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Methods in herpetological forensic work — clinical techniques

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Abstract
Biologists, law enforcement officials and veterinarians are routinely called upon to investigate reptile cases for abuse, neglect, illegal importation, and abandonment. While pursuing these situations, it is important that evidence is collected in an organized and systematic way to ensure successful prosecution or to mount a defense. There are different types of evidence that can be amassed to diagnose a disease/condition in a reptile case. Antemortem clinical investigations can be conducted for those cases where the animals are alive, while postmortem examinations should be pursued for animals that have expired. The purpose of this article is to review the common antemortem clinical techniques that can be used for forensic cases. There are a number of clinical diagnostics available for the forensic case, including the physical examination, clinical pathology, parasite diagnostics, infectious disease diagnostics, clinical toxicology, and diagnostic imaging. In addition to the clinical techniques, it is important to review and document the methods used to house and care for the animals. For this, a thorough review of the husbandry practices provided for the animal is needed.

Key words
Clinical techniques, diagnostic imaging, hematology, husbandry, physical examination, reptile.

Introduction

Biologists, law enforcement officials, and veterinarians are routinely called upon to investigate reptile (and sometimes amphibian) cases for evidence of abuse, neglect, illegal importation or abandonment. In many of these instances, there are discrepancies in the documented and undocumented communications between the individuals being charged and those processing the cases. To minimize the likelihood of encountering legal problems, the individuals processing these cases should follow an organized and systematic approach.

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There are different types of evidence that can be collected to diagnose a disease/condition in a reptile case. Antemortem clinical investigations can be performed for those cases where the animals are alive, while postmortem tests should be pursued for animals that have expired. Regardless of the types of diagnostics collected, standard protocols for evidence management, such as chain of custody documentation, should be followed to minimize the likelihood of legal discrepancies when the results are presented and cases are disputed (see Hart and Budgen, this series). The purpose of this article is to review the clinical antemortem methods that can be used to assist with the management of reptilian forensic cases.

Husbandry

Managing reptiles in captivity requires specific background information about an animal's life history. In many of the abuse or neglect cases encountered by wildlife enforcement officials, inappropriate husbandry conditions play an important role in the poor condition of the animal. Personnel investigating these cases should become familiar with the specific captive needs for a species to determine the level of neglect that can be attributed to inappropriate husbandry. There are numerous herpetoculture texts available that can provide specific captive husbandry information for reptiles (Advanced Vivarium Systems; BowTie Press Inc., Irvine, CA 92618, USA). When commercial literature is not available for a species that is being investigated, law enforcement officials might need to pursue specific life-history information available through the academic herpetological literature, natural climate and environmental data related to the country of origin of the species, or experts in the field (i.e., herpetoculturists, herpetologists, veterinarians).

Specific husbandry considerations

Reptiles are ectotherms and depend on the environmental temperature to regulate their metabolism, immune function and general behavior (Mitchell, 2006). Reptiles housed under inappropriate conditions are more susceptible to infectious diseases (decreased immune function), being undersized (reduced metabolism), not reproducing, and not displaying natural behaviors. When evaluating neglect cases, it is not uncommon to find animals being housed under ambient temperatures that do not meet their required environmental temperature range, resulting in their being hypothermic. It is also common to find cases where an external heat source is provided but not monitored. In these cases, animals commonly sustain first to third degree burns as a result of direct exposure to a heat source (fig. 1a).

Measuring environmental temperatures for animals that are being investigated is important. Environmental temperatures should be recorded in several sites within the reptile enclosure. Validating the thermometer being used is important in legal cases, and this can best be done prior to sampling by comparing it with at least one

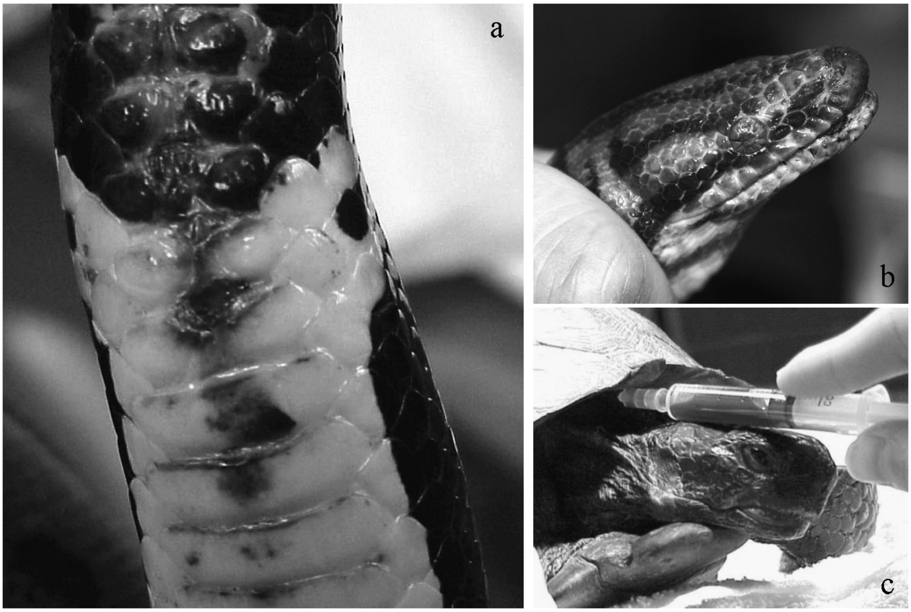

Figure 1. (a) Burning is common in reptiles with direct contact to a heat source. This boa constrictor (*Boa constrictor*) had direct exposure to an incandescent lamp and sustained thermal injury to its ventrum. (b) A rainbow boa (*Epicrates cenchria*) that was housed in a small cage at a low environmental humidity. It developed rostral abrasions while attempting to escape from its enclosure and was dysecdytitic. (c) Blood samples can be obtained from chelonians via the subcarapacial sinus, as in this gopher tortoise (*Gopherus polyphemus*).

or two other standards (e.g., mercury thermometer, digital thermometer). A minimum of three samples should be collected from the enclosure and the arithmetic mean and degree of sampling variability calculated. The methods used to provide heat for the reptile should also be recorded. Radiant heat from a light bulb, undertank heating pads, and "hot rocks" are commonly used.

Humidity should also be measured when evaluating a reptile's environment. Reptiles housed in low humidity environments may develop dysecdysis with secondary avascular necrosis, while animals housed in high humidity environments may develop moist dermatitis. A hygrometer can be used to measure environmental humidity in a case investigation. The hygrometer should also be validated using the method described for the thermometer.

The substrate on which an animal is housed can also have an effect on the animal's general health and well being. Rock-based and sand substrates are inexpensive and frequently used to house reptiles, but can lead to foreign body impaction if ingested. As some reptiles are geophagic, they have a natural tendency to ingest these materials to acquire trace minerals (Diaz and Mitchell, 2006). Underfed or nutritionally deprived animals may be more likely to ingest their substrate. Certain woody substrates can affect the environmental humidity of a reptile's enclosure. In many cases, these substrates absorb moisture and lower the humidity within the enclosure. If the environmental humidity is not monitored in these cases, and moisture supplemented as needed, dysecdysis and avascular necrosis of the digits or tail can occur. Substrate material should be changed regularly and inspected for feces and urine. In many cases of neglect, substrates are not changed regularly and the animals may be living in their own waste.

When evaluating the state-of-care being provided to a reptile, it is important to evaluate the size and type of the enclosure. There are recommendations for minimum cage sizes that can be used as a reference (Rossi, 2006). Animals housed in cages that are too small are more likely to develop injuries to the rostrum from attempting to escape from their enclosure (fig. 1b) or dermatologic conditions in cases where the animals are constantly exposed to their own wastes. The type of enclosure used can have an effect on the ventilation within the enclosure. Animals housed in enclosures with poor ventilation are more susceptible to respiratory ailments. Inadequate ventilation, in combination with poor sanitation, can lead to respiratory disease in reptiles.

The type of food(s) given to the reptile should also be examined closely. Animals offered an inappropriate diet are susceptible to developing metabolic diseases. The first step in characterizing the appropriateness of a reptile diet is to determine the normal feeding strategy of the animal (i.e., carnivore, insectivore, omnivore, or herbivore). In captivity, the two hardest groups of reptiles to provide nutrition for are the herbivores and insectivores. The primary reason for this is limited access to a diverse group of food items. The types of food, frequency they are offered, amount offered, and conditions under which the food is kept should all be recorded.

