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X-ray imaging as a time-saving, non-invasive technique for diet analysis

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ABSTRACT

Dietary patterns of animals have a long-recognized importance in ecology and evolution, with numerous and diverse applications. While many methods of diet assessment exist, the most common method of direct diet examination for most small vertebrates is stomach-content analysis, using labor-intensive surgical removal of the gut following death. Methods that can reduce the time required to collect diet information without necessarily sacrificing specimens could prove invaluable for a range of applications. We evaluated digital X-ray imaging as a non-invasive method for examination of stomach contents of small fishes. Based on both a feeding experiment and examination of field-collected preserved specimens, we found that digital radiography consistently revealed the presence of moderate- to high-density prey items in the stomach, such as small arthropods. Moreover, X-ray imaging allowed for rapid identification of some particular prey items such as detritus, dipteran larvae, ostracods, hard-shelled molluscs, and small fish. However, this method failed to detect some low-density prey items present in some stomachs, and could not be used for precise taxonomic identifications in most cases. Overall, we found that digital X-ray images can be quickly acquired from anesthetized or preserved animals, permit rapid identification of certain prey items, and facilitate digital data archives. Future studies that employ this method should first "ground-truth" the radiological signatures of prey items observed within a given study using stomach-content analysis, which then permits rapid data collection strictly using X-ray images. This method can provide information useful for determining the inclusion of certain prey items in diets, even quantifying particular taxonomic groups of prey (% occurrence, % by number). Thus our results indicate that for certain study goals, X-ray radiography may provide a time reducing, non-invasive technique for diet analysis of small vertebrates.

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1. Introduction

The measurement and understanding of dietary patterns in animals has central importance in ecology, evolutionary biology, conservation, and management. Diet analysis comprises a long-standing tool for addressing a range of questions, such as community assembly, trophic relationships among species, habitat use, management of threatened, game, or commercially harvested species, and resource competition's role in driving major ecological and evolutionary patterns (e.g. Bolnick et al., 2003; Collar et al., 2009; Morin, 2011; Odum, 1983; Polis and Winemiller, 1996; Schluter, 2000; Schoener, 1971). A number of methods exist for assessing animal diets, such as visual observations of feeding, morphological and molecular identification of prey taxa in feces and stomachs, stable isotope analysis, and lipid analysis (e.g. Hyslop, 1980; Peterson and Fry, 1987; Valentini et al., 2009). For small vertebrates, especially fishes, amphibians, and reptiles, morphological examination of stomach contents is the most commonly employed technique for direct diet analysis. Nonlethal techniques, such as stomach flushing using tubes or gastric lavage, is sometimes possible for larger individuals (e.g. Giles, 1980; Light et al., 1983), but post-mortem dissection represents the most common approach. In fisheries research and management, stomach dissections are regularly used for the analysis of diet.

Typically, the stomach/intestines are surgically removed from freshly-killed or preserved specimens, partially digested prey items extracted, and taxonomic identification of prey accomplished using microscopic examination. Once prey items have been identified, a range of approaches can be used for statistical analysis of diet (review of methods are beyond the scope of this paper, see Cortes, 1997; Hyslop, 1980). This method requires the death of the specimen and is time and labor intensive, and requires specialized training to process and identify the contents. Thus,

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alternative methods that can utilize live specimens or reduce the time required to collect diet data would be advantageous, as this would streamline the collection of diet information without sacrificing specimens.

Here we examined digital X-ray radiography as a rapid, non-invasive method for assessing diet of small fishes. A number of recent advances in digital radiography make this assessment timely: e.g. increases in resolution and magnification permit the detection of very small objects of low density, prices of digital radiography equipment has recently dropped considerably, portability of X-ray units has increased substantially, and many universities already have digital X-ray machines capable of at least moderate resolution of small vertebrates. While previous studies have employed X-ray radiography in the context of animal diets (e.g. rates of feed intake and gastric evacuation; Talbot and Higgins, 1983; McCarthy et al., 1992; Jobling et al., 1993, 2001), no previous study has examined the utility of this technique for identifying diet items of small vertebrates.

