AN ABSTRACT OF THE DISSERTATION OF

Orin C. Shanks for the degree of Doctor of Philosophy in Genetics presented on February 28, 2003.

Title: DNA Recovery and Identification from Stone Tool Microcracks

Abstract Approved

Redacted for Privacy

Lloyd W. Ream

The studies described here introduce a model for residue preservation on stone tools. They simulate stone tool manufacture in order to define parameters important for the study of DNA residues. Microscopic examination of stone tools has identified microcracks that trap DNA and protein from animal blood cells. Thorough investigation of different methods to recover residues from stone tools shows that surface washing leaves DNA and protein, trapped in subsurface microcracks. However, other extraction techniques are able to release 60-80% of DNA and protein residues.

Previous research documents the identification of protein from stone tools sonicated in 5% ammonium hydroxide, but it remains untested whether the same treatment yields useable DNA. Using this treatment, I identified 13-year-old DNA residues from experimentally manufactured stone tools. In addition, results clearly indicate that washing procedures typically used to curate stone tools removed only a small fraction of the DNA deposited during animal butchery.

Twenty-four pieces of chipped stone recovered from the Bugas-Holding site were studied to explore the validity of ancient DNA residue identifications. Nine tools

yielded DNA residues. Modern humans did not touch three of these tools, which suggests that the DNA recovered from them was present prior to excavation. One tool, which was handled by excavators without gloves, harbored DNA from three species, and these templates competed during PCR. On at least two tools, handling after excavation introduced animal DNA unrelated to tool use. Careful testing of Bugas-Holding chipped stone suggests that stone tools may harbor both ancient and modern DNA, and that investigators must take great care to exclude modern DNA from ancient specimens.

Ultimately, I developed and streamlined a method to analyze DNA-containing residues preserved on stone tools. This led to several technical improvements in ancient DNA residue analysis. These include a more effective DNA recovery protocol, methods to measure sensitivity and inhibition of PCR in each sample, and strategies to surmount competition between templates during amplification, which can occur in samples that contain DNA from multiple species. These new developments will help future investigators achieve the full potential of ancient DNA residue analysis.

DNA Recovery and Identification from Stone Tool Microcracks

by

Orin C. Shanks

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Head of the Genetics Program

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Orin C. Shanks, Author

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CONTRIBUTION OF AUTHORS

Chapter 2:

Dr. Robson Bonnichsen was cofounder of the "microcrack" model; he manufactured obsidian microblades, reviewed an earlier version of the manuscript, and supplied archaeological expertise. Dr. Anthony T. Vella provided useful discussions on fluorescence protein labeling and instruction in laboratory methods. Dr. Walt Ream was the principal investigator, helped compose manuscript, and secured funding for the project.

Chapter 3:

Dr. Marcel Kornfeld provided useful discussions; he manufactured experimental stone tools, butchered an animal with the same stone tools, stored the tools for 13 years, and reviewed an earlier version of the manuscript. Dr. Walt Ream was the principal investigator, helped compose the manuscript, and secured funding for the project.

Chapter 4:

Larry Hodges was a full-time laboratory technician on this project. He played an integral role in extraneous DNA precaution method designs, he helped build the DNA-free glove boxes, he purified the *taq* DNA polymerase, prepared silica used in DNA purification, performed DNA analysis on six bone samples and two stone tool artifacts, and reviewed an earlier version of the manuscript. Lucas Tilley was an undergraduate laboratory assistant on this project. He performed colony hybridization experiments on a stone tool DNA extract and provided assistance with screening transformants for canid sequences by PCR. Dr. Marcel Kornfeld was a source of archaeological expertise; he participated in the Bugas-Holding site excavations, supplied me with chipped stone for DNA analysis, proposed the "debitage" hypothesis, and reviewed an earlier version of the manuscript. Dr. Mary Lou Larson

was also a source of archaeological expertise; she participated in the Bugas-Holding site excavations, provided Bugas-Holding assemblage database files and spatial maps, and reviewed an earlier version of the manuscript. Dr. Walt Ream was the principal investigator, helped compose the manuscript, and secured funding for the project.

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This project is dedicated to my family.
To my sons, Brennon and Riley, who always make me smile and
remind me everyday what really matters.
And to my wife Trena, a final word of thanks.
Your love, support, and hard work have sustained me throughout this project,
without you it simply would not have happened.

DNA Recovery and Identification from Stone Tool Microcracks

Chapter 1

General Introduction

Orin C. Shanks

Identification of DNA residues in archaeological samples, including stone tools and bones, is the subject of this dissertation. Recovery, amplification, and sequence analysis of ancient DNA extracted from bone (25, 44, 64), soft tissue (28, 69), and coprolites (72) has allowed genetic characterization of animals present at archaeological sites. Organic residues on stone tools are another source of animal remains found at archaeological sites. Ancient DNA often shows extensive damage (51, 64, 69, 73). However, small regions of DNA have been recovered from ancient samples thousands of years old (9, 31, 72, 93). Thus if preservation is appropriate, DNA in ancient samples is accessible to genetic analysis.

Biological Residues on Stone Tools

Stone tools are commonly found at archaeological sites. They provide information regarding site chronology, manufacturing strategies, material procurement, and subsistence practices. Stone tools are particularly effective indicators of cultural practices when combined with studies of biological residues.

Residue analysis from stone tools offers several advantages over molecular analysis of other biological remains commonly found at archaeological sites. Stone artifacts are among the most well preserved materials in the archaeological record, and in many cases these are the only cultural remains recovered. Stone tools are easily accessible and are not destroyed during residue extraction.

Residue preservation. Traces of ancient DNA and protein preserve on stone tools (12, 27, 61, 88). Current models assume residues persist on surface flake scars produced during tool manufacturing (Figure 1, panel A) (20). As a result, it is common practice to examine stone tools under low power magnification prior to residue analysis (35, 53). However, others observe no correlation between the presence of visible surface residues and the ability to detect residues (62, 71, 83). These observations suggest that sites for residue preservation include subsurface microcracks (Figure 1, panel B).

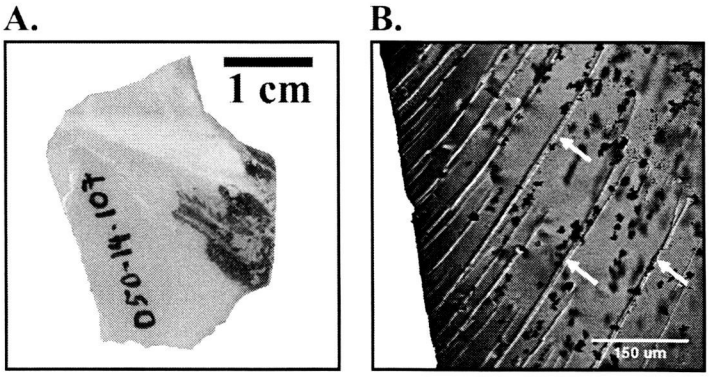

Figure 1: Utilized flake (panel A) with residues preserved on the tool surface and visible light micrograph of a microblade (panel B) with microcracks (arrows).

We propose that residues may preserve on both the tool surface and in subsurface microcracks. Each stone tool contains numerous microcracks (Figure 1; panel B). They are a byproduct of pressure and percussion flaking systems used by aboriginal artesians to fracture and shape fine-grained cryptocrystalline rocks into stone tools (8). These microcracks may fill with blood and tissue when tools are used to kill or process animals. Residues trapped in microscopic cracks may enjoy greater protection from the elements than residues deposited on the tool surfaces. Even tools that have been hand washed to remove sediment may retain biological residues in microcracks.

Residue recovery. The notion that microcracks may sequester ancient biological residues can explain inconsistencies between previous blood residue studies. Several studies report animal protein detection rates between 14-56% on stone tools, despite the absence of visible residues (21, 62, 83, 88). In each case, either guanidine hydrochloride treatments or sonication in 5% ammonium hydroxide were used to recover residues. In contrast, groups using alternative strategies to remove residues from stone tools succeeded less than 2% of the time (17, 35) unless visible surface residues were present (53). These conflicting reports could be reconciled if

microcracks harbor residues and specific treatments such as sonication effectively remove trapped DNA while other strategies do not.

Residue identification. Most residue analyses from stone tools have been limited to immunological characterization of proteins (21, 57, 61, 71, 88). Investigators have employed a number of immunological techniques including counter-immunoelectrophoresis, the Ouchterlony method, and radio, enzyme-linked, or gold immunoassays (16, 17, 62, 88). However, immunological methods cannot distinguish protein residues from closely related species, such as *Canis familiaris* (dog) and *Canis lupus* (wolf), nor can they distinguish subspecies. Also, immunological studies are limited to species for which specific antisera are available. More reliable residue identifications are possible with DNA sequence analysis, which is more sensitive and precise than protein detection.

Research Design

I initiated a series of experiments to: 1) test the "microcrack model" of residue preservation, 2) determine the most efficient strategy to release residues from stone tools, 3) establish an ancient DNA methodology, 4) demonstrate recovery of useable DNA from experimentally generated stone tools, and 5) test the validity of ancient residue identification from stone tools.

Microcrack model for residue preservation. We tested the hypothesis that microscopic fissures below artifact surfaces can trap biological residues. Obsidian microblades (Figure 2), which are small straight-sided flakes at least twice as long as they are wide, were produced and used in place of ancient tools to learn how microcracks absorb and retain residues. We measured the width and depth of microcracks and demonstrated their ability to trap DNA and protein from blood cells.

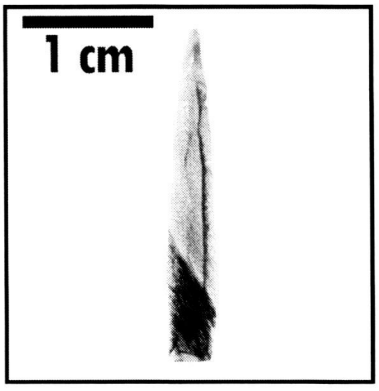

Figure 2: Photograph of an obsidian microblade.

Recovery of residues. Residue recovery from stone tool artifacts is critical to a successful residue analysis. If no residues are recovered, then even the most sensitive and sophisticated detection assays will fail. In earlier studies, protein residues were removed from stone tools by either surface washing with distilled water or buffered saline (35), soaking in 4M guanidine hydrochloride (87), or sonication in 5% ammonium hydroxide (62). To compare the effectiveness of these procedures, we used confocal microscopy to measure the release of fluorescently labeled DNA and protein from white blood cells lodged in obsidian microblade microcracks. First, all microblade surfaces were cleaned with distilled water or buffered saline (35). Second, trapped residues were documented by confocal microscopy. Third, microblades subjected to either 4M guanidine hydrochloride or sonication treatments or left untreated. After extraction, each microcrack was re-examined by confocal microscopy resulting in a series of confocal micrographs taken in one to five micron depth increments, both before and after extraction. We compared the fluorescence before and after extraction at a specific depth in each microcrack to estimate the fraction of residue removed by the treatment.

Ancient DNA methodology. Ancient DNA analysis presents a conundrum to the molecular biologist. DNA from aged samples is rare and often highly degraded (51, 64, 69, 73). Thus, ancient DNA detection requires a PCR assay that can amplify small

quantities of template DNA. The problem is that exceptional PCR sensitivity is achieved at the expense of detecting extraneous DNA templates. We targeted mitochondrial markers to enhance PCR detection of rare templates. To recover DNA from sample extracts, we used a series of precautionary steps that limited PCR contamination.

Marker selection. We examined mitochondrial DNA because eukaryotic cells usually contain hundreds of mitochondria and thousands of copies of the mitochondrial genome. In contrast, most animal cells contain a single nucleus and often just two copies of a particular gene. Therefore, due to its abundance, we are much more likely to recover mitochondrial DNA than a particular nuclear gene. Mitochondrial DNA normally evolves rapidly at the DNA sequence level and usually provides enough variation to make species identifications (91). Mitochondrial genes are also suitable for species identification because they are not affected by heteroplasmic substitutions and variable repeats found in rapidly evolving mitochondrial intergenic regions (10, 13). Finally, there is a considerable database available for sequence comparison.

Precautions to reduce extraneous DNA. Contamination with modern DNA is a significant problem in ancient DNA research (14, 67). DNA from equipment, other samples, and previously synthesized amplicons can contaminate PCR reactions. We limit extraneous DNA from these sources with physical barriers. DNA extractions, PCR cocktail assembly, PCR amplifications, and DNA sequencing occur in four separate buildings (Figure 3). We used separate glove boxes to mix reagents (Figure 3, box 1), recover DNA (Figure 3, box 2), concentrate extracts (Figure 3, box 3), set-up PCR reactions (Figure 3, box 4), and add template to PCR reactions (Figure 3, box 5). All glove boxes were equipped with an ultraviolet (UV) light source, an antechamber, and neoprene gloves to provide a DNA-free workspace (Figure 4). In addition, glove boxes contained dedicated equipment (see Appendix I). After each use, glove boxes were cleaned with 10% bleach and irradiated with UV light to

inactivate extraneous DNA templates (90), and equipment was treated with 10% bleach or 3% hydrogen peroxide (for bleach sensitive material), and ethylene oxide gas (65). Items (other than samples) that entered a glove box were cleaned with bleach, stored in sealed plastic bags, which were bleached (outside) before entry to and removal from the glove box antechamber (Figure 4).

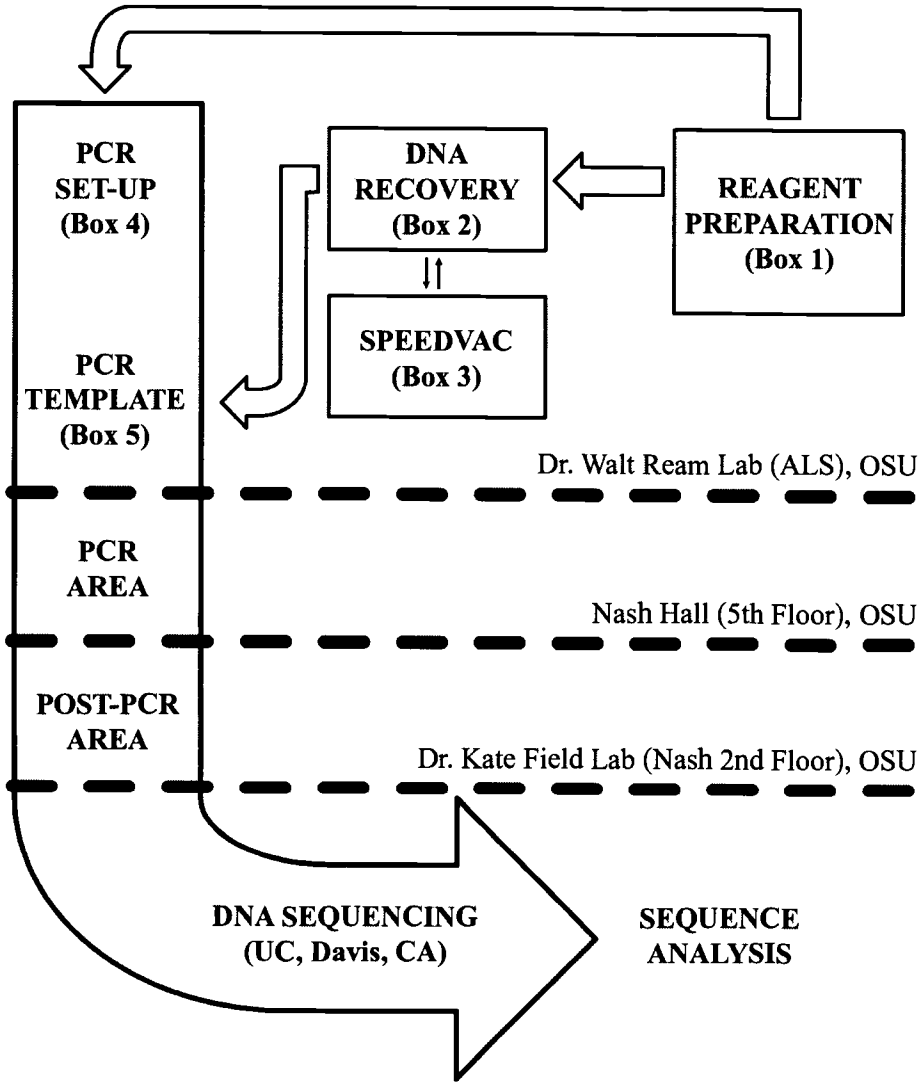

Figure 3: Physical barriers and uni-directional progression of analysis used in ancient DNA studies. A (box #) indicates a dedicated glove box workspace (Figure 4). Arrows indicate movement of reagents or samples and dashed lines indicate physical barriers.