Captive reptiles have different requirements for drinking water. The receptacle used to provide the water, the source of the water, the frequency at which the water is changed, and the addition of any supplements to the water should be recorded. Animals not provided access to water may become dehydrated, and in severe cases develop metabolic conditions attributed to chronic dehydration (gout, renal failure).

Aquatic species of reptiles should be provided with an environment that enables them to have continuous access to water. Removing aquatic reptiles from water for an extended period can be stressful to the animal. Red-eared sliders taken from water for extended periods were more likely to shed *Salmonella* than those animals maintained in water (DuPonte et al., 1978). Aquatic systems for reptiles should have regular water changes. The frequency of this will depend on the size of the system, number of animals, and organic load on the system. Biologic, chemical and mechanical filtration can all be used to limit the build-up of organic and inorganic toxins in an aquatic system. When evaluating aquatic systems, it is important to note the presence/absence of filtration, the quality of the water, and the condition of the animals. Reptiles kept in poor quality water are more likely to develop respiratory and skin infections.

All vertebrates, whether nocturnal, diurnal or crepuscular, are conditioned to a photoperiod that is seasonal. Because so many physiologic processes are linked to photoperiod, captive reptiles should be provided with exposure to light. Reptiles that are not given light in captivity or are exposed to excessive amounts of light (e.g., 24 hours of light) may be more susceptible to behavioral anomalies that affect appetite, water consumption, and reproduction, among others. Not providing light or allotting excessive amounts of light are considered to be forms of torture in humans, and should therefore not be considered appropriate for reptiles, with rare exceptions (e.g., cave species). In general, incandescent lighting provides radiant light or infrared heat. This type of lighting should be provided when radiant heat is the primary source for controlling the temperature within an enclosure. Fluorescent lighting is generally associated with visible light and ultraviolet radiation. Recent research suggests that snakes, chelonians and lizards all benefit from being exposed to ultraviolet B radiation (Acierno et al., 2007; Acierno et al., 2008). The types of light used (e.g., incandescent and fluorescent) and amount of light provided should be documented when evaluating the husbandry conditions of a reptile.

Captive reptiles should be provided with an environment (vivarium or outdoor enclosure) that mimics their natural habitat. This is important because it minimizes physiologic stress. Animals maintained under chronic stressful conditions are more susceptible to opportunistic infections because of a suppressed immune system. For example, arboreal species, such as the Old World chameleons, that do not have access to branches and foliage are more likely to have abnormal behavior patterns and die suddenly. A general environmental classification scheme for reptiles should start with whether the animal is terrestrial or aquatic. Next, the terrestrial grouping can be further classified into arboreal, above-ground, and fossorial species. This general classification scheme may be useful when evaluating an environment to determine if it is meeting the most basic needs of the reptiles.

A review of the husbandry practices being provided by a facility is necessary to determine the extent of neglect associated with a case. While policies may vary, one approach is as follows: All of the information obtained regarding the captive husbandry conditions provided to a reptile should be documented in triplicate and signed off by a witness. One copy of the document should remain with the party being investigated, the second copy should be reserved for review by specialists associated with the case, and the original should remain with the investigator's case files. Photographs should be collected of the animal's enclosure and husbandry methods and with the animal in its enclosure to confirm the animal was housed under a specific set of conditions. Photographs should be validated by being dated and signed off by a witness (see also Hart and Budgen, this series).

Physical examination

Live reptiles should be thoroughly examined during any forensic investigation. It is always recommended that a veterinarian familiar with reptiles performs this. The physical examination should be conducted in two parts: a hands-off examination and a hands-on examination. The hands-off examination will enable the examiner to evaluate the reptile's general demeanor, respiration, locomotion, and fecal/urine output. Animals suffering from neglect, such as dehydration from not being given an appropriate water source or abuse (e.g., trauma-induced fracture), may appear lethargic or have reducedambulation.

The hands-on examination should be performed in a similar fashion for every case to minimize the likelihood of missing a problem. Physical examinations in certain reptile species may be limited in the conscious patient because of anatomic (e.g., shell of a chelonian) or potentially hazardous reasons (e.g., venomous snakes). In these Situations, the animal may need to be sedated or anesthetized.

The head should be thoroughly palpated for any lesions or hidden fractures. Animals in poor body condition will often have a prominent sagittal crest, resulting from atrophy of the muscles covering the skull. This may be indicative of chronic disease or malnutrition. The eyes should be clear and free of discharge. Animals that are severely dehydrated (>8-10%) often have sunken eyes from a loss of moisture from the retro-orbital fat pads. Snakes and certain lizard species (e.g., gecko) have spectacles that cover the eyes. Retained spectacles may occur in these species when they are housed under less-than-optimal environmental humidity. The nares should be patent and free of discharge. Animals housed in small cages may develop severe rostral abrasions that can alter the nares. An external tympanum or ear can be found on chelonians and lizards, respectively. Aural abscesses are a common finding in chelonians housed under inappropriate environmental temperatures and offered a diet that is low in vitamin A. An oral examination should be performed to assess the general condition of the mucous membranes, teeth, jaws, choanae, and proximal gastrointestinal tract. It is important to limit the amount of force exerted when opening the oral cavity to minimize the likelihood of damaging the teeth or jaws. A soft-rubber spatula can be used to open the mouth of most lizards or snakes. Opening the oral cavity of a chelonian can be more difficult, and may require sedation. Thick, ropy mucus in the oral cavity is suggestive of dehydration. The mucous membranes of reptiles are often pale to pink in color. In some species, a pale color is indicative of anemia; this can be confirmed by collecting blood for a hematocrit and erythrocyte evaluation (see Hart and Budgen, this series).

After completing an examination of the head, the remainder of the body can be appraised. The epaxial muscles along the spine of lizards and snakes should be evaluated to assess body condition. Prominent vertebrae are suggestive of muscle atrophy and/or emaciation. The large muscle bellies associated with the limbs of crocodilians, lizards and chelonians can also be used to assess body condition. In certain species of lizards (e.g., fat-tailed geckos, *Eublepharis macularius*) the tail is a primary site of energy storage and loss of tail girth is suggestive of poor body condition. Crocodilians and other species of lizards will also have thin tails with prominent caudal vertebrae when they are in poor condition. The limbs should be palpated for any injuries. Fractures associated with blunt force trauma and metabolic bone diseases (fig. 2) are common in legal cases. Determining the cause of a fracture is best achieved by radiography. The digits and tails of reptiles

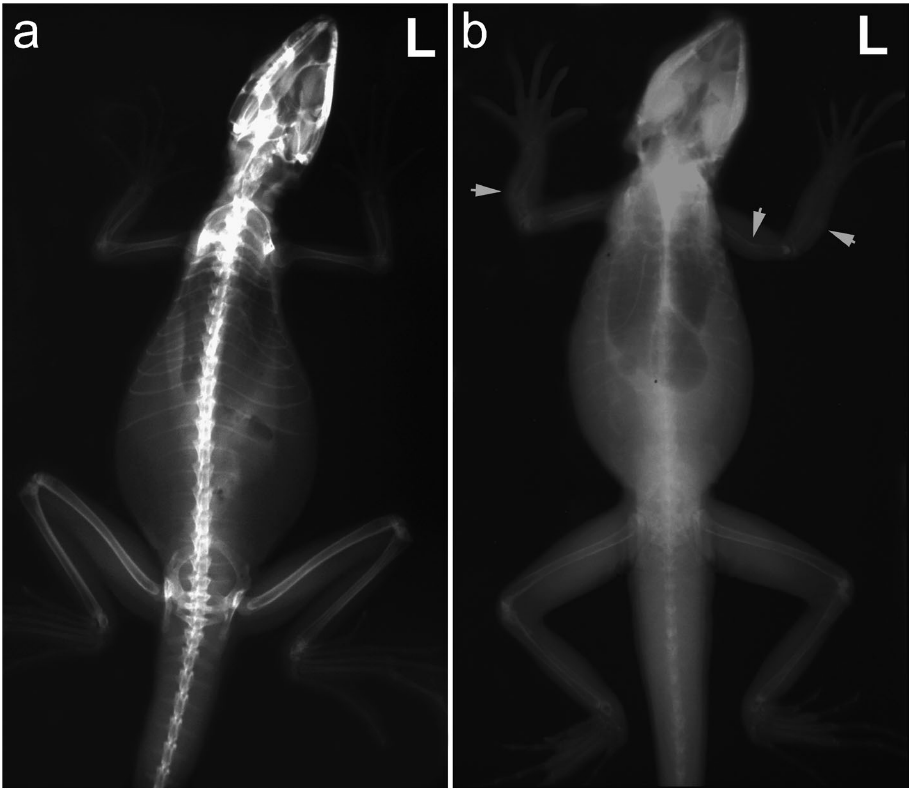

Figure 2. Dorsoventral radiographs of two Thai water dragons (*Physignathus cocincinus*) (L = left marker), demonstrating (a) normal appendicular and axial skeleton with acceptable bone opacity and cortical thickness; (b) abnormal skeleton with obvious reduction in bone opacity, poorly defined cortices, and several pathological fractures in the forelimbs (arrows). The radiograph is highly suggestive of secondary nutritional hyperparathyroidism; however, a definitive diagnosis in the live animal requires evaluation of husbandry and, in particular, environmental lighting, as well as determination of parathyroid hormone and 25-hydrox-vitamin D_3 values.

should be closely inspected for signs of compromise. Animals housed under adverse environmental conditions appear to be more susceptible to dysecdysis. When this occurs around digits or the tail, the blood supply to these structures may be compromised and the tip of the structure is lost.

