

Predator species identification from saliva at kill sites with limited remains

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Abstract

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Kill site investigations can provide valuable data about elusive predator species and predator-prey interactions for wildlife scientists and managers alike. However, the predator species must be definitively identified as the one responsible for the predation event. The traditional method of visually analyzing prey remains to identify predator species is prone to observer bias and may be particularly challenging in systems with congeneric predators. Often, so few remains are left behind that visual analysis is rendered impossible. Other potential evidence left by predators, such as scat or tracks, may be difficult to reliably link to the responsible predator or require adequate substrate conditions. Continuing advancements in molecular techniques provide a more objective option that is also feasible on limited remains: swabbing for predator DNA from saliva. This underutilized method has primarily been applied to larger carcasses that provide ample surface area to collect epithelial saliva cells. This study demonstrates the usefulness of a saliva-swabbing method for smaller prey with minimal surface area, including kill sites with almost no prey remains or even just a radio-collar left behind. This

is also the first study to compare saliva-swabbing success by sample type (carcass remains versus the radio-collar). From 2010-2013, I employed forensic techniques to increase certainty about predator species identification at snowshoe hare (*Lepus americanus*) kill sites for the ultimate purpose of elucidating predator-prey interactions. Predator saliva at kill sites was sampled with foam buccal swabs, stored in lysis buffer for shelf-stable preservation, and tested for predator mitochondrial DNA using the polymerase chain reaction (PCR) and species-specific primers. This saliva-swabbing method yielded definitive and objective predator species identification for the majority (58.5%) of sampled kill sites ($N = 31/53$). Not only were small amounts of remains (carcass and/or fur) often able to yield DNA from predator saliva, but saliva swabs from radio-collars proved to be significantly more successful for predator species identification (4.04 odds ratio). Saliva-swabbing also provided identifications at 65.5% of kill sites lacking unambiguous predator sign and contributed significantly more predator identifications throughout the year when compared to tracking methods. The expansion of saliva-swabbing methods to smaller prey, radio-collars, and limited remains allows for more definitive predator identifications at kill sites than have been possible with commonly employed methods, thus augmenting the potential to understand and manage for predators and prey.

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INTRODUCTION

Predator kill sites can provide a wealth of information about both predator and prey species. For example, if the responsible predator is known, for example, kill sites can provide insights into the presence of elusive predators (Mumma *et al.* 2014, Onorato *et al.* 2006), sources of interspecific conflict (Mattisson *et al.* 2011, Wengert *et al.* 2014), and foraging behavior (Husseman *et al.* 2003, Merrill *et al.* 2010). Kill site investigations are also important for resolving ambiguities following livestock depredations (Cavalcanti and Gese 2010, Sundqvist *et al.* 2008, Williams *et al.* 2003). For prey species, kill site data can reveal the relative importance of different sources of mortality and be the basis for demographic analysis and inference about patterns of predation risk (Bergman *et al.* 2006, Brand *et al.* 1975, Hebblewhite and Merrill 2007, Wirsing *et al.* 2002). Furthermore, kill site assessments that identify the predator can elucidate natural enemies for particular prey species and enable analysis of the impacts of different predator species on prey populations (Bergman *et al.* 2006, Clark *et al.* 2014, Knopff *et al.* 2010).

Kill sites are usually found either using clustered GPS/VHF locations from collared predators (*e.g.*; Kertson *et al.* 2011, McPhee *et al.* 2012, Svoboda *et al.* 2013, White *et al.* 2011), or by deploying radio-collars on prey species (*e.g.*, Ballard *et al.* 1981). These two approaches are not mutually exclusive; nonetheless, the prey-tracking method may be preferable if predators occur at low densities on the landscape, are difficult to trap, or are considered too sensitive to be studied solely with direct tracking methods due to the potential risks involved in immobilizing and collaring (Arnemo *et al.* 2006, Gommel *et al.* 2006, West *et al.* 2008). Moreover, while limited in only representing the prey species being tracked, the prey-tracking approach is unaffected by the amount of time spent by predators at kill sites, thus providing information on smaller prey species (or abandoned caches) that kill-site cluster analyses often miss (Bacon *et al.*

2011, Cavalcanti and Gese 2010, Donoghue *et al.* 1998, Neal *et al.* 1987, Ruth *et al.* 2010, Webb *et al.* 2008). When the prey rather than the predator leads the researchers to the kill site, forensic investigation may be needed to identify the predator. Such endeavors have been hindered by the difficulty in accurately and confidently determining the predator species when dependent on traditional methods.

Predator identification at kill sites has typically been obtained via scat collected at the kill site (*e.g.*, Ernest *et al.* 2002, Onorato *et al.* 2006) or visual diagnostics of remains (Hygnstrom *et al.* 1994, Neale *et al.* 1998, Sacks *et al.* 1999, Squires and Ruggiero 2007). However, in the absence of tracks or an unambiguous kill signature, these methods are often inadequate to definitively identify the predator responsible for the predation event (Larivière 1999, Ratz *et al.* 1999, Rosas-Rosas *et al.* 2008). Scat studies have dramatically improved because of molecular techniques for genetic testing, and, when seeking only presence data, scat can provide larger sample sizes than saliva or hair (Sugimoto *et al.* 2012). Nonetheless, using scat to link a predator to a specific kill site involves the oft-tenuous assumption that predator presence in the vicinity of the kill site equates to responsibility for the predation event (Kelly *et al.* 2011, Logan *et al.* 1996, Onorato *et al.* 2006, Pierce *et al.* 1999). Additional biases may arise as scat from certain predators can be easier to locate (Kelly *et al.* 2011, Onorato *et al.* 2006), an issue that is likely amplified by utilizing a large search-radius. These issues may be compounded by the length of time during which a scat may be present and the difficulty of accurately aging the scat (Hunter 2011, Mesa *et al.* 2014, Tsaparis *et al.* 2009). Scat is also not necessarily dependably present at kill sites (*e.g.*, Mumma 2014), particularly if the prey required minimal handling time. Scat deposition at a kill site is often related to handling time, but handling time can vary dramatically because of differences in a predator species' handling style, scarcity of prey, size/type of prey,

and even time of day (Fuller 1989, Sand *et al.* 2005, Smith *et al.* 2004, Tambling *et al.* 2010, Webb *et al.* 2008).

Visual diagnosis of the remains (*e.g.*, bite wound location and size, shearing of fur, amount of hemorrhaging, dismemberment, and amount of remains consumed) is another method commonly used to assign predator species at a kill site (Lyver 2000). Unlike scat, the carcass remains are clearly related to the predation event itself, so long as predation is the known proximate cause of mortality. Visual analysis of remains and/or scat is problematic, however, because misidentification based on visual characteristics is quite common (Farrell *et al.* 2000, Hernandez *et al.* 1997, Larivière 1999, Mumma *et al.* 2014, Rosas-Rosas *et al.* 2008, Sargeant *et al.* 1998; but see Prugh and Ritland 2005). Misidentification may be due to similar carcass-handling styles (particularly amongst congeneric predators), overlapping dentition morphology, minimal carcass remains with which to work, tampering of remains by small animals, scavenging, rarity of the predator, or observer inexperience (Cozza *et al.* 1996, Davison *et al.* 2002, Larivière 1999, Lyver 2000, Mumma *et al.* 2014, Williams and Johnston 2004).