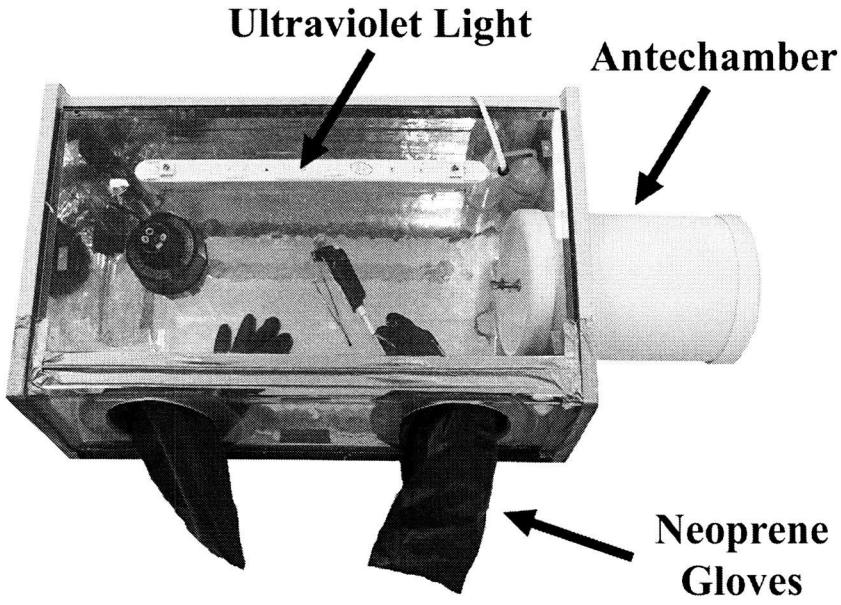

Figure 4: DNA-free glove box workspace used in ancient DNA studies. Arrows indicate location of UV light source, antechamber, and neoprene gloves.

Glassware and stainless steel instruments were baked for 12 hours at 230°C. Aerosol-resistant pipette tips were used for all liquid transfers, and dedicated pipettors were used for each operation. After use, pippettors were disassembled, cleaned with 3% hydrogen peroxide, and treated with ethylene oxide gas.

Contamination from previously synthesized amplicons is another potential problem that is avoided by physical separation. However, we substituted dTTP (2'-dexoythymidine 5'-triphosphate) with dUTP (2'-deoxyuridine 5'-triphosphate) in all PCR reactions in the event PCR amplicons from one reaction contaminated another reaction. Because PCR amplicons contained uracil instead of thymine, treatment with uracil-N-glycosidase (Ung) could inactivate these modern templates prior to amplification. Ung can then be inactivated by the addition of heat-stable Ung-inhibitor protein (Ugi) (52).

In addition to physical barriers, ancient DNA analysis progressed in one direction (Figure 3). Uni-directional progression of artifact analysis prevented backtracking of purified DNA and PCR amplicons from sample amplifications into

dedicated reagent, DNA recovery, sample concentration, PCR set-up, and PCR template areas. We also processed only one sample and mock extract each day to avoid cross-contamination between samples.

PCR reactions may also amplify modern DNA present in extraction and PCR reagents, which cannot be eliminated with physical barriers. Reagents were opened only in a dedicated reagent glove box (Figure 3, box 1) and were used exclusively for ancient DNA analysis. Whenever possible, reagents were manufactured in our lab using the same precautions used for ancient DNA analysis.

To screen for extraneous DNA in PCR reagents, we performed at least 20 no-template PCR reactions with the reagents prior to the initiation of a study. For each sample analyzed, we performed one mock DNA extraction, purification, and amplification using buffer alone. Mock extract controls determined whether plasticware, filters, extraction reagents, and handling procedures introduced extraneous DNA into PCR reactions. For every PCR reaction containing extract from a sample, we performed 4-10 no-template PCR reactions with purified water substituted for template DNA. Thorough routine testing was time consuming and expensive, but it was the only way to ensure that DNA detected from sample DNA extracts are genuine.

Recovery of useable DNA from experimentally generated stone tools. Rigorous tests comparing different methods to release residues from stone tools indicate that most commonly used residue extraction techniques leave DNA and protein behind, lodged in subsurface microcracks (Chapter 2). However, the use of sonication bath can release 60-80% of DNA and protein residues (Chapter 2). Previous research documents the recovery of protein from stone tools sonicated in 5% ammonium hydroxide (21, 62, 71), but it was unknown whether the same treatment allows recovery of useable DNA for residue identifications.

We identified DNA residues deposited on experimentally generated stone tools used to butcher a single animal. The species of the butchered animal was not disclosed until after we completed the residue analysis. Thus, analysis of

experimentally generated stone tools allowed us to authenticate our findings, which is not possible using excavated stone tools. In addition, we investigated the effects of standard artifact cleaning procedures used by archaeologists, because the majority of stone tools available for residue testing have been washed. Rigorous cleaning protocols used during tool curation (38, 80, 81) call for treatments that may interfere with DNA and protein recovery. These experiments laid the groundwork for a case study with ancient stone tool artifacts.

A case study. To test the validity of ancient DNA residue analysis, we examined DNA residues on 24 pieces of chipped stone excavated from a single well-characterized archaeological site, Bugas-Holding, Wyoming. We compared chipped stone touched by excavators with untouched tools to determine whether post-depositional artifact handling could introduce animal DNA residues unrelated to tool use. Our analysis included sediments and flakes lacking evidence of use, which served as controls for the presence of contaminating DNAs in the depositional environment. We also compared DNA preservation in bones and on stone tools from the same stratigraphic context. Finally, we introduced several methodological improvements for DNA residue analysis including a more effective DNA recovery protocol, methods to measure sensitivity and inhibition of PCR in each sample, and strategies to surmount competition between templates during PCR amplification.

The Bugas-Holding site is situated in northwestern Wyoming near the eastern border of Yellowstone National Park. Charcoal samples from the cultural layer yielded dates of 380 $\pm$ 100 BP, 490 $\pm$ 80 BP, and 200 $\pm$ 60 BP (74) placing the time of occupation during the Late Prehistoric Period (19). The site represents a single continuous winter occupation (74). Human activities within the excavated area include animal processing, storage, and consumption (6, 48, 74, 83). The archaeological record at Bugas-Holding consists of features (Figure 4; hearths and midden), chipped stone, ground stone, fauna, pottery, and ornaments. The fauna include bison (*Bison bison*), bighorn sheep (*Ovis Canadensis*), elk (*Cervus elaphus*),

pronghorn (*Antilocapra americana*), grizzly bear (*Ursus arctos*), and canid (*Canis lupus* and/or *Canis familiaris*) (74).

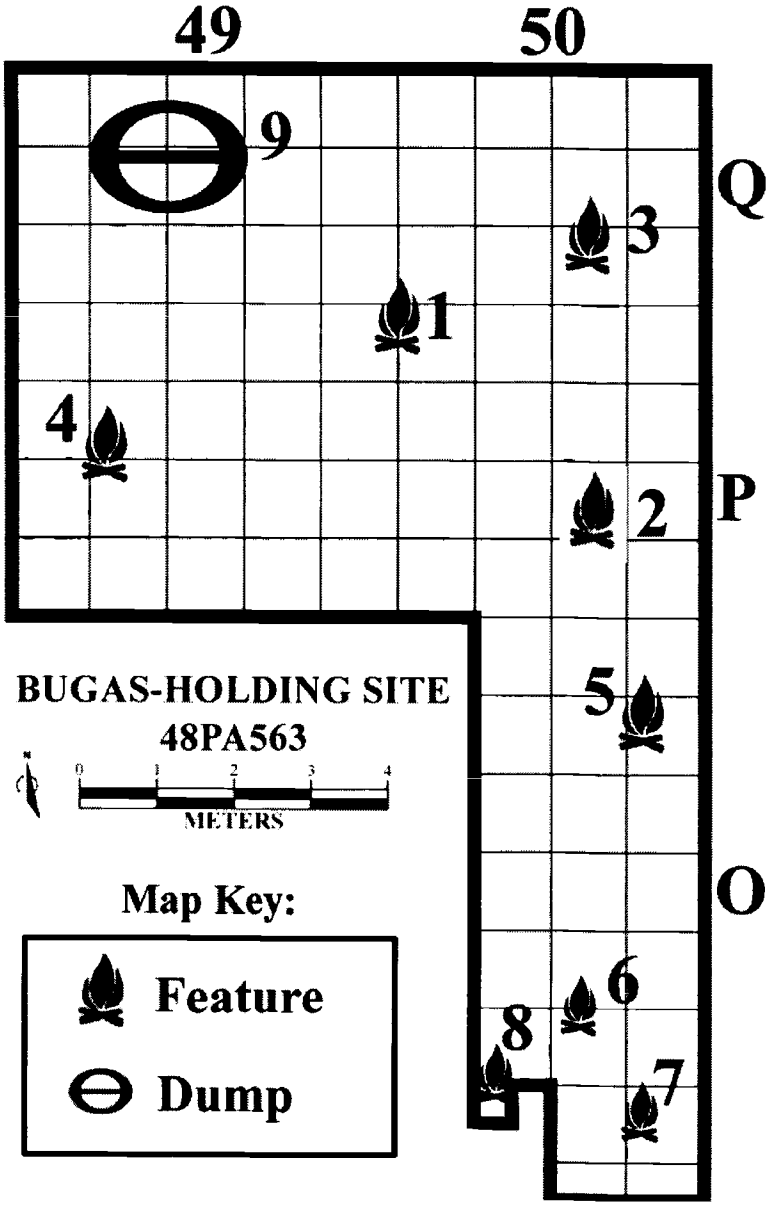

Figure 5: Spatial distribution of Bugas-Holding features and dump area (adapted from [83]).

The Bugas-Holding site was selected for analysis because it provided a large artifact assemblage of chipped stone, animal remains, and sediment samples. Previous

studies report residue preservation on stone tools (6, 83), and fine-scale excavation methods provided a well-documented history of artifact handling and storage. Many stone tools were collected by excavators wearing rubber gloves. Untouched tools were sealed in plastic bags to prevent contamination. Because site activities were established (2, 6, 34, 48, 59, 74, 83), our case study was designed to test and extend previous work where there is little controversy, thereby allowing us to test the validity of DNA residue identification.

Summary of Findings

Microscopic examination of experimentally generated stone tools has identified microcracks that trap DNA and protein from animal blood cells (Chapter 2). Thorough investigation of different methods to recover residues from stone tools shows that surface washing leaves DNA and protein, trapped in subsurface microcracks (Chapter 2). However, other extraction techniques are able to release 60-80% of DNA and protein residues (Chapter 2). Extensively washed stone tools can harbor biological residues (Chapter 3). Results clearly indicate that surface washing procedures typically used to curate stone tools removed only a small fraction of the DNA and protein deposited during animal butchery. I developed and streamlined a method to analyze DNA-containing residues preserved on stone tools. This led to several technical improvements in ancient DNA residue analysis (Chapter 4). Finally, I studied 24 pieces of chipped stone recovered from the Bugas-Holding site to explore the validity of ancient DNA residue identifications. Careful testing of Bugas-Holding chipped stone suggests that stone tools may harbor both ancient and modern DNA, and that investigators must take great care to exclude modern DNA from ancient specimens.

Chapter 2

Recovery of Protein and DNA Trapped in Stone Tool Microcracks

Orin C. Shanks, Robson Bonnichsen, Anthony T. Vella, and Walt Ream

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Introduction

Until recently, prehistorians seeking to explain stone tool use were restricted to the study of artifact shape and use-wear properties. A new and complementary approach focuses on organic residues extracted from flaked stone tool artifacts. Molecular techniques to identify ancient protein and DNA residues can provide important insights into how prehistoric people used individual tools to process specific animal species.

Traces of organic residues preserve on stone tools (12, 27, 61, 88). Current models assume residues persist on surface flake scars produced during tool manufacturing (20). As a result, it is common practice to examine stone tools under low power magnification prior to residue extraction (35, 53). However, others observe no correlation between the presence of visible surface residues and the ability to detect residues immunologically (62, 71, 83). These observations suggest that sites for residue preservation include subsurface microcracks.

We propose that residues are preserved on both the tool surface and in subsurface microcracks. Each stone tool contains numerous microcracks. They are a byproduct of pressure and percussion flaking systems used by aboriginal artisans to fracture and shape fine-grained cryptocrystalline rocks into stone tools (8). These microcracks fill with blood and tissue when the tools are used to kill or process animals. Residues trapped in microscopic cracks may enjoy greater protection from the elements than residues deposited on tool surfaces. Even tools that have been hand washed to remove sediment may retain biological residues in microcracks. This may explain why procedures that use an ultrasonic bath to release material from stone artifacts are more successful than methods directed toward surface residues (35, 57, 71).

In this study, we test the hypothesis that residues are trapped in microcracks below artifact surfaces. Obsidian microblades, which are small straight-sided flakes at least twice as long as they are wide, were produced and used in place of ancient tools to learn how microcracks absorb and retain residues. We measured the width and depth of microcracks, demonstrated their ability to trap DNA and protein, and

identified effective extraction techniques for DNA and protein residues. These experiments suggest that ancient residues trapped in artifact microcracks represent an important source of data.

Materials and Methods

We manufactured microblades (c. 300mm length x 50mm width x 2mm thick) using the Japanese Yebetsui pressure technique (42). Obsidian microblades were chosen for microcrack characterization for three reasons. First, microblades are small and easy to manipulate during microscopy (Figure 2, Chapter 1). Second, obsidian is translucent, allowing light to pass through the specimen during confocal and light microscopy (Figure 2, Chapter 1). Third, particular flows of obsidian contain fewer includsions than other types of stone used to manufacture tools (78). Many minerals, for example calcite, fluoresce under specific wavelengths of light (78), which can generate signals that interfere with detection of fluorescently labeled protein and DNA.

Obsidian was collected from the Hines Flow and Glass Butte localities in eastern Oregon. Except during their manufacture, all microblades were handled with latex gloves to minimize contamination.

Obsidian microblades were soaked for 24 h in a 2.5% suspension of Fluoresbrite yellow-green latex spheres (Polysciences) of different diameters (1, 3, 6, 10, and 20 μm). After soaking, microblades were dried for 72 h prior to a 5 min wash with water. Fluorescent beads were visualized by fluorescence and confocal laser-scanning microscopy using Leica instruments. Confocal imaging required a custom-built microscope slide holder (OEM, Inc., Corvallis, OR) to hold samples. Optical sections approximately 1 µm thick were recorded in 1 to 10 µm intervals. For each crack, 5 to 20 optical sections were compared.

White blood cells were isolated from 1 ml fresh cow blood and stained with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) as described (56). Red blood cells were lysed by centrifugation at 216 x g for 5 min at 4°C and the supernatant was discarded. Cells were suspended in buffered ammonium chloride (168 mM

ammonium chloride, 10 mM potassium bicarbonate, 0.00035 % phenol red, pH 7.2), incubated at room temperature for 10 min, and centrifuged at 216 x g for 5 min at 4°C. The supernatant was discarded and the cells were suspended in 2 ml of buffered salt solution (BSS; 20 liters of BSS, pH 7.2, contain 1.2 g KH_2PO_4, 3.8 g Na_2HPO_4, 3.8 g $CaCl_2 \cdot 2H_2O$, 8 g KCl, 160 g NaCl, 4 g $MgCl_2 \cdot 6H_2O$, and 2 g $MgSO_4 \cdot 7H_2O$ with glucose at 0.1% final concentration and 0.001% phenol red) and 20 μl of 5 mM CFSE (Molecular Probes) dissolved in dimethylsulfoxide (DMSO) and incubated at 37°C for 30 min. After staining, cells were washed with ice cold BSS and resuspended in 2 ml of ice cold BSS. Each microblade was immersed in 2 ml of CFSE-stained white blood cells and incubated at room temperature for either 5 min or 0.5, 6, 12, or 24 hours. Immediately following incubation, each microblade was washed in 5 ml distilled water for 5 minutes on a rotary shaker at 1,000 rpm, scrubbed by hand (with latex gloves), and dried for 24 h at room temperature. Untreated microblades made from both the Hines Flow and Glass Butte microblade samples were examined to assess auto-fluorescence and contamination due to handling.