The coelomic cavity of the reptile should be palpated for any abnormal masses. Animals that are used to transport illicit compounds, such as drugs, may have large firm palpable masses in their gastrointestinal tract. Confirmation of the source of the material can be achieved using diagnostic imaging.

Auscultating the lungs and heart of reptiles is often considered unrewarding; however, regular practice using a stethoscope is often helpful in identifying abnormalities in the heart rate or respiratory system. An ultrasonic Doppler can also be used to assess the heart. Animals housed under less-than-optimal conditions may be bradycardic and are more susceptible to pneumonia.

Clinical sampling

In the field of forensic medicine, the collection of antemortem clinical samples can prove invaluable in determining a diagnosis for a specific disease or injury, the duration of the disease or injury, and the overall physiologic condition of an animal (see Frye, and Hart and Budgen, this series). A number of clinical diagnostics can be used to answer the questions that are important to defining a forensic case. It is not possible here to go into detail regarding all of the diagnostics available for forensic cases, so the focus of this article will be on clinical pathology, infectious disease diagnostics, clinical toxicology, and diagnostic imaging.

When carrying-out any of the diagnostics described in this article, it is essential that all documentation is complete, legible, and recorded in triplicate (see also Hart and Budgen, this series). The original should be kept in a file that travels with the animal, a copy should always stay with the laboratory/veterinarian who is performing the diagnostic test, and a third copy should be held by the law enforcement official pursuing the case. A standard chain-of-custody should be followed when handling the samples, with each transfer of samples being signed-off in triplicate by the handlers. Minimizing the number of parties involved with sample management will minimize the likelihood of lost or misplaced samples (Cooper and Cooper, 2007).

When collecting samples for clinical evaluation, it is essential that these are managed in a fashion that does not compromise their integrity (see Frye, this series). For example, blood or tissue samples that are collected and transported at ambient temperature may autolyze and have little clinical value. Samples should also be stored in a locked enclosure if sample tampering is a concern. In some cases, laboratory accreditation policies may require this (see Hart and Budgen, this series).

Sample processing and analysis should be performed by the same persons, whenever possible, to minimize the likelihood of introducing bias into the results. All results should be signed off by the technician performing the analysis. When multiple individuals are involved with the analysis of samples, strict adherence to the protocol is required. Interpreting the results of multiple technicians can be difficult for certain diagnostics, especially histopathology, so it is important to discuss sample processing with the laboratory before submitting forensic samples (see Hart and Budgen, this series).

The chain of custody for clinical samples should include documentation regarding the individuals collecting the samples, processing the samples, analyzing the samples, disposing of the samples, and controlling and transporting the samples. The security methods practiced during the process should also be documented. It is essential, for legal purposes, that all documentation is complete and collated chronologically.

Clinical pathology

Clinical pathologic methods are generally used for such antemortem samples as body fluids and tissues (cytology). In this article, the emphasis is on blood and cytological samples. Various techniques can be used in forensic cases to describe the overall condition of an animal, to provide insight into the possible duration of the disease or injury, and to document the status of a case over time.

Blood sample collection

When obtaining blood from a reptile, it is important to collect and manage the samples in a timely fashion. Delays in collection can lead to the formation of microclots that alter the meaning of certain hematologic parameters. To limit the likelihood of clots, the syringe and needle can be pre-coated with heparin, and the blood should be stored in sample containers that are appropriate for the volume of blood (see Hart and Budgen, this series). Microtainer blood collection tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) are recommended for small volumes of blood. The venipuncture site should be decontaminated with an appropriate disinfectant (e.g., 70% alcohol) prior to collection. For most species, a 22-25 gauge needle fastened to a 3-ml syringe can be used to collect blood samples.

Common venipuncture sites in the snake are the ventral tail vein and the heart. The ventral tail vein can be approached by placing the animal in dorsal recumbency, grasping the tail distal to the vent, and inserting the needle at a 45 to 90° angle to the tail. The heart is generally located 1/4 to 1/3 of the distance from the head. It can often be seen when the animal is in dorsal recumbency. For cardiocentesis, the heart should be isolated between the index finger and thumb and the needle inserted at a 45° angle at the most distal point of the beating heart (ventricle).

The ventral tail vein, jugular vein, and ventral abdominal vein are common venipuncture sites in lizards. The approach to the ventral tail vein in the lizard is similar to that described for snakes, although care should be taken when working with animals that are subject to autotomy to prevent the loss of the tail. The jugular vein can be approached laterally. The landmarks for sample collection are the tympanum and the point of the shoulder. The ventral abdominal vein courses along the ventral midline between the xiphoid process and the pelvis. A 22-25 gauge needle fastened to a 3-ml syringe can be inserted at a 45° angle in between these landmarks to collect the sample from either of these sites.

The jugular vein, brachial plexus, subcarapacial vein, dorsal coccygeal vein and femoral vein can be used to collect blood samples from chelonians. The jugular vein is the site that is least likely to be contaminated with lymph, and is the preferred venipuncture site of the authors. Lymph-diluted samples can alter the results for certain hematologic measures (e.g., falsely decreased white blood cell count), so it is important to avoid this. Lymph-diluted samples have a “watered down” appearance compared with non-diluted samples. The chelonian jugular vein is located on the lateral aspect of the neck at the level of the tympanum. Placing a thumb at the base of the neck may help engorge the vessel, simplifying identification. The subcarapacial vein can be approached by inserting a needle at a 45-60° angle dorsal to the cervical vertebrae under the carapace (fig. 1c). The brachial plexus and femoral veins are located on the posterior and ventral surfaces of the forelimb and rear limb, respectively. Access to the dorsal coccygeal vein is by inserting a needle at a 45-60° angle over the caudal (tail) vertebrae.

The preferred site for venipuncture in crocodilians is the supravertebral sinus. This sinus is caudal to the skull on the dorsal midline and can be approached by inserting a needle at a 90° angle to the cervical vertebrae. Care should be taken not to thrust the needle through the vertebrae and into the spinal cord.

Complete blood count

The standard complete blood count (CBC) evaluates both the white (leukocytes) and red (erythrocytes) blood cells. Standard hematologic analyzers cannot be used to process CBC for reptiles (or amphibians) because of their nucleated red blood cells. Instead, the CBC must be performed using an estimation method. The estimation technique for white blood cells requires a two-step process. The first is estimating the number of white blood cells. There are two different methods for this. Historically, the Eosinophil Unopette (Becton Dickinson) method was preferred; however, in March 2007 the company discontinued this. Fortunately, it is possible to create a similar product using phloxine B stain or to purchase a replacement (Avian Leukopet; Vetlab, Miami, FL, USA). In either case, blood samples are mixed with the stain and loaded on a hemocytometer. Phloxine B stains heterophils and eosinophils red. The cells are counted and then applied to a formula, along with information obtained from the differential, to estimate the total white blood cell count. In cases where the stain is not available, an estimate can be obtained by counting the number of white blood cells on 10 fields at 400×, taking the average of the 10 fields, and multiplying the mean by 2,000. The white blood cell counts of reptiles can be affected by a number of physiologic and environmental parameters, but are generally between 5.0-15.0 × 10^3 cell/ml of blood. The second-step of the CBC is the cell differential. Most laboratories perform a 100 cell count under oil immersion (1000×).

Reptile white blood cells can be divided into the granulocytes and agranulocytes. The heterophil, eosinophil, and basophil are granulocytes, while the lymphocyte, monocyte, and azurophil are agranulocytes. The staining characteristics of the leukocytes can vary significantly between reptile species, so it is important to have samples screened by experienced personnel.

