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Research Note

New Methods for the Collection and Preservation of Spirorchiid Trematodes and Polystomatid Monogeneans from Turtles

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ABSTRACT: New techniques and protocols for the field collection and preservation of spirorchiid trematodes and polystomatid monogeneans are delineated for inclusion in existing postmortem examination protocols for the parasites of turtles. Spirorchiid trematodes are collected in body and organ washes of citrated saline solution concentrated by sedimentation in a separatory funnel. Polystomatid monogeneans are collected by precise dissection and examination of the urinary and accessory bladders, nasal and oral cavities, and the conjunctival sacs and nictitating membranes of the eye. Fixation by distilled water osmotony or heat followed by preservation and storage in 95% undenatured ethanol are recommended to produce specimens for subsequent morphological and molecular analysis.

KEY WORDS: Chelonia, turtle, platyhelminthes, Trematoda, Digenea, Monogenea, Spirorchiidae, Polystomatidae, blood fluke, eye, collecting techniques, parasite survey.

Turtles host a legion of described and undescribed endoparasitic species representing at least 4 phyla: Apicomplexa, Acanthocephala, Nematoda, and Platyhelminthes (see E. M. Ernst and C. H. Ernst (1977) and references therein). The ecology and evolution of freshwater turtles has been studied intensively, including multidecade monographic efforts on species ecology (e.g., Gibbons, 1990) and phylogenetic and biogeographical work across taxonomic levels using morphological, molecular, and paleontological characters (Bickham et al., 1996; Lamb and Osentoski, 1997; Shaffer et al., 1997; Iverson, 1998; Walker and Avise, 1998; Serb et al., 2001). Turtle–parasite systems offer rich systems for the exploration of biogeography, coevolution, and parasite speciation, but the taxonomy, ecology, and evolution of turtle parasites remain enigmatic. Few turtle species have been examined thoroughly for parasites; in fact, most turtle species worldwide have not been surveyed for parasites at all (Platt, 1992; Smith, 1997).

Standard techniques for the collection and preservation of parasites (Cable, 1958; Pritchard and Kruse, 1982) are satisfactory for most turtle parasites especially when the modifications and considerations of contemporary experts are brought to bear on specimens of Acanthocephala (Nickol and Ernst, 1987, Barger and Nickol, 2004; Barger et al., 2004), Nematoda (Kuzmin et al., 2003), *Eimeria* (Apicomplexa) (Bandoni and Duszynski, 1988; Duszynski and Wilber, 1997), and preservation methods for polystomatid monogeneans (Platt, 1977, 2000a, b; Pichelin, 1995). However, our experience in turtle parasite survey during the last 5 yr, including turtles from Nebraska, Iowa, Kansas, Missouri, Oklahoma, Texas, Louisiana, Arkansas, and Tennessee (U.S.A.), suggests that spirorchiid trematodes and polystomatid monogeneans are dramatically underreported in existing surveys, probably because existing techniques do not effectively collect these taxa during necropsy. Herein, we report new protocols and techniques developed to collect and preserve spirorchiid trematodes and polystomatid monogeneans during comprehensive chelonian necropsy.

Collection of spirorchiid trematodes

Members of the Spirorchiidae (Platyhelminthes: Digenea) live in the vascular, lymphatic, and nervous systems of turtles, but only 15% of the world's turtles have been examined for spirorchiids (Smith, 1997), suggesting that the majority of spirorchiid species have yet to be discovered. The greatest impediment to collecting spirorchiid trematodes has been simply finding the relatively small worms in large volumes of blood. Undiluted blood is opaque and quickly clots, making spirorchiid collection difficult if not impossible. Platt (1988) and Platt and Blair (1996) proposed the use of citrated saline to prevent clotting in pooled blood collected during turtle necropsy and examining the blood microscopically. Although an improvement, this technique imposes the time-consuming and often herculean task of scanning

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liters of diluted blood in small (<10 ml) aliquots and does not address the problem of specimens residing within organs. Our technique uses blood dilution with an anticoagulative saline, washing spirorchiids out of organs and tissues with agitation, and concentration of freed spirorchiids by gravity sedimentation.

