Clinical Evaluation, Common Diseases, and Veterinary Care of the Horseshoe Crab, *Limulus polyphemus*

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Abstract The American horseshoe crab, *Limulus polyphemus*, can be maintained in a wide variety of systems ranging from glass aquaria to fiberglass tanks with various types of mechanical and biological filtration. Adult horseshoe crabs are tolerant of a wide range of environmental conditions, with temperatures ranging from –5 to 35°C and salinities from 5 to 35 ppt, with optimal conditions between 15 and 21°C and 27 ppt salinity. Horseshoe crabs should be fed good-quality dead fish, squid, small crabs, clams, frozen brine shrimp, and artificial shrimp/fish diets. Clinical evaluation of a horseshoe crab can be problematic as the hard carapace makes examination and sample collection difficult; however, non-lethal clinical assessment can include external examination, radiology, and hemolymph chemistries and cultures. Biochemical parameters of the horseshoe crab's hemolymph parallel those seen in many other marine species, but several parameters are notably different from ambient seawater, i.e., calcium, magnesium. Survey and contrast radiographic studies of the cardiovascular and gastrointestinal systems of the horseshoe crab can be undertaken using conventional and fluoroscopic techniques. Infectious etiologies include algae, fungus, colonial and filamentous cyanobacteria, Gram-negative bacteria, and a variety of protozoan and metazoan parasites. Non-infectious problems range from water quality problems to developmental syndromes and traumatic injuries. Little is known of the therapeutic options for treatment of horseshoe crab diseases; however, a few treatments have been suggested for the removal of ectocommensals and external parasites, and the pharmacokinetics of oxytetracycline following intracardiac and oral dosing have recently been investigated.

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1 Introduction

Although the horseshoe crab is not a commonly kept laboratory animal, this species is an important one. Accordingly, the captive maintenance of these animals has proven invaluable to researchers using horseshoe crabs as experimental models in such varied fields as conservation biology, hematology, and ocular research. As with other laboratory species, proper husbandry is key to horseshoe crab health. But mastery of husbandry techniques is not enough; successful rearing of captive horseshoe crabs also relies on the scientist's ability to detect and manage disease. This chapter reviews the clinical approach to assessing the health of individual adult horseshoe crabs, describes some of the more commonly encountered diseases of captive animals, and concludes with a discussion of both preventative medicine and therapeutic veterinary care of the horseshoe crab.

2 Clinical Evaluation

2.1 Clinical History

The clinical history is the most basic, and often the most informative part of the clinical evaluation. The clinical history has three basic components: signalment, chief complaint, and patient history.

Signalment defines the individual patient's identification. In the case of horseshoe crabs, the species should be defined as being *Limulus polyphemus*, *Tachypleus gigas*, *T. tridentatus*, or *Carcinoscorpius rotundicauda*. Although there are no specified breeds of horseshoe crabs, there are distinct genetic strains defined by the physical environment from which the animals are derived. As such, the physical (e.g., wild vs. captive, brackish vs. saltwater) and geographical (e.g., Atlantic vs. Pacific, subtropical vs. temperate) environment where the crab lives should be described. The age of the animal should be estimated (or if raised in captivity, the age can be specified), and the sex of adult animals should be noted. Finally, the identity of the individual animal should be defined (i.e., tank number, tag number).

The chief complaint is defined by the client or researcher and should become the focal point of the comprehensive clinical evaluation; it is the main reason the animal is being presented to the clinician. The chief complaint should include description of the manner of disease onset, clinical signs noted, and duration of those signs. The chief complaint is more clearly identified by the clinician as the evaluation progresses, with the eventual goal being identification of an etiology (or a combination of etiologies) that explains the chief complaint.

The patient history should include description of diseases (and any treatments) which have previously affected the individual, its family, or its cohorts (in the wild this would be defined as diseases which previously affected the individual's

population; in captivity this is defined as pathologies that have affected tankmates). The patient history should also include description of the physical microenvironment, including time in captivity, housing, water quality (salinity, temperature, nitrogenous waste, etc.), diet, water filtration, light cycle, etc.

2.2 Physical Examination

After acquisition of a clinical history, the physical examination should be initiated. The examination should be systematic and thorough; all body systems should be investigated. The examination should begin with a "hands-off" examination, consisting of visual observation of respiration, ambulation, feeding behavior, etc. The examination should then progress to the "hands-on" portion of the examination. The entire carapace should be visually inspected, then palpated. Note any obvious problems such as crush injuries, fractures, and epibiont fouling, but also note abnormal coloration, texture, and hardness. Be sure to evaluate the joints between the prosoma and opisthosoma, opisthosoma and telson, and opisthosoma and spines; manipulate the joints to assess range of motion and to identify untoward resistance to manipulation of the joint and to identify abnormal flexion/extension. In inspecting these joints, evaluate the color, texture, and general integrity of the arthrodial membranes. Next, inspect the two lateral compound eyes; one should be on each side of the prosoma, and the lenses should be free of lacerations and/or ulcerations. Turn the horseshoe crab over and examine the ventrally located mouth at the center of the base of the legs and feeding appendages. Moving to the gills, inspect the operculum and individual book gills. Note any emphysema, hemorrhage, parasitic infestation, traumatic injury, etc., to the gill leaflets. Finally, examine the anal slit and genital pores; distal patency of these tubular tracts can be confirmed with passage of a blunt probe into the orifices.

The goal is to compile data from the history and physical examination in order to formulate a list of differential diagnoses. These differentials should guide the clinician in arriving at a diagnosis. Before resorting to postmortem diagnostic evaluation, the diagnostic plan should employ non-lethal techniques capable of ruling out differentials low on the list and/or confirm (or at least increase the suspicion of) a specific disorder.

2.3 Antemortem Diagnostics

2.3.1 Tissue and Fluid Sampling

Clinical pathology includes analysis of body fluids and tissue samples. Carapace and gill booklet scrapings, gill leaflet biopsies, fecal analysis, and hemolymph sampling are among the most rewarding of pursuits in clinical pathology of the horseshoe crab.