In this study, we investigated gut/stomach contents using X-ray imaging in four small fish species (15–80 mm standard length): Eastern mosquitofish (*Gambusia holbrooki*, Girard 1859), Bahamas mosquitofish (*G. hubbsi*, Breeder 1934), Trinidadian guppy (*Poecilia reticulata*, Peters 1859), and Hart's killifish (*Anablepsoides hartii*; formerly *Rivulus hartii*, Boulenger 1890). Collectively, these omnivorous species are known to exhibit a broad diet, including detritus, algae, aquatic and terrestrial insects, crustaceans, molluscs, and even juvenile fishes. We hypothesized that X-ray images would reveal the presence of prey contents in the stomach, and permit the detection and identification of some broad groups of prey taxa based on their dense body parts (e.g. shells, exoskeletons, bones), such as molluscs (e.g. gastropods, bivalves), crustaceans (e.g. ostracods, shrimp), insects (e.g. chironomids, beetles), and vertebrates (e.g. fish, tadpoles).

2. Materials and methods

Our goal was to determine whether X-ray imaging could reveal the presence or absence of prey in stomachs of small fishes, and allow the identification of 5 different types of prey items that vary in density (mass per unit volume): (1) soft homogeneous prey (e.g. algae, detritus), (2) weakly shelled arthropods (e.g. small shrimp, ants), (3) moderate-density arthropods (e.g. ostracods, beetles), (4) hard-bodied prey (e.g. shelled molluscs, crabs) and (5) vertebrates (e.g. small fish, anurans). We took a two-pronged approach to accomplish this: we conducted a feeding experiment with live fish to directly assess the accuracy of diet identification using X-ray imaging, and we examined preserved, wild-caught fish specimens to evaluate the utility of the approach for the examination of natural dietary patterns.

2.1. Feeding experiment

We performed a feeding experiment using 21 live individuals of *G. holbrooki* and 3 individuals of *A. hartii*. All fish were collected from the wild (*G. holbrooki*: North Carolina, USA; *A. hartii*: Trinidad) and housed in 38-L aquaria in common laboratory conditions. Prior to the feeding experiment, fish were placed individually into separate 8-L tanks and starved for 48 h. For *G. holbrooki*, we assigned three adult females at random to each of seven diet treatments: (1) no prey: starved, (2) soft, low-density homogeneous prey: TetraMin Pro flakes, (3) low-density crustaceans: live *Artemia* sp. nauplii, (4) low-density insects: live ants, (5) moderate-density insects: thawed bloodworms (*Chironomus tetans*), (6) hard-shelled prey: live snails (*Physa acuta*), and (7) vertebrate: one live *G. holbrooki* juvenile. We fed two live *P. reticulata* juveniles (3–4 mm SL) to *A. hartii* to test for detection of vertebrate consumption in this species.

Within 1 h of feeding, we X-rayed each individual and saved a digital image. We placed each live fish into a small, moist plastic bag, laid the fish on its side within a petri dish, and set the dish in the X-ray machine to capture a lateral image. We used a custom-built digital X-ray unit comprising a micro-focus X-ray source (Hamamatsu L6731-01) and a digital X-ray detector (PaxScan 2520E) housed in a lead-shielded cabinet, set to 45 kV and 40 μA. Radiation exposure to each fish was low, approximately 25–50 mrem – roughly equal to a human dental X-ray for comparison. Fish were then immediately placed into a recovery tank. Removal from water, X-ray imaging, and return to recovery tank typically only required approximately 30 s. Identification of all stomach contents based on digital X-ray images was conducted blind of fish ID.

Table 1
Collection and sample size information for wild-caught adult specimens examined in this study.

Species	Collection location	*N*
Gambusia holbrooki	Melbourne, Florida, USA	60
	James Island Park, South Carolina, USA	60
Gambusia hubbsi	East Twin blue hole, Andros Island, Bahamas	40
	West Twin blue hole, Andros Island, Bahamas	40
	Hubcap blue hole, Andros Island, Bahamas	40
Poecilia reticulata	Kahala, Oahu, Hawaii, USA	120
Anablepsoides hartii	Arima Valley, Trinidad	23

2.2. Preserved specimens

We examined digital X-ray images (using method and equipment described above) of wild-caught specimens preserved in 70% ethanol to assess the ability to detect natural dietary patterns of preserved small fish with X-ray images. We examined 120 *G. holbrooki*, 120 *G. hubbsi*, 120 *P. reticulata*, and 23 *A. hartii* (see Table 1 for collection and sample size details).