Extraction of spirorchiids during necropsy: Examination for spirorchiid blood parasites (Digenea: Spirorchiidae) immediately follows decapitation and pithing, a technique of euthanasia acceptable according to guidelines published by the American Veterinary Medical Association (Beaver et al., 2001). A citrated saline solution (CSS [aqueous, 0.5% NaCl, 0.2% $Na_3C_6H_5O_7 \cdot 2H_2O$]) is used throughout the necropsy to prevent blood clotting and dilute the otherwise opaque blood. Pour a generous quantity of CSS into the body cavity and collect the diluted blood in an enamel necropsy pan. Remove the plastron by cutting the plastron bridges with aviation shears (Wiss 9-3/4″ Metalmaster® Straight-cut Compound Action Aviation Snips M-3R, Cooper Hand Tools Division, Cooper Industries, Inc., Raleigh, North Carolina, U.S.A.) and slicing dermal and muscle attachments with a small, flexible fillet knife (Rapala® 4″ Signature Fillet Knife BPSFN4, Rapala-Normark VMC Corporation, Minnetonka, Minnesota, U.S.A.), irrigating the body cavity at frequent intervals with CSS. Remove organs to individual beakers of CSS, liberally irrigate the internal surfaces of the carcass, and collect the CSS–blood solution or "body wash" from the necropsy pan into labeled 1- or 2-liter beakers or screw-top jars. Process individual organs to free spirorchiids as follows. Cut the intestinal mesenteries at intervals to allow spirorchiids to escape the mesenteric blood vessels, place the prepared intestine and mesenteries in a 1-liter screw-top jar with ca. 500 ml CSS, cap the jar and shake vigorously for 1 min. If the intestine is overly packed with chyme or feces, small hemostats or rubber band ligatures can be used to seal the intestinal lumen before agitation. This is not required but can significantly reduce fecal contamination of the CSS. Remove the intestine to a container of CSS and collect other parasites from the intestine using standard postmortem techniques after collecting spirorchiids. Cut the spleen, heart, liver, and lungs several times and place them in individually labeled 500-ml screw-top jars containing ca. 250 ml CSS and shake vigorously for ca. 1 min to liberate spirorchiids. Remove the spleen, heart, liver, and lungs to individual containers of CSS and collect other parasites from these organs using standard postmortem techniques after collecting spirorchiids. Bisect the skull with aviation shears, taking care not to damage the eyes, and remove the brain to a labeled 500-ml screw-top jar containing ca. 250 ml CSS and shake vigorously for ca. 1 min. Place the remaining halves of the skull in CSS for later examination and collection of polystomatid monogeneans. Jars and beakers containing organ or body washes can be refrigerated or held on ice temporarily but should be examined before proceeding to the rest of the necropsy. Spirorchiid extraction before postmortem examination of organs has no effect on the subsequent position or collection of other platyhelminths in the intestine, spleen, heart, liver, and lungs.

Postnecropsy sedimentation, concentration, collection, and preservation of spirorchiids: Individually agitate organ and body washes and place them in individual glass separatory funnels (sizes ranging from 250 ml to 2 liters, as appropriate) and allow the wash to sediment for at least 5 min. Remove 30-ml aliquots through the stopcock of individual separatory funnels, transfer in 10-ml aliquots to a glass petri dish, and examine the wash for spirorchiid trematodes under a dissecting microscope. Remove individual spirorchiids to a holding dish of clean CSS with a small pipette. As the examination progresses, swirl each separatory funnel gently to release worms adhering to the side of the funnel. Continue to examine 30-ml aliquots of wash until 2 consecutive aliquots yield no additional spirorchiids.