The heterophil is analogous to the neutrophil in mammals. In most reptiles, heterophils have fusiform, eosinophilc granules, which can lead to confusion when differentiating them from eosinophils. This, however, should not be a problem since the heterophils are much more common on a blood smear than are eosinophils and will be the predominant granulocyte. An elevation of heterophils (heterophila) is common in acute inflammatory disease and physiologic stress. Seasonal changes in the heterophil count are possible, with higher levels noted in the summer months. Heterophils are the predominant leukocyte in many species of lizards and snakes, accounting for 50-80% of the leukocytes in the differential. Eosinophils typically have round granules, and these cells are associated with parasitism and hypersensitivity. Eosinophil counts in reptiles are generally less than 0.5×10^3 cell/ml of blood. Basophils have large metachromatic, basophilic-staining granules that cover the nucleus. These cells are associated with histamine release. Basophils are a common finding in chelonians. Basophil counts in reptiles are generally less than 0.5×10^3 cell/ml of blood.

Reptile lymphocytes are similar in appearance to mammalian lymphocytes, and are characterized by a high nuclear to cytoplasmic ratio. These cells serve many different functions, from antibody production to being natural killer cells. A reduction in the circulating lymphocytes may be observed with stress, viral infections, neoplasia, and seasonal changes (e.g., winter). Elevations in lymphocytes can be associated with an immune-mediated response to an infectious disease, traumatic injury or neoplasia.

Monocytes are the largest of the white blood cells. These cells have a higher cytoplasmic to nuclear ratio than lymphocytes. Monocytosis is most often observed with chronic inflammatory responses. It is not uncommon to see monocytosis in cases of chronic infection and traumatic injuries. Monocyte counts in reptiles are generally less than 0.5×10^3 cell/ml of blood. Elevations greater than 1.0×10^3 cell/ml of blood are suggestive of chronic inflammation.

Erythrocytes are generally measured via the packed cell volume (PCV) or from screening and estimating the numbers on a stained slide. The PCV can provide insight as to the general condition of the animal. The PCV of reptiles varies with physiologic and environmental factors, but generally ranges between 20-35% (0.20-0.35 in SI Units) (Mitchell, 2008). The PCV of reptiles is routinely used to assess hydration status and general physiologic condition. Reptiles that are dehydrated can become hemoconcentrated, with values exceeding 40%. Anemia is a common finding in reptiles with chronic inflammatory disease. In the history of these cases, it is not uncommon to find that the animals are housed in a suboptimal environment (for instance, with a low environmental temperatures) and are given a low quality diet.

Chemistries

Chemistry profiles provide insight into the working physiology of a reptile. Electrolyte, enzyme, mineral, protein, and glucose levels can be used to assess a reptile's health status, while also providing insight into the animal's most recent experiences. For example, reptiles that have been injured from a gunshot wound may present with elevated creatine kinase (CK) and alkaline phopshatase (ALP) levels associated with muscle and bone damage, respectively.

The primary enzymes analyzed on a chemistry profile include the aspartate aminotransferase (AST), CK, and ALP. AST can be found in multiple tissues, but is predominantly in muscle and liver tissues. CK is primarily located in skeletal, smooth and cardiac muscle. Elevations in both of these enzymes are a common finding with muscle damage due to injury and muscle loss observed with starvation. ALP is an enzyme found in many different tissues. Higher levels of ALP seen in juvenile animals are often attributed to bone growth. Elevations in this enzyme might also be expected with bone-healing.

The electrolytes can be used along with the physical examination and PCV to assess a reptile patient's hydration status. Elevations in sodium and chloride are common in dehydrated reptiles. Sodium, potassium, and chloride levels may all be decreased in animals that are given a poor quality diet or are deprived of food.

Calcium and phosphorus are important minerals in the body. Both are obtained through the diet; therefore, animals offered low quality diets often have inadequate value. In captivity, most of the diets offered to reptiles are more likely to be deficient in calcium than in phosphorus. Because of the way that the body stores and mobilizes calcium, it is not possible, in most cases, to assess calcium storage levels in the reptile by chemistry analysis. Instead, radiographs or other diagnostic image techniques (e.g., computed tomography) should be used to assess bone structure and integrity. Animals on poor quality diets are more susceptible to metabolic diseases that can affect the bone structure, increasing the risk of pathologic fracture. Calcium and phosphorus values can also be used to assist with gender determination in non-sexually dimorphic species during a breeding season, as both will increase in females.

Protein values can be used with other findings to assess hydration (total protein, albumin) and to help determine an animal's well-being. Hypoalbuminemia is common in starved reptiles. Protein electrophoresis can be used to measure the different protein fractions to further assess the inflammatory response of a reptile.

Glucose levels in reptiles are naturally lower than those reported in mammals and birds. The slow metabolic rate and decreased activity of reptiles, in contrast to those of these higher vertebrates, is probably responsible for this. Animals that are held under poor conditions, such as may occur when they are being smuggled, are often hypoglycemic. Animals with poor energy reserves may succumb under such circumstances.

Cytology

Fine-needle aspirates and tissue biopsies can be collected to characterize the antemortem (and postmortem — see Cooper, J.E., this series) status of cells or tissues. The sensitivity of fine-needle aspirates is low to moderate, while that of full-tissue biopsies is generally higher. Data collected from these diagnostic samples may be useful in directing additional tests and providing insight into the duration of a lesion.

Parasite sampling

Reptiles naturally harbor a variety of ecto- and endoparasites. The most common ectoparasites found on terrestrial reptiles are mites and ticks. In aquatic species leeches are a common finding. Ectoparasites are often readily diagnosed during thorough physical examination.

The number of potential reptile endoparasites is greater and includes hemoparasites, protozoa, trematodes, cestodes, and nematodes. Hemoparasites are commonly identified while screening a blood smear. Both protozoa and nematodes (filarids) are seen in reptiles. Fecal-screening by direct saline smears and zinc sulfate flotation can be used to diagnose most gastrointestinal parasites. Serial samples may be required to confirm the presence of parasites as shedding can be transient.

In many forensic cases, determining the source of the animal, wild versus captive-bred, is important (see Frye, this series). Collecting and identifying parasites can be helpful in this respect. For example, if *Amblyomma marmoreum*, the African tortoise tick, is found on a group of leopard tortoises, *Geochelone pardalis*, being shipped within the United States, it is highly likely that the animals are illegal as the United States Department of Agriculture placed a prohibitive ban on the importation of all African tortoise species in 2000.

Infectious disease sampling

Reptiles have evolved to mask illness so as to avoid predation. Although this method of self-preservation is valuable to these animals in the wild, it can make disease diagnosis in captive animals a challenge. Historically, bacteria were considered the primary pathogens associated with reptiles because microbiological culture was the only diagnostic test available. In the past two decades, there has been a movement to develop diagnostic assays specifically tailored to diagnosing infectious diseases in reptiles and amphibians. The following assays are currently available for reptiles: paramyxovirus (snakes), *Mycoplasma* spp. (chelonians), iridovirus (chelonians), herpesvirus (chelonians), *Salmonella* spp. (all species), and West Nile virus (all species). Certain pathogens can be disseminated via aerosols or close contact (e.g., paramyxovirus, herpesvirus, mycoplasmosis, *Salmonella*, iridovirus) and those managing confiscated animal collections should consider the use of testing and quarantine to minimize exposure and disease dissemination.

Clinical toxicology

Toxicologic sampling may be necessary in cases where a known exposure to a toxin has occurred or in a situation where other diseases have been ruled out and a toxic exposure is then suspected (Rotstein, 2008). In cases of a known exposure, sampling should be directed at measuring the toxin in blood or (antemortem) tissue samples (e.g., biopsy of liver). If a toxin is not known but suspected, it is important to use other available clinical data to direct the case. Blood and physical examination results may be used to identify a specific organ system that is affected, which will help further to refine the search for a toxin.

There are variables that can affect one's success in diagnosing toxicity, including the type of sample collected, the method used to collect the sample, the techniques employed to transport and store the sample, and the actual assays used to test the samples. To maximize the likelihood of success in diagnosing a toxin, it is important to follow an organized and systematic approach.

The types of samples required for testing will depend on the suspected toxin. In reptiles, whole blood, serum, urine, gastric contents, parenchymal organs and skin samples may be useful. Discussing the case with a clinical toxicologist may help clarify which samples are needed.

Because of likely legal ramifications, a standard chain of custody should be followed when handling toxicological samples. Examples of chain of custody forms for cases related to non-traditional species are available (Rotstein, 2005; Cooper and Cooper, 2007). These forms should include information about the sample type, amount or number, date of transfer, name of the person and organization sending or receiving the sample, and the method of transportation (Rotstein, 2005).