Advances in molecular techniques may allow saliva-swabbing methods to present a viable alternative to traditional forensic approaches (Ernest *et al.* 2002, Kilgo *et al.* 2012, Mumma *et al.* 2014, Sundqvist *et al.* 2008, Wengert *et al.* 2014, Williams and Johnston 2004). Saliva swabbing provides an objective method for testing the evidence most reliably associated with the predation event: the carcass remains. The few studies that have employed the modern forensic technique of saliva collection at a kill site to obtain predator identity have examined carcass remains with a relatively large surface area (Appendix Table 2). Some of the earliest work to obtain predator saliva from prey remains served to identify the predator responsible for livestock depredations (Blejwas *et al.* 2006, Ernest and Boyce 2000, Williams *et al.* 2003) and

the practice is being successfully implemented in newer livestock-depredation studies as well (Caniglia *et al.* 2013). Recent studies have successfully applied a saliva-swabbing method to kill sites of wild ungulate prey (Kilgo *et al.* 2012, Mumma *et al.* 2014), providing valuable information on predator-prey interactions. Saliva swabbing has proven effective when investigating cause of death of a sensitive predator species, the fisher (*Martes pennanti*) (Wengert *et al.* 2013, Wengert *et al.* 2014). Saliva has also been used to identify ungulates to species by testing browsed twigs (Nichols *et al.* 2012). Steffens *et al.* (2012) identified a suite of both aerial and terrestrial predators that were depredating black-fronted tern (*Chlidonias albobriatus*) nest sites using a combination of methods, including saliva swabbing. Saliva-swabbing has also been utilized in environments that were partially manipulated for research purposes. A recent study used saliva to help identify predators of woylies (*Bettongia penicillata*) in predator-baited areas (Marlow *et al.* 2015), and Harms *et al.* (2015) examined how nuclear DNA from the saliva of captive wolves (*Canis lupus*) and lynxes (*Lynx lynx*) degrades over time on roe deer (*Capreolus capreolus*) carcasses.

The ability to successfully amplify predator DNA from saliva collected at kill sites of smaller animals and/or small surface area has not been extensively studied. The relative novelty of saliva swabbing as a method in predator-prey research, and the assumption that it will be less effective in cases where the prey species is small and/or mostly consumed, may have limited its study, particularly with wild species in natural systems. Validation of this approach at kill sites where prey remains are limited would offer a new tool for understanding the ecology of predators targeting small-bodied species. Accordingly, I applied a saliva-swabbing technique to help identify predators of a prey species with low body mass – the snowshoe hare (*Lepus*

americanus) – and whose carcass remains at kill sites are typically minimal. Specifically, I addressed the following questions:

(1) Can limited or small-prey remains yield sufficient saliva DNA for predator identification?

(2) Can saliva swabbing be used to assign predator species when visible predator sign is absent or ambiguous?

(3) Can predator saliva be obtained from just a radio-collar, and, if so, is there a significant difference in the ability to obtain predator saliva by sample type (radio-collars vs remains)?

I also provide enough detail about the DNA collection methodology for it to be reproducible by other researchers.

MATERIALS AND METHODS

Study Area

I evaluated the efficacy of saliva-swabbing small prey species in north-central Washington, on the northeastern slopes of the Cascade Mountains, near the city of Loomis, WA (48°53'35"N, 119°49'20"W). I chose six trapping sites 17-20 ha in size based on adequate sign of snowshoe hare use. Sites were also chosen from within a region utilized by Canada lynx (*Lynx canadensis*), a carnivore that is listed as federally threatened under the Endangered Species Act and as threatened in several states of the United States, including Washington. Elevation within the study area ranges from 1,383-1,932 m. The trapping sites were in the Loomis State Forest and Okanogan National Forest, managed by the Washington Department of Natural Resources and USDA Forest Service, respectively. The study region is characterized by mixed southern boreal forest dominated by lodgepole pine (*Pinus contorta*), subalpine fir (*Abies lasiocarpa*), and Engelmann spruce (*Picea engelmannii*), along with scattered Douglas-fir (*Pseudotsuga menziesii*), sitka alder (*Alnus sinuata*), and western larch (*Larix occidentalis*). All trapping sites have experienced minor to heavy levels of timber management over time.

Field Methods

From 2010-2013, I affixed mortality-sensitive VHF radio-collars (24 g, Advanced Telemetry Systems, Isanti, MN, USA) and numbered ear tags (No. 3, National Band and Tag Co., Newport, KT, USA) on 223 individual snowshoe hares weighing at least 575 g (below 5% of total body mass¹). I captured snowshoe hares using Tomahawk live traps (32" × 9" × 9" double-door, Tomahawk Live Trap Co., Tomahawk, WI, USA), baited with alfalfa-based rabbit feed and covered with tree branches for camouflage and insulation. During each trapping session, I set 40-50 traps as a grid, with trap lines spaced approximately 50 m apart from each other and any roads. I set the trapping grids in the late afternoon or evening and checked them each morning. I occasionally set out partial (*e.g.*, ~50%) grids to maintain sufficient numbers of collared hares while balancing logistical needs. Snowshoe hare capture and handling procedures were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC) under protocol 4226-02.

I monitored hare radio-collar frequencies for mortality signals a minimum of three times per week each summer, fall, and winter to allow for timely investigations. Mortalities were not monitored from April to June because of access issues from snow-melt. Upon detection of a mortality signal (double-pulse mode, activated after 8 hours with no movement), which was usually within 24-72 hours from the last detected "live" signal, I used ground-based VHF radio-telemetry to locate the mortality site (typically found within a few hours). In order to avoid cross-contamination (*i.e.*, potentially transferring predator DNA from another area by touching or stepping on the radio-collar or remains), I exercised extreme caution in the final stages of searching and subsequent processing of kill sites because of the camouflaged nature of snowshoe

¹ One additional hare weighing 550 g was fitted with a collar weighing 22 g.

hares. Specifically, I abruptly slowed my hiking/snowshoeing pace when signal strength indicated close proximity to the radio-collar, and I visually verified the absence of remains/fur or tracks before taking additional steps forward. Upon discovery of the kill, I assessed the proximate cause of mortality and whether the hare died of predation. Predation was determined based on tracks, signs of a struggle, sub-dermal hemorrhaging, and amount of carcass/fur remaining. The specific location of the kill site was determined based on the location of the collar and other evidence (*e.g.*, signs of a struggle). I documented all evidence potentially related to predator identity in datasheets and with photographs. Collectable prey evidence relevant to the predation event (carcass pieces, bone fragments, matted fur, and the radio-collar) as well as any predator evidence (predator hair(s), scat, raptor feather(s)) was transferred using uncontaminated materials (*e.g.*, disposable chopsticks, sterile disposable gloves, sterilized tweezers) for collection in sterile freezer bags. Bags were labeled and kept on ice packs during warmer months for the return trip to the field station, at which point they were either swabbed immediately or placed in a freezer to be swabbed when more time was available (typically within a week).