Two strategies were used to stain cow blood DNA with 4,6-diamidion-2-phenylindole (DAPI). DNA in CFSE-labeled white blood cells was stained with 10 μg DAPI in 2 ml BSS as outlined above. Alternatively, microblades were soaked in unstained whole blood, washed, and DNA in the blood residue was stained *in situ* with DAPI. Microblades were soaked in 1 ml of a 1:1 mixture of BSS and whole blood in 4.5 mM EDTA. After incubation for 24 h at room temperature without agitation, the microblades were removed and dried for 24 h. Blood-stained microblades were soaked for 24 h at room temperature without agitation in 1 ml of BSS containing 5 μg DAPI. Next, each microblade was washed with 5 ml distilled water as described previously and dried for 24 h at room temperature. Untreated microblades were stained in the same way to test for contamination due to handling and autofluorescence. All steps involving DAPI were performed in a darkroom.

Stained DNA and protein resident in microcracks were detected using confocal microscopy to distinguish between surface and subsurface fluorescence. Each microcrack was divided into sections approximately 2 μm thick to track penetration of

organic residues. The number of sections examined per microcrack ranged from 10 to 30. CFSE-stained protein in cells was visualized with a fluorescein isothiocyanate (FITC) band-pass optical filter, and DAPI-stained DNA was detected with a 440 nm band-pass filter. Dual-channel scanning of labeled DNA and protein was achieved by aligning both filters.

Residues harbored in microcracks were stained with CFSE and DAPI as described above. After surface washing but prior to extraction of subsurface blood residues, the location and quantity of fluorescent material were documented using confocal microscopy. A series of confocal images of the same cracks was taken after extraction of subsurface residues. Magnification, laser level, voltage, pinhole size, offset, and image averaging remained constant between confocal images taken before and after residue extraction. Unstained microblades were examined for autofluorescence and contamination during manufacture. Fluorophore longevity and experimental variation were estimated using stained microblades not subjected to extraction.

Two extraction methods were tested; microblades were (1) soaked in 40 ml of 4 M guanidine hydrochloride, 0.5 M Tris, pH 7.4 with agitation at 900 rpm for 18 h (87), or (2) submerged in 3 ml of 5% ammonium hydroxide and cleaned in an ultrasonic bath for 3 min followed by agitation at 900 rpm for 30 min (62). Immediately following each extraction, microblades were washed for 5 min with agitation at 1,000 rpm in 5 ml phosphate buffered saline (PBS) (Sigma) or distilled water. After washing, microblades were dried at room temperature for 24 h.

Confocal images of a particular crack at a specific depth, taken before and after residue extraction, were aligned using Adobe PhotoShop (version 5.0). Visible light images of DNA and protein lodged in microcracks were converted from an indexed color image to a red/green/blue (RGB) color image and superimposed by matching three reference points, for example microcracks, inclusions, and the blade edge. Fluorescent images were linked to the corresponding light images. Fluorescence from microcracks was measured by enclosing the area of interest inside a computer-generated cropping box. Intensity of fluorescence within a boxed region was

compared, before and after extraction, using IP Lab (version 3.2) for Macintosh (Scanalytics, Inc.). Only red pixels were used for comparisons; the intensity of each pixel was rated from zero (no color) to 255 (color saturation), and the sum of all pixel scores was calculated for each image.

Results

Microcracks produced during microblade manufacture extended below the surface. A light micrograph of a typical microblade showed numerous microcracks perpendicular to the tool edge and several parallel to it (Fig. 6, panel A). Microcracks both on and below the microblade surface diffract light as it passes through the transparent obsidian, causing the striations visible in Figure 6, panel A. A scanning electron micrograph (SEM), which only detects the microblade surface, was taken at the same magnification as the light micrograph and showed that the surface was smooth (Fig. 6, panel B). Comparison of these images indicates that most microcracks lie beneath the microblade surface.

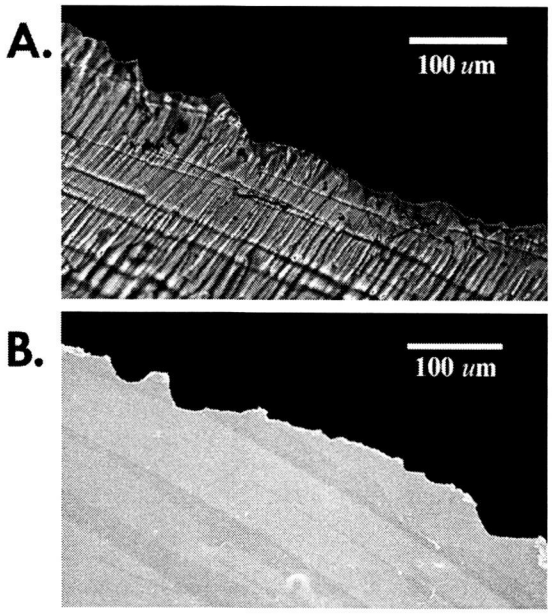

Figure 6: Visible light (panel A) and scanning electron (panel B) micrographs of a microblade surface.

We used fluorescently labeled latex beads to determine whether these microcracks could trap small particles. Beads 1 μm in diameter entered microcracks by capillary uptake (4) and remained trapped during extensive surface washing. Several microcracks were detected by light microscopy (Fig. 7, panel A), and a fluorescence micrograph of the same area showed latex beads lodged in these fissures (Fig. 7, panel B). Fluorescence microscopy cannot distinguish beads trapped on the microblade surface from those resident in subsurface microcracks.

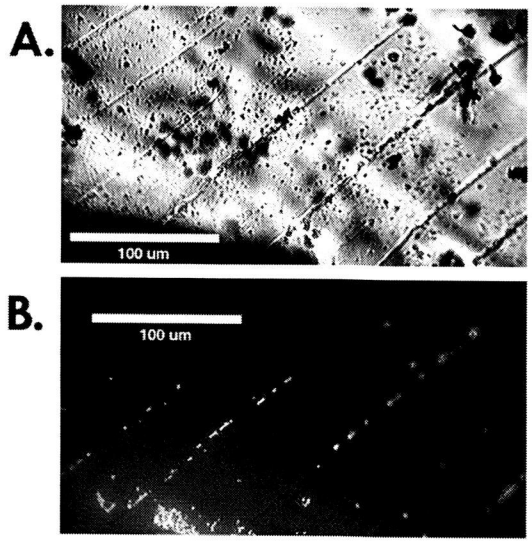

Figure 7: Microcracks in obsidian. Microcracks were photographed under visible (panel A) or xenon laser (panel B) light. Panel B shows 1-μm-diameter fluorescent latex beads trapped in microcracks.

We used confocal microscopy to measure the depth to which latex beads penetrated. Figure 8 shows a series of confocal micrographs of a single microcrack taken at the surface and at depths of 18, 36, 51, and 75 μm below the microblade surface. The 6-μm-diameter fluorescently labeled latex beads were detected at each depth as fluorescent points arrayed in a linear fashion. However, the position of the fluorescence moved from the upper left of the field in the surface image (Fig. 8, panel A) progressively down and to the right as the depth increased (Fig. 8, panels B-E), indicating the direction that the fissure penetrated from its origin on the dorsal surface

of the microblade. A visible light image showing fissures, without regard for their depth below the surface, was superimposed upon a fluorescent confocal image taken 51 μm beneath the microblade surface; the beads were located in a clearly visible microcrack within a relatively planar region of the microblade (Fig. 8, panel F).

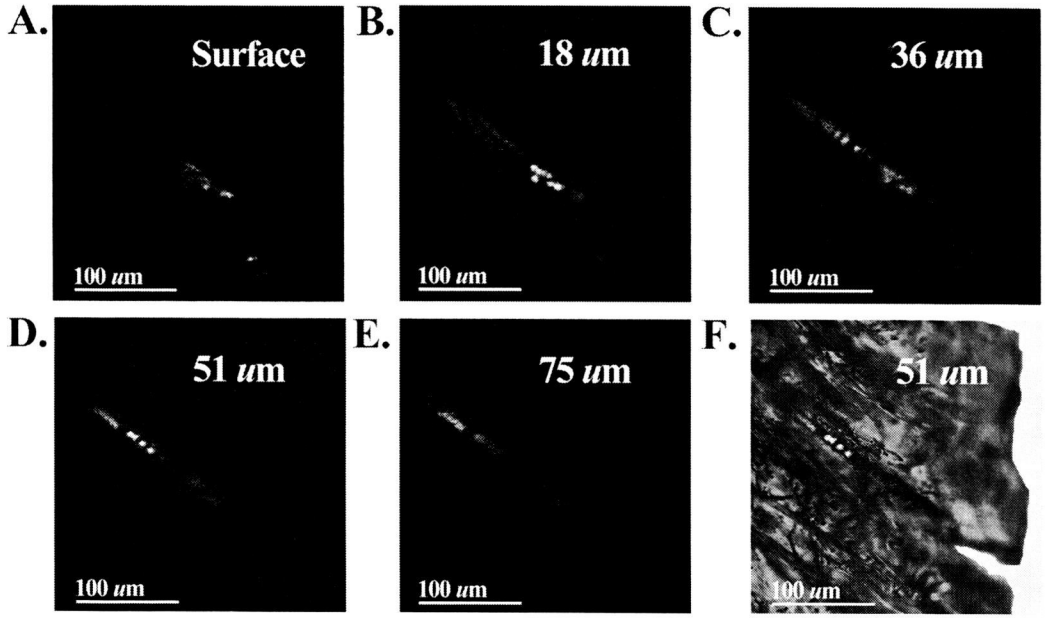

Figure 8: Confocal micrographs of latex beads trapped in a microcrack. Panels A through E show 6-μm-diameter latex beads at the microblade surface and at depths of 18, 36, 51, and 75 μm beneath the surface. Panel F shows a visible light image superimposed on the confocal micrograph taken 51 μm beneath the microblade surface.

The smallest latex beads, 1 μm in diameter, exhibited the deepest subsurface penetration: beads were detected 140 μm below the surface of one microblade (data not shown). Beads 10 μm in diameter - the approximate size of lymphocytes and granulocytes (77) - readily entered microcracks by capillary diffusion and were not removed by surface washing (data not shown). In contrast, 20 μm beads were rarely retained on microblades after washing (data not shown). Microblades that were not soaked in a suspension of fluorescent latex beads did not emit a fluorescent signal (data not shown), indicating that autofluorescence of inclusions did not contribute to

the signal observed on treated microblades. These observations show that rigid cell-sized particles entered microcracks in obsidian microblades and were not removed by surface washing.

Because rigid particles the size of white blood cells entered fissures in obsidian microblades, we expected microcracks to sequester blood cells as well. Due to their deformability, the most abundant white blood cells, neutrophils and lymphocytes, can move readily through pores 5 μm in diameter (1, 60), and this flexibility should facilitate their diffusion into microcracks. Upon dessication, white blood cells release proteins and DNA; these molecules likely diffuse into microcracks much more readily than intact cells. White blood cells isolated from fresh whole cow blood were labeled with CFSE, a fluorescent dye that stains proteins. Twelve obsidian microblades were soaked in CFSE-stained cells for 24 h, washed extensively, dried, and examined by confocal microscopy. CFSE-stained blood proteins were trapped in subsurface microcracks on all microblades examined (Fig. 9, panels A, D, and G). Comparison of these confocal fluorescence micrographs with the corresponding visible-light images (Fig. 9, panels B, E, and H) showed CFSE-stained proteins in linear streaks that co-localized with visible fissures. Thus, microcracks that extend below the microblade surface harbored fluorescently-labeled blood proteins.

Stone tools used for hunting and processing may contact animal blood for short periods of time. To test whether capillary uptake of blood proteins into microcracks required prolonged exposure to blood cells, we immersed microblades in CFSE-labeled white blood cells for either 5 min or 0.5, 6, 12, or 24 h. Immediately after soaking the microblades in white blood cells, they were washed, dried, and examined by confocal microscopy. A 5 min incubation in white blood cells allowed CFSE-stained protein to diffuse at least 30 μm below the microblade surface (data not shown), indicating that blood proteins entered microcracks rapidly. Longer incubation did not affect the results appreciably, which suggests that diffusion of blood proteins into microcracks was complete within 5 min.

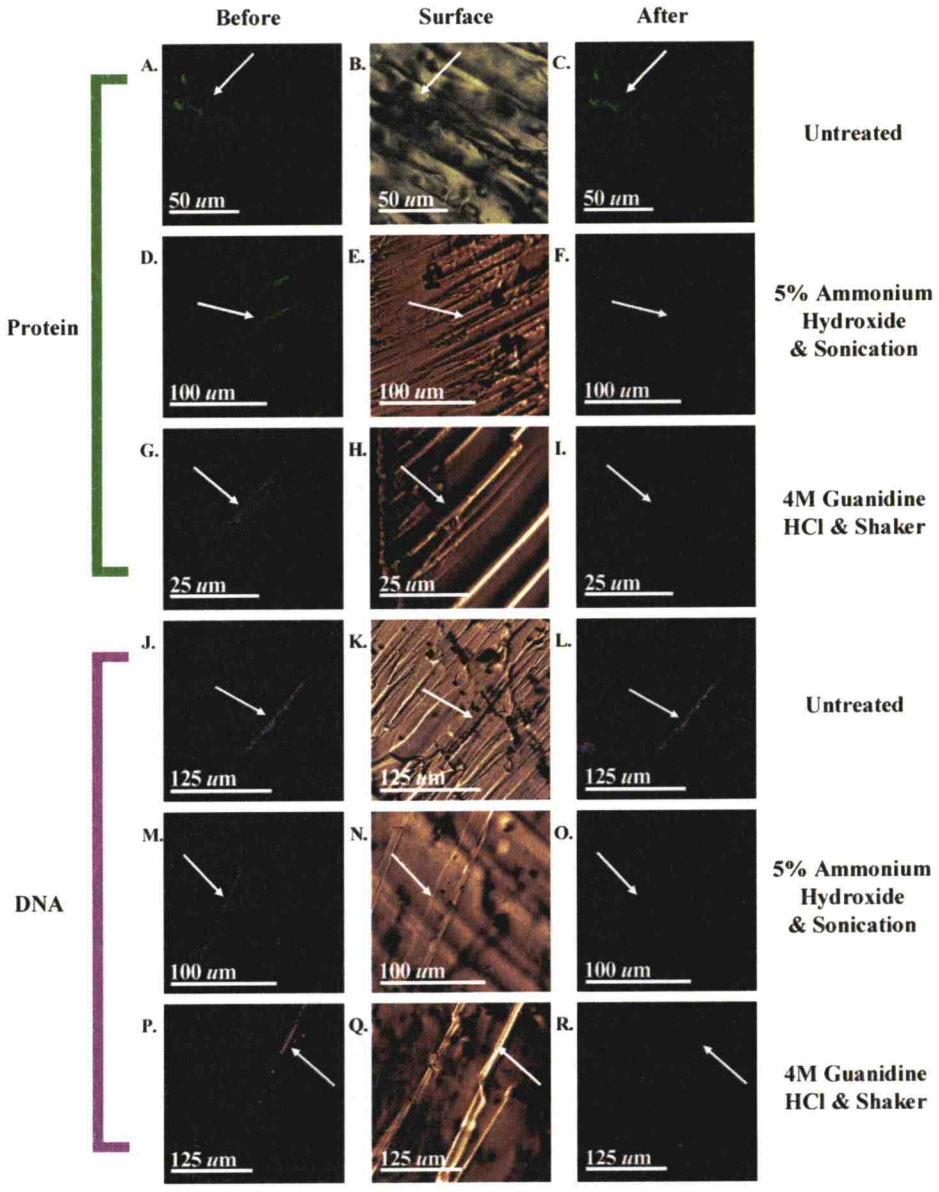

Figure 9: Confocal micrographs of CFSE-labeled proteins and DAPI-stained DNA contained in microcracks before and after extraction. The top three rows show CFSE-labeled proteins, and the bottom three rows show DAPI-stained DNA. Confocal images in the left- and rigt hand columns were taken before and after extraction, respectively, at depths of 16 (A,C), 41 (D,F), 7.5 (G,I), 24 (J,L), 18 (M,O), and 48 (P,R) µm beneath the microblade surface. Corresponding visible light micrographs appear in the center column. Arrows indicate microcracks. Extraction treatments used are indicated to the right of each row of images.