Carapace and gill booklet scrapings involve scraping the surface of these structures with a glass coverslip and making a wet mount preparation of the mucus, isolated cells, and debris. This technique can be utilized to identify parasites, fungi, and certain bacteria (namely those of the genus *Flexibacter*). Taking a gill leaflet biopsy is a simple method by which either a wet mount can be made to examine gross morphology and cytology or tissues can be prepared for histopathologic examination (refer to Section 2.4.2).

Fecal analysis is most often performed by direct smear and/or fecal flotation and is used to identify gastrointestinal parasites. Feces can be collected directly from the animal by insertion of a small fecal loop into the posterior end of the intestine; if this is not possible, an alternative, but suboptimal, method involves collecting feces from the tank.

Hemolymph can be non-lethally sampled from the interdigitating membranes of the legs or from the cardiac sinus, the latter location providing larger volumes of hemolymph and for ease of access is often the preferred site of sampling. Hemolymph can be used for bacterial culture if such an infection is suspected or to evaluate for hemoparasites. Clinical chemistries may be obtained by analysis of horseshoe crab serum and can be used to gain specific information regarding the function of various body systems. Serum is obtained by centrifuging whole hemolymph in a sterile glass vial and pipetting the serum away from the cellular components which pellet at the bottom of the vial. Serum samples can be analyzed using automated clinical chemistry systems found in human or veterinary diagnostic laboratories. Reference intervals have been reported for healthy adult horseshoe crabs (*L. polyphemus*) (Smith et al., 2002).

It is a common misconception that horseshoe crabs are resistant to development of systemic bacterial infections; this theory arose because horseshoe crabs have a specific lysate found within amebocytes that demonstrates anti-endotoxin properties. The presence of endogenous antibacterial compounds reduces the incidence of sepsis, but cannot prevent septicemia from developing, as is demonstrated each time a vertebrate animal dies of sepsis despite chemical and cellular mechanisms for destruction of bacteria within their blood. Therefore, it is important not to discount the potential for bacterial sepsis in the case of a lethargic, anorectic animal. If septicemia is suspected, culture and identification of bacteria from the hemolymph should be attempted. Whole hemolymph can be streaked onto marine agar (1% NaCl in any standard media, such as brain heart infusion, trypticase soy, or Luria-Bertani) and incubated at 25°C for 1–4 days. Alternatively, whole hemolymph can be passed through a sterile, stainless steel syringe filter holder and over an encased piece of sterile filter paper; the filter paper can be used to directly inoculate marine agar, which is then incubated at 25°C for 1–4 days.

2.3.2 Diagnostic Imaging

Various modalities exist by which the living horseshoe crab can be imaged. Among these are conventional radiology, contrast radiology, fluoroscopy,

ultrasound, computed tomography, and MRI. To date only radiographic/fluoroscopic techniques have been described in the literature (Melchior et al., 1995; Spotswood and Smith, 2007).

Conventional radiography can be performed to obtain films of living animals. In the event that the animal is active, sedation may be needed to minimize motion artifacts. Sedation can be achieved, without chemicals and without apparent physiologic harm, by removing the horseshoe crab from water for 5–15 minutes prior to handling and imaging.

The simplest type of radiographic imaging study is accomplished by taking at least two survey films; the minimum of two films includes both a lateral and a dorsoventral radiograph (Fig. 1). Because the majority of the internal viscera of the horseshoe crab have similar radiodensities, it is difficult to ascertain morphologic pathology from survey films alone. Positive contrast radiography has been reported; included were results from gastrointestinal and angiographic studies (Melchior et al., 1995; Spotswood and Smith, 2007). To perform a positive contrast gastrointestinal series, either static radiographs or fluoroscopy can be employed (Fig. 2). If conventional radiography is used, the time series should include a pre-injection radiograph and images immediately, 6, 18, and 30 minutes after administration of the contrast medium. Spotswood and Smith (2007) demonstrated similar results when either iodinated contrast media (15 mL of 300 mg/mL iohexol or 15 mL of 370 mg/mL sodium amidotrizoate/meglumine) or barium sulfate (15 mL of a 30% solution) was used to perform the study. These media can be administered via gavage using a #8 French polyvinyl catheter. Because non-ionic iodinated contrast media is preferred for angiographic studies (Fig. 3), it may prove more cost-effective to keep only one contrast medium in stock and use a compound such as iohexol for both gastrointestinal and angiographic studies. Spotswood and Smith (2007) injected 12 mL/kg iohexol into the cardiac sinus over a 10 second period. Using fluoroscopy, computerized digital subtraction was used to improve

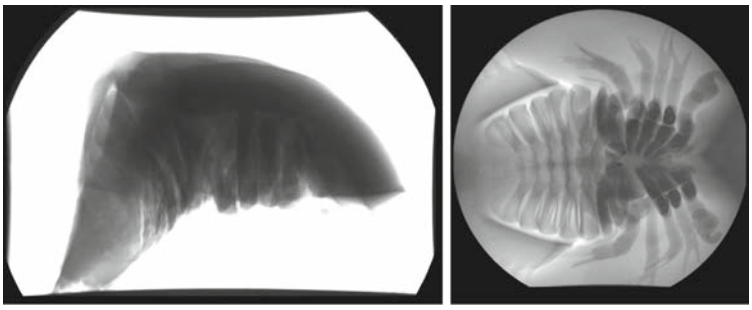

Fig. 1 Survey radiographs. These survey films are fluoroscopic still images (*left* = lateral; *right* = dorsoventral); similar information could be obtained with conventional survey radiographs