For each preserved specimen, we attempted to determine contents of the stomach based on the X-ray image. After viewing a number of images, six natural categories emerged from our identifications: (1) no prey contents, (2) soft homogeneous prey (e.g. algae, detritus), (3) low-density prey (e.g. branchiopods, ants), (4) moderate-density prey (e.g. ostracods, dipterans), (5) shelled mollusc prey, and (6) vertebrate (fish) prey. To determine the accuracy of diet identification, we dissected three randomly selected specimens of each species from each diet category using the traditional method of surgically removing the gut and identifying the contents under a microscope (Leica S8 APO stereoscope). We then compared the diet classification from X-ray images to that from direct morphological identification.

3. Results

3.1. Feeding experiment

The guts of all six starved fish appeared empty in X-ray images (Fig. 1a and b). We could not detect flakes or *Artemia* sp. nauplii with our X-ray images (Fig. 1c). In two out of the three fish fed ants, small hard parts of prey were visible in their guts in the X-ray Images – presumably reflecting broken pieces of the ants – but these were difficult to identify as ants or even as insects (Fig. 1d). Based on X-ray images, we accurately detected prey items in all fish fed bloodworms (Fig. 1e), *P. acuta* snails (Fig. 1f), newborn *G. holbrooki* (Fig. 1g), and juvenile *P. reticulata* (Fig. 1h). Thus, we consistently could not detect the two lowest density prey types, but could detect the three highest density prey types; ants appeared