Both proteins and DNA contained in blood residues can be used to identify animal species (27, 53, 62, 71, 88). We used DAPI, a fluorescent dye that stains DNA, to detect DNA residues on obsidian microblades. Two strategies were used to stain DNA with DAPI. Isolated CFSE-stained white blood cells were treated with DAPI so that proteins and DNA were labeled with dyes that emit different wavelengths of light. Then, microblades were soaked in these doubly labeled white blood cells. This allowed us to detect both protein and DNA residues harbored at the same location. Alternatively, microblades were soaked in unstained whole blood, washed, and DNA residues were stained in situ by treating the microblade with DAPI. Microblades not deliberately exposed to blood were treated with DAPI to test for DNA from other sources; fluorescence was detected only on microblades that had been soaked in blood. Confocal microscopy documented DNA trapped beneath microblade surfaces. As was the case with protein residues, stained DNA occurred in linear streaks (Fig. 9, panels J, M, and P) that corresponded to fissures observed with visible light microscopy (Fig. 9, panels K, N, and Q).

In the course of our analysis of trapped protein and DNA residues, we examined 91 residue-containing microcracks in 45 microblades. For each microcrack, a series of confocal images were recorded, beginning at the surface and continuing at regular 2 μm intervals until no more fluorescence was detected. This analysis produced 2,297 images and indicated that the maximum depth to which blood residues penetrated varied around a median of 44 μm (Fig. 10). Residues were seldom detected deeper than 60 μm, although one specimen harbored DNA 145 μm beneath the microblade surface.

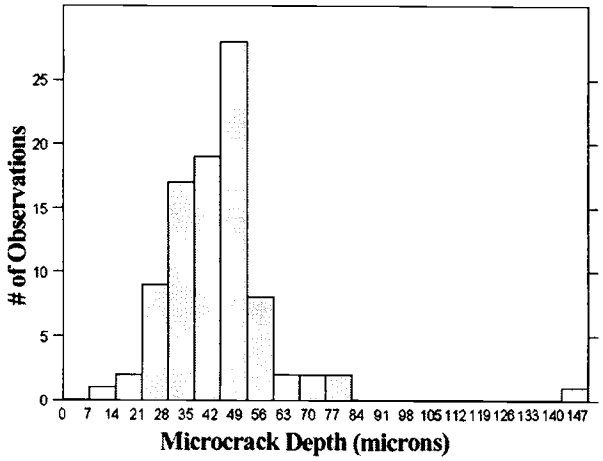

Figure 10: Maximum depths of trapped blood residues.

Subsurface microcracks in obsidian microblades protected both protein and DNA from removal by surface washing with distilled water or buffered saline solutions. In earlier studies, protein residues were removed from stone tools either with an 18 h incubation in 4 M guanidine hydrochloride (87) or with 3 min of ultrasonic vibration in 5% ammonium hydroxide (62). To compare the effectiveness of these procedures, we used confocal microscopy to measure the release of CFSE-labeled protein and DAPI-stained DNA from subsurface microcracks in obsidian microblades. Trapped residues were documented by confocal microscopy as described (Fig. 9). Microblades were then subjected to one of the extraction treatments or left untreated, and then all specimens were scrubbed with water for 5 min and dried. After extraction, each microcrack was re-examined by confocal microscopy (Fig. 9, panels C, F, I, L, O, and R). A series of confocal micrographs were taken in 2 μm depth increments, both before and after extraction. To estimate the fraction of residue removed by the treatment, we compared the fluorescence before and after extraction at a specific depth in each microcrack.

Table 1: Comparison of Extraction Methods

	DNA Released				Protein Released		
Extraction Method	**Mean (%)**	**SD (%)**	**Sample Size**		**Mean (%)**	**SD (%)**	**Sample Size**
Water	0	16	15		0	8	6
4M Guanidine HCl	61	18	19		80	17	12
5% Ammonium Hydroxide w/ sonication	78	13	15		75	12	11

Treatment of 8 microblades with 4 M guanidine hydrochloride removed 61 ± 18% of the DNA from 19 microcracks and 80 ± 17% of the protein from 12 microcracks (Fig. 9; Table 1). Ultrasonic cleaning of 5 microblades in 5% ammonium hydroxide released 68 ± 13% of the DNA from 15 microcracks and 75 ± 12% of the protein from 11 microcracks (Fig. 9; Table 1). Several microblades were washed with water but not treated with guanidine hydrochloride or ammonium hydroxide. Fluorescence intensities measured in microcracks before and after the water wash were remarkably similar (Table 1) indicating that labeled protein and DNA residues remained trapped in microcracks in these microblades (Fig. 9). This also proved that the fluorescence measurements were reproducible and not diminished by photo-bleaching of the CFSE and DAPI dyes upon exposure to the laser light used to produce the fluorescent emissions.

Discussion

In this study of obsidian microblades, we documented subsurface microcracks that trapped blood residues containing protein and DNA. These microcracks were able to harbor rigid cell-sized latex beads 10 μm in diameter, although beads 20 μm in diameter were too large to enter these cracks readily. On average, blood residues penetrated microcracks to a maximum depth of 44 μm; both blood residues and latex beads occasionally diffused 140 to 145 μm beneath the microblade surface. Blood residues entered microcracks by capillary uptake within 5 min of exposure. Thus, residue trapping did not require extreme force or lengthy contact with blood. Although blood residues were not removed from microcracks by thorough washing of the microblade surface with water or buffered saline solutions, treatment with 4M

guanidine hydrochloride, a strong denaturant, or sonication in 5% ammonium hydroxide removed 60 to 80% of the DNA and protein harbored in microcracks. These observations suggest that microcracks in stone tools used for killing or processing animals may sequester biological residues from the elements and from washing after excavation. These data also demonstrate that two extraction procedures used in earlier studies (62, 87) can release most of the DNA and protein harbored in subsurface microcracks.

Our work demonstrates the importance of the extraction step in residue analysis, because some procedures remove only surface residues whereas others also dislodge protein and DNA from subsurface microcracks. The notion that microcracks may sequester ancient biological residues can explain inconsistencies between previous blood residue studies. Several studies report animal protein detection rates between 14-30% on stone tools, despite the absence of visible residues preserved on tool surfaces (62, 83, 88). In each case, either guanidine hydrochloride or sonication/ammonium hydroxide methods were used to release trapped residues. In contrast, groups using alternative strategies to remove protein from stone tools succeeded less than 2% of the time (17, 35) unless visible surface residues were present (53). Our observations, which show that surface washing does not remove blood proteins trapped in microcracks, reconcile these conflicting reports. Recovery of protein and DNA residues from previously washed tools greatly expands the number of artifacts suitable for biological residue analysis because stone tool assemblages housed in museums and other collections may retain useful molecular information.

In summary, our observations support the hypothesis that microcracks can harbor protein and DNA residues. These data suggest that ancient residues in prehistoric artifacts are preserved in subsurface microcracks. Ancient residues may be much more common than previously anticipated and represent a significant, yet frequently overlooked, source of information about the past. Although we used obsidian microblades for this study due to their transparency, cryptocrystalline rocks – used by ancient peoples to make flaked stone tools – contain microcracks (84), which

are routinely produced with each fracture or flake removal during tool manufacture (8). Thus, an individual microblade, flake, projectile point, or scraper may contain hundreds or thousands of subsurface microcracks that can harbor ancient animal residues. Analysis of protein and DNA recovered from ancient remains can identify animals at the subspecies level (9). Residue studies, in conjunction with other lines of evidence such as tool form and use-wear data, can indicate how specific tools were used to process animals and extend our understanding of ancient human cultures.

Chapter 3

DNA and Protein Recovery From Washed Experimental Stone Tools

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Introduction

Recovery, amplification, and sequence analysis of ancient DNA extracted from bone (25, 31, 44), soft tissue (28, 68), and coprolites (72) has allowed genetic characterization of animals present at prehistoric sites. Residues on stone tools are another source of biological remains (50). Tools that came into contact with animals can preserve useable DNA and protein (12, 20, 27, 61, 88). Identifiable residues on tools can support and extend interpretations of archaeological sites by supplying significant information about tool use and species diversity not apparent from morphological analysis.

Residue analysis from stone tools offers several advantages over molecular analysis of other biological remains commonly found at archaeological sites. Stone artifacts are among the most well preserved materials in the archaeological record, and in many cases these are the only cultural remains recovered. Stone tools are easily accessible and are not destroyed during residue extraction.

Residue identifications also extend traditional stone tool use-wear and faunal studies. Use-wear studies require lengthy contact with animal tissues to leave behind detectable polishes. For example, a tool used to cut meat for 90 minutes did not acquire a visible polish (39). In contrast, stone tools trap blood in microcracks within minutes of exposure (82). Thus, residue analysis provides a means to identify expedient tool use which is not possible by conventional use-wear methods. Faunal reports are often skewed toward remains from larger animals because bones from small animals are difficult to identify (22). Residues on stone tools provide a means to identify small animal butchery events that cannot be described by traditional faunal analysis.

Both immunological detection and DNA sequencing can identify an unknown residue. However, most residue studies have been limited to immunological characterization of proteins (21, 53, 61, 71, 88). Investigators have employed a number of immunological techniques including counter-immunoelectrophoresis, the Ouchterlony method, and radio, enzyme-linked, or gold immunoassays (16, 17, 62, 88). However, immunological methods cannot distinguish protein residues from

closely related species, such as *Canis familiaris* (dog) and *Canis lupus* (wolf), nor can they distinguish subspecies. Also, immunological studies are limited to species for which specific antisera are available. More reliable residue identifications are possible with DNA sequence analysis, which is more sensitive and precise than protein detection.

Several studies report the recovery of DNA from residues on excavated stone tools (27, 41, 53, 55). However, methodological advances warrant a reassessment of the recovery of DNA from residues deposited on stone tools. Microscopic examination of stone tools has identified microcracks that trap DNA and protein from animal blood cells (82). Rigorous tests comparing different methods to release residues from stone tools indicates that most commonly used residue extraction techniques leave DNA and protein behind, lodged in sub-surface microcracks (82). However, stone tools submerged in 5% ammonium hydroxide and sonicated release 60-80% of DNA and protein residues (17). Previous research documents the identification of protein from stone tools sonicated in 5% ammonium hydroxide (21, 62, 71), but it remains untested whether the same treatment yields useable DNA. This issue must be addressed before sonication in 5% ammonium hydroxide treatments can be applied to authentic stone tool artifacts.

In this study, we identified DNA residues deposited on experimentally generated stone tools used 13 years ago to butcher a single animal. The species of the butchered animal was disclosed after we completed our residue analysis. Thus, analysis of experimentally generated tools allowed us to authenticate our findings, which is not possible using excavated stone tools. We also investigated the effects of artifact cleaning on DNA and protein recovery because the majority of stone tools available for residue testing have been washed. Cleaning protocols (38, 80, 81) used during stone tool curation call for treatments that may interfere with DNA and protein recovery. Finally, we demonstrate that tools with no visible signs of organic material can yield sufficient DNA for identification.

Materials and Methods

In 1987, three stone flakes were manufactured for an experimental butchery. The tool kit was made from Morrison chert collected in Northwestern Wyoming. The tools were used to butcher an animal then placed in a box and stored in an unheated garage. The storage environment provided shelter from direct sunlight. After 12 years, tools were sealed in individual polyethylene bags and stored indoors at room temperature. During manufacture and use, all tools were handled without gloves.

Contamination precautions. Mammalian DNA that contaminates reagents and equipment, contact of the sample with extraneous DNA, and cross contamination of samples with other DNAs being processed in the lab are significant problems in ancient DNA research (14, 67). Physical precautions provide our first line of defense against contamination. DNA extractions and assembly of PCR cocktails, PCR amplifications, and DNA sequencing occur in three separate buildings. We used separate glove boxes to mix reagents, do extractions, concentrate DNA extracts, mix PCR reactions, and add template to PCR reactions. Only one sample and mock extract were processed each day to avoid cross-contamination between samples. When not in use, each glove box was irradiated with ultraviolet (UV) light to inactivate DNA templates (90). Equipment was treated with 3% hydrogen peroxide and ethylene oxide gas (65). Work surfaces were cleaned with 10% bleach. Aerosol-resistant pipette tips were used for all liquid transfers, and dedicated pipettors were used for each operation. After use, pipettors were cleaned with bleach, disassembled, and treated with ethylene oxide gas. Reagents were stored in small aliquots, used once, and discarded.

To identify animal species, we (9) and others (15, 49, 63) have found mitochondrial cytochrome *b* sequences useful. Animal-specific PCR primers (L15684 = 5'-CTCCACACATCCAA ACAACG-3' and H15760 = 5'-TGTTCGA CTGGTTGTCCTCC-3' (36) anneal to highly conserved sequences that flank a variable region of the cytochrome *b* gene, thereby providing 76 base pairs (bp) of useful sequence between the primers that contain sufficient information to distinguish

most species. The PCR products were 116 bp long. These primers rarely amplify human DNA, which prevented human DNA in PCR reagents from interfering with species identifications (9).

Prior to initiation of this study, we performed 10 no-template PCR reactions with the reagents we intended to use. We did not detect contamination in these controls and proceeded with our study. For every PCR reaction, we performed four no-template PCR reactions with purified water substituted for template DNA. For each sample analyzed, we performed one mock DNA extraction, purification, and amplification using buffer alone.

Residue extraction. Tools A and B underwent a series of washing steps to recover DNA and protein and monitor residue recovery after artifact cleaning. Each tool was placed in a sealed polyethylene bag (0.2 mm thickness). Tool A was soaked in *ultrapure* water (Specialty Media) for 10 minutes and then scrubbed by hand (inside the sealed bag) to remove visible surface residues. Next, Tool A was submerged in 3 ml 5% ammonium hydroxide in a fresh sealed polyethylene bag and cleaned as described (62).

Tool B underwent eight sequential extractions. All extractions were performed in *ultrapure* water (Specialty Media). Washes one through 5 included a 10 minute soak followed by a 2 minute scrub. For wash 6, Tool B was soaked for 1 hour and then agitated for 30 minutes at 180 rpm. Next, Tool B was soaked for 48 hours and then agitated for 16 hours at 180 rpm. The final extraction entailed a 72-hour soak followed by 24 hours of agitation at 180 rpm. Immediately following each extraction, samples were divided for DNA (1/3 extract volume) and protein (2/3 extract volume) analyses. Protein samples were vacuum dried and reconstituted in 100 µl phosphate buffered saline (Sigma).

DNA purification. DNA extracts were centrifuged at 3000 rpm for 10 minutes in an Eppendorf 5415 C microcentrifuge. The supernatant was concentrated in a Microcon 30 (Amicon, Beverly, ME) and eluted in 50 µl water. Insoluble material was air dried

for 2 hours and mixed with the Microcon 30 eluate. Next, DNA samples underwent proteinase K (Sigma) digestion (1.2 U) in 300 µl extraction buffer (10 mM Tris, 10 mM EDTA, 2% SDS, 100 mM NaCl, 39 mM DTT, pH 8.0) at 56°C for 6-12 hours. The solution was then extracted with water-saturated phenol and chloroform/isoamyl alcohol (24:1). The aqueous phase was removed and concentrated in a Microcon 30 to a final volume of 60 µl. DNA samples were purified further by a silica extraction method (33).

DNA amplification. PCR products were amplified as described (62) with primers L15684 and H15760. PCR sensitivity was measured using a cytochrome *b* template containing a 20 bp insert. The 136 bp PCR products amplified from this template were easily distinguished by agarose gel electrophoresis from 116 bp products amplified from wild-type cytochrome *b*. To construct 136 bp template DNA, a 116 bp PCR product derived from cow cytochrome *b* (coordinates 15,432 to 15,547) was mutagenized by overlap extension PCR (29). Two PCR products were amplified in separate reactions using primer pairs L15684 and H68 = (5'-ACCGCGGTGG AGCTCCAGCTCATTGGCTGAGTGGTC GGA-3') or H15760 and L32 = (5'-AGC TGGAGCTCCACCGCGGTCCTAT TCTGAGCCCTAGT-3') generating PCR products that overlap by 20 nucleotides. The two PCR products containing the 20 bp insertion from pBluescript SK+ vector (Stratagene) (5'-AGCTGGAGCTCCACCGC GGT-3'), were mixed and amplified using primers L15684 and H15760 to generate the full-length (136 bp) target DNA. The target DNA was inserted into pCR2.1 (size with 136 bp insert is 4,044 bp) via a topoisomerase-mediated reaction performed according to the manufacturer's instructions (Invitrogen). Plasmid DNA containing the 136-bp construct was diluted in 10 mM Tris, 0.1 mM EDTA, pH 8.0 to generate samples that contained approximately 1, 10, 10^2, 10^3 and 10^4 molecules of template DNA (10^4 molecules equals 4.2×10^{-5} ng of DNA).