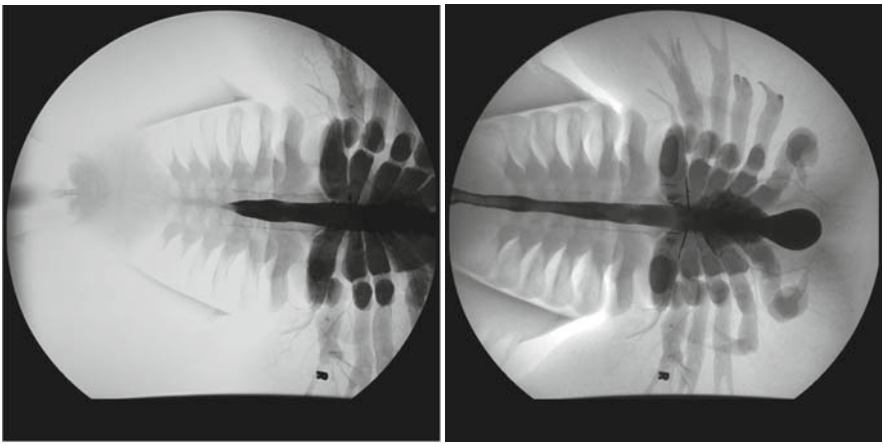

Fig. 2 Still images from a fluoroscopic positive contrast gastrointestinal series (*left* = dorsoventral, 6 minutes; *right* = dorsoventral, 18 minutes); iohexol was used as the contrast medium

visualization of peripheral vasculature. However, if fluoroscopy is not available, similar data can be collected by taking several static radiographs at timed intervals following injection of the contrast media.

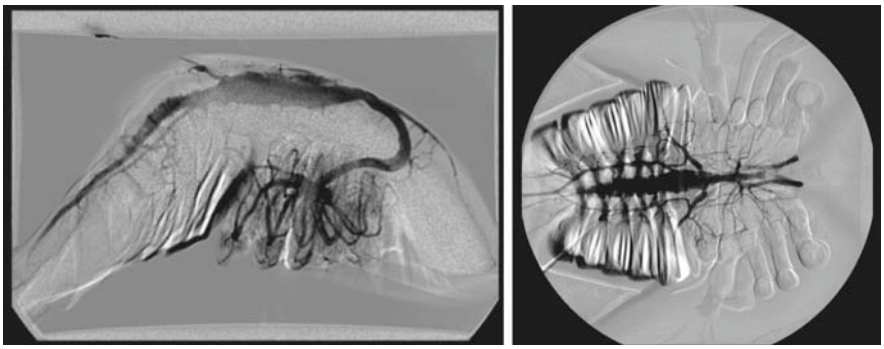

Fig. 3 Still images from a fluoroscopic positive contrast angiogram (*left* = lateral, 4.5 seconds; *right* = dorsoventral, 1.0 second)

2.4 Postmortem Diagnostics

2.4.1 Necropsy

As with physical examination, a necropsy (the veterinary equivalent of an autopsy) should include a systematic and thorough review of all body systems. The goal of a necropsy is evaluation and identification of gross pathologies

not identifiable via non-lethal methods and acquisition of tissue samples for histopathologic analysis. Because internal viscera rapidly autolyze, the necropsy should always be performed as soon after death as possible. If the animal is alive, euthanasia can be achieved by injection of pentobarbital (390 mg per animal) into the cardiac sinus. Cardiac, respiratory, and cerebral arrest should occur within approximately 30 seconds of injection of the euthanasia solution.

A necropsy is begun with a full, non-invasive physical examination. Internal examination involves dissection of the animal, starting with removal of the dorsal half of the prosoma. Use bone cutters or heavy-duty shears to trim a few millimeters proximal to the free margin at the union of dorsal and ventral prosoma. Use a scalpel to connect the two ends of this incision by cutting through the dorsal prosomal exoskeleton a few millimeters cranial to the joint between the prosoma and opisthosoma. A curved probe is then used to bluntly separate the dorsal exoskeleton from any underlying tissues. Once the carapace is freed from underlying soft tissue connections, scissors may be used to cut the optic nerve. Lift the dorsal prosomal exoskeleton away from the body, exposing the hepatopancreas, gonadal tissue, brain, and cardiac sinus. At this point, blunt dissection can be used to access other internal viscera, including those of the digestive, circulatory, excretory, nervous, and reproductive systems. Tissues should always be collected as atraumatically as possible to minimize artifact on histopathology.

2.4.2 Histopathology

In the clinical setting, histopathologic evaluation of tissue specimens is most often used to characterize the microscopic details of a lesion. Alone, results of such analysis cannot be used to make a clinical diagnosis. Rather, formulation of a descriptive diagnosis should arise from correlation of histopathologic findings with other results from the comprehensive clinical evaluation.

Several obstacles exist which may hinder the utility of histopathology in horseshoe crab diagnostics. The first problem encountered is often the investigator's lack of knowledge regarding proper methods for tissue collection and preservation. But perhaps the weightiest hindrance to histopathologic analysis of horseshoe crab tissues is a lack of available reference materials that describe normal histologic anatomy in the horseshoe crab. This section attempts to remedy that problem, providing histological descriptions of normal, clinically relevant horseshoe crab tissues.

Postmortem tissue sampling for histologic analysis can be performed using the approaches described in Section 2.4.1. Antemortem sampling can be more challenging, and surgical biopsies are possible with the assistance of proper instrumentation. Aseptic technique should be employed; use alcohol swabs to cleanse the carapace and sterile surgical equipment for all invasive procedures. Starting from the outside and working inward, the first tissue which can be sampled for histologic analysis is the carapace. If the target piece of carapace

is located at the lateral border of the prosoma, bone cutters or shears can be employed to remove a small sample; otherwise, carapace biopsies should be cut, using a #22 sterile, stainless steel surgical scalpel blade, as small squares (ideally <1 cm^2) in the carapace. Remove the square of chitinous carapace by gently undermining the tissue with a probe to remove it from the attached soft tissue structures (i.e., vasculature, nervous structures, organ capsules). If biopsy of internal viscera is desired, surgical access to those organs must be attained. The previously described biopsy technique used to sample exoskeletal tissue should be used to make a small window for surgical access to underlying tissues. The simplest place to make such a window is just anterior to the legs on the ventral prosoma, as the carapace is thinnest and only minimally mineralized in this anatomic region. Once a window is prepared, blunt dissection can be used to visualize superficial organs. If the target tissue is deeper, introduction of a laparoscope (with saline, not gas insufflation) may be necessary. After collection of all necessary biopsy specimens, the body wall (carapace) must be closed. If access to the body was attained by making a surgical window in well-mineralized carapace, use a small amount of surgical epoxy to close the wound. If access was gained in the region of relatively less mineralized chitin and if the window was small enough, sutures may provide sufficient closure. It should be noted that, as in most other veterinary species, mucosal biopsy samples can also be obtained non-lethally from the gastrointestinal tract by means of endoscopy.