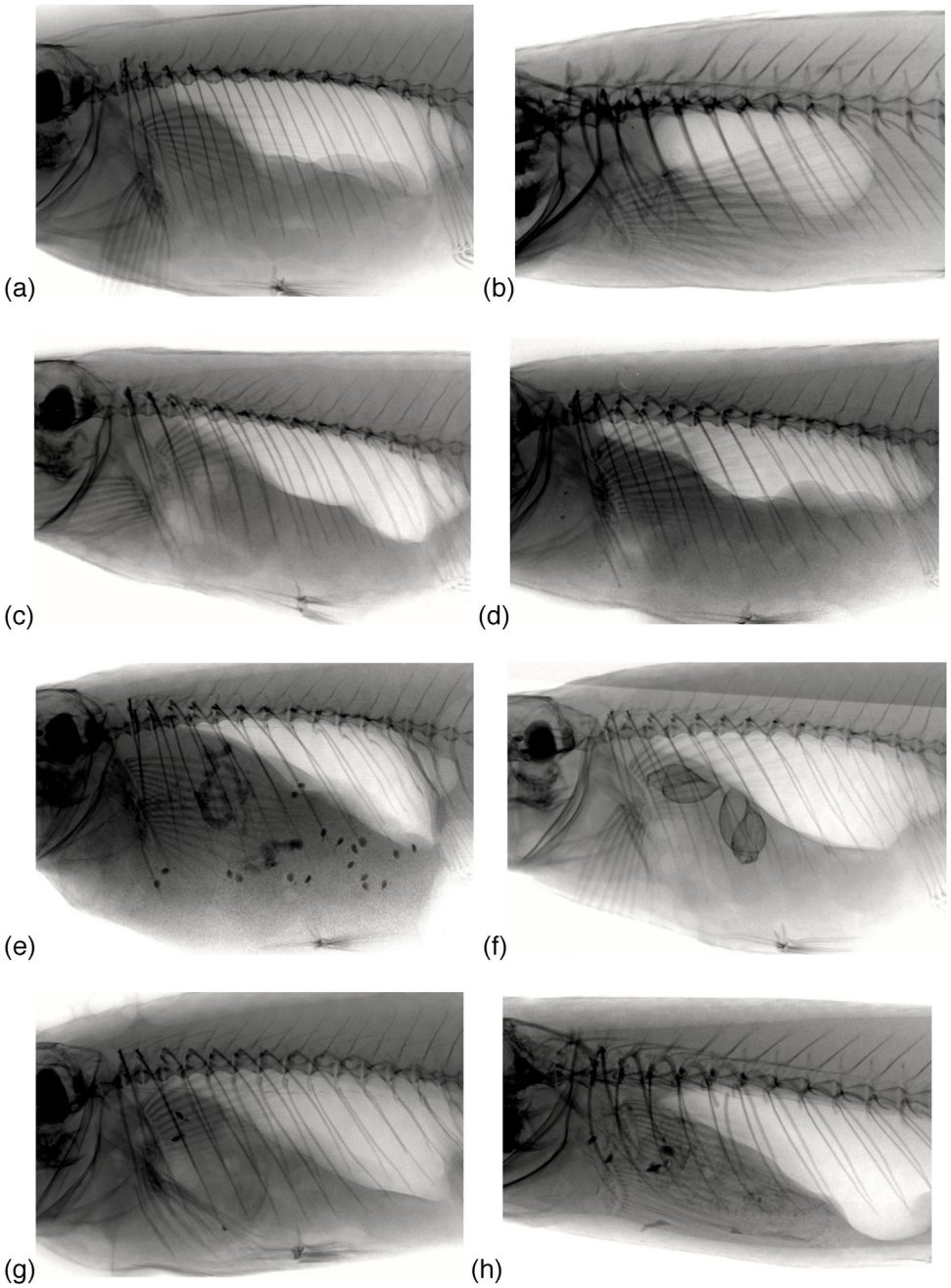

Fig. 1. Representative X-ray images from each diet treatment in the feeding experiment showing only the intestinal region of each image (anterior to the left). (a) Starved female *G. holbrooki*, depicting no apparent object signatures within the gut (only yolked eggs, spherical objects visible in the image, were observed in the relevant body region). (b) Starved female *A. hartii*, depicting no apparent object signatures within the gut. (c) Female *G. holbrooki* fed TetraMin Pro flakes, depicting no apparent object signatures within the gut. (d) Female *G. holbrooki* fed ants, depicting several small low-density items within the gut (yolked eggs also visible in image). (e) Pregnant female *G. holbrooki* fed bloodworms, clearly depicting the shape of the *Chironomus tetans* larvae (embryo otoliths appear as paired black spots in the image). (f) Female *G. holbrooki* fed *Physa acuta*, depicting the consumed snail shells. (g) Female *G. holbrooki* fed a newborn *G. holbrooki*, depicting the otoliths and body outline of the fish prey. (h) Female *A. hartii* fed two juvenile *P. reticulata*, depicting the otoliths, vertebrae, and parts of the skulls of the fish prey.

to represent prey densities around the threshold of detectability using this method.

3.2. Preserved specimens

First, we found that stomachs we identified as empty based on X-ray images actually contained small, low-density prey items, such as soft-bodied prey, weakly shelled prey, or filamentous plant material (Fig. 2a). We further frequently encountered these prey items in dissected stomachs that we identified to contain other prey types, but these prey were typically not visible in X-ray images. Second, guts that we identified as containing soft, homogeneous prey or low- to moderate-density arthropod prey were filled with a variety of prey items, including ants, dipteran larvae, small adult dipterans, and filamentous plant material (Figs. 2b and c and 3a and b). Guts of *P. reticulata* in particular appeared to be filled homogeneously with low-density prey, which we confirmed to comprise detritus and algal material; interspersed higher-density items within these guts represented small arthropods (Fig. 2c). Third, among the moderate-density items visible in radiographs, dipteran larvae were occasionally identifiable based on their characteristic size and elongate shape (Fig. 3b), and