Saliva-Swabbing Protocol

There was a paucity of relevant literature on saliva swabbing at wildlife-attributable kill sites at the beginning of my study (Ernest and Boyce 2000: $N_{kill\ sites} = 4$; Blejwas *et al.* 2006: $N_{kill\ sites} = 37$ kill sites; Sundqvist *et al.* 2008: $N_{kill\ sites} = 2$)², which led me to develop my techniques from human forensics methods. I utilized the double-swabbing technique, which entails rubbing a wet swab followed by a dry swab on the same surface because it can improve collection rates by loosening the epithelial cells (Sweet *et al.* 1997), and a wetted sampling device improves cell

² All of these studies were on domestic sheep (*Ovis aries*) depredation.

transfer in general (Goray *et al.* 2010). I also chose synthetic foam swabs over the commonly used cotton swabs because I was concerned about degradation of non-target organic compounds, and synthetic swabs have been shown to be superior to cotton when collecting saliva-based DNA (Mitsouras and Faulhaber 2009, Rutledge *et al.* 2009). Furthermore, I specifically chose foam swabs specialized for buccal collection over other synthetic options because of their large surface area and porous composition (EpiBio Catch-All™ Collection Swabs, Epicentre, Madison, WI, USA). I utilized amber glass vials with the caps pre-attached to protect samples from light deterioration and to avoid handling contamination (*i.e.*, accidental transfer of predator DNA from a previously visited site). I chose lysis buffer solution as my method for mtDNA stabilization and storage after experimenting with air-drying of swabs, freezing, desiccation with silica packets, and the lysis buffer solution. Air-drying proved inconvenient due to risk of contamination in a cramped field station, and freezing was not ideal given the risk of thawing and the need for eventual processing in a distant lab. Initial results of PCR analysis indicated that lysis buffer and silica storage methods yielded similar amplification rates, and the lysis buffer was determined to be the more convenient of the two options for lab processing. Additional information can be found in the Appendix.

I prepared a sanitized workspace at the field station prior to processing samples. Small amounts of 1% Phosphate Buffered Saline (1x PBS, Teknova, Hollister, CA, USA) and lysis buffer (Buffer ATL, Qiagen, Valencia, CA, USA) were each transferred to two separate high-density polyethylene/polypropylene bottles (Nalgene Wide-Mouth Bottle, HDPE/PP, 30 mL, Nalgene, USA). After setting out, labeling, and opening all of the supplies, I would don a fresh pair of sterile gloves. A foam swab (EpiBio Catch-All Collection Swab) was then dipped in PBS, rubbed thoroughly over the sample while keeping it inside the bag, and broken off into a

sterile vial (8mL Borosilicate Amber Glass Vial with Phenolic 14B Rubber Lined Cap Attached, Wheaton Industries, Millville, NJ, USA). This process was repeated over the same sample area with a second foam swab with no PBS (dry). After breaking the second swab into the same vial as the first, 100 µl of lysis buffer were deposited onto the swabs using a needleless syringe (1cc syringe, Monoject, St. Louis, MO, USA). Material lists and step-by-step kill site analysis, collection, and swabbing methods are located in the Appendix.

Laboratory Analysis

In collaboration with the Rocky Mountain Research Station (Kristy Pilgrim/Michael Schwartz), genomic DNA was extracted from swab samples using the QIAGEN Dneasy Blood and Tissue kit according to manufacturer's instructions for tissue samples. The 16S rRNA region of mitochondrial DNA (mtDNA) was amplified using conserved, universal primers 16sL 5'-TTAAACGGCCGCGGTATCC-3' and 16sR 5'-GAATTACGCTGTTATCCCT-3' modified from Hoelzel and Green (1992) as well as felid and canid primers developed by Wengert *et al.* (2013). Reaction volumes of 50 µl contained 50–100 ng DNA, 1× reaction buffer (Applied Biosystems, MA, USA), 2.5 mM MgCl₂, 200 µM each dNTP, 1 µM each primer, and 1 U AmpliTaq Gold polymerase (Life Technologies, CA, USA). The PCR program was 94°C/5 min, [94°C/1 min, 55°C/1 min, 72°C/1 min 30s] × 34 cycles, 72°C/5 min. The quality and quantity of template DNA were determined by 1.6% agarose gel electrophoresis. PCR products were subjected to restriction fragment analysis as described in Mills *et al.* (2000). PCR products from samples that were analyzed for species using DNA sequencing analysis were purified using ExoSap-IT (Affymetrix-USB Corporation, OH, USA) according to manufacturer's instructions. DNA sequence data were obtained using the Big Dye kit and the 3700 DNA Analyzer (ABI; High Throughput Genomics Unit, Seattle, WA, USA). DNA sequence data were viewed and aligned with Sequencher (Gene Codes Corp., MI, USA).

Statistical Analyses

Data Preparation

All statistical analyses were conducting on fresh kill sites unless otherwise specified. My *a priori* intentions, as well as the frequency distribution of the data, determined what constituted a fresh kill site. Old kill sites (based on maximum time elapsed since the hare was last known to be alive) had only been processed opportunistically, during collar salvages after the spring hiatus from field work, and were not originally intended for analysis. I did not expect to obtain saliva from older kills, nor would I be able to compare the saliva results with other definitive predator sign (*e.g.*, tracks) by including older kills. However, samples from older kill sites were still processed in the lab, primarily to evaluate the potential longevity of salivary mtDNA. To determine the threshold for fresh versus old kill sites, I examined the distribution of kills by age. The distribution of kill-site age for all sites, including old kill sites, was left-skewed and roughly bimodal, with very few kill sites collected between 11-95 days ($N = 5/78$) in comparison to 96-167 days ($N = 18/78$) (Fig. 1). Most kill sites (70%) were processed within 10 days of the hare's last "known-alive" date ($\bar{x} = 2.7$ days since last live date), and older kill sites were only processed opportunistically during recovery of radio-collars after the spring hiatus. In contrast to the mean maximum age of kill sites ≤ 10 days (2.7 days), the mean age of old kill sites was 99.3 days. After binning the kill sites for relative evenness in sample size (see Appendix: Supplemental Information), I examined whether there was a difference in success rates between these age groups using an ANOVA ($F_{4,770} = 4.13$, $p = 0.002$) followed by *post hoc* Tukey HSD tests (Zar 2010) using R v 3.1.3 (R Core Team 2015). Only those kill sites older than 10 days exhibited significant differences in mean success compared to kills ≤ 10 days (Appendix Table 3). Although predator saliva DNA was successfully extracted from one of the oldest kill sites

(139 days old), the likelihood of a successful predator identification using saliva significantly decreased with age of the kill when including older kill sites (GLM, $Z = -2.87$, $p = 0.004$). Thus, in addition to my *a priori* intent, the outlier nature and significant difference from kill sites ≤ 10 days warranted removal of older kills from the main dataset.