Collection of polystomatid monogeneans

The 16 polystomatid (Platyhelminthes: Monogenea: Polystomatidae) species known from Nearctic turtles constitute 3 described genera, *Neopolystoma* (5 spp.), *Polystomoides* (8 spp.), and *Polystomoidella* (3 spp.) and comprise the subfamily Polystomatinae. Polystomatids of turtles are reported primarily from the urinary and accessory bladders (Yamaguti, 1963; E. M. Ernst and C. H. Ernst, 1977) but specimens are also collected from the oral mucosa and nasal cavities (Timmers and Lewis, 1979) or the conjunctival sac (Strelkov, 1950; Pichelin, 1995; duPreez and Lim, 2000; Platt, 2000a, b). We suspect that the primary impediment to collection of polystomatid monogeneans from turtles is simply that standard examination protocols do not include the primary sites of infection. Our protocol includes techniques for the examination of urinary and accessory bladders, oral mucosa and nasal cavities, and the inner surface of the nictitating membrane and conjunctival sacs of the eye.

Examination of the urinary and accessory bladders: The urinary bladder is often filled with urine and easily damaged during necropsy while removing

the plastron or internal organs. Spirorchiids are not found in association with the urinary bladder and so it is removed before the other organs and placed in CSS. With a small pair of scissors, carefully cut the urinary bladder free at a point close to its connection with the cloaca. Be sure to include the accessory bladders, small bladders attached laterally to the urinary bladder that are rarely filled and frequently missed in dissection (Wyneken, 2001). Everhart (1958) reported polystomatid infections more frequently from the accessory bladders than from the urinary bladder, and our experience suggests that they are the site of polystomatid infection at least as frequently as the primary urinary bladder. Although polystomatids are often visible through the wall of a filled bladder with the unaided eye, bladders should be individually teased or cut open and examined microscopically. Remove individual polystomatids to a holding dish of clean CSS with a pipette or soft forceps.

Examination of the oral mucosa and nasal cavities: After removing the brain for extraction of spirorchiids (see above), the nasal cavities are flushed with CSS using a small syringe. In many cases, the nasal cavities can be directly inspected under a dissecting microscope. Inspect the oral mucosa for polystomatids under a dissecting microscope. Remove individual polystomatids from the oral mucosa, nasal cavities, and nasal cavity wash to a holding dish of clean CSS with a pipette or soft forceps.

