manipulating species *in situ* and the high cost to perform these searches.

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