Diagnostic imaging

In this context, diagnostic imaging refers to radiography, ultrasonography, computed tomography (CT), magnetic resonance imaging, and endoscopy. These imaging techniques have become instrumental in the description and identification of pathologic lesions in the live animal, and therefore in assisting in making a definitive diagnosis. In the field of forensic medicine, they can also serve to document pathologic changes, their duration, resolution, or deterioration over time for legal purposes. The collection of radiographs from deceased animals is often useful immediately prior to necropsy examination, and can be valuable, for example, in the evaluation of bone density or the location of metal foreign bodies such as gunshot (see Cooper, J.E., this series).

Documentation and record-keeping

Accurate and infallible documentation is of the utmost importance in forensic cases (see Hart and Budgen, this series). Material for diagnostic imaging must be readily identifiable (by, for example, microchip or ID tag), and be accompanied by a signed and dated consent or submission form as part of maintaining the chain of custody. The submission form can also include a report section for the results and interpretation.

Diagnostic imaging interpretations should be made based solely upon the image(s) obtained and recorded, without assumption or bias. Consequently, re-examination and re-interpretation can be performed later by others using the same images, which can lead to differences of opinion. While some images may be definitive and without question, others are often open to interpretation and debate. It is therefore often advisable to perform serial examinations and complement imaging with histopathology, microbiology, parasitology, and toxicology to confirm the diagnosis. Furthermore, although images may be taken by any qualified veterinarian, it is wise to seek an opinion from recognized specialists. With respect to diagnostic images from reptiles or amphibians, Diplomates or Diploma holders of recognized radiology and zoological medicine boards, or those with relevant PhD or other higher degree qualifications should be consulted for an expert opinion. Lists of such specialists are usually maintained by the jurisdictional veterinary licensing board, or expert legal witness register (Cooper and Cooper, 2007).

Most diagnostic imaging systems now record results electronically, and DICOM (Digital Imaging and Communications in Medicine) standard formatting should be used preferentially. Such images should also be securely archived using the industry-standard PACS (Picture Archiving and Communication System). Hardcopy photographic films should be placed inside protective folders, labeled, and filed. The originals should be maintained by the veterinarian, with copies provided until the originals are legally required. When a diagnostic image becomes legally important, it is wise to move photographic films (or electronic copies of digital images) into a fire-proof safe.

As diagnostic images are increasingly likely to be digital in nature, it is important that state/provincial and national legislation relating to the use of electronic records and signatures is understood. For example, in the US State of Georgia (and probably other US States) the following points apply (Secretary of State, 2002):

(a) Electronic diagnostic images are not denied legal validity solely on the grounds that they are electronic.
(b) Electronic signatures on images suffice for the purposes of a legal signature and verification.
(c) The precise authentication or identification of the image is required and, if challenged, the burden of proving the reliability of a digital image rests on the proponent of the electronic record.

While some similar regulations exist in other regions of the World, it is nonetheless vital that local requirements be thoroughly researched and followed (see Hart and Budgen, this series).

Radiography

The majority of radiology machines in small animal veterinary practices can produce radiographs of diagnostic quality for most species of reptile or amphibian. Slower, high detail, non-screen films achieve the best resolution — certainly greater than that produced by most veterinary computed-radiography (CR) or digital-radiography (DR) units, and are preferred for small reptiles that weigh <100 g.

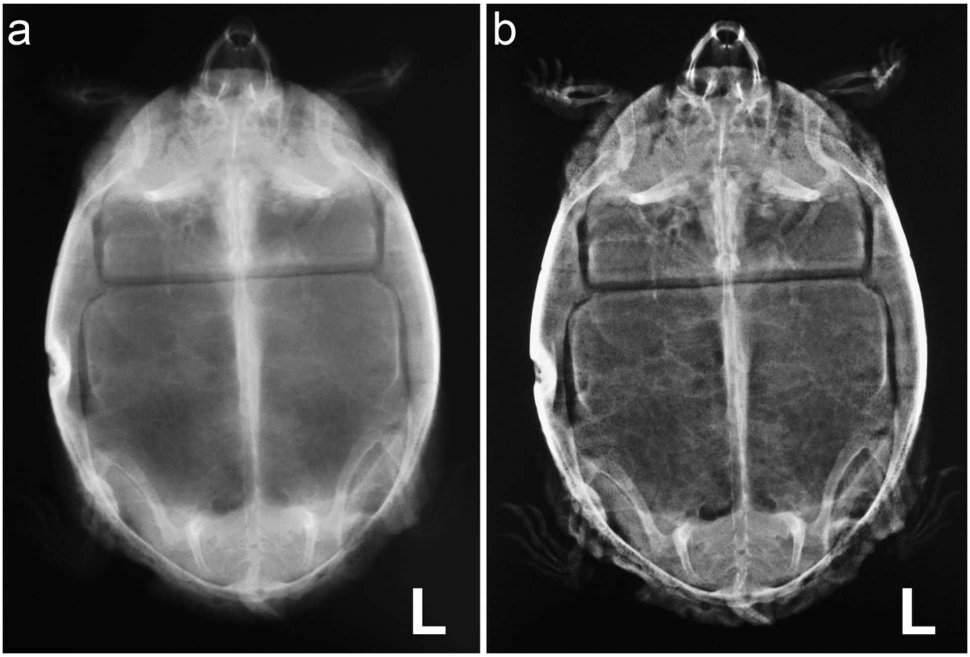

Figure 3. Whole body dorsoventral radiographs of a box turtle (*Terrapene carolina*), taken using traditional film (a) and digital technology (b). Note the improved contrast generated by edge enhancement of the digital radiograph (L = left marker).

Reptiles of between 100 g and 100 kg in weight can be adequately radiographed using most small animal (film, CR and DR) machines, although CR/DR often produces greater contrast, which often assists in radiographic interpretation (fig. 3). Reptiles >100 kg generally necessitate the use of a more powerful large animal machine in order to penetrate the large coelom of crocodilians or komodo dragons (*Varanus komodoensis*), or shell of giant tortoises or sea turtles. There are several key points to consider:

(a) Human safety. Radiography uses X-ray radiation to produce a visual image on photographic film or digital media. Therefore, due care and regard are required in the execution of radiography. In general, animals are not manually held under the primary X-ray beam, and all persons should leave the immediate area while the radiology unit is activated. In some countries it is illegal for persons to be in the immediate vicinity when the radiography is being activated (Cooper and Cooper, 2007), while others require a log of persons involved with the procedures — such a log can also serve as evidence to demonstrate the date and time when the examination was performed.
(b) Reptile positioning. Radiography should be performed with the animal in precise anatomical position. Lateral and dorsoventral radiographs must be straight, and without rotation or twisting. Positioning is aided by anesthesia, but may be difficult postmortem when rigor has become established.

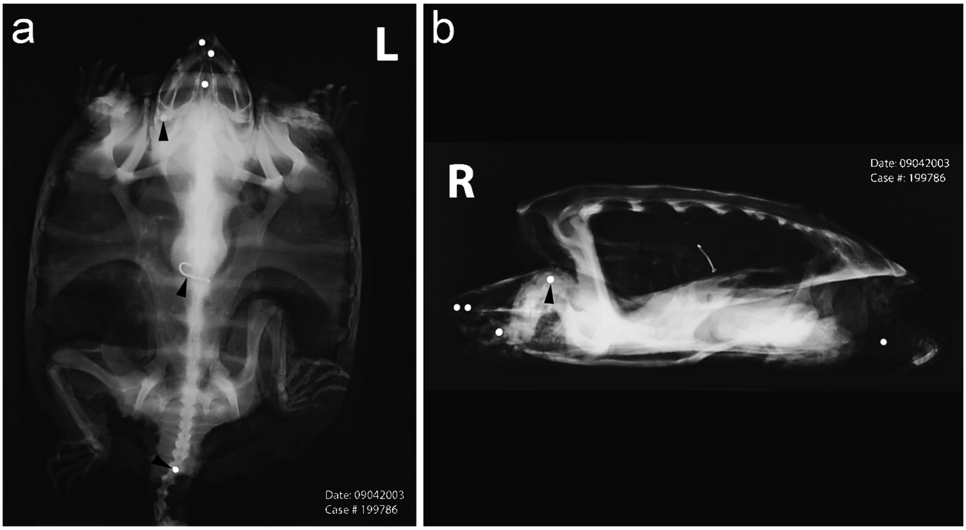

Figure 4. Whole body dorsoventral (a) and lateral (b) radiographs of a common snapping turtle (*Chelydra serpentina*) (L = left and R = right markers, case numbers and date of examinations included). The dorsoventral radiograph (a) demonstrates three obvious shotgun pellets in the head; however, two other shotgun pellets and a swallowed fishing hook (black arrows) are less apparent. The lateral radiograph (b) not only demonstrates the three shotgun pellets in the head, but also more clearly shows the hook and the two other gunshot associated with the caudal skull and the tail. This case demonstrates the absolute importance of taking at least two radiographic views in order to prove that abnormalities lie within the body, and to reduce the likelihood of lesions being missed.