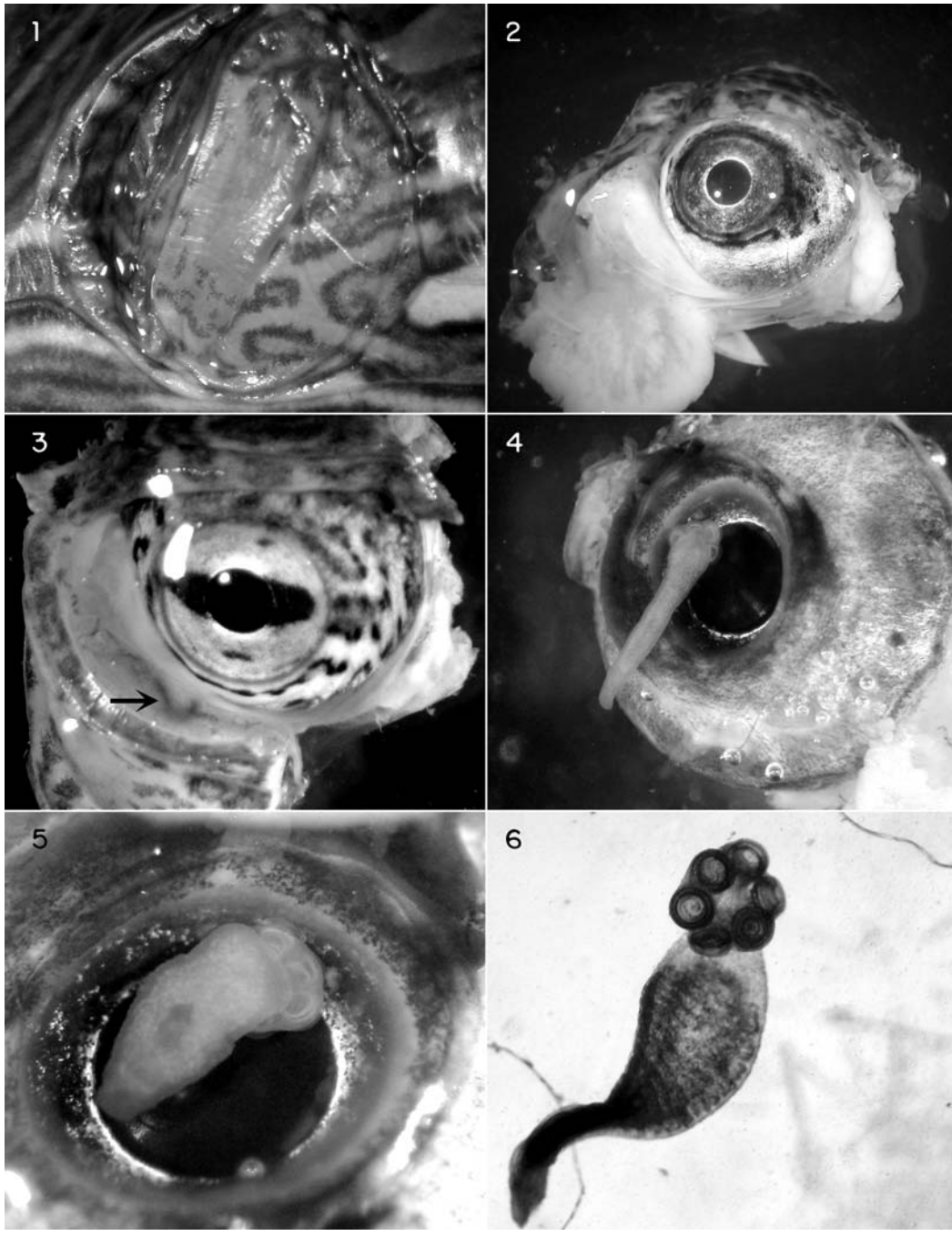
Examination of the nictitating membrane and conjunctival sacs of the eye: Simple inspection of the eye and conjunctival sacs does not reveal most ocular polystomatid infections. A thorough examination for ocular polystomatid infection requires removal of the eye from the orbit and careful dissection of the eyelids and nictitating membrane. This dissection follows removal the brain for extraction of spirorchiids and examination of the oral mucosa and nasal cavities for polystomatid monogeneans. Using a #11 scalpel blade, carefully cut through the skin surrounding the eye, following the inner edge of the orbit and circumscribing the entire eye (Fig. 1). Carefully slide the tip of the scalpel between the eye and the inner wall of the orbit to sever the dorsal and ventral oblique muscles at the anterior ocular angle and the dorsal and ventral rectus muscles at the posterior ocular angle. Carefully slide a partially open pair of forceps under the eye and gently lift the eye from the orbit. This exposes the lateral rectus and retractor bulbi muscles inserted deeply on the eye. Sever these muscles and remove the freed eye to a dish of clean CSS (Fig. 2). Carefully bisect the posterior ocular angle with a pair of fine scissors to free the upper and lower eyelids. Reflect the eyelids and carefully inspect their inner surfaces for polystomatid monogeneans, which usually appear orange but translucent against the pale host tissue. Remove individual polystomatids to a holding dish of clean CSS with a pipette or soft forceps. Carefully bisect the nictitating membrane at the posterior ocular angle, reflect the nictitating membrane, and inspect the inner surface and the surface of the exposed conjunctiva (Fig. 3) and remove individual polystomatids to a holding dish of clean CSS with a pipette or soft forceps. Polystomatids are quite mobile and often move across the surface of the cornea (Figs. 4, 5) or even into the dissecting dish once the eyelids and nictitating membrane are reflected. Some individuals are recalcitrant and remain tightly affixed to the mucus accumulated in the conjunctival sac. They are easily removed with the mucus to a holding dish and will usually abandon the mucus if left undisturbed for several minutes (Fig. 6).