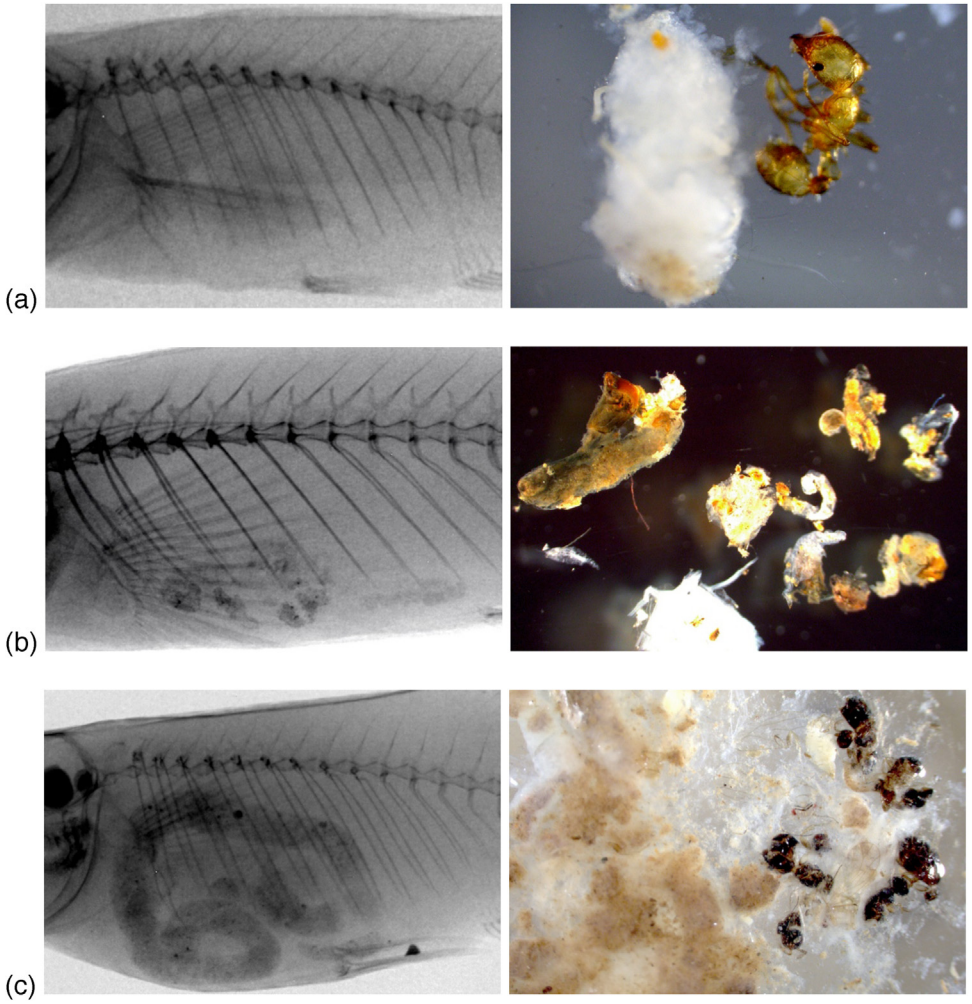

Fig. 2. Representative X-ray images of the intestinal regions of adult wild-caught specimens (left) and the low- to moderate-density prey removed from their stomachs (right). (a) Female *G. hubbsi* incorrectly identified to have an empty stomach, while it actually contained a soft-bodied insect larva and an ant. (b) Male *A. hartii* identified as containing soft-bodied prey and low- to moderate-density arthropods according to the X-ray, and dissected contents revealed Chironomidae and Simuliidae larvae and filamentous plant material. (c) Female *P. reticulata* identified as containing homogeneous low-density items, such as detritus, and several higher-density items, such as arthropods; dissected contents revealed detritus and homogeneous algal material, along with several small adult hymenopterans.

ostracods were always easily identified (Fig. 3a and b). Fourth, snail shells were among the most easily identifiable prey item in X-ray images, as the shells appear highly defined in radiographs and all dissected stomachs confirmed our identifications via radiographs (Fig. 3c). Finally, fish prey were generally readily identifiable (Fig. 3d), with the exception that pregnant live-bearing fish carrying one or a few late-stage embryos can appear on an X-ray image to have instead consumed small fish (we encountered one instance here, where a pregnant *G. hubbsi* female contained a single embryo at the final stage of development, which would generally be difficult to distinguish from consumed fish prey).

4. Discussion

Diet analysis holds central importance in a range of ecological and evolutionary applications. Traditional methods for diet analysis of small vertebrates are time and labor intensive, and typically involve the death of the animal. This situation can prove problematic in the face of time constraints, or when the animals of interest need to remain alive, such as in the cases of endangered species, captive breeding programs, the need to minimize impacts on populations, and to avoid negative public perceptions of management or conservation programs (Crossman and Hamilton, 1978; Hartleb and Moring, 1995; Light et al., 1983). Small vertebrates pose special challenges to nonlethal diet analysis, as techniques commonly applied to larger animals are typically not feasible with small individuals (Hyslop, 1980; Strange and Kennedy, 1981; Waters et al., 2004). Thus, a need exists for methods of diet analysis amenable to small vertebrates that reduce the required time and labor, and can be applied to live animals.