For standard histopathology, tissues should be immersed in buffered 10% formalin; the volume of formalin in which these tissues are placed should exceed the volume of the specimen by at least ten times. And although ultrastructural morphology will not be discussed, tissues can be collected for electron microscopy (scanning or transmission) by immersion in 5% glutaraldehyde, 4.4% formaldehyde, and 2.75% picric acid in 0.05 M sodium cacodylate buffer at pH 7.36. Whether preparing tissues for light or electron microscopic evaluation, the tissues should be immersed in fixative for no less than 24 hours.

Once the tissues have been properly preserved in formalin they can be prepared for sectioning and staining. Soft tissues can be processed using standard histological techniques (Luna, 1968). For optimal results, chitinous structures (namely the carapace, gills, and anterior portion of the gastrointestinal tract) should be demineralized prior to sectioning. Rinse formalin-fixed tissues in phosphate-buffered saline, then in deionized water. Place rinsed tissues in a solution containing 30 mL 0.5 M EDTA (pH 8.0), 70 mL ddH_2O and 1.2 mL 6 N HCl with agitation, at room temperature, for 2 or 3 days. Remove tissues and rinse with water several times before proceeding to the paraffin embedding process (Moore et al., 2002). Standard histologic stains such as hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), silver stains, and trichrome stains can be employed.

The chitinous carapace (Fig. 4) is variable in thickness and is defined by three distinct layers. The outermost layer, the epicuticle, is quite thin, refractory to staining, and has a slight greenish coloration. It is acellular and lacks chitin. It provides a hard, waterproof surface to protect the horseshoe crab's body.

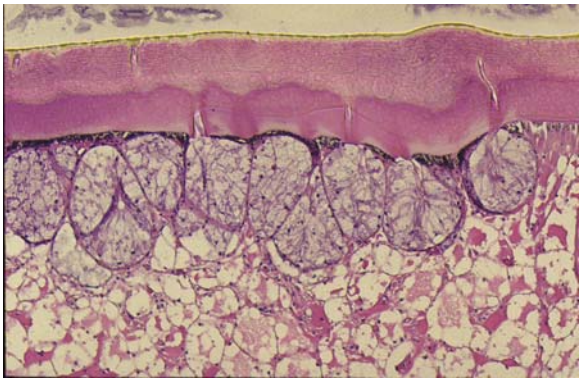

Fig. 4 Normal histology: carapace (H&E; 10×)

The middle layer of carapace, the exocuticle, is typically much thicker than the epicuticle. It is chitinous, with a pale, eosinophilic, and largely acellular appearance. The deepest layer is the endocuticle which is more eosinophilic than the epicuticular layer; it has a laminated appearance, which results from layering of chitin within a scant protein matrix. Beneath these layers of the exoskeleton lies the epidermal layer; the epidermis is composed of a single layer of columnar cells that contains small "packets" of black pigment near the apex. The epidermal cells produce chitinous matrix. Intertwined within the epidermal layer are dermal glands and trichogen cells. The dermal glands are large and globoid in shape; they are secretory in nature and are just deep to channels called dermal gland ducts. The ducts extend to the outer surface of the carapace and allow for release of products, such as pheromones, from the gland cells. The trichogen cells are vacuolated and basophilic and have a striking resemblance to nerve bundles; from these cells, bristles are projected through channels in the carapace and to the surface of the horseshoe crab.

Each book gill (Fig. 5) is composed of numerous gill leaflets. Each leaflet is made of two parallel lamellae, which are connected by chitinous pillars. The

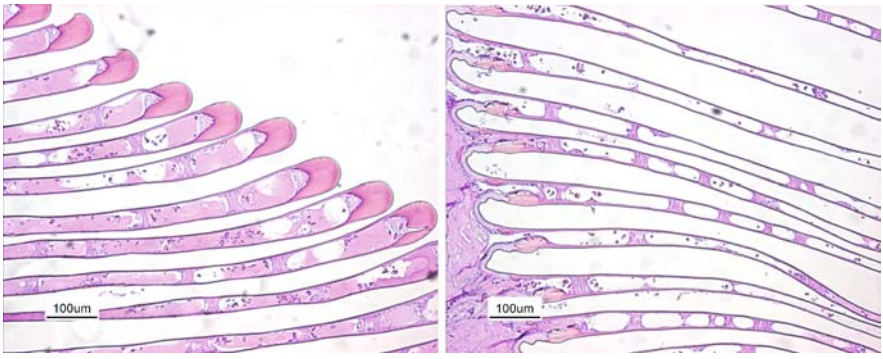

Fig. 5 Normal histology: gills (*left* = distal gill tips; *right* = proximal gill attachments; H&E)

pillars provide structural support; spaces between the pillars serve as vascular channels, which presumably provide the horseshoe crab with large amounts of surface area for both respiration and osmoregulation. The tips of the leaflets are blunt and composed of a thick layer of proteinaceous matrix. Cytoplasmic structures of the gills stain darkly with eosin while nuclei stain more basophilic. The proximal gill attachment is highly muscular, and the caudal half of each leaflet has a small muscle bundle between the proximal attachment and the first pillar; contraction of this bundle allows for gill movements responsible for both movement of water across the gills (aiding in respiration) and ambulation. The distal tip of each gill leaflet displays a thick, blunt, and acellular cap which connects the two lamellae of the individual leaflet and protects the leaflet from physical damage.

The hepatopancreas (Fig. 6) is a large accessory digestive organ. Its tubules are lined with simple columnar cells, allowing the tubules to act as secretory acini. Interstitial cells are large, angulated, and arranged in a loose cord-like structure. The interstitium occupies more of the organ than does the tubular network.