Relaxation, fixation, and storage of spirorchiid trematodes and polystomatid monogeneans

There are numerous techniques for the relaxation and fixation of platyhelminths (Cable, 1958; Knudsen, 1972; Pritchard and Kruse, 1982), and choice of a technique depends on personal experience, available facilities, and the amount of material to be preserved. Our techniques are designed to process specimens relatively rapidly in rudimentary laboratory or field conditions with the caveat that specimens must be available for both morphological and molecular analysis.

Relaxing spirorchiids: Maintain spirorchiids alive in a small dish or glass plate well of CSS until you are ready to relax and fix them. Replace the CSS with distilled water, repeating the change once or twice over 5 min. After 5 min in distilled water most worms relax and are suitable for fixation. Changing the solution in the holding dish rather than moving the spirorchiids themselves avoids unnecessary handling of these small fragile worms, which are easily lost or damaged. Move relaxed specimens directly to 95% undenatured ethanol for fixation and storage.

Relaxing polystomatids: Polystomatid monogeneans are much larger and more muscular than spirorchiids and will not relax in distilled water. Techniques that involve placing pressure on the worms during fixation are time consuming and usually cause significant anatomical distortion. Place individual polystomatids on the distal third of a clean glass slide in a small drop of CSS or distilled water and gently but quickly heat the slide from underneath with a butane lighter until the worm straightens.



Figures 1–6. Collecting polystomatid monogeneans from the eye of a turtle. **1.** Eye in situ, circumscribed with scalpel before removal from orbit. **2.** Eye removed from orbit to citrated saline solution; posterior ocular angle bisected to reflect lower eyelid. **3.** Nictitating membrane bisected at posterior ocular angle and reflected to expose polystomatid trematode on inner surface (arrow). **4–5.** Polystomatids moving across the cornea after being freed from the nictitating membrane or conjunctival sac. **6.** Free ocular polystomatid in a dish of citrated saline solution.

Quickly remove the heat before the liquid boils and move the fixed specimen to 95% undenatured ethanol for final fixation and storage. The heat source and timing are critical in this procedure, which admittedly requires some practice but produces superior morphological specimens. We use microtorch-style windproof refillable butane lighters such as the Cabela's Alaskan Outfitter All-Weather Lighter® (Cabela's Outfitters, Sidney, Nebraska, U.S.A.) or the Colibri Quantum® series (The Colibri Group, Providence, Rhode Island, U.S.A.), which produce an instant, intense, pinpoint flame that is faster, more predictable, and easier to control than a disposable cigarette lighter, match, or alcohol burner.

Fixation and storage: Formalin-based fixatives preserve specimens suitable for light microscopy, histology, and electron microscopy but severely damage DNA and render specimens unsuitable for use in molecular analyses (Dessauer et al., 1996). Fixation in 95% undenatured ethanol maintains the molecular utility of the parasites and produces specimens suitable for light microscopy and histology. Specimens preserved in ethanol should be stored at temperatures of −20°C or lower to extend the utility of DNA. Ethanol specimens used for light microscopy or histology sometimes stain poorly: hematoxylin stains are often imprecise or "muddy," and analine and carmine dyes lack brilliance. Postfixation of ethanol specimens for 15 to 60 min in Bouin's fluid or an appropriate ethanol-based picric acid fixative before staining yields specimens that stain as well or better than specimens initially preserved with a formalin-based fixative.

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