We found that X-ray imaging can be used to quickly identify some particular moderate- to high-density prey taxa within stomach contents of both live and preserved small fishes. Although identification of these prey taxa with X-ray images is quite rapid, the taxonomic resolution will generally be much lower than that obtained via microscopic examination of stomach contents. However, the method can detect very small prey items within small organisms – the entire body cavity length for the fish examined in this study typically only spanned 8–15 mm, with prey parts as small as 0.1 mm long identified in images.

In our feeding experiment, we found that X-ray imaging could be employed to accurately identify chironomids, snails, and small fish prey, as we correctly identified these prey in all cases. Moreover, small ants were often detectable as small, low-density items within the stomach; although, these items could not be identified as hymenoptera or even insects without examination of the

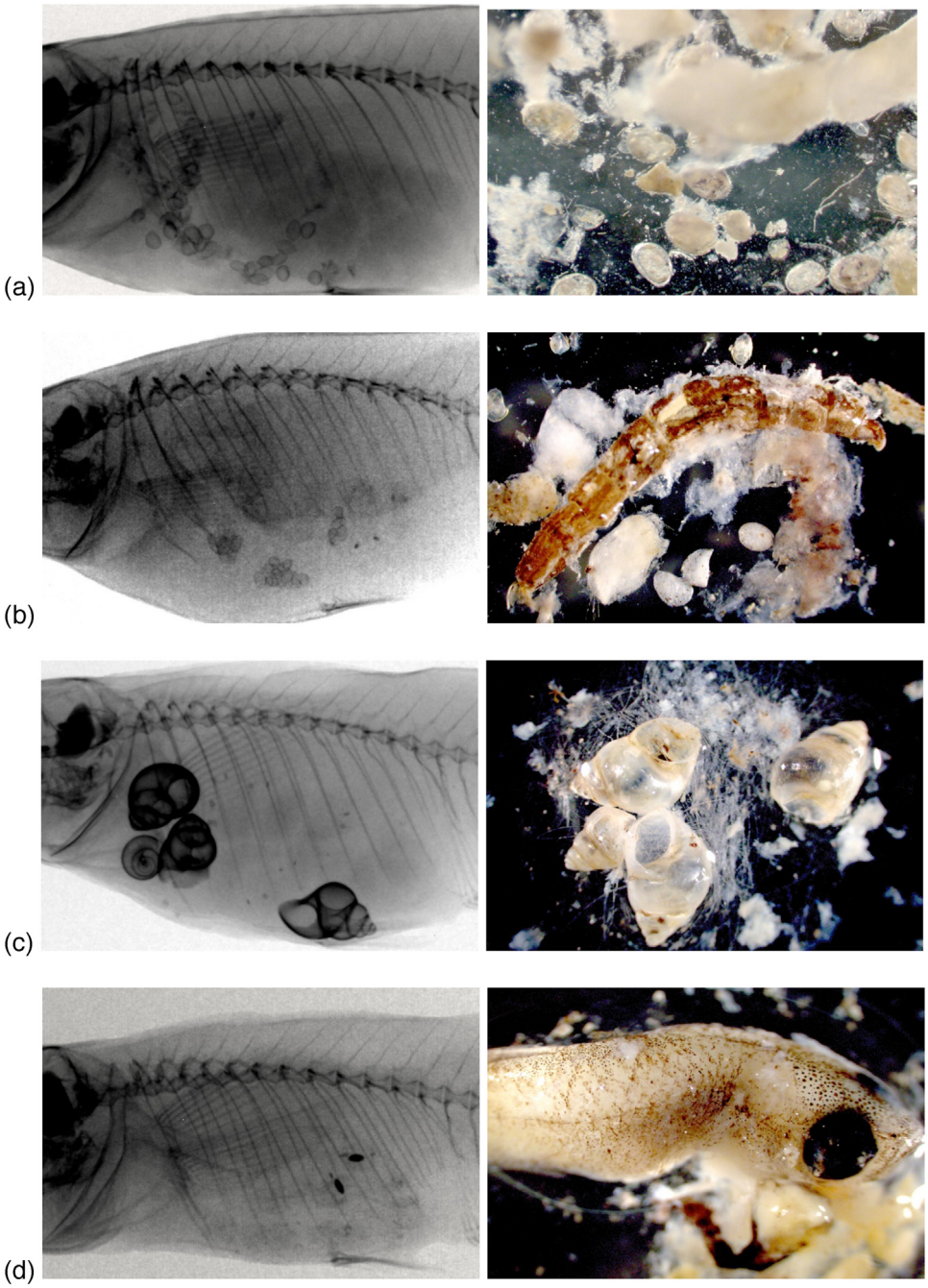

Fig. 3. Representative X-ray images of the intestinal regions of adult wild-caught specimens found to contain moderate- to high-density prey items (left) and the prey removed from their stomachs (right). (a) Female *G. holbrooki* identified as containing many ostracods, and several low- to moderate-density items in its stomach (yolked eggs also visible); dissected contents revealed many ostracods, along with plant material. (b) Female *G. holbrooki* identified as containing several clumps of ostracods and an elongate moderate-density item indicative of a dipteran larva in its stomach; dissected contents revealed ostracods, a Brachycera larva, a small annelid, and plant material. (c) Female *G. holbrooki* identified as containing four