(c) Labeling. Radiographic films must be uniquely labeled to identify the animal, date of examination, and include anatomical markers (i.e., left, right, dorsal, ventral) where appropriate (fig. 4).
(d) Correct exposure. Over- and under-exposed radiographs, even if adequate for clinical diagnosis, may not stand up to the rigors of cross-examination and should be repeated to obtain the best possible image.
(e) Minimum of two views. It is essential to take at least two views at 90° to each other. Failure to do so can lead to legitimate criticism of radiographic interpretation as one could argue that abnormalities were merely artifacts or present outside the body and superimposed on the radiograph (fig. 4).
(f) Distortions and artifacts. Radiographs are a 2-dimensional image of a 3-dimensional structure and consequently they often create distortions. For example, lesions are magnified on the radiographic film, and therefore absolute measurements taken on the radiographs are not necessarily accurate. The further the lesion is away from the film, the greater the potential distortion. To make a precise measurement, it is necessary to include a marker of known length on the cassette next to the animal. This can then be used as a standard by which to calculate true anatomical dimensions from radiographic measurements. This is especially true when working with digital images that can be

viewed as various magnifications. CR can create radiolucency artifacts around metal implants which may be mistaken for infection and loosening of screws and pins.

Extensive reviews on reptile radiography have been published and should be consulted for further details (Hernandez-Divers and Hernandez-Divers, 2001; Hernandez-Divers and Lafortune, 2004; Raiti, 2004; Silverman, 2006). However, radiographs are especially useful for determining the presence of gunshot, fractures and other skeletal abnormalities (figs 2, 4 and 5) (Cooper and Cooper, 2007). Radiography can also be useful for evaluating soft tissues; however, interpretations are generally descriptive rather than definitive.

In many forensic cases, especially those relating to charges of animal cruelty, there is a need to determine the duration of a particular disease process. This can present problems for the reptile clinician because, unlike birds and mammals, reptile metabolism is greatly affected by ambient temperature. Therefore, it is not possible to precisely determine the duration of a metabolic condition or healing of a fracture without detailed husbandry information and serial radiographs (fig. 5). Consequently forensic interpretations should be conservative.

Ultrasonography

Ultrasonography requires greater skill to perfect, especially in reptiles, where the heavily keratinized skin, bony osteoderms, and chelonian shells can hamper examination (see Cooper, J.E., this series). Detailed reviews exist that should be consulted for methodology and interpretation (Raiti, 2004; Stetter, 2006). Ultrasonography permits an evaluation of soft tissues, and has been particularly valued for cardiac and urogenital evaluations. Modern machines are capable of determining accurate and absolute tissue/organ dimensions. Such measurements can be useful for measuring the size of fat bodies as an objective assessment of body condition, or the size of gonads to determine reproductive status (fig. 6).

In addition, like radiography, interpretations are generally descriptive because a definitive diagnosis relies upon the demonstration of a host pathological response and identification of the causative agent using histopathology and other laboratory methods (i.e., toxicology, microbiology, parasitology) (see Frye, this series). While ultrasound-guided fine-needle aspirates for cytology and microbiology are safe and easy to obtain, the lack of tissue architecture associated with cytologic samples makes them inferior to tissue biopsy. Larger biopsies can also be collected using ultrasound-guidance; however, such procedures carry significantly greater risks of iatrogenic trauma (Ramiro et al., 1993).

Much of the interpretative power of ultrasonography relies in being able to move back and forth over an area, changing angle and plane to obtain a 3-dimensional idea of the region of interest. However, such examinations can be difficult to document adequately when only still images are recorded. The ability to record video

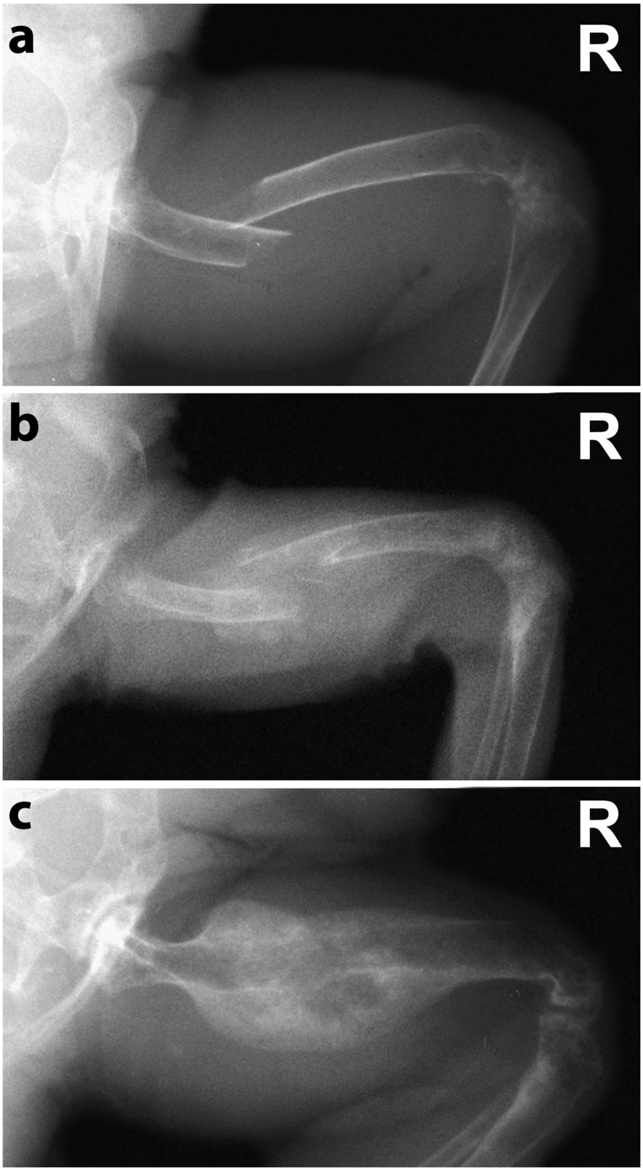

Figure 5. Dorsoventral radiographs depicting various stages of fracture-healing without surgical intervention in the right femur of a green iguana (*Iguana iguana*) (R = right marker); (a) Traumatic fracture three days old, but a similar appearance could be seen for up to 10 days depending on husbandry; (b) Traumatic fracture 22 days old showing evidence of fibrous callus formation, but a similar appearance could be seen as late as 60 days depending on husbandry; (c) Traumatic fracture 90 days old showing evidence of major remodeling, but a similar appearance could be seen as early as 60 days and may be present many months or even years after the initial incident, depending on husbandry.

segments is therefore important to fully document ultrasonographic examinations and findings.