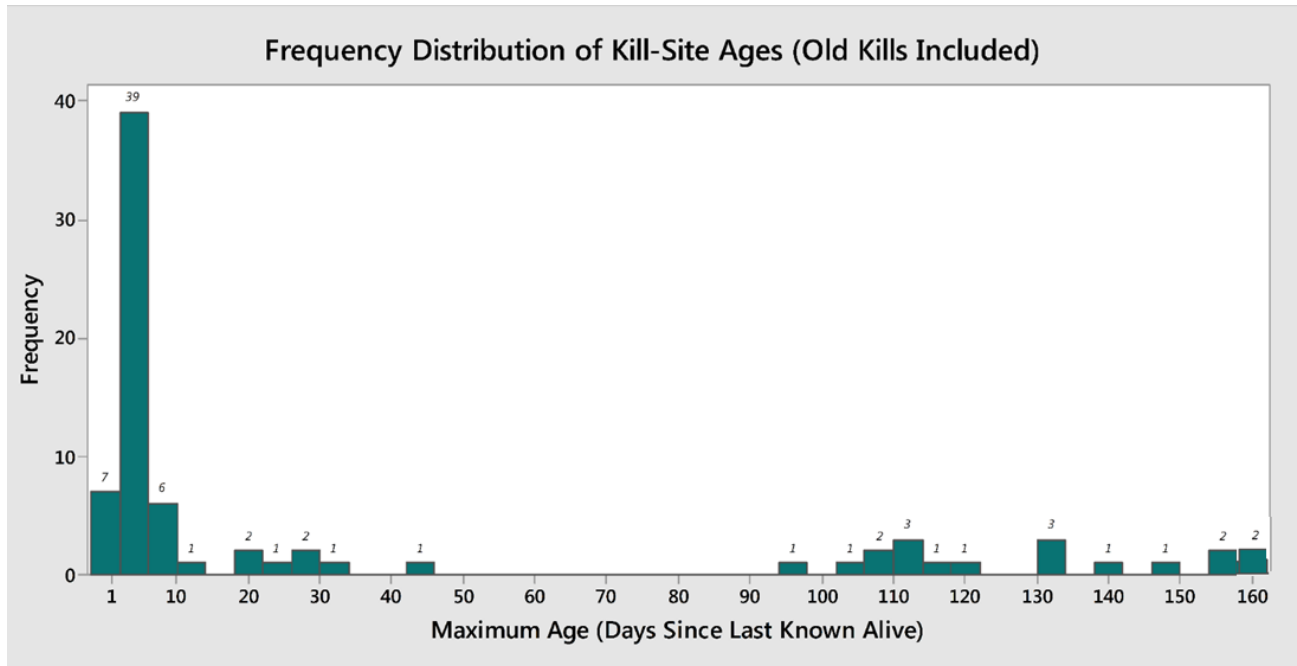
After establishing criteria for inclusion, I analyzed the main dataset in three stages. First, I examined the overall rate of successfully identifying a predator at a kill site. Then, I identified whether any extraneous site-level variables (number of swabs taken, snow, study-year, and/or age of kill site) had a significant influence on likelihood of successful predator identification. Finally, I assessed success rates by sample type – the radio-collar (“Collar”) compared to the carcass remains (“Remains”) – considering data only from fresh kill sites.

Overall Success Rates

To assess whether saliva swabbing could be at least nominally successful when applied to kill sites with limited remains, I examined the overall rate of identifying a predator at a snowshoe hare kill site using saliva. I additionally noted which predator species were detected using this method and whether cross-contamination may have occurred, using detection of human DNA as a proxy for accidental transfer of trace predator DNA.

To convey these overall success rates in the context of the limited kill-site evidence available for swabbing in this study, I quantified the potential amount of carcass remains encountered by calculating the last-known weight of snowshoe hares that were eventually depredated. I also used photographic documentation and written mortality reports to classify the amount of carcass remains found at each kill site into three broad categories: scant (1-25%), few (26-40%), moderate (41-60%), and most (61-100%) of the carcass present. I used Fisher’s exact test for small sample sizes to examine whether the likelihood of a successful predator

Figure 1. Frequency distribution of all kill sites ($N = 78$) by maximum days elapsed since the hare was last known to be alive.



identification from saliva varied by the approximate amount of remains at the kill site.

Predator Sign Comparisons

I determined whether saliva could identify the predator when tracks were absent by examining whether saliva swabbing had been successful when the traditional method of predator identification using definitive sign (tracks) was not possible. Predator sign was considered definitive if found in the immediate vicinity of the kill site (*e.g.*, alongside the struggle site, within a few meters of the remains, etc.) and if it was unambiguous enough to be verified in the field by any observant biologist with a tracking guide. It was necessary to omit first-year results for all track analyses because only a subset of the saliva samples were processed in the lab in the first year, and I had to prioritize sample-testing for kill sites with no other evidence to provide a definitive identification.

I used McNemar's test for paired data to compare the proportion of definitive predator identifications obtained by tracks versus those obtained by saliva swabs. I did not compare the saliva results with my inferences about predator identity based on less-verifiable visual methods (*e.g.*, carcass-handling characteristics) because my ability to deduce the responsible predator improved continually over time with more experience and iterative confirmations from the saliva-swab results.

Site-Level Variables Potentially Influencing Saliva Detection

The number of swabs I took at each kill site tended to vary with the amount of matted fur or bite marks present. To address this issue for the subsequent analysis (Success by Sample Type, pooled across successful kill sites by individual swab), I used a generalized linear model (GLM) to test whether the number of swabs taken at each kill site was a strong predictor for obtaining a predator identification. I also modeled whether presence of snow, study year, and/or

maximum possible age of the kill site (1-10 days only) influenced the likelihood of a successful predator identification from saliva. Number of swabs and age of kill site were treated as continuous variables while study year and snow were treated as categorical. I calculated variance inflation factors (VIF) to evaluate collinearity between variables using the “Car” package in R (Fox and Weisberg 2011). I then performed stepwise logistic model selection to test whether predator identification from saliva (binary: success/fail) was influenced by any of these four variables, together or separately. I added two-way interactions separately to a full main-effects model to avoid over-parameterization. Model-based comparisons to the null model were performed with an ANOVA. I did not perform model selection using Akaike’s Information Criterion (AIC) (Akaike 1974) because I did not expect any of these site-level variables to be strong predictors of success and therefore had no *a priori* candidate models (Burnham and Anderson 2004). I considered variables for inclusion at $\alpha = 0.10$.