snail shells in the gut (embryos with otoliths also visible); dissected contents revealed four snail shells and filamentous plant material. (d) Female *G. hubbsi* identified as containing one small fish prey (the pairs of otoliths are the darkest spots, while the jaw and vertebrae are also evident); dissected contents revealed a single small fish prey.

stomach contents. Our analysis of preserved wild-caught specimens confirmed that we could use X-ray imaging to accurately identify homogeneous algae/detritus, ostracods, snails, and fish, while we could typically also identify the presence of dipteran larvae. Moreover, the presence of some other prey, such as small arthropods, could often be determined from X-ray images, although the taxonomic status could not be determined without stomach-content analysis.

Because species-specific, morphological diagnostic characters are often not visible in X-ray images, identification to species level was not reliable in most cases in this study. In some simple natural systems, there may only be a single species of a particular prey type, in which species-level identification is possible. If a study requires detailed information regarding the taxonomic identity of a variety of food items consumed by an animal, or requires volumetric or weight information, then X-ray imaging will not be suitable to accomplish the study goals. Thus, X-ray imaging can provide rapid information useful for certain types of diet analysis, but not others. For instance, the method can rapidly acquire presence/absence of particular prey taxa in stomachs of

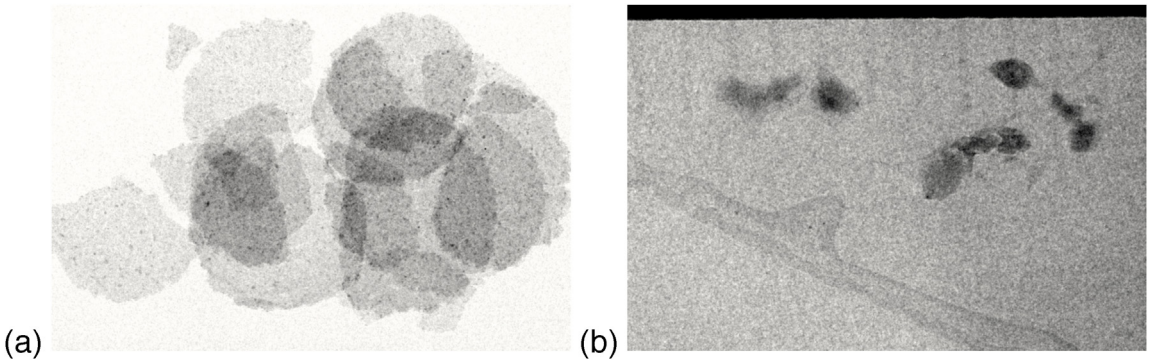

Fig. 4. (a) X-ray image of TetraMin Pro flakes when examined alone (not within fish stomach). (b) X-ray image of three ants when examined in isolation.