DNA sequencing. PCR products were either sequenced directly or inserted into pCR2.1 and transformed into *Escherichia coli* strain NR8052 [*Δ(pro-lac), thi, ara,*

trpE977, ung-1] (45, 46). Prior to sequencing, PCR products and plasmid DNAs were purified using a QIAquick PCR Purification Kit and a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The resulting DNAs were sequenced with primer L15684 by the dye-terminator method using an Applied Biosystems Model 373A automated sequencer.

Protein characterization. Protein extracts were centrifuged at 16,000 x g for 5 minutes. Soluble proteins were quantified with Bradford assays (11) and silver stained after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (75).

Results

To identify the butchered animal, we analyzed DNA extracted from residues washed from the surface of a flake used in the experimental butchery. Blood residue was visible on the surface of Tool A (Figure 11).

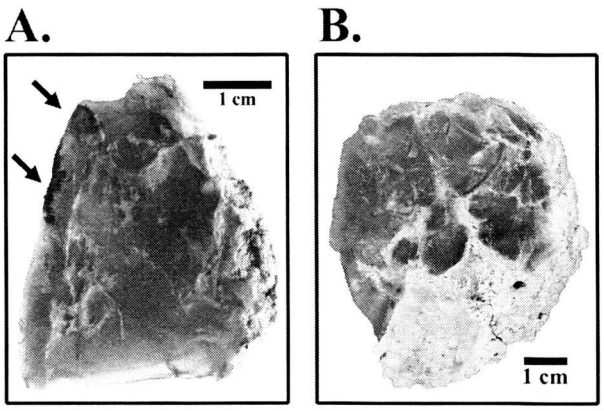

Figure 11: Experimentally generated stone tools. Panel A shows visible residues (arrows) deposited on the surface of Tool A. Tool B (panel B) underwent eight washes to remove preserved residues.

An initial cleaning with water removed a total of 17 mg of protein, as determined by Bradford assay. SDS-PAGE revealed a single protein species with an electrophoretic mobility identical to that of serum albumin, an abundant blood protein (Figure 12, lane 1). Direct sequencing of a PCR product amplified using DNA recovered from Tool A identified the butchered animal as *Antilocapra americana* (antelope) (Figure 13). Five

independent clones derived from the same PCR product also yielded identical *A. americana* sequences (Figure 13).

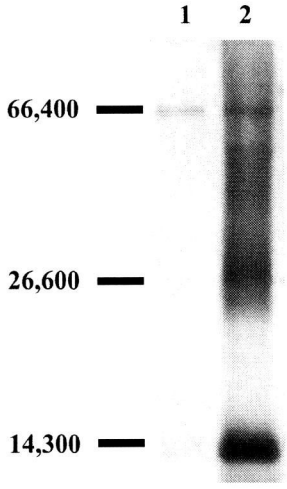

Figure 12: Proteins released from stone tools. SDS-PAGE of proteins recovered by surface washing (Lane 1) [17 µg] and sonication (Lane 2) [252.5 µg].

Immediately following the initial wash, Tool A was cleaned in an ultrasonic bath as described (62). DNA from the resulting extract was amplified by PCR and identified as *A. americana* by DNA sequence analysis. Proteins recovered from the second extraction were quantified using a Bradford assay, which showed that 252.5 mg of protein were released by ultrasonic cleaning. SDS-PAGE demonstrated four protein components with electrophoretic mobilities that correspond to those of abundant whole blood proteins including serum albumin (MW = 66,400), immunoglobulin heavy and light chains (MW = 50,000 and 25,000, respectively), and hemoglobin α and β chains (MW ~ 15,000) (Figure 12, lane 2) (24, 37, 58).

```
          15685                                                    15735
A. americana      AAGCATAATATTCCGACCATTCAGCCAATGCTTATTCTGAATCCTAGTAGC
B. taurus         ....................C...........C........GC.........
S. scrofa         ............T......C.A..T......C..........A........

          15736                                15760
A. americana      AGACCTACTCACCCTAACATGAATC
B. taurus         .........G..A..C........T
S. scrofa         ......CA.T..A...........T
```

Figure 13: Partial cytochrome *b* nucleotide sequences. *Antilocapra americana* sequences were obtained from PCR products amplified from DNA extracted from stone tools (top line), whereas PCR products produced in 4 of 64 no-template control reactions yielded cow and pig sequences. Capital letters show positions where *Antilocapra americana* differs from cow and pig. Dots indicate bases identical to the *Antilocapra americana* sequence. Coordinates are based on those of the complete human mitochondrial genome sequence (5).

To test the effect of washing on recovery of DNA and protein, we performed eight sequential washes on Tool B (Table 2). The initial tool cleaning was designed to simulate washing protocols commonly used by archaeologists to prepare tools for morphological examination and storage (80, 81). The initial wash removed 0.9 mg of protein (Table 2). Four additional surface washes released 20.9 mg, 17.3 mg, 13.5 mg and 2.8 mg of protein, respectively (Table 2). After the fifth wash, more rigorous wash procedures were used to remove residues still adhering to the tool. Wash six and seven removed 5.3 mg and 20.8 mg of protein, respectively (Table 2). The eighth wash dislodged 0.9 mg of protein (Table 2). Overall, Tool B was washed for 162.5 hours in 65 ml of water, which released 82.5 mg of protein. Each wash yielded enough *A. americana* mitochondrial DNA for PCR amplification and sequencing.

Table 2: Tool B Extractions.

Extraction #	Extract Volume	Soluble Protein Recovery	Percent Protein Recovered
1	5 ml	0.9 mg	1.1%
2	5 ml	20.9 mg	25.3%
3	5 ml	17.3 mg	21%
4	5 ml	13.5 mg	16.4%
5	5 ml	2.8 mg	0.75%
6	10 ml	5.3 mg	6.4%
7	15 ml	20.8 mg	25.2%
8	15 ml	0.9 mg	1.2%
Totals	**65 ml**	**82.4 mg**	**100%**

During our study, PCR reactions performed with primers L15684 and H15760 yielded detectable products from approximately 10 copies ($4x10^{-8}$ ng) of target DNA (Figure 14). Sixty-four no-template controls were performed, and 4 yielded PCR products including 1 pig and 3 cow sequences (Figure 13). No contaminating DNA was detected during mock extractions and amplifications.

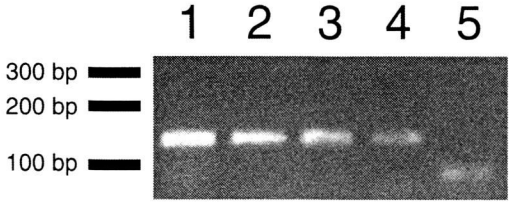

Figure 14: PCR products obtained with primers L15684 and H15760 (expected size, 136 bp). Lane 1, ~10^4 copies; Lane 2, ~10^3 copies; Lane 3, ~10^2 copies; Lane 4, ~10 copies; Lane 5, ~1 copy of pBOVS target DNA. The ~80 bp band resulted from interactions between primer oligonucleotides during PCR.

Discussion

We report the simultaneous recovery of protein and DNA from blood residues on experimental stone tools. Sonication in 5% ammonium hydroxide did not compromise our ability to recover useable DNA. To gauge the performance and reliability of this method, we extracted residues from stone tools used by M.K. to butcher an *A. americana*. Because this researcher did not reveal the identity of the animal to the other authors until the study was completed, this was a blind test. Sequence analysis

of DNA recovered from blood residues correctly identified the animal as *A. americana*.

Tools used in this study were 13 years old, and preservation conditions do not model those of a depositional environment. Residues that survive for hundreds or thousands of years at an archaeological site are most likely less abundant and more difficult to remove than the residues present on the tools studied here. Nevertheless, our results clearly indicate that surface washing procedures typically used to curate stone tools removed a small fraction of the DNA and protein deposited during the butchery. The initial cleaning released only 1% of the protein that we eventually recovered from the tool, and a second surface wash removed an additional 25%. In addition, a tool soaked, scrubbed, and agitated for over 6 and a half days still yielded enough useful biological residue to make a DNA identification.

Residues recovered from a tool after ultrasonic cleaning contained 14.8 times more protein than visible residues adhering to the tool surface, which were removed by a surface wash. Furthermore, surface protein preservation was poor yielding a single protein species resembling serum albumin (Figure 12, lane 1) compared to four protein species (Figure 12, lane 2) recovered from subsurface residues. This suggests that residues on the tool surface were more quickly degraded and less favorable for residue identification analyses than those protected in microcracks beneath the tool surface.

Due to the primers used, human DNA did not affect our analysis of non-primate mammalian cytochrome *b* sequences. Four PCR products amplified from no-template control reactions yielded either cow or pig mitochondrial DNA sequences. Mitochondrial DNA from those species was the only contaminants detected in no-template PCR reactions. The absence of cow and pig sequences in mock extract controls suggests that these contaminates originate from PCR reagents used in this study, most likely deoxynucleoside triphosphates (dNTPs). Water, $MgCl_2$, and 10x buffer are inorganic PCR reagents unlikely to contain animal DNA. There is no obvious source for cow or pig DNA in primers, which were chemically synthesized and purified by HPLC. We purified our own *Taq* DNA polymerase in a sterile hood

supplied with HEPA filtered air, and we used chromophotography equipment decontaminated with UV light. dNTPs are prepared by humans, probably from cow and pig tissues, and DNA from these species may contaminate dNTPs. Because dNTP synthesis protocols are proprietary information, it was difficult to prove the source of PCR reagent contamination. Cow and pig DNA did not confound analysis of tool residues, because cow, pig, and antelope sequences are easily distinguished from one another (Figure 13).

In conclusion, our study shows that tools used for animal butchery may harbor sufficient residues for protein and DNA analyses even after extensive surface washing. In addition, wash techniques used to clean stone tools for morphological examination only remove poorly preserved surface residues and leave behind sheltered protein and DNA trapped below the tool surface.

Chapter 4

DNA from Ancient Stone Tools and Bones Excavated at Bugas-Holding, Wyoming

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Introduction

Stone tools are commonly found at archaeological sites. They provide information regarding site chronology, manufacturing strategies, material procurement, and subsistence practices. Stone tools are particularly effective indicators of cultural practices when combined with other forms of analysis, including studies of biological residues. Traces of DNA and protein are thought to preserve on stone tools used to process animals (12, 20, 27, 50, 62, 88). Microcracks produced during stone tool manufacture trap blood residues beneath the tool surface (82). These residues are not removed by surface washing, but sonication can release 60-80% of the trapped DNA (82). Residues deposited in microcracks may enjoy greater protection from the elements than surface residues.

DNA in ancient samples is accessible to genetic analysis if preservation is adequate. Ancient DNA often shows extensive damage (32, 51, 67, 73), but small fragments of DNA (< 400 base pairs) were recovered from samples thousands of years old (9). Several investigators have reported recovery of DNA from residues deposited on stone tools (27, 41, 53, 54). To extend this pioneering work, we examined DNA residues on 24 stone tools excavated from one well-characterized site. Our analysis included flakes with no evidence of use and sediments, which served as controls for the presence of contaminating DNAs in the depositional environment. Here we describe several technical improvements in DNA residue analysis. These include a more effective DNA recovery protocol, methods to measure sensitivity and inhibition of PCR in each sample, and strategies to surmount competition between templates during PCR amplification, which can occur in samples that contain DNA from multiple species. These new developments will help future investigators achieve the full potential of DNA residue analysis.

Bugas-Holding is located in northwestern Wyoming near the eastern border of Yellowstone National Park. A group of Shoshone occupied this site during one winter of the Late Prehistoric Period (19, 74). Charcoal from the cultural layer yielded uncalibrated radiocarbon dates of 380 $\pm$ 100 years before present (ybp), 490 $\pm$ 80 ybp, and 200 $\pm$ 60 ybp (74). Humans processed, stored, and consumed a variety of animal

species at this site (6, 48, 74, 83). The archaeological record at Bugas-Holding consists of hearths, a midden, chipped and ground stone, pottery, ornaments, and remains from bison (*Bison bison*), bighorn sheep (*Ovis canadensis*), elk (*Cervus elaphus*), pronghorn (*Antilocapra americana*), bear (*Ursus arctos*), and canids (dog [*Canis familiaris*], coyote [*C. latrans*], or wolf [*C. lupus*]) (74).

The Bugas-Holding site yielded a large assemblage of chipped stone, animal remains, and sediment samples. Previous studies detected protein residues on stone tools from Bugas-Holding (83), suggesting that tools in this assemblage may harbor DNA too. The history of artifact handling and storage was well documented. Excavators wearing rubber gloves collected fourteen stone tools, and these tools were sealed in separate plastic bags to prevent contact with modern DNA. Because site activities were established by previous work (2, 6, 34, 48, 59, 74, 83), our study was designed to test the validity of DNA residue analysis. We tested 24 chipped stone artifacts for the presence of DNA residues, and we compared DNA preservation in bones and stone tools from the same stratigraphic context. Nine tools and four bones yielded DNA; some of the DNA showed evidence of lesions often found in ancient DNA. Archaeologists did not touch three of these tools, which suggests that the DNA recovered from them was present prior to excavation. We recovered DNA from three species on a single tool and observed competition between these templates during PCR. Also, we detected modern DNA deposited on stone tools by archaeologists after excavation. Stone tools may harbor both ancient and modern DNA, and investigators must take great care to exclude modern DNA from ancient specimens.

Materials and Methods

Contamination precautions. Contamination with modern DNA is a significant problem in ancient DNA research (14, 69). DNA introduced during excavation and storage may contaminate samples. DNA from equipment, other samples, and previously synthesized amplicons may contaminate PCR reactions. We limited extraneous DNA from these sources with physical barriers. Excavators wore rubber gloves when they collected twelve stone tools, which were immediately sealed in

plastic bags. DNA extraction, PCR cocktail assembly, PCR amplification, and DNA sequencing occurred in three separate buildings. We used separate glove boxes to mix reagents, prepare samples, extract DNA, and set-up PCR reactions. We processed only one sample and mock extract each day to avoid cross-contamination between samples. After each use, glove boxes were cleaned with 10% bleach and irradiated with ultraviolet light to inactivate DNA templates (90), and equipment was treated with 3% hydrogen peroxide and ethylene oxide gas (65). Reagents were stored in small aliquots, used once, and discarded.

Prior to the initiation of this study, we performed 20 no-template PCR reactions with the reagents we used. For each sample analyzed, we performed one mock DNA extraction, purification, and amplification using buffer alone. For every PCR reaction containing extract from a sample, we performed 4-10 no-template PCR reactions with purified water substituted for template DNA.

Residue extraction. We recovered DNA residues from stone tools in several steps. Each tool was sealed in a polypropylene bag (0.2 mm thickness) and soaked in 2-10 ml of 5% ammonium hydroxide for 30 minutes. Next, the submerged tool was vacuum infiltrated (/28.5 mmHg) for 20 minutes and sonicated (Branson 1210, 50/60 kHz) for 3 minutes (62). Finally, the tool was gently agitated on a wavetable (36 oscillations/min) for 30 minutes. The ammonium hydroxide solution was then collected and vacuum dried in a SpeedVac concentrator (-28.5 mmHg). The dried extract was dissolved in 1 ml extraction buffer [5 mM EDTA, 0.5% SDS, pH 7.8] and incubated at 56°C with 1.2 U of proteinase K (Sigma) for 6-12 hours. The solution was extracted once with water-saturated phenol and again with chloroform/isoamyl alcohol (24:1). The aqueous phase was concentrated in a Microcon 30 microfiltration cartridge (Amicon) to a final volume of 60 µl. Samples were purified further by a silica extraction method (33).

