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Best Practices for Ancient Rodent Midden Collection, Processing, and Curation

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Abstract

As new midden researchers enter the field and new field campaigns are conducted, standardization of methods will greatly improve our ability to utilize middens to address a variety of research questions spanning midden series, regions, and taxa. This protocol provides a workflow with best practices for fossil rodent midden field collection, lab processing, sample curation, and data archival.

Troubleshooting

Introduction

- 1 Although there was some debate early on about the methods and assumptions of midden analysis, as well as differences with purpose of individual studies, the field has largely conformed to similar methods for field collection in the Americas, including subsampling, specimen processing, sorting, identification, and curation of reference material across labs (e.g., Spaulding et al., 1990). Standardized methods help ensure that midden series are generally comparable across laboratories and studies, but they must be revisited and tested to ensure information in middens remains accessible and uncontaminated. Uniformity and understanding standardized methods are especially important as new researchers engage in midden research and in anticipation of ongoing advancements in microscopy, biogeochemistry, genomics, bioinformatics, and ecological and evolutionary theory and modeling. In addition to tapping existing archives of dated midden materials for ancillary studies, answering new ecological and evolutionary questions will spur new field campaigns and customized sampling protocols. This primer seeks to provide a general workflow (Figure 1) and update best-practice field and laboratory standards, with expected iterations, in the use and preservation of middens moving forward.