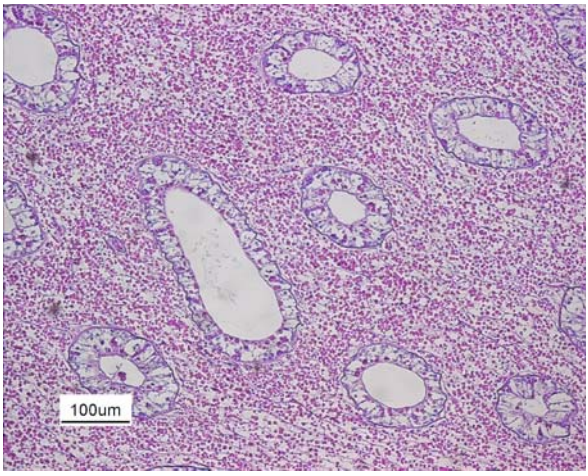

Fig. 6 Normal histology: hepatopancreas (H&E)

Based on gross morphology, the gastrointestinal tract (Fig. 7) of horseshoe crabs can be divided into four segments: esophagus, proventriculus, ventriculus, and intestines. The basic morphology of the tubular tract is similar to that of vertebrates, with each segment displaying several distinct layers, including a mucosal layer, mucosa muscularis, submucosa, and tunica muscularis externa. The most anterior segment is the esophagus, which begins at the mouth and extends caudally to the proventriculus. Identifying characteristics of this segment include epithelial crypts which produce the sclerotized lumenal surface of the esophagus. These epithelia are well perfused, as is evidenced by the innumerable

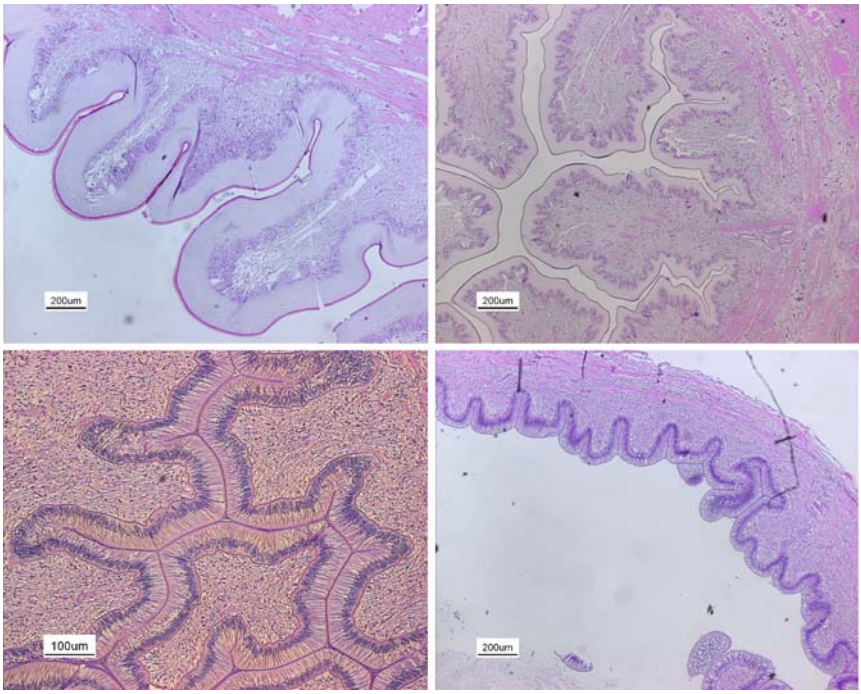

Fig. 7 Normal histology: gastrointestinal tract (*top left* = esophagus; *top right* = proventriculus; *bottom left* = ventriculus; *bottom right* = intestinal tract; H&E)

vascular channels in the submucosa. The proventriculus is also sclerotized, but its protective coating is of much greater thickness than can be found in the esophagus. The tunica muscularis externa is also far thicker in the proventriculus than in the esophagus, ventriculus, or intestines. It should be noted that the proventriculus is highly plicated, which allows this organ to expand when accepting digesta. Moving distally, the ventriculus is the first digestive segment which is not sclerotized. It is composed of a tall columnar epithelium lying atop a thick submucosal layer; the muscularis layers are thin in the ventriculus. Continuing to move distally, the next and final segment is a short, straight intestinal tract with multibranched digestive diverticulae interdigitating into the hepatopancreas. The intestines are similar to the large intestines of mammals in that they are lined with a columnar epithelium which displays crypts but not villi. The submucosal and muscular layers are intermediate in thickness.

Muscle tissue (Fig. 8) in the horseshoe crab is also much like that of vertebrates. Skeletal muscle is striated, with eccentric nuclei, a few satellite cells, and occasional vascular channels. The cardiac sinus is located in a dorsomedial position just deep to the prosomal carapace. The horseshoe crab has an open circulatory system; the heart pumps hemolymph into a well-defined arterial system which delivers and baths organs with hemolymph; hemolymph is

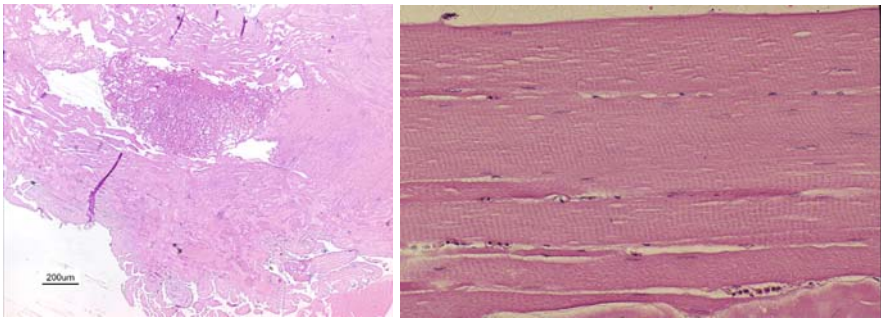

Fig. 8 Normal histology: muscle (*left* = cardiac muscle; *right* = skeletal muscle; H&E)

returned to the heart via venous shunts. Like skeletal muscle, the cardiac muscle is striated and has eccentric nuclei; however, the muscular bundles are less organized and tend to have a more pronounced interstitial matrix than appears in skeletal muscle bundles. Smooth muscle cells are fusiform and have central nuclei and, in comparison with skeletal muscle, are not highly organized.