Success by Sample Type (Collar Swabs vs Remains Swabs)

To determine whether a Collar swab or a Remains swab was more likely to yield a positive predator species identification when there was predator saliva present and available to be collected in the first place (*i.e.*, among kill sites with at least one successfully amplified saliva swab), I took swab samples separately from the radio-collar (“Collar”) and fur/carcass pieces (“Remains”) whenever possible. I was interested in making this comparison between Collar swabs and Remains swabs for multiple reasons. I sought to improve efficiency for future studies by examining ways to reduce the extensive time required for thorough kill-site processing and swabbing. A simple way to reduce time spent (and lab expenses) was to prioritize the evidence type being swabbed. Not only would this procedure reduce the number of swabs needed overall, but I also observed differences in processing effort of Collars and Remains that warranted a

comparison of success between the two sample types. The collection and swabbing of a radio-collar was generally much less time-consuming than searching for small amounts of remains from a prey species with camouflaged pelage. In contrast, contamination of the collar was easier to avoid because its location was continually transmitted. If saliva could be collected from the collar as well as the remains – or even just the collar in the absence of substantial remains, as was often the case – then search time could be focused on these more obvious pieces of evidence. Furthermore, the radio-collar's location at the neck of the animal arguably makes it the most dependable item to have encountered the predator's mouth at some point during the kill event.

For the analysis of Collars vs Remains swabs, I excluded kills with no predator saliva detected to avoid biasing analyses against Remains swabs because of their dramatically higher sample-size contribution. Specifically, there were many kill sites for which many Remains swabs were collected, but no predator saliva identification was obtained (Fig. 2). There were also many sites at which both a Remains and Collar swab had provided the ID, making comparisons across all sites very difficult (Appendix Fig. 2). Therefore, to compare success by sample type, I only included kill sites with verified predator saliva present (*i.e.*, at least one successful swab). Additionally, I only considered kill sites for which samples were taken on both Collars and Remains in order to compare their success rates. The final consideration was whether to use the kill site or individual swab as the sampling unit. The range of swab samples taken per kill site differed between Collars and Remains because of the nature of the swabbing method. Collar size, and therefore surface area, was the same at each kill site. Thus, the number of Collar swabs taken was low and relatively constant ($n = 34$ swabs; range = 1-2 taken per kill site; $\bar{x} = 1.3$ swabs/kill site). In contrast, the number of Remains swabs taken was dependent on

the area of bite wounds and/or saliva-matted fur available for swabbing and was therefore more varied ($n = 70$ swabs; range = 1-6 taken per kill site; $\bar{x} = 2.6$ swabs/kill site). Thus, I could not evaluate success of Collar vs Remains samples by simply comparing whether at least one swab of each sample type was successful at the kill-site level. Instead, I examined the data hierarchically to take “effort” – that is, the varied number of swabs taken per sample type, per site – into account. I accomplished this step by examining success by sample type on a per-swab basis (n) across kill sites (N).

My statistical method selection for the Collars vs Remains analysis was primarily driven by concerns that swabs at kill sites were likely correlated with each other and therefore not fully independent. Pooling the swab samples by type and testing with comparatively more common methods was deemed inappropriate because it would not account for within-kill-site correlation. I expected that the amount of saliva deposited at a kill site will vary based on characteristics of that particular predation event, with some kill sites harboring more saliva than others. Ignoring this clustering could underestimate the true standard error, thus increasing the risk of Type I error (Cameron *et al.* 2008) and potentially producing inaccurate results (*e.g.*, Marucco *et al.* 2008). I also eschewed a mixed-models approach because I was more interested in obtaining a population average than a site-specific parameter (Fitzmaurice *et al.* 2012, Hubbard *et al.* 2010).

To address these issues, predator saliva detection by sample type (Collar vs Remains) was analyzed with a logistic Generalized Estimating Equations (GEE) model using the package *geepack* in R (Halekoh *et al.* 2006, Liang and Zeger 1986). An extension of generalized linear models, GEE models use quasi-likelihood estimation instead of maximum likelihood estimation to estimate parameters and quantify marginal (population-averaged) changes in outcome as a function of covariates. GEE models would also adjust my model estimates according to

correlation of swabs from the same kill site and allow for correct inference of the parameter of interest, in my case success by sample type. A GEE model was determined to be most appropriate overall because: (1) it does not depend on a correctly specified error distribution (correlation matrix), (2) it is reliable even when the size of clusters is small in comparison to the number of clusters (3) it requires fewer assumptions (such as homoscedasticity), and (4) it accounts for clustering of non-independent data (*e.g.*, swabs taken at the same kill site) while treating such clustering as noise (a nuisance parameter) (Halekoh *et al.* 2006, Hanley *et al.* 2003, Hubbard *et al.* 2010, Liang and Zeger 1986). Additionally, the variance estimator used for a GEE is the empirically based “sandwich” estimator, which is robust even if the correlation matrix is misspecified (Halekoh *et al.* 2006, Hubbard *et al.* 2010). Problems may arise with GEE models if there are many missing observations or if cluster size itself is related to the outcome (Hanley *et al.* 2003). However, the dataset used in this analysis had already been reduced to remove missing observations (*i.e.*, kill sites for which only one sample type had been swabbed). As described earlier, the potential issue of cluster size affecting the outcome was addressed by examining whether success was related to the number of swabs taken.

I chose an independent working correlation matrix for the GEE analysis. Independent covariance matrices are considered preferable if the number of clusters is relatively small (Diggle *et al.* 1994). There was no *a priori* biological reason to expect non-independence between clusters (kill sites), and preliminary testing confirmed this assumption (GLM, $Z = 1.13$, $p = 0.26$). However, the GEE is still robust even with misspecification of the correlation matrix. I applied a jackknife estimator for the standard error (SE), per the recommendations of Halekoh *et al.* (2006) and Paik (1988), because this subset of kill-site data fell slightly below the threshold of 30 clusters.

To test whether sample type (Collar vs Remains) influenced my ability to identify the predator species at kill sites, I used an ANOVA (Zar 2010) to compare the GEE model to a null (intercept-only) model. Sample type was the only reasonable explanatory variable to consider when examining the success/failure by individual swabs within “successfully identified” kill sites, as was done for this (the Collar vs Remains) analysis. In comparison, the kill site is the statistical unit of interest for all of the other analyses described in this paper. Quasi-likelihood under Independence Criterion (QIC) weights (analogous to AIC) are presented for the fitted and the null models (Pan 2001).

RESULTS

Predator saliva was successfully collected and identified to species at 58.5% of all fresh kill sites ($N = 31/53$ sites). No human handling contamination was detected; all DNA amplified from saliva swabs was attributable to an indigenous wildlife species, indicating a low likelihood that trace amounts of non-target predator DNA were accidentally transferred from another field site via hands or gear. Predator species identified using this saliva-swabbing method and species-specific primers included Canada lynx (*Lynx canadensis*), bobcat (*Lynx rufus*), western coyote (*Canis latrans*), pine marten (*Martes americana*), and cougar (*Puma concolor*). DNA of any species (including snowshoe hare) was amplified from 100% of all kill sites ($N = 53$) and 89.3% of all swabs ($n = 159/178$).