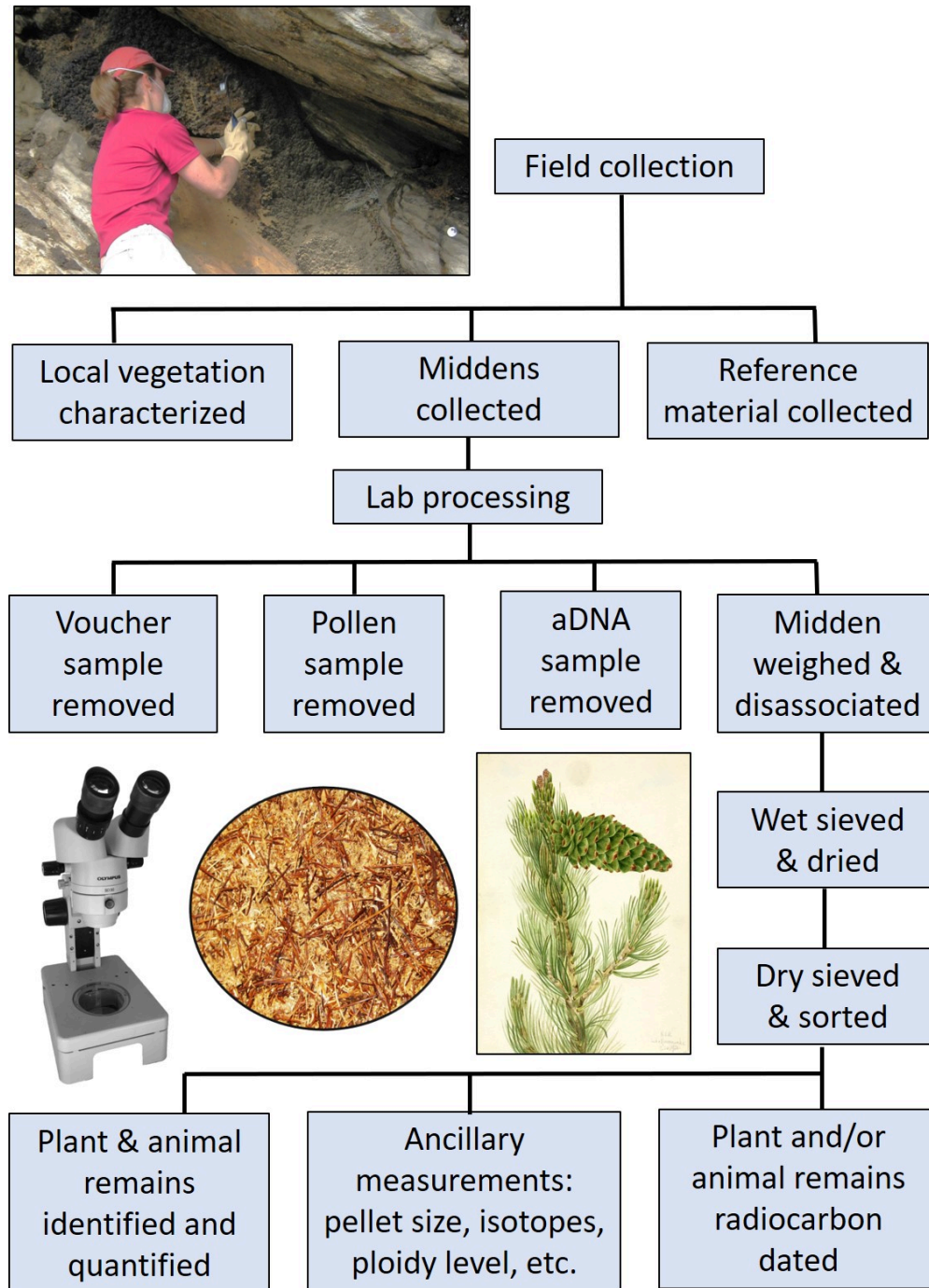


Figure 1. General workflow for field collection and laboratory processing of middens. Top left photo shows a midden being collected at City of Rocks National Reserve, Idaho. Lower photo panel depicts a microscope, a view of midden material from City of Rocks with *Pinus flexilis* needles (limber pine) at low magnification, and a watercolor depicting a *Pinus flexilis* branch and cone. *Pinus flexilis* watercolor by Mary Vaux Walcott, Public domain, via Wikimedia Commons; microscope image by Sarah Greenwood, CC BY-SA 4.0 via Wikimedia Commons.

Field Collection

- 2 Early midden series were typically collected by one or just a few researchers, and due to processing (i.e., labor) and ¹⁴C dating costs, were limited to one or two dozen middens. Over the past three decades, focused field campaigns have relied on larger field crews (often 6-10 people) collecting 50-100 or more middens from a specific area or region in a few days or weeks. Some notable examples include Latorre et al (2002); Lyford et al (2003); Jackson et al (2005); Holmgren et al (2006; 2014); and Norris et al (2016). Pretreatment of materials for Accelerator Mass Spectrometry (AMS) ¹⁴C dating by midden researchers reduces costs and makes dating a larger number of middens more economical (Betancourt, 2004). Still, one of the biggest challenges in fieldwork is finding areas that preserve a suitable number of fossil middens for detailed midden series.

Finding middens in the field invariably involves prior experience and some trial and error, so reconnaissance to identify areas with suitable rock outcroppings and good midden potential is advisable before engaging a larger field crew. Middens can be found in caves and rock shelters and crevices in different substrates (Figure 2); the diversity of rock types yielding older and abundant middens increases with aridity, but usually come from canyons and escarpments with more resistant bedrock like granites, basalt, and well-consolidated limestones and sandstones. Consulting geological and topographic maps can aid in targeting areas, as can talking to local landowners and managers who may be familiar with the location of middens. Predictive GIS models for locating middens are rare. One exception is Mensing et al. (2000), who used a weights-of-evidence method, borrowed from mineral exploration, that relied on geology, elevation, and aspect to determine areas with high midden probability in central Nevada. Still, there is no substitute for driving around a region of interest, inspecting the slopes with binoculars (which may help distinguish between shadows and holes), and hiking up to check rock cavities and contacts to identify areas to comb later with a larger field team. Field campaign length depends upon the amount of area to be covered and size of the field team. Key steps are outlined below.



Figure 2. Examples of middens in the field. Top: midden from shelf at contact zone between rock layers, La Jara Canyon, Colorado. Bottom: midden from granite inselberg, Joshua Tree National Park, California.

- 3 Characterizing the local vegetation. Characterizing the plants growing in the area today, both locally at the midden collection sites and within the overall region, is key to establishing the midden-vegetation relationship and assessing changes in species composition in middens through time. For example, midden-vegetation comparisons can help determine if extralocal species detected in middens are found in close proximity today or if their appearance or disappearance in fossil middens indicate a larger displacement downslope or regional migration.
- 3.1 Plants growing within ~50 m of each midden (i.e., the foraging distance of the rodents) should be documented. A description of the vegetation using five dominance classes (1 = rare, 2 = more than one plant, 3 = subdominant, 4 = common, 5 = dominant) is typically recorded, along with approximate ground cover of dominant species.
- 3.2 Because midden preservation spanning more than a few years or decades is restricted to rocky hillslopes, inferences about past vegetation should pertain only to these habitats. Still, to provide a more complete picture of vegetation in an area, nearby species found at higher or lower elevations along hillslopes, on different aspects, substrates, or growing in the valley floor where middens are not preserved should also be recorded. Here relative abundance, general dominance classes, or percent cover should be recorded.
- 3.3 A list of plants growing in the overall region should also be compiled. Field identifications may be supplemented with herbarium specimens and vegetation lists to capture winter

vs. summer annuals, ephemeral plants, etc. Visiting the field site to identify plants during various times of the year is ideal, but not always feasible.