DNA amplification. PCR reactions (50 µl) contained 10 mM Tris, pH 8.5, 50 mM KCl, 200 µM (each) dATP, dCTP, and dGTP, 400 µM dUTP (Pharmacia), 1 µM of

each HPLC purified primer (Invitrogen), 2.5 U *Taq* DNA polymerase, and 2.5 mM $MgCl_2$. Incubation temperatures were 92°C, 52°C, and 72°C for 1 minute each; 40 cycles were performed for each reaction. Templates were amplified in two sequential 40-cycle PCR reactions. Initial PCR reactions contained 5 µl of undiluted extract (or 5 µl from a 5x, 30x, or 100x dilution). Five microliters of PCR product from each reaction was amplified for an additional 40 cycles. PCR reactions were performed in low-retention reaction tubes (0.2 ml) using a MJ Research PTC-200 thermal cycler. All amplifications were repeated.

Table 3: List of Primers

Primer	Sequence (5' to 3')	Marker	Reference
L15684*	CTCCACACATCCAAACAACG	cytochrome *b*	(43)
H15760*	TGTTCGACTGGTTGTCCTCC	"	(43)
L32	AGCTGGAGCTCACCGCGGTCCTATTTCTGAGCCCTAGT	"	This study
H68	ACCGCGGTGGAGCTCCAGCTCATTGGCTGAGTGGTCGGA	"	This study
HC1700	GCCCCTTAGCCAATGCCTA	"	This study
L16S04*	TCTCTTACTTCCAATCCGTG	16S rRNA	This study
H16S06*	CGGAGGTTGTTYTGTTCTCC	"	This study
FelidY	TCTCTTACTTCCAATCCGTGAAATTGACCTTCCCGTGAAGCTTTAAT TAACCGACCCAAAGAGACCATATGAACCAACCGACAGG	"	This study
FelidZ	CGGAGGTTGTTTTGTTCTCCGAGGTCACCCCAACCTAAATTGCCGG CCCATATAGAGGTTTGTTGTTCCTGTCGGTTGGTTCATA	"	This study
12S01*	AGAGTGGYCAYATGTTATC	12S rRNA	This study
12S03*	TTGTAYCCTRGCTTTCGTGG	"	This study
FowlY	AGAGTGGYCAYATGTTATCTACACCAGCTAAGATCAAAATGCAACC AAGAGCCCAACCTAAACCCATCTTAGCCTC	"	This study
FowlZ	TTGTATCCTAGCTTTCGTGGGTTAAAATTAGTCGCTGAGGCTAAGA TGGGTTTAGGTTGGGCTCTTGGTTGCATTT	"	This study
L15984	AGAAGCTCTTGCTCCACCAT	D-loop	This study
H16238	ATGGTGATTAAGCCCTTATT	"	This study
L16021	AGAAGCTCTTGCTCCACCATCTTCTTAAACTATTCCC	"	This study
L16173	GTTTGCCCCATGCATATAACCTTACATAGGAC	"	This study
H16122	TTATATGCATGCATGGGGCAAA	"	This study

Primers used in study that target mitochondrial DNA sequences. Sequence nomenclature: Y = C or T and R = A or G. An * depicts primers purified by HPLC for ancient DNA testing.

Primers. Primers used in this study are listed in Table 3. All DNA extracts were amplified with cytochrome *b* (cytb) primers L15684 & H15760, which yield a 116-base-pair (bp) amplicon. Extracts that contained amplifiable DNA or visible surface residues were also amplified with primers (L16S04 & H16S06) designed to amplify a

191-bp region of the mitochondrial 16S ribosomal RNA gene (16S) from the Felidae, Cervidae, and Ovidae families. This primer pair does not amplify canid, cow (*Bos taurus*), pig (*Sus scrofa*), and human DNA templates. Birds from the Phasianinae subfamily were identified by amplifying 137-bp of the mitochondrial 12S ribosomal RNA gene (12S) with primers 12S01 & 12S03. To distinguish wolf from dog, we amplified 292 bp of mitochondrial D-loop sequence with primers L15984 & H16238.

PCR sensitivity. PCR sensitivity was measured using modified templates containing 20 to 40-bp insertions or deletions (Table 4). The PCR products amplified from these control templates were easily distinguished by agarose gel electrophoresis from products amplified from wild-type DNAs.

Table 4: Modified Control Templates

				PCR Product Length		
Plasmid Name	**Parent Species**	**Marker**	**Primers**	**Wildtype Template**	**Control Template**	**Coordinates***
PBOVS	*B. taurus*	cytochrome *b*	L15684/H15760	116 bp	136 bp	15,432 to 15,547
pK9	*C. familiaris*	D-loop	L15965/H16257	292 bp	272 bp	15,965 to 16,257
Pfelid	*F. catus*	16S rRNA	L16S04/L16S06	192 bp	152 bp	2,955 to 3,146
Pfowl	*G. varius*	12S rRNA	L12S01/H12S03	137 bp	112 bp	1,594 to 1,730

* Wild-type DNA sequence coordinates listed in order of appearance include *Bos taurus* (accession #: NC_001567), *Canis familiaris* (accession #: NC_002008), *Felis catus* (accession #: NC_001700), and *Gallus gallus* (accession #: NC_001323).

pBOVS (*Bos taurus*; cytb; Figure 15, panel A) and pK9 (*C. familiaris*; D-loop; Figure 15, panel B) control templates were derived from wild-type sequences (Table 4) and mutagenized by overlap extension PCR (29). pBOVS contains a 20-bp insertion from pBluescript SK+ vector (Stratagene) (5'-AGCTGGAGCTCCAC CGCGGT-3') and pK9 has two 20-bp deletions (Figure 15). To build pFelid and pFowl, long (85 or 76 nucleotide) primers were designed from wild-type sequences such that their 3' ends overlapped and one primer in each pair lacked 40 (FelidY) or 25 (FowlY) bases of the wild-type sequence (Figure 15, panel C).

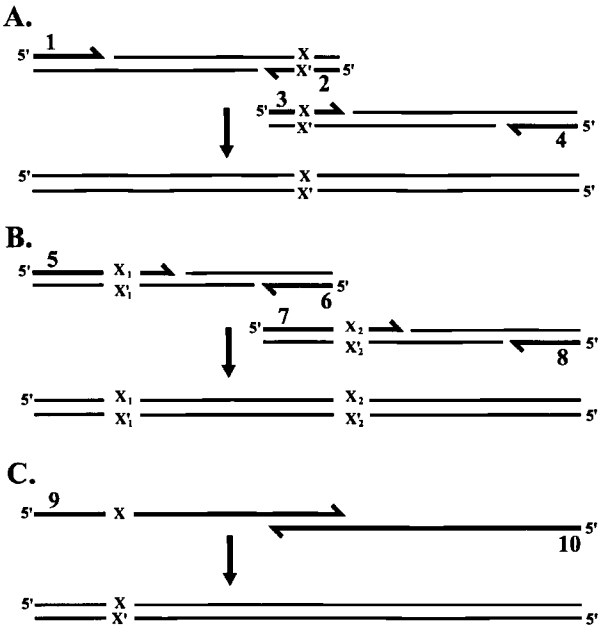

Figure 15: Construction of control templates. Panels A and B illustrate overlap extension PCR (Higuchi *et al.* 1988) to construct pBOVS (panel A; primers: 1 = L15684; 2 = H68; 3 = L32; 4 = H15760) and pK9 (panel B; primers: 5 = L16021; 6 = H16122; 7 = L16173; 8 = H16238) (Table 1). Panel C shows the strategy used to build pFelid (primers: 9 = FelidY; 10 = FelidZ) and pFowl (primers: 9 = FowlY; 10 = FowlZ) control templates. X and X^1 indicate the location of deletions or insertions.

All control templates were inserted into pCR2.1 TOPO vector (Invitrogen) via a topoisomerase-mediated reaction performed according to manufacturer's instructions; recombinant plasmids were transformed into *Escherichia coli* NR8052 [*Δ(pro-lac), thi, ara, trpE977, ung-1*] (45, 46). Control template plasmids were diluted in 10 mM Tris, 0.1 mM EDTA, pH 8.0 to generate samples that contained approximately 1, 10, 10^2, 10^3, and 10^4 molecules of template DNA (10^4 molecules of pCR2.1 [3,908-bp] equals 4.2 x 10^{-5} ng of DNA).

PCR inhibition. PCR inhibition was assessed in test reactions with primers L15684 & H15760 that included 100 copies of pBOVS and 5 µl of DNA extract. Samples that

inhibited PCR were diluted 5, 10, 30, and 100-fold and tested again for PCR inhibitors. Dilutions that relieved inhibition were tested for ancient DNA.

Detection of rare amplicons in multiple template amplifications. PCR products were inserted into pCR2.1 TOPO and transformed into *E. coli* NR8052. Individual transformants were screened for canid inserts by PCR or colony hybridization (23). For PCR screening, 38 transformants were picked from each library, added to individual 25 µl PCR reaction mixtures, and incubated at 94°C for 2 minutes to lyse cells. PCR reactions were performed in 10 mM Tris, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 200 µM (each) dNTPs, and 2.5 U *Taq* DNA polymerase. These reactions contained three primers (1 µM each): HC15700 (Table 3) and two primers that flank the multiple cloning site of pCR2.1 (5'-GTAAAACGACGGCCAGT-3' & 5'-AAC AGCTATGACCATG-3'). Incubation temperatures were 92°C, 58°C, and 72°C for 1 minute each; 30 cycles were performed for each reaction.

For colony hybridization, we used a ^{32}P-labeled canid-specific cytochrome *b* oligonucleotide (Hcanid; 5'-CCTTAGCCAATGCCTATTCTGACTTTTAGTC-3', coordinates 15,722 to 15,752, accession NC_002008). The oligonucleotide was end-labeled with γ-^{32}P ATP (ICN Biomedicals, Inc.) and T4 polynucleotide kinase (GIBCO) according to manufacturer's instructions. Individual colonies were fixed to Gene Screen Plus membranes (NEN Life Science Products, Inc.) and hybridized to radiolabeled probe in 50% formamide, 1% SDS, 1M NaCl, 10% dextran sulfate, 0.01% salmon sperm DNA at 42°C for 12 hours. Membranes were washed as described (75) and exposed to Kodax BioMax Film at 25°C for 8 hours.

DNA sequencing and alignment. PCR products were either sequenced directly or inserted into pCR2.1 TOPO and transformed into *E. coli* NR8052. Prior to sequencing, PCR products and plasmid DNAs were purified using QIAquick PCR Purification Kit or QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The resulting DNAs were sequenced at Davis Sequencing (Davis, CA) by the dye-terminator

method using an Applied Biosystems Model 373A automated sequencer. Sequences were aligned with BLASTN (3) and ClustalW (DNASTAR Version 5.00).

Burial environment controls. Sediment samples collected during excavation were tested for animal and bacterial DNA. DNA was extracted from 10 mg sediment samples and amplified with primers L15684 & H15760 and L16S04 & H16S06 (Table 3). To show that sediments did not trap DNA irreversibly, a sediment DNA extract was amplified with bacterial 16S primers 1406F (5'-TGYACACACCGCCCGTC-3'; Y = C or T) and 1492R (5'-GGTTACCTTGTTACGACTT-3') (47).

Assessment of protein and DNA preservation in bone. Amino acid composition of collagen protein in bones from Bugas-Holding was determined by AAA Service Laboratory (Portland, OR) using high performance liquid chromatography (HPLC). Each analysis measured total protein (nanomoles/milligram of bone) and residues per thousand (R/1000) values for each amino acid. DNA was purified from bone using the silica purification method (33) or phenol-chloroform (49).

Results

DNA and protein preservation in ancient bones. We measured protein preservation in three bones from Bugas-Holding (Table 5). Protein levels ranged from 51% to 78% of that in modern bone and glycine/aspartic acid ratios were also similar to the ratio in modern bone. All three bones yielded bison DNA (Table 5) and independent DNA analysis of samples P50-3-94 and Q49-16-256 confirmed our sequences. We tested three additional bones for useable DNA and one yielded a bison sequence.

Table 5: DNA and Protein Recovered from Bones Excavated at Bugas-Holding

Catalog No.	Nearest Feature	Maximum Length (mm)	Gly/Asp	Total Protein (nmols/mg)	DNA Identification
O50-18-37	6	93	6.2	1251	*B. bison*
P50-3-94	3	50	6.4	1734	*B. bison**
Q50-23-95	3	61	6.6	1924	*B. bison*
Q50-16-256	1 & 3	63	---	---	*B. bison**
Q49-12-99	9	60	---	---	No product
Q50-25-28	9	109	---	---	No product
Modern Cow	---	---	6.7	2475	---

Nearest feature numbers refer to feature locations diagramed in Figure 2. A * indicates that the identification was reproduced by Lenoard and Wayne. A dash (---) indicates no data.

DNA residues recovered from stone tools. We tested 24 chipped stone artifacts from Bugas-Holding for DNA residues, fourteen of which were not handled (Figure 16; Table 6). Nine stone tool extracts contained sufficient DNA for detection by PCR. DNA was recovered from three tools not touched by excavators and from six tools handled during excavation and storage. All tools yielding DNA were manufactured from chert except tool P49-15-499, which was made of quartzite. DNA was not recovered from the three tools with visible surface residues. PCR inhibition was observed in 54% of the extracts (Table 6). Dilutions ranging from 10x to 30x removed 90-97% of the inhibitors from the extracts and allowed amplification of control template DNA.

Formal tools. DNA analysis of extracts from 17 formal tools (Table 6) produced a 47% success rate. Formal tools are normally generated to perform specific functions such as scrape hide or cut flesh, so they are more likely to have been used than flakes. The eight formal tool extracts that yielded DNA included five bifaces, two side scrapers, and one end scraper. All of these tool extracts harbored canid DNA; two tool extracts also contained domestic cat DNA, and one of these (O48-24-37) had mule deer DNA too (Table 6).

Utilized flakes. Four utilized flakes were tested for presence of DNA residues (Table 6). Utilized flakes are usually expedient tools that are manufactured, used, and discarded in quick succession. One quartzite flake (P49-15-499) extract had canid DNA.

Flakes. Flakes are pieces of chipped stone that are intentionally removed from a tool or core during manufacture, sharpening, or resharpening. Three flakes that lacked evidence of use (Table 6) were included as negative controls to screen for DNA introduced during excavation, storage, and handling of chipped stone. No DNA was recovered from these flakes.

Table 6: Chipped Stone Attributes and DNA Analysis Summary

Catalog #	Nearest Feature	Tool Type	Material Type	Touch	Visible Residue	PCR Inhibition	DNA ID
P49-13-803	4	Biface	Chert	Yes	No	None	*Canid* & Felid numt*
P50-19-1172	2	Biface	Chert	Yes	No	30x dilution	Canid
O48-24-37	Test pit	Biface	Chert	Yes	No	10x dilution	*Canid*; dog, mule deer & cat
P49-18-222	4	Biface	Chert	No	No	30x dilution	*Canid*
P49-15-73	4	Biface	Chert	No	No	None	*Canid*
P50-17-209	2	Biface	Chert	No	No	Complete	----
P49-12-69	4	Biface	Quartzite	No	No	None	----
Q50-18-199	3	Biface	Obsidian	Yes	No	10x dilution	----
P50-13-92	2	Endscraper	Chert	Yes	No	None	*Canid*
P49-4-362	4	Formal Tool	Chert	Yes	Yes	None	----
O50-18-1	6	Formal Tool	Chert	No	No	10x dilution	----
Q49-18-2718	9	Projectile Point	Chert	No	Yes	None	----
O50-12-134	5	Side Scraper	Chert	No	No	10x dilution	*Canid*
P50-23-521	5	Side Scraper	Chert	Yes	No	None	*Canid*
P49-4-176	4	Side Scraper	Chert	No	No	10x dilution	----
P49-16-38	4	Side Scraper	Chalcedony	No	No	30x dilution	----
O50-4-93	5	Side Scraper	Obsidian	No	No	None	----
P49-15-499	4	Utilized Flake	Quartzite	Yes	No	None	*Canid*
O50-18-3	6	Utilized Flake	Chert	No	No	30x dilution	----
P49-4-125	4	Utilized Flake	Obsidian	No	No	None	----
O50-14-107	5	Utilized Flake	Chert	Yes	Yes	10x dilution	----
O50-7-59	5	Flake	Chert	No	No	None	----
O50-8-171	5	Flake	Chert	No	No	Complete	----
P49-12-207	4	Flake	Chert	Yes	No	30x dilution	----

Nearest feature numbers refer to feature locations diagramed in Figure 2. Complete under PCR inhibition column indicates that the DNA extract prevented PCR amplification of 100 copies of pBOVS control template at a 100x dilution. A dash (---) indicates that PCR reactions did not amplify animal DNA from tool extract.