The small body size of hares³ combined with high carcass-consumption rates led to minimal remains at almost all (94%) of the kill sites. Specifically, 64% percent ($N = 34$) of fresh kill sites had only scant carcass/fur remains, 30% ($N = 16$) had few remains, 0% ($N = 0$) had moderate remains, and less than 6% ($N = 3$) had most or all of the carcass left. Not only were

³ The mean last-known weight of hares that were eventually depredated was 1090 g (range: 575-1675 g).

these small amounts of remains often successful at yielding detectable predator DNA, but the kill sites with the least remains appeared to be as successful as those with more remains (Fisher's exact test, $p = 0.107$).

This saliva-swabbing method provided species identifications that would have been ambiguous or unobtainable otherwise. Specifically, 65.5% ($N = 19/29$) of kill sites lacking clear predator sign in the field yielded detectable predator saliva. When other forms of definitive evidence were present, the saliva DNA results did not conflict with those species identifications (see Appendix: Supplemental Information). Furthermore, for the two study-years during which saliva swabs were tested for all kill sites could be compared with predator sign, saliva swabs contributed a significantly higher proportion of definitive identifications compared to the null than tracks (McNemar's test with Yates' continuity correction, $\chi^2 = 5.76$, $p=0.016$, OR: 3.17 [95% CI: 1.26-7.93]).

Successful predator identification at a kill site using this saliva-swabbing method did not vary significantly by any of the site-level variables (study year, snow presence, maximum age of the kill site (≤ 10 days), or number of swabs taken) (Table 1). I also did not find evidence of collinearity from VIF calculations (none greater than 10; Belsley *et al.* 1980). Further model-building was not considered due to lack of significance of main effects or interactions.

A significant amount of variation in predator identification success was explained by sample type (Collar vs Remains) when compared to a null model, with a negative relationship observed for Remains swabs (Table 2) ($n = 104$, $N_{\text{clusters (kill sites)}} = 27$, \bar{x} clusters = 3.85, Wald $\chi^2 = 10.27$, $p = 0.0013$). Only a third of the samples taken at successfully identified kill sites were Collar swabs, yet they comprised half of the resulting successful swabs ($n_{\text{Collar}} = 21/41$) (Fig. 2).

Within the kill sites with at least some detectable predator saliva, the success rate for Collar swabs was 0.618 [95% CI: 0.435 - 0.772], whereas the success rate for Remains swabs was 0.286 [95% CI: 0.221 - 0.360] (Fig. 3). Following a back-transformation, the odds of successfully obtaining predator DNA from a Collar swab were 4.04 times higher than for Remains swabs.

DISCUSSION

Overall Success

My findings demonstrate the feasibility of a forensic saliva-swabbing method for predator identification in cases involving small prey and limited remains, an area largely unexplored in previous work. To my knowledge, this is the first study to use DNA to identify predators at kill sites of snowshoe hares or other similarly small mammalian prey as well as compare success by sample type (Collars vs Remains) in a natural system. Despite the minimal physical remains at a typical snowshoe hare kill site, our saliva-swabbing method was effective for most predation events. Specifically, I successfully identified the responsible predator at the majority of kill sites (58.5%), despite having scant or few remains available to swab in 94% of the kill sites for a prey species averaging a little over 1000g. My findings provide further evidence that modern molecular techniques are enabling the expansion of field-based wildlife methods, including forensic kill site analysis, far beyond what was previously possible (Kelly *et al.* 2011, Long *et al.* 2011, McKelvey *et al.* 2006, Mills *et al.* 2000, Oliveira *et al.* 2010, Pilgrim *et al.* 1998, Pilgrim *et al.* 2005, Prugh *et al.* 2005).

Increased certainty about predator identity at kill sites can improve the scope and rigor of predator-prey studies that would otherwise depend solely on more traditional methods. In my study, saliva swabbing provided objective predator identifications that were often unobtainable

Table 1. ANOVA comparison of models for successful collection of predator saliva at a kill site as a function of age, number of swabs, snow, and year.

MODEL	df	Deviance	$p(<\chi^2)$
Null	52	71.938	
Age	1	0.609	0.435
<i>Error</i>	51	71.329	
Number of Swabs	1	0.570	0.450
<i>Error</i>	51	71.368	
Snow	1	0.078	0.781
<i>Error</i>	51	71.861	
Year	2	0.303	0.860
<i>Error</i>	50	71.635	
Age + NumSwabs + Snow + Year	5	1.855	0.869
<i>Error</i>	47	70.083	
Age + NumSwabs + Snow + Year + Age*NumSwabs	6	2.457	0.873
<i>Error</i>	46	69.481	
Age + NumSwabs + Snow + Year + Age*Snow	6	2.532	0.865
<i>Error</i>	46	69.406	
Age + NumSwabs + Snow + Year + Age*Year	7	6.604	0.471
<i>Error</i>	45	65.334	
Age + NumSwabs + Snow + Year + NumSwabs*Snow	6	3.581	0.733
<i>Error</i>	46	68.357	
Age + NumSwabs + Snow + Year + NumSwabs*Year	7	3.352	0.851
<i>Error</i>	45	68.586	
Age + NumSwabs + Snow + Year + Snow*Year	7	8.778	0.269
<i>Error</i>	45	63.160	

Table 2. Parameter estimates, p -values, and quasi-likelihood criterion (QIC) results from a model of successful saliva collection by sample type (Collar swabs vs Remains swabs) using generalized estimating equations and a jackknife estimator.

Generalized Estimating Equation (GEE) Model Results					
	β	Estimate	SE	QIC	p
Success ~ Sample Type	Intercept	0.480	0.378	132	0.2040
	Covariate (Remains)	-1.396	0.435		0.0013**
Null Model	Intercept	-0.430	0.164	141	0.0086

Figure 2. Proportional contribution to success by sample type (Collar swabs vs Remains swabs). Each bar shows the proportional number of saliva swabs taken for each sample type, and the green section indicates the proportion of swabs that successfully yielded predator mtDNA. One-third of the samples taken at successfully identified kill sites were Collar swabs, yet they comprised half of the resulting successful swabs ($n = 21/41$). The odds of successfully obtaining predator DNA from a Collar swab were 4.04 times higher than for Remains swabs.