The testes (Fig. 9) have a thin tunica albuginea lined with islands of spermatogonia. The male gonadal primordia have large basophilic nuclei, with sparse basophilic cytoplasm. The primordial cells migrate toward the lumen as they mature, allowing the cells to move into the lumen for storage; mature spermatocytes residing within the testicular lumen are tailless and stain deeply basophilic.

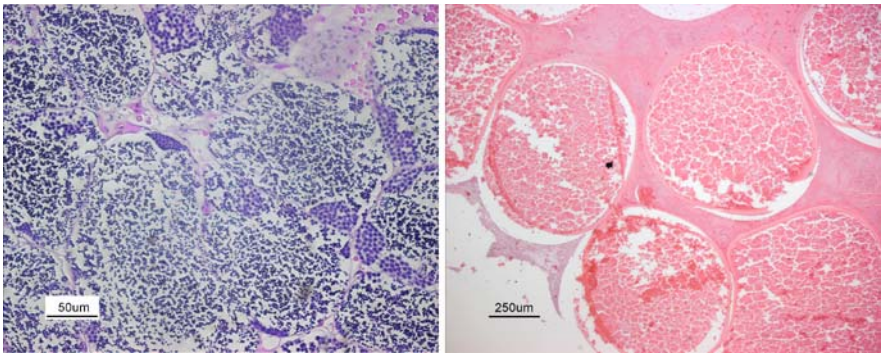

Fig. 9 Normal histology: gonads (*left* = male; *right* = female; H&E)

Ovaries are the largest organ in the body of a gravid adult female. Histologic description of this organ is challenging, as the eggs are encased within a thick shell which make sectioning and staining of the ovarian tissues difficult. In contrast to the testes, ovarian tissue (Fig. 9) retains far more eosin than hematoxylin. The ovaries are lined with columnar epithelium which appears to be oogenetic. The

eggs are large (>500 μm) and surrounded by a thick cuticle; within the cuticle lies granular cytoplasm and a nucleus which varies in size depending upon stage of egg maturity. And unlike other invertebrates, the individual eggs of the horseshoe crab do not lie within their own follicles. A tunica propria lies outside the ovogenetic epithelium; it is between this connective tissue layer and the oogenetic tissue where eggs mature and are stored (Kingsley, 1892).

Neuroanatomy and ocular anatomy of the horseshoe crab have been described at length by other investigators and for sake of brevity will not be discussed here (Chamberlain and Barlow, 1980; Chamberlain and Wyse, 1986; Fahrenbach, 1981; Weiner and Chamberlain, 1994).

Amebocytes are the only cellular constituent of hemolymph. These cells are nucleated and granulated; the granules contain coagulogen and are variable in number and size. Amebocytes first appear within the hemocoel cavity of the embryonic horseshoe crab at the fifteenth embryonic stage in *T. tridentatus* (Liang et al., 1990) and during the eighteenth embryonic stage in *L. polyphemus* (Coursey et al., 2003). Although amebocytes are often seen in tissues and within vascular channels, to date, a hematopoietic organ has yet to be identified.

3 Common Diseases

3.1 Non-infectious Diseases

A significant non-infectious cause of morbidity and mortality in captive adult horseshoe crabs is panhypoproteinemia. A specific etiology has yet to be identified, but suggested causes include (in order of descending likelihood) nutritional imbalance/deficiency, protein-losing enteropathy, hepatic insufficiency, and protein-losing nephropathy. This syndrome appears to affect all adult animals in captivity and generally results in 100% mortality. The total protein levels in the hemolymph of these animals begin to fall within 3–4 weeks of wild harvest and commencement of captive maintenance. Protein levels drop below the reference interval (3.4–11.7 g/dL; Smith et al., 2002) within 3–4 months. Morbidity is not noted until about 5 weeks before an individual horseshoe crab ultimately succumbs to the disease; clinical signs include anorexia and lethargy. Despite this syndrome having nonspecific signs, progressive worsening of panhypoproteinemia can be monitored by measuring the total protein concentration using a clinical refractometer. As the disease progresses, changes in hemolymph protein concentrations will also become grossly observable; the hemolymph clotting time will increase significantly, and the serum will be clear and colorless rather than the normal opaque and blue (Fig. 10). At this point it is not known whether the primary cause of death in affected animals is respiratory failure (resulting from loss of hemocyanin), secondary infection (due to compromised innate immunity arising from loss of acute phase proteins), or distributive shock (or relative hypovolemia, resulting from increasingly severe edema due to loss of oncotic pressure).

Fig. 10 Gross appearance of hemolymph from a hypoproteinemic horseshoe crab; the tube on the *left contains* clear, colorless hemolymph from a hypoproteinemic animal, while the tube on the *right contains* opaque, blue hemolymph from a normal animal

Other non-infectious problems of captive horseshoe crabs range from water quality problems of ammonia toxicity, gas supersaturation and high turbidity, to molting problems of the shell, legs, or telson. In addition, traumatic injuries such as puncture wounds, fractures of the carapace, and crushing of the exoskeleton have been documented. Hemorrhage from these lesions can often appear significant but is rarely fatal. Wound repair in horseshoe crabs is facilitated by the migration of amebocytes from the hemolymph followed by wound healing (Bursey, 1977; Clare et al., 1990).

3.2 Infectious Diseases

Only scattered reports of infectious diseases affecting horseshoe crabs exist in the literature. These include algae, fungi, colonial and filamentous cyanobacteria, Gram-negative bacteria, and a variety of parasites (Bang, 1956; Leibovitz and Lewbart, 2004, Smith, 2006). Shell disease is probably the most common problem in both wild and captive horseshoe crabs. This syndrome is usually manifested by discoloration of the carapace or erosion of the exoskeleton (Bullis, 1994). Chlorophycophytal (green algal) infection of the surface of the prosoma is probably the most common pathogen identified from the horseshoe crab (Leibovitz and Lewbart, 1987, 2004). Infections may manifest as a greenish to grayish discoloration of the superficial surface and deeper tissues of the exoskeleton.