- 4 Collection of reference materials. Identification of fossil materials relies heavily on reference materials from extant vegetation. Collection of plant materials from the study area is frequently done in concert with activities for characterizing the local vegetation and midden collection.
 - 4.1 Paper coin envelopes are the mainstay for collecting smaller plants and plant parts in the field. These hold sufficient material for reference purposes and are easy to transport and store. Furthermore, the paper helps to draw out moisture and prevent decay. Seeds, fruit, florets, flowers (especially for Asteraceae), needles, stems, and leaves are all valuable for reference.
 - 4.2 Additionally, entire plants may be collected and pressed in the field, especially if identification is not possible without taxonomic keys.
 - 4.3 Some herbariums allow minimal sampling of plant fragments to supplement reference collections.
 - 4.4 Collection site location information should be documented for all of the above, as well as the collector and any relevant herbarium ID numbers.
 - 4.5 Plant reference materials can be stored long-term in the lab in their paper envelopes. These can be placed into small plastic bins to protect them from water damage or pests. They can also be transferred to plastic vials and arranged into cabinets, although this requires more storage space.
- 5 Collection of middens.
 - 5.1 Assign teams of two or three individuals to areas of a slope or boulder field to systematically cover areas of interest. Ideally, an experienced team member will accompany each group, especially for the first few days to train others and ensure proper collection.
 - 5.2 Once a midden is located, data collection should proceed using field forms that include:
 1. Project name
 2. Date
 3. Site Name
 4. Midden number
 5. Latitude, Longitude, and Elevation from GPS
 6. Slope aspect and angle
 7. Lithology

8. Collector(s)
9. Comments and sketches, including direction to the site, notes of midden location and orientation, location relative to other middens, and a sketch of midden and context. Digital photos can also be use to supplement the sketches
10. Modern vegetation within ~50 m of the midden. This should include plant taxa as well as approximate ground cover of dominants. Dominance classes are also used where 1 = rare, 2 = more than one plant, 3 = subdominant, 4 = common, and 5 = dominant.
11. Notes on nearby vegetation, but from >50 m. These observations can supplement the overall characterization of vegetation at a site.

- 5.3 Midden identification is done using numbered metal tags distributed to teams and hammered into the rock using masonry nails to mark the collection location. All tags should have unique numbers to ensure each midden has its own designation. Fixing a nail into a crack or small fissure is usually necessary; loose tags tend to be moved by animals, particularly avid collectors like packrats.
- 5.4 Middens should be examined carefully to ensure they represent a single time unit. If a midden has a layered appearance, contains an internal weathering rind (see also #7 below), or appears as multiple chunks in very close proximity that may or may not comprise a single midden, layers/chunks should be collected separately and a letter added to the number for each (e.g., #1A, 1B, 1C). This is preferable to giving them individual numbers so that it is clear once back in the lab that these may or may not represent a single midden. Given the expense of radiocarbon dating, if contents appear identical in the lab after washing, radiocarbon dating of more than one layer/chunk may not be warranted.
- 5.5 Ideally, a midden sample approximately the size of a loaf of bread or shoebox should be collected to provide enough material for analysis and vouchers, while leaving enough material in situ for the future whenever possible.
- 5.6 Middens are often indurated and the urine matrix may form a strong bond with rock walls. A flooring chisel and rock hammer are used to free the midden sample. Dust masks (KN 95 or N95) and safety glasses are advised to protect against dust and fragments of midden and rock.
- 5.7 The outer dusty, tar-like weathering rind (Figure 3a) should be removed to the extent possible in the field. This weathering rind forms when the amberat matrix is subject to humidity and can incorporate modern contaminants. The weathering rind is removed with the flooring chisel (and hammer when necessary) to scrape and chop off the other rind, taking care to also remove any fresh, modern vegetation adhering to the surface. In the case of middens that can only be removed in blocky pieces, waiting to remove the weathering rind in the lab is an option.
- 5.8 The midden should be placed into a heavy polyethylene bag along with an index card with the midden site name, number, collectors, and whether or not it was cleaned in the field. The bag is then folded around the midden and wrapped generously to protect it

during transportation. The midden site and number should be labeled with a permanent marker on the outside Figure 3b.