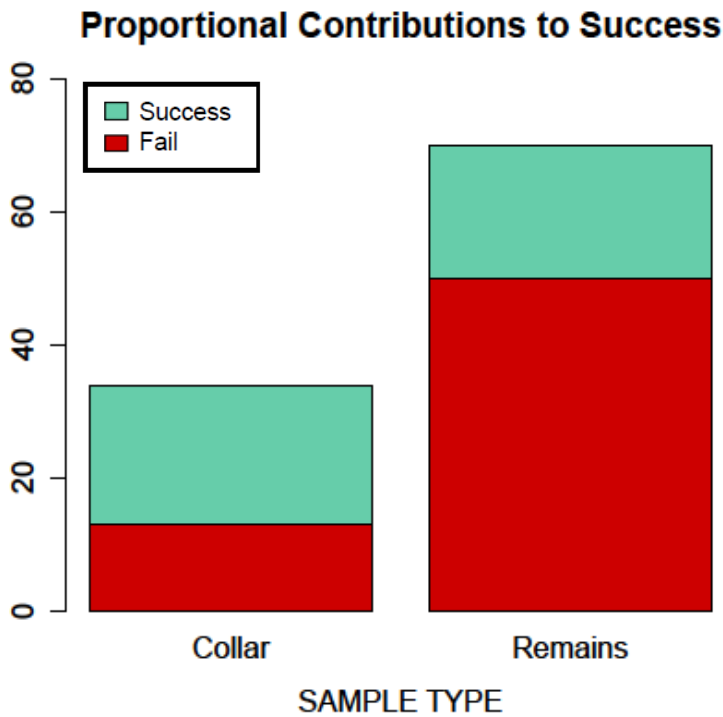
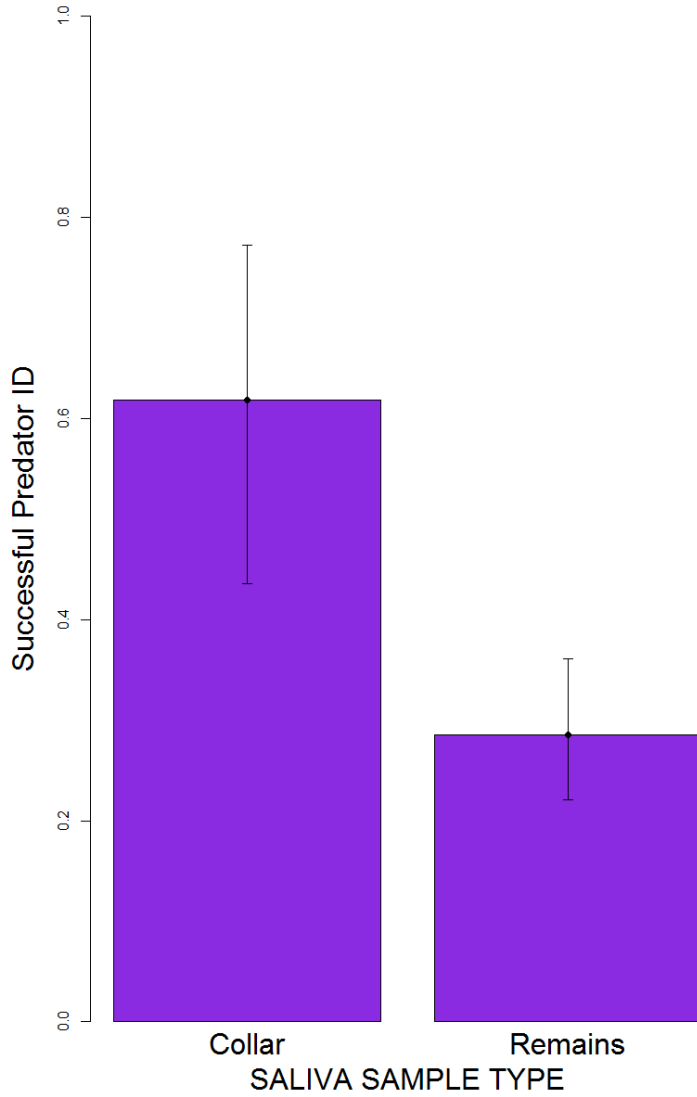


Figure 3. Percentage of successful predator identifications from saliva by sample type (Collar swabs or Remains swabs) within kill sites where one or more swabs yielded predator mtDNA ($n = 104$), shown with 95% confidence intervals. Collar swab success = 0.618 [95% CI: 0.435 - 0.772]; Remains swab success = 0.286 [95% CI: 0.221 - 0.360].



with other methods, such as when snow was absent or tracking conditions were suboptimal. For the two study-years during which I tested saliva swabs at all of my kill sites, thus allowing me to compare the saliva-swabbing method to a more traditional one (definitive observable predator sign in the field), I was able to obtain unambiguous species identification from saliva at 19 of 29 kill sites lacking clear predator sign, which more than doubled the total number of definitive predator identifications in my broader predation study.

Site-Level Variables Potentially Influencing Saliva Detection

None of my measurable site-level variables were expected to be strong drivers of saliva collection success *a priori* within the main dataset of kill sites ≤ 10 days old. Study-year was considered the least likely predictor because my saliva-swabbing protocol was formed early in the study, leading me to assume that results would be similar across years. Previous saliva-swabbing predation studies did not examine and/or did not report saliva success by year, and the insignificance of year in my study substantiated my *a priori* assumptions. However, I did not anticipate the observed absence of even weak effects for the variables of snow presence, number of swabs, and/or age of the kill site.

I did not detect differences in successful amplification of predator saliva DNA as a function of the presence/absence of snow. Given the negative effects of both heat exposure and moisture on the integrity of DNA (Arismendi *et al.* 2004, Höss *et al.* 1996, Pääbo *et al.* 2004, Steadman *et al.* 2006, Smith *et al.* 2003; but see Crainic *et al.* 2002, Nelson and Melton 2007), it is possible that degradation due to warmer temperatures was counterbalanced by the aridity of snow-off seasons in the study region (Mitchell 1976). My failure to reject the null hypothesis that snow influences saliva retention has an important implication; namely, that saliva-swabbing is equally effective when it is needed the most, during snow-off periods when there is rarely any

form of unambiguous predator sign that can be linked to the kill, as was observed in my study system.

I did not observe a significant decrease over time in the retention of mtDNA from predator saliva on hare carcasses ≤ 10 days old, despite the effects of age on saliva success for the much older kills that were eliminated from the main dataset *a priori* (see Methods and Appendix). These findings are consistent with other recent wildlife studies that have examined the relationship between saliva mtDNA and kill-site age; for example, Mumma (2014) found no difference in species identification success rates from predator saliva on caribou calf carcasses when monitored every other day, once a week, or opportunistically. However, rapid recovery of saliva would have been more important if I had chosen to conduct microsatellite analysis because problems with allelic dropout and false alleles increase over time (Harms *et al.* 2015), while analyses using mitochondrial DNA will not create false answers, so long as contamination has been avoided (Foran *et al.* 1997).