Fungal infections of the horseshoe crab appear to be limited to reports from captive individuals. Adult horseshoe crabs with branchial mycosis were reported by Leibovitz and Lewbart (2004), and mycotic infection of juvenile horseshoe crabs has been reported in captive individuals (Densmore, pers com).

Horseshoe crabs are also commonly infected with blue-green cyanobacteria (Leibovitz, 1986). These filamentous organisms (*Oscillatoria* spp.) colonize and penetrate the chitinous surfaces of the gill tissue. The disease can progress to

involve deeper tissues of the gill and vasculature sinuses, sometimes resulting in tissue necrosis, swollen and ruptured gills leaflets, and death. A similar bacteria (*Beggiatoa* spp.) also colonizes the surface of the gill leaflets, but does not appear to be as invasive as *Oscillatoria* spp. (Leibovitz and Lewbart, 2004). Other bacteria identified from shell and gill lesions of the horseshoe crab included *Leucothrix* sp., *Vibrio* sp., *Flavobacterium* sp., *Pseudomonas* sp., and *Pasteurella* sp.

A number of parasites have been reported and include a variety of protozoa, a digenetic trematode, a couple of nematodes, and several turbellarid worms. Debilitated horseshoe crabs are often affected with protozoan species belonging to the ciliate genera *Pananophrys* spp., the flagellate genera *Hexamita* spp., or amoeba of the family Paramoebidae (Leibovitz and Lewbart, 2004). An unidentified protozoan was also reported from the hemolymph of an Asian species of horseshoe crab (Chen et al. 1989). The digenetic trematode, *Microphallus limuli*, of the herring gull (*Larus argentatus*) uses the horseshoe crab as a second intermediate host for its life cycle (Stunkard, 1950, 1951, 1953, 1968). The encysted metacercarial stage can be found in the connective tissue, muscle, brain, and eye of juvenile and adult horseshoe crabs. Though it has been postulated that these parasites may interfere with normal body functions, clinical significance has not been reported. Nematodes (i.e., *Monhysteria* spp. and *Grathponema* spp.) have been reported to invade the carapace of the horseshoe crab (Leibovitz and Lewbart, 2004) and several species of triclad turbellarid worms have been described from the horseshoe crab (Groff and Leibovitz, 1982; Kawakatsu, 1989; Ryder, 1882; Wheeler, 1894). The most significant of these, *Bdelloura candida*, commonly resides between the gill leaflets, on the ventral appendages, and on the external surface of the ventral carapace and obtains some of its nutrition from hemolymph acquired from lesions on the gill tissue. In addition, stalked cocoons of these parasites are located on the surface of the gill leaflets where they may interfere with respiratory activity of the gills.

Finally, there are a number of ectocommensals that frequent the external surfaces of the exoskeleton. These include bryozoans, sponges, barnacles, blue mussels, lady slippers, snails, oysters, whelks, and a variety of coelenterates, annelids, and free-living nematodes (Botton, 1981; Turner et al., 1988; Deaton and Kempler, 1989; Grant, 2001). Rarely do any of these organisms cause harm to the horseshoe crab, except when they directly interfere with normal functions such as mobility or respiration.

4 Veterinary Care

4.1 Preventative Medicine

4.1.1 Husbandry

Appropriate husbandry of adult horseshoe crabs requires knowledge and understanding of the housing, water quality, and nutritional requirements of

the animal. Routine health checks are a good way of monitoring the overall health of the captive animals.

A recent review detailed housing and water quality requirements for the adult horseshoe crab (Smith and Berkson, 2005). To summarize their findings, tank choice is largely dependent upon the user's preference; anything from glass aquaria to fiberglass tanks, depending on the size and number of horseshoe crabs, may be used with success. Substrate may include sand or crushed coral, but are often forgone without sacrificing animal health. Culture systems must incorporate both biological and mechanical filtration; use of protein skimmers, UV filters, and ozonators are optional additions to the filtration system. The ultimate goal of filtration is to remove and maintain low concentrations of nitrogenous and organic wastes and minimize turbidity. Water quality should be monitored daily to assure that proper filtration is occurring. Of importance are temperature (15–21°C), salinity (25–27 ppt), ammonia (<1.2 mg/L), nitrite (though of less importance in saltwater than freshwater, nitrites should be kept below 0.15 mg/L), and pH (7.4–8.0). Saltwater can be either obtained from natural water sources and filtered to remove potential contamination and infectious agents or made using commercially available artificial marine salts.

In the wild, horseshoe crabs consume a variety of foods including marine mollusks and worms (Walls et al., 2002, Smith, 2006). The goal of feeding captive animals is to mimic the nutritional composition of diets of wild animals and maintain health. Larval stages of the horseshoe crab readily feed on new hatched brine shrimp. Captive adult horseshoe crabs are typically fed good-quality raw fish fillets, squid, crabs, clams, and shrimp or artificial diets such as commercially extruded shrimp diets and sinking, bottom-feeder pellets. Feeding is typically done at a rate of 0.75% body weight every 2–3 days. Unfortunately, the aforementioned problems with panhypoproteinemia in captive adult horseshoe crabs seem to suggest that these diets may be nutritionally incomplete.

Appropriate monitoring of horseshoe crab health and performing frequent "health checks" is essential. These examinations are a miniature version of the complete physical examination and should include monitoring weight gain/loss, observing behavior (feeding, ambulation, sexual, etc.), and completing a non-invasive hands-on physical examination. Measurement of total serum proteins is an inexpensive adjunct to the health check and provides important information pertaining to the long-term health of the horseshoe crab (refer to Section 3.2.1).

4.1.2 Biosecurity

Equally important as providing adequate husbandry and regularly assessing horseshoe crab health is establishment and enforcement of strict biosecurity protocols for disease prevention. Such protocols should describe requirements for animal identification, protocols for disinfection and quarantine, and other procedures for limiting spread of pathogens.