individuals, and presence/absence of broad groupings of prey types where taxonomic identities are difficult to discern with X-ray images and are also less important for the study. In some cases, numerical information may also be obtained from X-ray images. Thus, digital radiography might often allow for collection of data for two commonly employed dietary metrics, percent frequency of occurrence and percent by number of prey taxa in stomachs (Cortes, 1997). While a range of prey taxa were detectable using digital radiography, our study also revealed that not all types of prey items were visible in X-ray images. For instance, we could not detect flake food within fish stomachs in our feeding experiment, and sometimes could not detect ants. These prey items, when imaged in isolation, are clearly evident in X-ray images taken with our equipment (Fig. 4). However, when contained within surrounding fish tissue, these low-density food items become very difficult to detect. Further, our examination of wild-caught preserved specimens confirmed that guts appearing empty on X-ray images could actually contain soft-bodied, low-density food items such as ants, soft-bodied insect larvae, and plant material. Consequently, this method cannot accurately distinguish empty stomachs from stomachs that contain certain types of low-density prey.

In addition to the limitations of this method discussed already regarding detection and identification of particular prey items, this method may also be affected by variable processing (e.g. prey crushing and tearing) and digestion rates of different prey within different species (e.g. Macdonald et al., 1982). Prey densities can decline during digestion, making the method more amenable to foregut analyses and prey items that are swallowed whole where moderate-density parts are passed in feces.

Compared to more labor-intensive methods, such as gut dissection and stomach-content analysis using identification with a microscope, taking and analyzing X-ray images provides a dramatic improvement in the time required for analysis, and permits the examination of live animals. For instance, digital X-ray images usually take about 1 min per specimen to acquire, with images immediately available for inspection (no film development required), and digital files ready for archiving for long-term storage and future reference. After initial training for identification of the relevant prey categories from radiographs, scoring stomach contents from images usually only requires a few minutes per specimen. Meanwhile, surgical extraction of a stomach and microscopic analysis of diet typically require much more time per specimen. Microscopic identification of prey taxa extracted from stomachs demands considerable skills that require substantial training for the identification of a wide range of possible prey taxa, while identification of a smaller set of prey categories on X-ray images necessitates much less training. The use of X-ray imaging versus direct stomach-content analysis for diet studies of small vertebrates largely represents a tradeoff between time, training, precision, and lethality: X-ray imaging allows the rapid extraction of broad dietary information requiring minimal training where the specimens can remain alive after examination, while direct stomach-content analysis represents a more time-consuming extraction of detailed dietary information requiring substantial training where the individuals must typically die prior to examination. Thus, a researcher or manager must weigh these four primary factors when deciding whether X-ray imaging might represent the most appropriate method for their study.

The methods described here should apply to small vertebrates other than fish, such as reptiles, amphibians, and small birds and mammals. Indeed, during the course of other projects that involved X-ray imaging of small lizards and frogs, we have witnessed a number of obvious diet items in the images, such as terrestrial arthropods and vertebrates (RBL, unpublished data). Further, portable X-ray applications are becoming more commonplace today, and this implies that diets of live animals could be examined directly in the field. Because the method requires little time and handling, and can be employed with extremely low levels of radiation exposure, this method could represent a suitable solution for diet studies of threatened species, or in situations where it is important not to impact the study population, including small populations, breeding programs, zoo animals, or lab animals.

We envision other uses of digital radiography for ecologists and evolutionary biologists as well, such as the use in reproductive and developmental studies of viviparous fishes. For instance, X-ray images provide highly accurate information regarding whether a fish is pregnant or not, and can often permit the counting of the number of developing embryos within some species. Thus, studies examining the fecundity of females might employ X-ray imaging to rapidly collect information on the frequency of pregnancy, number of offspring, and perhaps estimates of the size and developmental stage of embryos.

5. Conclusion

Overall, our study revealed that X-ray imaging can provide an appropriate tool for diet analysis of small vertebrates, depending on the goals of the study. The two most obvious advantages of the approach are the reductions in time required for data collection and the ability to non-invasively examine live animals. This method is probably most useful for studies that seek rapid collection of broad dietary information, where detailed taxonomic information or prey-size information is less important for the goals. This non-lethal method can rapidly and accurately provide information on certain types of prey items contained within the stomachs of specimens, and should thus receive increased use in diet studies in the future.

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