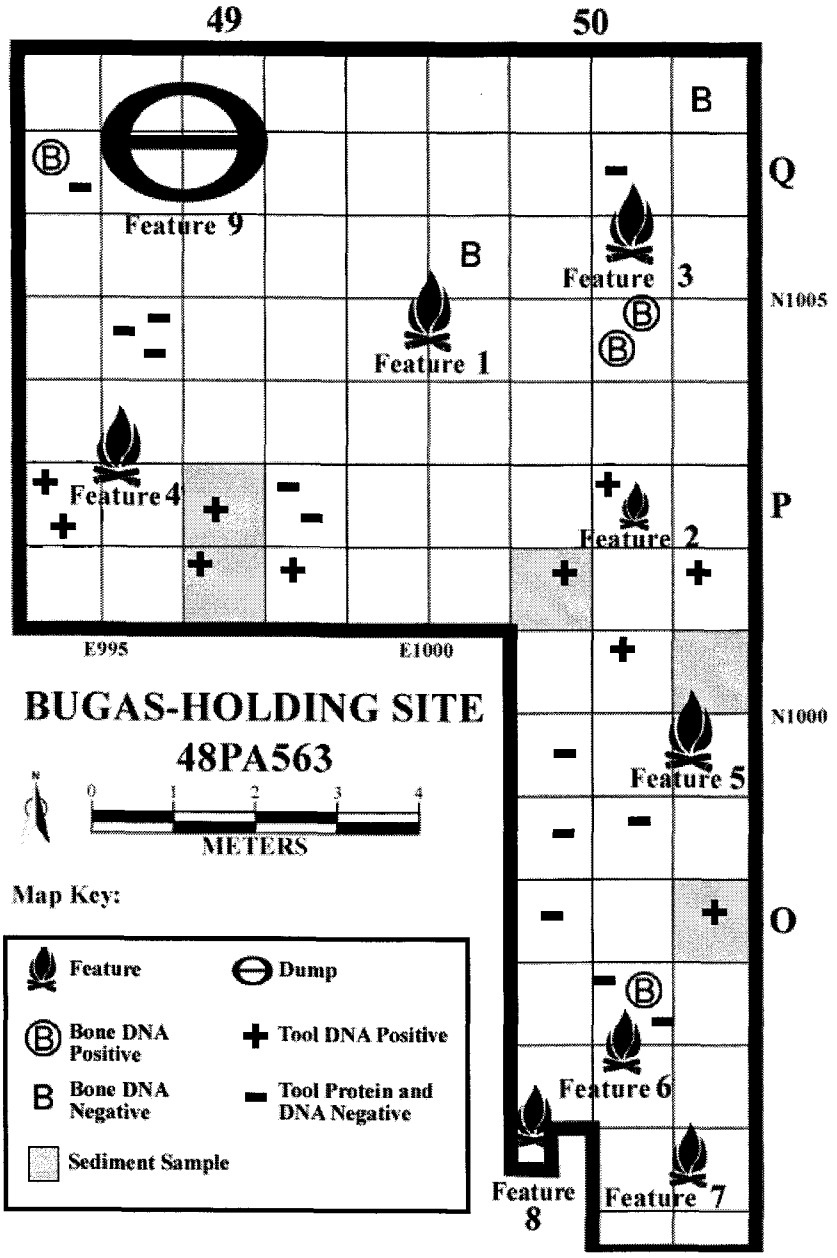

Figure 16: Spatial distribution of features and samples (chipped stone, bones, and sediments) tested for DNA (adapted from [83]).

Canid sequences exhibited high PCR error frequencies. *Taq* DNA polymerase is error prone because it lacks proofreading activity: it cannot remove incorrectly incorporated nucleotides from newly synthesized DNA. PCR products amplified from modern

templates contain misincorporated bases at a frequency of approximately 2.1 x 10^{-4} errors per nucleotide (40). Greater-than-expected rates in PCR products amplified from ancient templates are often attributed to the insertion of incorrect nucleotides by *Taq* DNA polymerase when it encounters lesions in template DNA (26, 30, 44, 70). Modern DNA present in PCR reagents (see Methods, Extraneous DNA in PCR reagents) provided an opportunity to compare error frequencies in canid amplicons with those derived from modern DNAs. Among 15 independently synthesized canid amplicons, seven contained at least one nucleotide change (7.9 x 10^{-3} errors/nucleotide; Figure 17). We analyzed 20 independent cow amplicons derived from extraneous DNA templates found in no-template PCR reactions: one of these amplicons contained a single nucleotide change (6.6 x 10^{-4} errors/nucleotide). The 12-fold higher error frequency in the canid amplicons suggests that canid DNA, which was recovered from stone tools, contained more lesions than the cow DNA present in the PCR reagents.

		Arg	Ser	Met	Met	Phe	Arg	Pro	Leu	Ser	Gln	Cys	Leu	Phe	Trp	Leu	Leu	Val	Ala	Asp	Leu	Leu	Thr	Phe
Artifact No.	**PCR No.**	**C**	**AGC**	**ATA**	**ATA**	**TTC**	**CGG**	**CCC**	**CTT**	**AGC**	**CAA**	**TGC**	**CTA**	**TTC**	**TGA**	**CTT**	**TTA**	**GTC**	**GCC**	**GAT**	**CTT**	**CTC**	**ACT**	**TT**
P49-13-803	0069-16	.	...	...	...	...	T..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..
O48-24-37	0157-7	.	...	...	...	...	...	...	...	...	...	...	...	C.	...	...	...	...	...	...	...	...	...	..
	0157-8	.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	C.	...	..
P49-18-22	0081-1	.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	C.
P50-13-92	0120-8	A	G..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	C.
O50-12-134	0156-17	.	...	...	...	...	...	...	...	...	G.	...	...	...	...	...	...	...	...	...	...	...	...	..
P49-15-499	0058-12	.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	A..	...	...	...	..

Figure 17: Canid amplicons with errors. The top two lines show wild-type canid cytochrome *b* amino acid and nucleotide sequences. Only the nucleotide changes are shown for each amplicons.

Rare PCR products in mixed template amplifications. PCR products amplified from DNA extracted from artifact O50-12-134 (Table 6) were inserted into pCR2.1 and transformed into *E. coli*. Individual transformants were screened for canid sequences by PCR. One of 38 transformants screened by PCR harbored a canid DNA insert; six additional amplicons were sequenced yielding one pig, two cow, and three human sequences. We performed a second PCR using this extract and inserted the amplicons into pCR2.1. To detect the rare canid amplicons from this second PCR, we used colony hybridization to screen 420 transformants. This identified 29 canid clones,

which were confirmed by PCR. Three of these canid amplicons were also sequenced. An additional 20 clones were sequenced yielding three pig, five human, and 12 cow amplicons.

Competition between templates during PCR. DNA recovered from artifact O48-24-37 (Figure 18) contained genes from dog, mule deer, and cat (Table 6). Two independent PCR reactions designed to amplify a portion of the cytochrome *b* gene produced amplicons that contained canid sequences; dog and wolf are identical in the region analyzed. To distinguish these species, a third PCR reaction was performed using primers that amplify the hypervariable D-loop region of both dog and wolf. This sequence identified the animal as a dog with haplotype D3 from clade I (89).

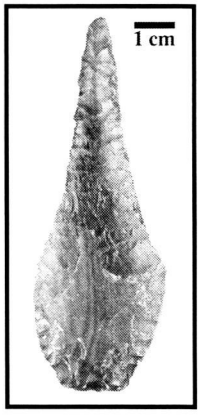

Figure 18: Biface (O48-24-37) excavated from a test pit located 10 meters west of Feature 8 (see Figure 2).

DNA templates from more than one species can compete with one another during PCR, allowing the DNA from one species to interfere with the detection of other species (76). Our cytochrome *b*-specific PCR primers are each 20 nucleotides long and based on a consensus sequence of non-primate mammals (36). This primer pair does not match any species perfectly; differences in primer:template mismatches cause the optimum annealing temperature to vary from one species to another. These primers have 4 or 5 mismatches with felids and New World deer (family Odocoileus),

but each primer has only 1 or 2 mismatches with canid templates, which dominated the PCR amplifications. Mule deer and domestic cat (*Felis catus*) DNA sequences were detected in PCR reactions with primers that closely match 16S rRNA sequences from mule deer (*Odocoileus hemionus*), white-tail deer (*Odocoileus virginianus*), felids, bighorn sheep, bison, bear, and antelope.

Animal DNA was absent in sediment samples. Five sediment samples (P49-13, P50-17, P49-18, O50-12 and P50-12) were tested for the presence of mammalian DNA in the burial environment (Figure 16). Each sediment sample was analyzed in the same way as a stone tool. No mammalian DNA was found. Negative results were not due to PCR inhibitors commonly found in DNA sediment extracts (92). Diluting sediment extracts 10x to 30x alleviated inhibition; comparable dilutions were necessary to eliminate PCR inhibitors from 11 extracts recovered from chipped stone artifacts (Table 6). Bacterial sequences encoding 16S rRNA were recovered from a 30x dilution of sample P49-13, clearly demonstrating that it was possible to recover and amplify DNA from this sediment sample (data not shown).

PCR reagent contamination. To test for the presence of extraneous DNA in PCR reagents and the laboratory environment, we performed 944 no-template and mock-extract PCR reactions: 887 (94%) were negative. We sequenced the 16 PCR products from PCR reactions reported in Table 4 and found DNA from cow (10), guinea pig (3), human (2), and chicken (1). Guinea pig DNA likely originated from extraction reagents or the lab environment because it was detected only in mock extract controls and one tool extract. Cow and chicken DNA present in no-template PCR reactions apparently came from PCR reagents, which cannot be eliminated with physical barriers (see Materials and Methods Contamination Precautions). Water, $MgCl_2$, and 10x buffer are inorganic PCR reagents, unlikely to contain animal DNA. There is no obvious source for cow or pig DNA in primers, which were chemically synthesized and purified by HPLC. We purified our own *Taq* DNA polymerase in a sterile hood supplied with HEPA filtered air, and we used chromophotography equipment

decontaminated with UV light and 10% bleach. Deoxynucleoside triphosphates (dNTPs) are prepared by humans, probably from animal tissues, and DNA from these tissues may contaminate dNTPs. Because dNTP synthesis protocols are proprietary information, it was difficult to prove the source of PCR reagent contamination. Human DNA in no-template reaction and mock-extracts may come from PCR reagents or laboratory personnel. Extracts from stone tools may also contain excavators' DNA and genuine ancient human DNA. Because human cytochrome *b* sequences do not vary between populations, we were unable to resolve the source of human DNA.

During our study, PCR performed with cytochrome *b* primers routinely detected 10 copies of pBOVS template DNA, while the 16S, 12S, and D-loop primer sets consistently amplified 100 copies of pFelid, pFowl, and pK9 templates, respectively. The most sensitive and universal primer set (cytb) accounted for all but one of the PCR products produced in no-template and mock-extract PCR reactions. Contaminating DNA sequences did not confound analysis of chipped stone and bone samples, because they were easily distinguished from bison, canid, and mule deer sequences recovered from Bugas-Holding artifacts.

Discussion

Protein and DNA preservation in ancient bones from Bugas-Holding. Ancient bones often contain sufficient organic material to permit measurement of protein degradation. Amino acid composition of ancient organic material indicates the degree of protein preservation in the sample (18, 86). For example, bones that contain less than 100 nmols/mg of protein are too poorly preserved to radiocarbon date (85). Because similar environmental conditions promote degradation of both protein and DNA (73), protein preservation may correlate with DNA preservation sufficient for PCR amplification. Three bone fragments from Bugas-Holding contained 1251 to 1924 nmols/mg of protein and useable ancient DNA (Table 5). Because bones from Bugas-Holding contained well preserved protein and amplifiable DNA, we inferred that stone tools (that had been used) would likely harbor viable ancient DNA.

Canid residue authentication. We detected canid DNA on nine tools, confirming other evidence that humans processed canids at Bugas-Holding. Excavators drew the same conclusion from a canid femur (Q50-25-28) with three cut marks recovered near Feature 1 (Figure 16) (74). Many Native American groups relied on canids as a food source during difficult times (79). A harsh six-month winter in the Wyoming High Plains may have forced the Bugas-Holding site occupants to such drastic measures.

Several lines of evidence indicate that canid DNA detected on three untouched stone tools (P49-18-222; P49-15-73; O50-12-134) was ancient. First, we performed 579 no-template and mock-extract PCR reactions and never amplified a canid sequence. In contrast, amplification of canid sequences (from specific extracts) was reproducible, but canid sequences were not detected in the burial environment. In addition, canid residues occurred on formal tools and utilized flakes, but flakes that lacked evidence of use did not harbor DNA. Modern DNA introduced during excavation or in our laboratory would be distributed randomly among all types of chipped stone. Canid amplicons exhibited higher PCR error rates than amplicons derived from extraneous modern DNA. PCR errors occur more often when the template DNA contains lesions, which are common in ancient DNA (30, 32). Finally, the presence of canid DNA at Bugas-Holding makes archaeological sense. Two canid teeth were found at Feature 4 near four tools that contained canid DNA, and a canid femur with butchery marks was found nearby at Feature 1 (Figure 16) (74).

Multiple residues detected on a biface. Unlike bones and other animal tissues, tools may contain residues from more than one animal species (83). A chert biface (O48-24-37; Figure 3) excavated from a test pit located 10 meters west of Feature 8 (Figure 18) contained dog, mule deer, and domestic cat DNA. The presence of domestic cat DNA is significant because these animals are not indigenous to the Rocky Mountain geographic area. Domestic cat DNA could be present because an archaeologist handled a cat and then touched the biface. Because the archaeologist may have also handled a dog before touching the tool, the authenticity of the dog DNA is ambiguous. However, it is unlikely that archaeologists encountered mule deer DNA during artifact

handling, because no mule deer remains were discovered at the Bugas-Holding site. Detection of multiple DNAs on a single tool demonstrates the versatility of our method and the potential to generate data regarding tool use and diet diversity not apparent from more traditional lithic analyses.

Amplification from DNA extracts with multiple templates. PCR reactions that use "universal" primer sets are designed to amplify DNA from more than one species. Mixed templates compete with one another under these conditions. In some cases, one template may dominate a PCR reaction and block or severely reduce amplification of other templates. Ancient people probably used their stone tools to process more than one animal. Although sharpening a stone tool between uses may remove some biological residues, stone tools may harbor DNA from multiple species. Even PCR cocktails with template extracted from a single animal may contain DNA from two or more species because PCR reagents contain extraneous DNA, primarily from cows and humans. A universal primer set designed to amplify a portion of the mammalian cytochrome *b* gene detected canid DNA in an extract from a chert biface (O48-24-37). We detected mule deer and cat DNA in the same extract when we used primers (16S) designed to amplify these species preferentially. Extraneous DNA in PCR reagents contributed significantly to amplifications sensitive enough to detect less than 100 copies of template. DNA extracted from a chert side scraper (O50-12-134) yielded canid amplicons, but 93% of the PCR products were derived from cow and other extraneous templates. The possibility that PCR cocktails contain template DNAs from multiple species necessitates the use of multiple primer sets. Also, clone libraries of PCR products must be constructed and screened for rare amplicons, which can be identified by sequencing. Because PCR reagents contain extraneous DNA, we performed more no-template reactions than extract-containing reactions, and we sequenced the products of all positive no-template PCR reactions reported in Table 4. Thorough testing is costly, but it is the only way to identify multiple residues deposited on stone tools and to ensure that DNA detected on stone tools is genuine.

Useable DNA survived post-depositional processes in bones and on stone tools at the Bugas-Holding site. DNA recovered from stone tools and bones identified animals at the subspecies level. Examination of PCR sensitivity, inhibition, and template competition led to several advances. These include internal controls to monitor PCR sensitivity and inhibition, and a strategy to relieve PCR inhibition by diluting extracts. We also developed strategies to recover rare DNAs from PCR reactions that contained template DNA from several species. Our study shows that artifact handling by archaeologists can introduce animal DNA unrelated to tool use; domestic cat DNA was detected on two bifaces touched during excavation and storage (Table 6). To help eliminate extraneous DNA contamination on chipped stone, excavators must wear clean sterile latex gloves during excavation and immediately seal the untouched artifact in a plastic bag. Residues recovered from stone tools are an important source of ancient DNA. The ability to identify residues on stone artifacts will allow researchers to link tool use with animal species to support inferences about human cultural practices.