Figure 3. a) Midden showing weathering rind along top and sides, lower inner material with juniper seeds. b) Middens wrapped in plastic for transportation.

Lab Processing

- 6 In the past, lab processing has varied among labs. Some researchers merely identified macrofossils visible on the outer surface of middens, while others soaked, disaggregated, and sorted the bulk of the middens with vouchers and other subsamples retained. For anyone attempting aDNA or other sensitive chemical analyses, it is critical to note that most middens are soaked and rinsed in tap water then dried in low-temperature ovens (generally 25°C or less but exact temperatures have rarely been recorded). Thus, preservation of genetic or other material may have been compromised. Sensitive analyses should utilize unprocessed midden subsamples, with aDNA subsamples becoming a standard part of processing as discussed below. A general workflow for lab processing can be found in Figure 1 and illustration of specific steps in Figure 4.
- 7 After cutting through the plastic and tape with scissors, the midden should be inspected again for layers (see #4 above under "Collection of Middens") and any remaining weathering rind removed to the extent possible (Figure 4, top left).
- 8 Although beyond the capabilities of many researchers and institutions, the use of X-ray computed tomography (CT scan) or other internal imaging technique to record midden structure prior to processing could be explored, in the event that this structure proves informative (e.g., identify areas of remixing).
- 9
 1. Small pollen, aDNA, and voucher samples (typically the size of a golf ball to tennis ball for pollen and aDNA and a softball for the voucher) should be removed from the midden before the rest of it is washed. In the case of very small middens and depending on study goals, these subsamples may be omitted. Because midden vouchers represent all remaining unwashed material for any new techniques or analyses to draw on in the future, it is critically important that the intact voucher

midden is preserved for future work as much as possible. Vouchers should be stored in sealed polyethylene (PE) bags with labels inside, and the bags then stored inside high-density polyethylene (HDPE) bins or pails for protection from dust, moisture, pests, and light.

- 10 After pollen, voucher, and aDNA samples are removed, the remaining midden should be weighed, then placed into a labeled plastic bucket with lid (Figure 4, top right) and filled with tap water to dissolve the urine matrix and release fossils. Given the copious amount of water needed for soaking and rinsing middens, the use of deionized or ultra-pure lab water is prohibitive.
- 11 Once middens have soaked long enough to dissociate (typically a few days to a week), they are wet sieved through stacked 2-mm (#10) and 0.5-mm (#35) metal mesh screens. The finer mesh screen ensures small seeds, bones, or arthropods are captured. Midden materials need to be poured in gradually and with no more than ~3 mm deep to allow for adequate washing. This avoids the foamy water that forms when washing overtopping the sieves. A spray nozzle or tubing can be used to direct water (Figure 4, bottom left) and should be gentle enough that it does not break up the wet pellets or destroy plant and arthropod remains. Materials should be washed until the water is clear and no longer foamy and runs clear. At this point, the #10 and #35 screens are turned out onto labeled paper plates. In most cases, this will need to be repeated multiple times for each midden.
- 12 Paper plates may be left out to air dry (Figure 4, center) or placed into a very low temperature oven (25°C). This temperature is generally below temperatures the materials would have been subjected to in nature. Higher temperatures that may degrade aDNA should be avoided.
- 13 Dried materials are again sieved using 2-mm (#10), 1-mm (#18), and 0.5-mm (#35) mesh screens to separate plant, bone, and arthropod fragments by size and facilitate sorting.
- 14 Plant or animal remains are removed for radiocarbon dating. For conventional dating, ~10 g is used. For Accelerator Mass Spectrometry (AMS) dating, ~3 mg is generally sufficient.
- 15 Materials are sorted, identified using a binocular stereozoom microscope (Figure 4, bottom left), and quantified. For plant macrofossils, there has been a profusion of categories used by different researchers for subfossil quantitation. Although the number of identified specimens (NISP) is the most information-rich method, it is extremely labor and time intensive. Coupled with the fact that midden materials are subject to collection bias by the rodents, the use of relative abundance is generally favored by researchers. This allows researchers to pull example fragments only for very abundant species and scan the rest of the midden for new or less abundant species. A typical relative abundance scale used is from 0 to 5 in which 0=0, 1=1, 2=2-24, 2.5=25-49, 3=50-74, 3.5=75-99, 4=100-149, 4.5=150-199, 5=200+ fragments.