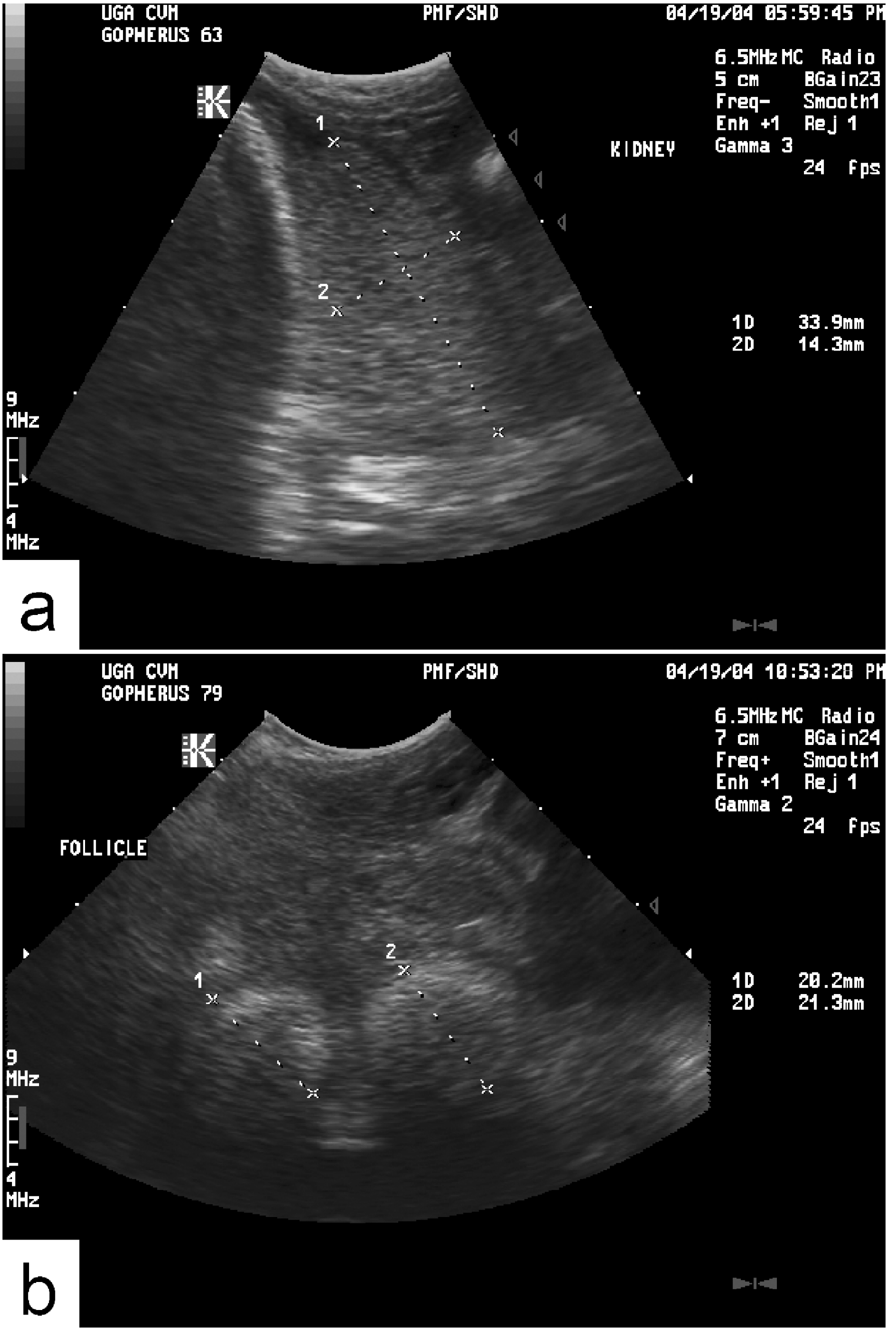

Figure 6. Caudodorsal ultrasonographs taken via the left prefemoral area of two Gopher tortoises (*Gopherus polyphemus*) as part of a reproductive assessment for a conservation project; (a) sub-adult male with a testis measuring 33.9 mm by 14.3 mm; (b) mature female with two ovarian follicles measuring 20.2 mm and 21.3 mm.

CT and MRI

Computed tomography (CT) and magnetic resonance imaging (MRI) are advanced imaging techniques that are seldom available outside large referral hospitals (Raiti, 2004; Silverman, 2006). CT is a sophisticated radiographic procedure that is particularly useful for examining mineralized or calcified structures (skeleton, shell, hard-shelled eggs) or at bone/air interfaces (i.e., skull) (fig. 7). MRI relies on the use of powerful magnets to affect proton alignment and is more useful for soft tissue detail, especially the central nervous system. Both CT and MRI require the animal to be motionless for 15-45 minutes, and so general anesthesia is often essential. CT-guided fine needle aspirates can be collected for microbiology and cytology; however metal needles cannot be used in proximity to an MRI unit.

CT and MRI data stored in the standard format known as DICOM (Digital Imaging and Communications in Medicine) can be used to create a variety of 3D reconstructions that, unlike the original scans, can often be more easily understood by non-imaging specialists (figs 7 and 8). OsiriX is one software package specifically designed for navigation and visualization of multimodality and multidimensional images: 2D viewer, 3D viewer, 4D viewer (3D series with temporal dimension) and 5D viewer (3D series with temporal and functional dimensions). The 3D viewer offers all modern rendering modes: multiplanar reconstruction, surface rendering, volume rendering and maximum intensity projection, 2D MPR (multiplanar reconstruction) both curved and orthogonal, 3D maximum intensity projection, 3D volume rendering, 3D surface rendering, virtual endoscopy and anonymization. This software is freely available from the Macintosh web site (http://www.apple.com/downloads/macosx/imaging.3d/osirix.html), but is only available for Macintosh Apple computers. Like radiography and ultrasonography, lack of histologic and other laboratory confirmation should caution against making a definitive diagnosis based solely on CT or MRI images.

Any metallic object can create artifacts on CT, or be displaced causing serious tissue damage under the influence of MRI magnets. Additionally, strong magnetic fields can damage microchips. Therefore, animals must be radiographed first to check for the presence of microchips, metal foreign bodies, or implants before performing CT or MRI. Microchips should also be read and recorded prior to MRI; with additional identification (i.e., second microchip or tag) applied if the original chip becomes inactivated.

Endoscopy

Endoscopy has proved to be an extremely useful technique for the minimally-invasive examination of internal structures in reptiles (Hernandez-Divers, 2004a, 2004b; Hernandez-Divers et al., 2004; Hernandez-Divers et al., 2005a, 2005b; Taylor, 2006; Hernandez-Divers et al., 2007). Rigid and flexible endoscopes permit the transmission of images through a terminal lens positioned inside the reptile through a camera to a recording device and video monitor. Endoscopes can be inserted

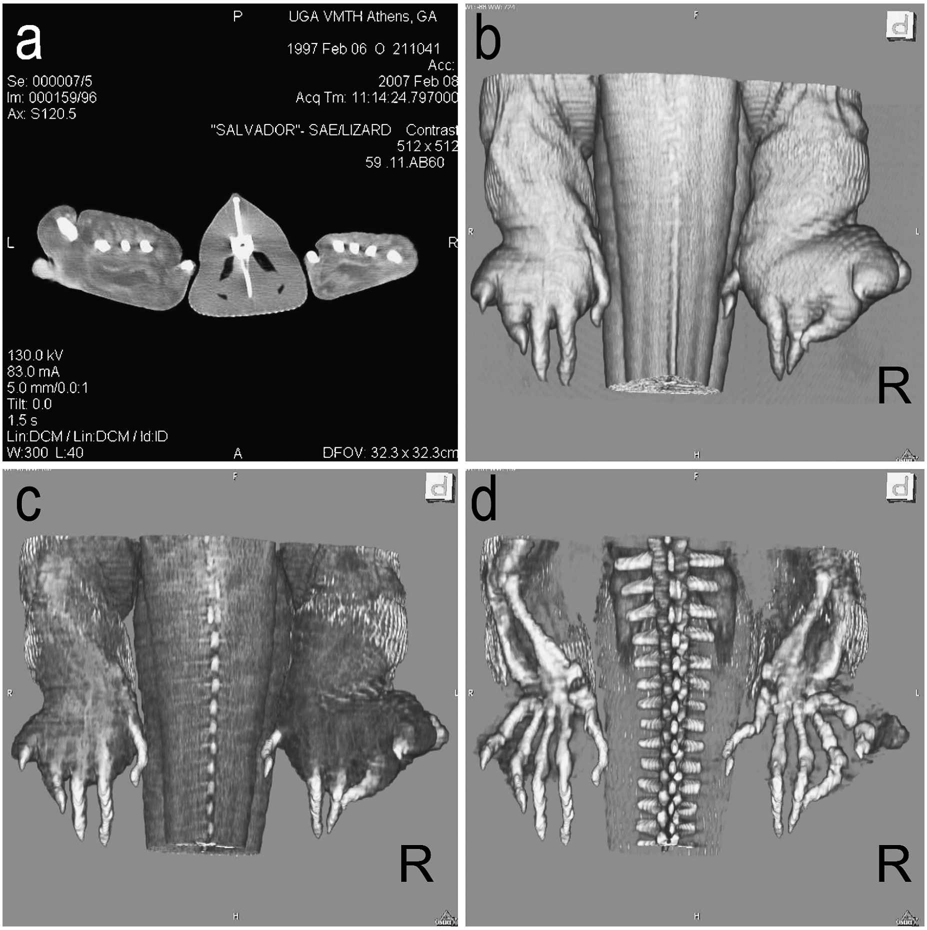

Figure 7. CT images of a Nile monitor (*Varanus niloticus*) that presented with gross distortion of the right hindlimb; (a) Original CT data presented as an anatomical 'slice'; (b) 3D reconstruction of the surface contours; (c) 3D reconstruction demonstrating the underlying soft tissues; (d) 3D reconstruction of the deepest bone tissues. Despite clearly demonstrating gross abnormalities of the right limb, a definitive diagnosis requires biopsy of the affected tissues for histopathology and microbiology.