Identification of individual animals is an important aspect of disease prevention, as it provides a means by which individual medical records can be

maintained. It would be impossible to track a single animal's health without individual identification; in turn, this would make it impossible to institute preventative measures such as repeated treatments and quarantines. Tagging is a simple means of attaching numerical identifiers to individual animals. Two simple and effective methods for attaching tags to the adult horseshoe crab are drilling the carapace and using marine epoxy (Fig. 11). In drilling the carapace,

Fig. 11 Individual identification can be achieved by tagging (*left* = drilled carapace; *right* = marine epoxy)

use a small diameter drill bit and make the holes as close to the lateral border of the prosoma as possible. This helps to avoid the hemolymph-filled body cavity and prevents trauma to internal organs. Affix the tag to the animal by threading plastic cable ties through the hole in the tag and then through the freshly drilled holes in the carapace. A less invasive method for affixing tags to the carapace involves using marine epoxy. Choose a quick-setting epoxy that is minimally exothermic; dry the carapace and place enough epoxy on the exoskeleton to allow for adhesion of the tag. Although slightly more labor intensive and more invasive, these authors prefer the drilling method, as epoxy-adhered tags tend to fall off the carapace after a couple of months. Note that drilling through the carapace to affix a permanent identification marker should only be performed in the adult animal; anecdotal evidence suggests drilling of the carapace of juvenile animals could interfere with growth and molting (Gore et al., 2006).

It is also important to have established quarantine measures. These must be predetermined in order to ensure that all staff members of a facility are familiar with the protocol and can institute them in the face of an outbreak of infectious disease. Individual quarantine protocols can be developed for different diseases, but this is a cumbersome task and can become problematic in the event of initial misdiagnosis or delay in proper diagnosis. Instead, it is recommended that a single, conservative protocol be developed that provides suitable isolation for an infected animal or population of animals. These authors recommend quarantining sick animals in a designated "hospital" tank located at least 8 m from any other horseshoe crab tank; this distance minimizes risk of waterborne or aerosol

transfer of disease. The hospital tank should have its own set of nets, water quality testing equipment, etc. There should also be a suitable perimeter around the hospital tank; anyone entering this buffer zone should be required to step through a footbath containing an appropriate disinfectant. The final step in setting up a quarantine protocol is deciding on length of quarantine. Any sick animal which enters quarantine should remain in isolation for a minimum of 45 days after the cessation of clinical signs of disease; this amount of time should be adequate to ensure the animal has cleared the infection and is not harboring an infectious pathogen. Additionally, a preemptive 60 day quarantine for any new animals entering a facility is recommended; this allows ample time for recrudescence of latent disease following the stress of shipment to the new facility.

As was alluded to earlier, when discussing the development of quarantine protocols, limiting the spread of potential pathogens between animals is an essential part of disease prevention. Quarantine can aid in limiting spread of disease from an individual to all its tankmates. Limiting spread of pathogens between tanks should be approached in a similar manner; each tank should have its own set of accessory equipment (nets, water quality equipment, etc.), and tanks should be physically separated by a suitable distance to minimize the risk of spreading pathogens through the air or through splashing water.

In the event that disease does occur, there should be a standardized disinfection protocol to help limit the spread of disease to other animals in the facility and to animals that will occupy the affected tank at some point in the future. Nets and other tank-side accessories can be disinfected by submersion in Roccal (1:256 dilution; Roccal-D Plus disinfectant, Pfizer Animal Health) or another similar water-based disinfectant. Saltwater tanks can be disinfected by draining the saltwater and rinsing with freshwater. After rinsing, fill the tank with 50,000 ppm bleach and expose for at least 30 minutes; drain the tank, spray down with 70% ethanol. Allow the tank to dry, and sit for at least 24 hours before refilling with freshwater. After refilling, sodium thiosulfate (2.8% per 1 ppm chloride) can be added for the purpose of inactivating chlorine in the water. After 2 hours, saltwater can be added to the system and animals can be reintroduced (Mainous and Smith, 2005).

4.2 Therapeutics

Little information is available in the literature to guide drug therapy in the horseshoe crab. Successful treatment of microbial disease (algal, bacterial, and fungal) has not been reported. As such, attempts at using antimicrobial drugs in the horseshoe crab should be attempted only after reviewing reports of drugs used in other invertebrate species. The only antimicrobial which has FDA approval for use in an invertebrate is oxytetracycline, which is approved for treatment of gaffkemia in *Homarus americanus*, the American lobster. These authors have studied the oral and intracardiac pharmacokinetics of oxytetracycline in the horseshoe crab (Nolan et al., 2007). They found that intracardiac

administration of 25 mg/kg oxytetracycline sustains a plasma concentration over 10 g/mL for at least 5 days and has a terminal half-life of 128.3 hours. Intracardiac administration of oxytetracycline is simpler for the user and less stressful for the animal; together with the fact that the aforementioned plasma concentrations are higher and maintained longer than is accomplished with oral administration, we suggest that the intracardiac route of drug administration in the horseshoe crab is more appropriate than the oral route.

Three treatments have been suggested for external parasites; these include a 3–12 minute freshwater bath, a 15–60 minute formalin bath (1–1.5 ppt formalin), and a 60 minute acetic acid bath (3–5% acetic acid) (Bullis, 1994; Landy and Leibovitz, 1983). Because formalin is not highly soluble in saltwater, formalin treatment should be combined with a freshwater bath treatment whenever attempted. Animals should be monitored for adverse reaction for the duration of any of these bath techniques; at the first sign of distress, the animals should be removed from the bath, quickly rinsed in freshwater, and returned to their saltwater tanks.

The final available disease management option in horseshoe crabs is euthanasia. There are no AVMA-approved techniques for terminating invertebrate species; however, the most rapid and atraumatic method reported involves injection of pentobarbital (390 mg/animal) into the cardiac sinus of an adult horseshoe crab. As noted in Section 2.4.1, cardiac, respiratory, and cerebral arrest should occur within approximately 30 seconds of injection of the euthanasia solution.

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