- 16 Ancillary measurements (aDNA, arthropods, bones, isotopes, pellet size, ploidy level, pollen, etc.) should follow guidelines published elsewhere as appropriate for the measurement type.
- 17 Macrofossils are then quantified and stored in plastic vials and/or gelatin capsules.
- 18 f mixing is suspected, such as in the case of an incongruous species occurrence, additional AMS dates on the materials may allow for resolution if sufficient material exists.
- 19 Lastly, in regions where more than one rodent species may contribute to middens over time, the midden agent species should be identified using pellet morphology and aDNA to aid interpretation of data and potential depositional biases. In most cases this will necessarily first involve sequencing small mammal DNA from modern museum vouchers at highly variable mtDNA loci, developing targeted mini-barcodes with the power to differentiate candidate species, and building a reference library of variation within and between species by depositing sequences on GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and BarCode of Life when applicable (<http://www.barcodinglife.com/>). For vertebrates, the standard barcode locus is a 650 base pair section of the mitochondrial COI gene (<https://www.ncbi.nlm.nih.gov/genbank/barcode/>). With a sufficient genetic reference library available, it would be the researcher's choice to pursue barcoding of the midden DNA via traditional PCR and Sanger sequencing (pro: relatively simple and cheap; con: requires good aDNA fragment length and/or quantity) or next generation sequencing with or without target enrichment (pro: more effective for degraded or low-quantity aDNA; con: more expensive and complicated both methodologically and bioinformatically). While sequencing technology is a quickly evolving field, even new approaches that emerge will be faced with the same challenge of short, degraded DNA fragments.



Figure 4. Midden processing steps illustrated.

Curation

- 20 Archiving middens should be a key priority as it provides materials for additional morphological, isotopic, geochemical, aDNA, and trait-based analyses. Archived materials also allow for additional radiocarbon dating and cross-checking of identifications. Acknowledging that it will be impossible to implement the following immediately across midden collections, we have several recommendations for curation aimed at enabling future analyses. Many of these are common to the curation of organic specimens.
- 21 Middens should be stored separate from modern specimens or samples that could contaminate them, and away from labs that have dealt with radioactive substances that might prohibit ^{14}C dating in the future.
- 22 Middens should be stored in sealed polyethylene bags (PE) and nested inside high-density polyethylene (HDPE) bins or pails for protection from dust, moisture, pests, and light.
- 23 Consistent cold storage ($4\text{--}10\text{ }^{\circ}\text{C}$) to avoid molecular degradation would be ideal. However, given the large amount of storage space required for midden, this is not feasible for most materials. Nevertheless, storing in a temperature-controlled environment to avoid high humidity and freeze-thaw cycles is recommended.



- 24 The integrity of labels should be considered. Permanent markers on gelatin capsules can degrade from oils transferred from the skin and may need to be refreshed after being handled. Similarly, permanent markers may fade over time from plastic bags. Additional printed paper labels placed within vials and bags is recommended. Samples should be periodically checked for fading and integrity of storage materials.

Data and Publication

- 25 Future midden publications should be very explicit about the methods used to process middens. Uploading new publication information into the Neotoma Database Publications group library (<https://www.zotero.org/groups/2321378/neotomadb/library>) is encouraged.
- 26 Data should be archived and accessible to the global community at the time of publication. Plant macrofossils present the most comprehensive dataset in the USGS/NOAA North American Packrat Midden Database (2022, <http://geochange.er.usgs.gov/midden/>), which includes information from hundreds of midden publications. Work is also underway to integrate midden data with other types of paleoecological data through the Neotoma Paleoecology Database (NeotomaDB, <http://www.neotomadb.org>).
- 27 For the USGS/NOAA North American Packrat Midden Database, standards are published in a data dictionary (<https://geochange.er.usgs.gov/midden/ofr-01-0022.pdf>). Going forward, data should be compatible with NeotomaDB and Darwin Core to facilitate data sharing and discovery.
- 28 Informatic challenges include cataloging—whether by individual specimens or groups of specimens (e.g., of the same species or morphotype), imaging, and keeping pace with taxonomic updates. The latter requires linkages between databases, an issue that other groups [VertNet, GenBank, etc.] are working to resolve.

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