Chapter 5

General Conclusions

Orin C. Shanks

The studies reported here integrate traditional archaeological methods with molecular techniques to recover and characterize ancient DNA recovered from stone tools and bones. My research addressed several issues:

1. Characterize microcrack model for residue preservation on stone tools.
2. Identify extraction methods that remove residues trapped in subsurface microcracks.
3. Establish precautions to allow detection of trace amounts of DNA while minimizing DNA contamination.
4. Demonstrate recovery of useable DNA from experimentally generated stone tools sonicated in 5% ammonium hydroxide.
5. Test the validity of DNA residue analysis from ancient stone tools and bones recovered from the same stratigraphic context.

I streamlined techniques to maximize DNA recovery from chipped stone. I show that artifact handling by archaeologists can introduce animal DNA unrelated to tool use. Examination of PCR sensitivity, inhibition, and template competition led to several method advances. These include internal controls to monitor PCR sensitivity and inhibition, and a strategy to relieve PCR inhibition by diluting extracts. I also developed strategies to recover rare DNAs from PCR reactions that contained template DNA from several species. This research provides strong evidence for the reliability of residue identification from ancient stone tools.

Microcracks Rapidly Trap DNA and Protein

Experiments support the hypothesis that microcracks harbor DNA and protein residues. Microcracks penetrate below the surface of a tool and readily trap blood residues containing DNA and protein (Chapter 2). My model for residue preservation in subsurface microcracks was characterized with experimentally generated obsidian microblades. However, all cryptocrystalline rocks – used by ancient peoples to make flaked stone tools – contain microcracks (84), which are routinely produced with each

fracture or flake removal during tool manufacture (8). Thus, an individual microblade, flake, projectile point, or scraper may contain hundreds or thousands of subsurface microcracks that may harbor ancient animal residues.

Blood enters microcracks by capillary uptake within 5 minutes of exposure (Chapter 2). Thus, residue trapping does not require lengthy contact with blood and provides a means to identify expedient tool use, which is not possible by traditional use-wear methods.

Residue Recovery from Microcracks

My work demonstrates the importance of the extraction step in residue analysis, because some procedures remove only surface residues whereas others dislodge DNA and protein from subsurface microcracks. Blood residues are not removed by thorough washing of a microblade surface with water or buffered saline. However, treatment with 4M guanidine hydrochloride, a strong denaturant, or sonication in 5% ammonium hydroxide removed 60-80% of DNA and protein harbored in microcracks (Chapter 2). These observations explain why residue identification studies where tools were washed or soaked in water or buffered saline had low residue identification success rates (17, 66). Simulation experiments suggest that microcracks in stone tools used for killing or processing animals may sequester biological residues from the elements and from tool cleaning after excavation. The potential to recover DNA and protein residues from previously washed stone tools greatly expands the number of artifacts suitable for biological residue analysis.

Precautions to Reduce Extraneous DNA

I used physical barriers, dedicated equipment, and rigorous cleaning regimes to limit extraneous DNA from equipment, other samples, and previously synthesized amplicons (Chapter 1). To test for the presence of extraneous DNAs, I performed 1,029 no-template and mock extract control PCR reactions, 968 (5.9%) were negative. Positive no-template control PCR reactions suggest that PCR reagents may contain modern DNA (Chapter 4). Extraneous DNA sequences did not confound analysis of

stone tool residues and bones, because they were easily distinguished from bison, canid, antelope, and mule deer sequences recovered from experimentally generated tools (Chapter 3) and Bugas-Holding artifacts (Chapter 4).

To monitor the introduction of previously synthesized amplicons into new PCR reactions, I used control template DNAs containing insertions or deletions. The PCR products amplified from these control templates were easily distinguished by agarose gel electrophoresis from products amplified from wild-type DNAs. During our studies, each PCR set-up included one control template positive reaction and five test reactions (1, 5, 10, 30, and 100-fold dilutions) to detect PCR inhibition in extracts. Control template PCR products were handled in the same way as sample extract PCR products. Due to the unique size of control templates, contamination from previously synthesized control template amplicons would be obvious in no-template, mock extract, or sample extract PCR reactions. No previously synthesized control template PCR amplicons were detected in these reactions.

The ability to detect trace quantities of extraneous DNA required excessive numbers of no-template and mock extract control PCR reactions. Dedicated equipment, physical separation, and contamination characterization were costly, but it was the only way to ensure that DNA detected in ancient samples was genuine.

DNA Recovery from residues deposited on washed experimental stone tools

To gauge the performance and reliability of DNA recovery with sonication in 5% ammonium hydroxide, I extracted residues from stone tools used by Dr. Marcel Kornfeld (University of Wyoming) to butcher an antelope (*A. Americana*). Because Dr. Marcel Kornfeld did not reveal the identity of the animal until after the DNA study was completed, this was a blind test. Sequence analysis of DNA recovered from the unknown animal residues correctly identified the butchered animal. This study suggests that tools used in animal butchery harbor sufficient residues for DNA and protein analyses. Sonication in 5% ammonium hydroxide did not compromise recovery of useable DNA.

I also performed eight sequential washes on one tool to test the effect of artifact cleaning on recovery of DNA and protein residues for molecular analysis. The initial tool wash was designed to simulate washing protocols commonly used by archaeologists to prepare tools for morphological examination (80, 81). These experiments showed that surface cleaning techniques typically used by archaeologists only remove a small fraction of the protein deposited during butchery. Artifact cleaning removed poorly preserved surface residues and left behind sheltered residues trapped below the tool surface.

A Case Study: The Bugas-Holding Site

To test the validity of residue analysis, I studied 24 pieces of chipped stone for the presence of DNA residues, I compared DNA preservation in bones and chipped stone from the same stratigraphic context, and I analyzed five sediment samples recovered from the Bugas-Holding site in northwestern Wyoming.

DNA and Protein preservation in ancient bones. Many archaeological sites could benefit from DNA residue identifications from chipped stone. However, it is difficult to predict whether a specific site will yield useable ancient DNA. Randomly selecting sites can be expensive and offers little assurance of residue preservation. To estimate the level of organic preservation at Bugas-Holding, we measured protein preservation in three bones associated with chipped stone. The three bone fragments yielded near modern levels of protein preservation and useable ancient DNA. Independent DNA analysis of two bone samples by Drs. Jennifer Leonard and Robert Wayne (UCLA) confirmed the DNA sequence identifications. Characterization of protein preservation in bones from Bugas-Holding was an inexpensive and reliable strategy to infer the probable condition of DNA on chipped stone.

DNA residues recovered from chipped stone. I tested 24 chipped stone artifacts for DNA residues. Nine tool DNA extracts from Bugas-Holding contained sufficient DNA for detection by PCR including five bifaces, two side scrapers, one end scraper,

and one utilized flake. The excavators did not touch three of these tools. One tool extract contained DNA from three species, and these templates competed during PCR amplification. On two artifact DNA extracts, handling after excavation probably introduced animal DNA unrelated to tool use.

Although this study was designed to test the validity of DNA residue identification methods, results provide another set of data relevant to site interpretation. The presence of canid DNA in chipped stone DNA extracts suggests that these animals were processed at Bugas-Holding and might play an important role in Rocky Mountain winter subsistence strategies. Thus, faunal (74), chipped stone (7, 48), and DNA studies all suggest that animal processing occurred at the Bugas Holding site.

Animal DNA was absent in sediment samples. Compounds in the sediment, such as animal feces, may lead to DNA identifications unrelated to tool use. Five sediment samples (P49-13, P50-17, P49-18, O50-12, and P50-12) were tested for DNA. No mammalian DNA was found suggesting that DNAs recovered from chipped stone extracts (canid, mule deer, and domestic cat) are not extraneous sequences from the burial environment.

Future research at the Bugas-Holding site. My work at the Bugas-Holding site has raised an interesting archaeological question. Despite the relative abundance of bighorn sheep bones, I did not detect DNA from this species on chipped stone. Instead, I found DNA from other animals including dog and mule deer. Although the absence of bighorn sheep DNA on stone tools could be a function of small sample size, I expected to find this DNA on stone tools at Bugas-Holding because: 1) bighorn sheep bones are abundant and 2) the PCR conditions work well on ancient bighorn sheep (9). Where is the bighorn sheep we expected to find on these tools?

Faunal seasonality and mortality studies suggest that bighorn sheep were butchered early in the site occupation (74). Analysis of 75 fetal bison bones combined with tooth eruption and wear studies of bighorn sheep, bison, and elk remains

recovered from Bugas-Holding indicates a four to five month winter occupation extending from October or November until March or April (74). Based on tooth eruption and wear patterns of bighorn sheep, Rapson (1990) concludes that a distinct mortality pattern of faunal use emerges with bighorn sheep being killed during a restricted period of the fall (September-November).

In addition, excavators point out the extremely small average size and the high frequency of broken stone tools deposited at the site (48, 74). Many of the stone tools show evidence of resharpening, which probably removed the tool surface that contained blood from the first animals (perhaps bighorn sheep) processed with the tools. Stone tools may contain primarily (or only) DNA deposited after the last resharpening. By the same reasoning, debitage produced by sharpening probably contain most (or all) of the DNA deposited by butchery events that occurred prior to sharpening. The dull surfaces removed during sharpening are precisely those that had the most forceful contact with animal tissues, and these surfaces likely contain substantial amounts of DNA.

The abundant debitage excavated around specific features at Bugas-Holding offer an opportunity to test this novel hypothesis, first suggested by Dr. Marcel Kornfeld, that debitage produced by resharpening stone tools may be an excellent source of DNA residues. If bighorn sheep DNA are more abundant on debitage than on intact stone tools, residue analysis may support the current interpretation that bighorn sheep were butchered early in the occupation.

Methodological Advancements

Thorough investigation of residue preservation in obsidian microblades, the release of residues trapped in subsurface microcracks, DNA analysis of artifacts from Bugas-Holding, and examination of PCR sensitivity, inhibition, and template competition led to several method advances. Procedures established through our studies will help future investigators achieve the full potential of DNA residue analysis.

DNA recovery from chipped stone microcracks. Studies indicate that submerging a stone tool in a 5% ammonium hydroxide while sonicating can release up to 80% of trapped DNA and protein containing residues from experimentally generated obsidian microblades (Chapter 2). To enhance the sonic action, we forced solvent into microcracks with vacuum infiltration. Improved wash regimes led to the successful recovery of residues preserved on stone tools from the Bugas-Holding site (Chapter 4).

Guidelines for field collection of chipped stone artifacts for DNA analysis. Artifact handling by archaeologists may introduce non-human animal DNA unrelated to tool use. We detected domestic cat DNA on two bifaces recovered from the Bugas-Holding site that were touched during excavation and storage (Chapter 4). These animals are not indigenous to a Rocky Mountain geographical area. Domestic cat DNA could be present because an archaeologist handled a domestic cat and then touched these tools. To help eliminate extraneous DNA on chipped stone, excavators must avoid touching the artifact by wearing clean latex gloves during excavation and immediately sealing the untouched artifact in a plastic bag. In a separate bag, seal a small amount (~ 10 grams) of sediment from the adjacent area where the artifact was found. Sediment samples are needed to identify extraneous DNA present in the burial environment.

Amplification from DNA extracts with multiple templates. PCR amplifications that use "universal" primer sets are designed to amplify DNA from more than one species. Mixed templates compete with one another under these conditions (76). In some cases, one template may dominate or severely reduce amplification of other templates (Chapter 4). Ancient people probably used their stone tools to process more than one animal. Although sharpening a stone tool between uses may remove some biological residues, stone tools may harbor DNA from multiple species.

PCR reagents contain extraneous DNA, primarily from cows and humans (Chapter 4). Thus, even PCR cocktails with template extracted from a single animal may contain DNA from two or more species. Our research indicates that extraneous

DNA in PCR reagents contribute significantly to amplifications with universal primers sensitive enough to detect less than 100 copies of template DNA (Chapter 4).

The possibility that stone tool DNA extracts and PCR cocktails contain template DNAs from multiple species necessitates the use of multiple primer sets. Also, clone libraries of PCR products must be constructed and screened for rare amplicons, which can be identified by sequencing. Because PCR reagents contain extraneous DNA, one must perform more no-template reactions than extract-containing reactions, and all products from positive no-template reactions must be sequenced.

Monitoring PCR performance with control templates. DNA template containing PCR reactions either yield detectable PCR product or they do not. There could be several reasons for no detectable amplicons. It is possible that a reagent expires or an experimental error occurred during PCR set-up. Maybe the DNA extract contains uncharacterized compounds that interfere with PCR amplification. To monitor PCR amplification sensitivity and inhibition, we added control templates to PCR test reactions. Amplification of PCR reacts with approximately 1, 10, 10^2, 10^3, and 10^4 molecules of control temple DNA established PCR sensitivity levels for each primer set (Chapter 4). Test reactions containing control template and DNA extract identified the presence of uncharacterized inhibitors that prevented amplification (Chapter 4).

Removal of PCR inhibitors from DNA extracts. PCR inhibition was observed in more than half of the extract PCR amplifications. Dilutions ranging from 10 to 30-fold removed 90-97% of the inhibitors from chipped stone DNA extracts and allowed amplification of control template DNA (Chapter 4). For example, a DNA extract from an untouched chert biface (P49-18-222) required a 30-fold dilution to alleviate inhibition of control template DNA. Amplification of the same diluted extract with our universal cytochrome *b* primers yielded a canid DNA sequence. Thus, diluting extracts was a simple and effective strategy to remove uncharacterized PCR inhibitors from DNA extracts.

Closing Statement

Experimental studies and analysis of ancient bones and stone tools from Bugas-Holding have laid the groundwork for future ancient DNA research. I show that microcracks produced during stone tool manufacture trap blood residues beneath the tool surface. I optimized methods to maximize DNA recovery, detect DNA from extracts with multiple templates, monitor PCR sensitivity, and alleviate PCR inhibition. In addition, I outline specific guidelines for archaeologists to collect artifacts for DNA analysis. Ultimately, these studies demonstrate the validity of DNA residue identification from chipped stone and the potential to generate data regarding tool use and diet diversity not apparent from more traditional lithic analyses.

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Appendix

Appendix: DNA-Free Glove Box Dedicated Equipment

Box 1 (Reagent Preparation). Pippetors (p20, p200, and p1000); Sartoris BP615 analytical balance; plastic microtube rack; sharpie; stainless steel forceps; squeeze bottle; low retension barrier pippetor tips (p10, p20, p200, and p1000); 50ml falcon tubes; kimiwipes; medium ziplock bags; low retension microtubes (0.65ml, 1.6ml, and 2.0ml)

Box 2 (DNA Recovery). Pippetors (p20, p200, and p1000); VWR heat block; stainless steel scissors; stainless steel forceps; 1210 Branson Ultra Sonic bath; plastic microtube rack; Eppendorf Aerosal-tight Capsule rack; Eppendorf Aerosal-tight Capsule bodies, springs, and safety caps; Eppendorf Aerosol-tight Fixed-angle Rotor; Eppendorf Microcentrifuge 5415C; squeeze bottle; Lab Quake Rotisserie Shaker; sharpie; polypropylene bags (0.2 mm thickness); low retension barrier pippetor tips (p10, p20, p200, and p1000); 50ml falcon tubes; kimiwipes;; low retension microtubes (0.65ml, 1.6ml, and 2.0ml)

Box 3 (Speed Vac Concentratrion). Savant SC110 Speed Vac; Sargent-Welch DirecTorr Vacuum Pump; stainless steel cold trap; vacuum pump filter (2" diameter x 24" length PVC pipe packed with calcium carbonate and sodium bicarbonate); medium ziplock bags; kimiwipes; Parafilm; 0.22um filtration disk (Fisherbrand 09-719A)

Box 4 (PCR Set-Up). Pippetors (p20 and p200); mini centrifuge; stainless steel forceps; squeeze bottle; plastic PCR microtube rack; low retension barrier pippetor tips (p20 and p200); kimiwipes

Box 5 (PCR Template). Pippetor (p10); mini centrifuge; stainless steel forcep; low retension barrier pippetor tips (p10); kimiwipes, medium ziplock bag; squeeze bottle