through the buccal cavity to examine the esophagus, stomach and small intestine, via the glottis to examine the trachea, bronchi and lungs, and via the vent to examine the cloaca, bladder, oviducts, and large intestine. Sterile rigid endoscopes can also be inserted via a small surgical incision into the coelomic cavity. Following coelomic insufflation using carbon dioxide, air or sterile saline, the major internal organs can be visualized, including the heart, liver and gallbladder, stomach and intestines, gonads, kidneys, pancreas, spleen, and if present, the urinary bladder (Hernandez-Divers, 2004a; Hernandez-Divers et al., 2004; Taylor, 2006). In most cases, tissue biopsies can be collected, that would otherwise require a major surgical approach to obtain in the live animal (Hernandez-Divers and Shearer, 2002;

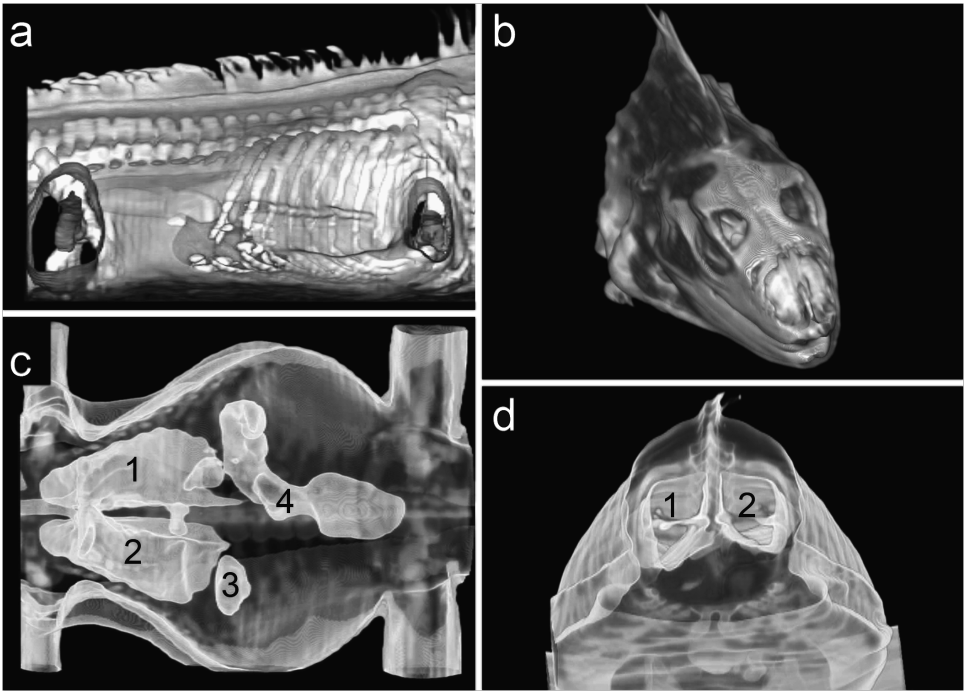

Figure 8. Three-dimensional MRI reconstructions of a green iguana (*Iguana iguana*); (a) Lateral projection demonstrating the rib cage and axial skeleton; (b) Craniodorsal oblique projection demonstrating the skull; (c) Dorsoventral projection demonstrating the air cavities of the right (1) and left (2) lungs, stomach (3) and large intestine (4); (d) Cranial cut-away view of a craniocaudal projection demonstrating the right (1) and left (2) lungs.

Hernandez-Divers, 2004b; Hernandez-Divers et al., 2005b; Hernandez-Divers et al., 2007). Precise positioning, endoscopic technique, and the extent of organ visualization are largely determined by species, the organ(s) of specific interest, and the preference of the endoscopist.

The ability to visualize (and record) internal structures in a 3-dimensional color format and safely collect tissue biopsies to confirm the diagnosis sets this modality apart from radiography, ultrasonography, CT and MRI (fig. 9). Endoscopic interpretation is less open to subjective opinion or controversy because endoscopic biopsies are taken to confirm the visual diagnosis. Unlike the other imaging techniques that can only guide the safe collection of fine needle aspirates for cytology, endoscopy facilitates the collection of tissue biopsies for microbiology, histopathology and toxicology (Hernandez-Divers, 2004b; Hernandez-Divers et al., 2005b; Hernandez-Divers et al., 2007). Endoscopic biopsies maintain tissue architecture, and have been shown to be comparable to tissue samples collected at necropsy (Hernandez-Divers et al., 2005b; Hernandez-Divers et al., 2007). However, endoscopy is obviously painful and therefore, unlike as in radiography and ultrasonography, general anesthesia of the animal is mandatory.

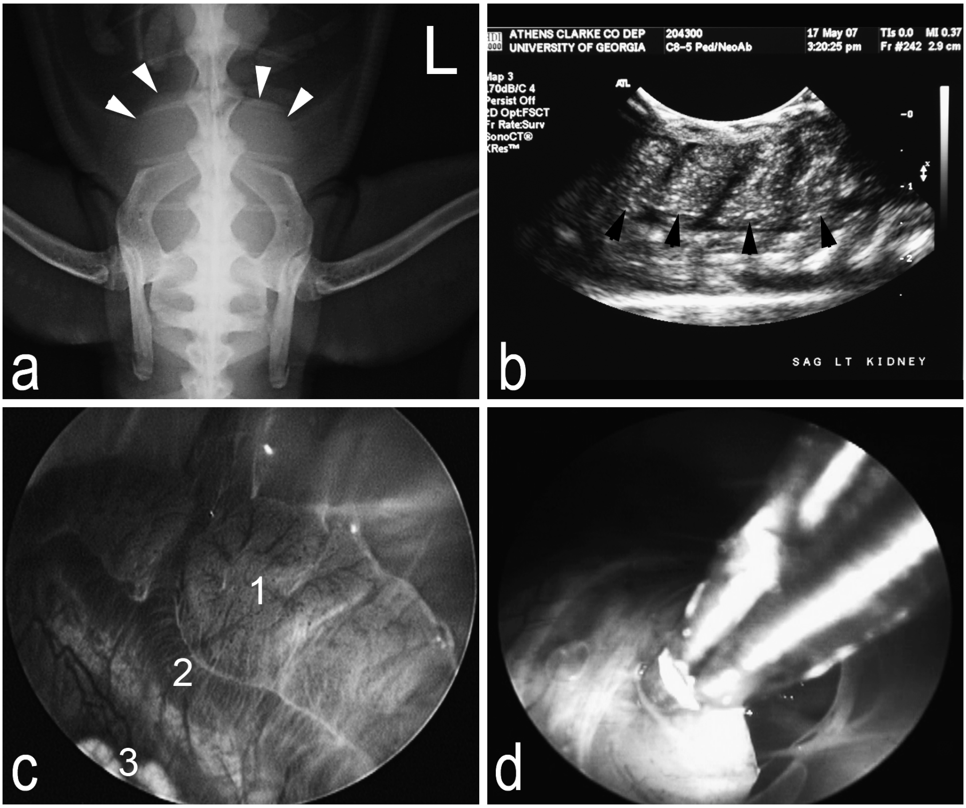

Figure 9. Comparison between radiography, ultrasonography and endoscopy for renal evaluation in reptiles; (a) Dorsoventral radiograph of a green iguana (*I. iguana*) demonstrating a bilateral soft tissue opacity in the caudodorsal coelom — although this is suggestive of renal enlargement and disease, a precise diagnosis cannot be made without biopsy; (b) Sagittal ultrasonograph of the caudal coelom of a yellow rat snake (*Elaphe obsoleta quadrivittata*) demonstrating multiple lobes of the left kidney (arrows) each containing small hyperechoic multifocal areas — although this is suggestive of renal disease with either mineralization or gout, a precise diagnosis cannot be made without biopsy; (c) Endoscopic view of the left kidney of a male red-eared slider (*Trachemys scripta elegans*) demonstrating excellent visual detail of the renal surface (1), renal blood vessels (2), and edge of the epididymis (3); (d) View of the same kidney, demonstrating the ease of endoscopic biopsy that can subsequently be used to confirm the nature and extent of any disease process.

Summary

To have success in managing herpetological forensic cases, it is important to be prepared. There are a number of different diagnostic tests available so it is important to discuss options with experts in the field and the laboratory to ensure that the samples are collected, transported, and tested appropriately. It is also essential to design all of the documentation that will accompany the case prior to getting started. Following a standard evidence management protocol using chain of custody documentation will minimize the likelihood of problems later.

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