The number of swabs taken had no significant relationship with the likelihood of successfully amplifying predator DNA at a kill site. This result is intriguing because limiting the number of swabs taken would improve efficiency of the sampling process. However, the inability to detect a relationship between success and the number of swabs must be treated with caution; even among kill sites with detectable saliva DNA present, only 41.3% of those pooled swabs yielded detectable saliva. Thus, if I had tested fewer swabs per kill site, I expect that overall success rates may have been lower. Moreover, predator saliva DNA was not detected at 11 kill sites where other definitive evidence implicated a terrestrial predator. Saliva must have been present at those kill sites, but it was not successfully collected and/or amplified, and the collection of too few swabs is one possible explanation among many unknowns. For most

studies, this potential for a missed detection by taking too few swabs is likely of greater consequence than the extra effort required to collect and process more swabs than necessary. Furthermore, oversampling should not detract from the conclusions attained during PCR analysis and would only be expected to strengthen results, assuming no cross-contamination occurred. Finally, some evidence from human forensics studies does suggest a positive relationship between number of swabs and the ability to forensically detect trace amounts of DNA (*e.g.*, Bright and Petricevic 2004). For these reasons, I would recommend taking more swab samples when possible. However, if taking additional swabs is not justified because all areas that appear likely to yield saliva DNA have already been swabbed, as was usually the case for my study, then combining more swabs per sample (*i.e.*, combining more than just the dual swabs in one sample vial) might also increase success rate. I did not combine more than two swab samples primarily because of concerns about handling contamination as well as the potential for losing the singled combined sample to DNA degradation or other causes. I also aimed to maintain consistency in my saliva-swabbing protocol after choosing the Sweet *et al.* (1997) double-swab technique. Nonetheless, cautious handling and swabbing of the remains/radio-collar apparently avoided handling contamination, and no more than one predator species was detected from predator saliva at a kill site (but see Appendix: Supplemental Information regarding rare detections of more than one predator in the immediate vicinity using other methods). Thus, it might not be necessary to keep the samples separate for smaller-prey kill sites and could potentially improve upon my success rates.

Success by Sample Type (Collar Swabs vs Remains Swabs)

Radio-collar swabs comprised half of the successful swabs despite representing only a third of the samples. While I did not initially expect this result, a hard, non-porous surface has

been found to be the most effective substrate for collecting human saliva DNA when collected using a porous material (Goray *et al.* 2010). The considerably higher success of the Collar swabs over Remains swabs may perhaps have been due to predator epithelial cells captured on Remains swabs being diluted by the profusion of hare DNA sloughing off the carcass remnants as well as the fur; the snowshoe hare fur was noticeably difficult to remove completely from a swab after sample collection without risking handling contamination.

Whereas Collar swabs were more successful than Remains swabs overall within kill sites that had obtainable predator saliva, it is important to note that Remains swabs still provided valuable information on predator identification when comparing success at the kill-site level. Specifically, Remains swabs alone successfully yielded predator DNA at seven kill sites for which Collar swabs were unsuccessful. Conversely, Collar swabs alone successfully yielded predator DNA at ten kill sites for which Remains swabs were unsuccessful. Among an additional four kill sites yielding predator saliva DNA where only one sample type was tested, three of the successful identifications were from Collar swabs and one from Remains swabs. Not including Year 1, when only a subset of kill sites and swabs were tested overall, this single-sample testing was usually due to an extreme scarcity of remains available to swab (*e.g.*, a tuft or two of unmatted fur). Both sample types contributed substantially to successful identifications at the kill-site level, but the apparently lower success of Remains swabs was not detected until I examined the results on a per-swab basis; this inability to detect a difference in success by sample type at the kill-site level was primarily due to the number of kill sites for which both a Remains swab and a Collar swab provided the predator identification (Appendix Fig. 2). Yet, the Remains swabs were vitally important for the additional data they provided, particularly because the per-kill-site (predation event) success rate is a more ecologically meaningful result

than individual swab success. These results suggest that, although collar-swabbing should perhaps be prioritized when field time is limited and/or lab costs must be reduced, sampling of carcass remains should not necessarily be excluded, particularly if the number of kill sites available for sampling in a study is low. If feasible for the study, any potential source of saliva DNA should be sampled.

Proximate Cause of Mortality

Scavenging by a species of predator that could be misidentified as the one responsible for the mortality is a concern for any method of predator identification at a kill site, including molecular methods, regardless of whether the proximate cause of mortality can be attributed to predation or another causes (Larivière 1999). Still, erroneous identification of the predator at a kill site can be reduced with frequent monitoring as well as swabbing only antemortem (*i.e.*, hemorrhaged) wounds, and most studies have found scavenging to be generally low (Boutin *et al.* 1986, Brand *et al.* 1975, Keffins *et al.* 2012, Mumma 2014, Murray *et al.* 1994, Murray *et al.* 1997, Squires and Ruggiero 2007, Wengert *et al.* 2014). When snowshoe hares die from natural causes other than predation, the majority of the carcass is left behind (Murray *et al.* 1991), the scavengers are almost always small animals (Murray *et al.* 1997), and scavenging on hares by their natural predators is rare (Boutin *et al.* 1986, Brand *et al.* 1975, Murray *et al.* 1997, Squires and Ruggiero 2007). I found predation to be the cause of death for almost all mortalities, consistent with other snowshoe hare studies (Abele *et al.* 2013, Brand *et al.* 1975, Griffin *et al.* 2005, Hodges *et al.* 2001, Murray *et al.* 1997, Wirsing *et al.* 2002). For older kills, it is possible that the hare died of disease or another cause and was later scavenged, but it seems unlikely that the majority of the remains (including larger bones) would be removed in their entirety if this were the case (Keffins *et al.* 2012, Wengert *et al.* 2014). In addition, epithelial cells from saliva

seem to be deposited differently than other sample sources. In human forensics, the first individual to deposit saliva is typically the primary source of saliva DNA, even after contact with subsequent deposition sources (Warshauer *et al.* 2012). In a wildlife forensics application, this retention of saliva from the first “depositor” could suggest that saliva from the predator responsible for the kill persists as the major source of DNA, despite later exposure to scavengers.

Saliva-Swabbing Compared to Other Methods

Traditional predator-identification methods, particularly visual assessment of the carcass, might not accurately categorize a kill site if portions of the carcass have been removed by scavengers. In my study, kill-site tampering by red squirrels (*Tamiasciurus hudsonicus*), southern red-backed voles (*Myodes gapperi*), and gray jays (*Perisoreus canadensis*) was observed on multiple occasions. Other studies on snowshoe hares have also documented scavenging by small animals, including red-backed voles, meadow voles (*Microtus pennsylvanicus*), deer mice (*Peromyscus maniculatus*), and black-billed magpies (*Pica pica*) (Brand *et al.* 1975). Of particular concern is the fact that kills with less visual evidence might be erroneously attributed to predators with a tendency to consume, or remove, more of the carcass (*e.g.*, coyotes and cougars, or raptors, respectively) if small scavengers have tampered with the remains, highlighting the increased value of more definitive molecular techniques like the saliva-swabbing method. Although the likelihood of scavenging/tampering by small animals seemed to increase with time, it also often occurred for kills that were collected promptly. However, scavenging by predators potentially attributable to the kill was not observed or detected by saliva-swabbing, despite rare evidence of visitation by secondary predators (see Appendix: Supplemental Information).

I originally anticipated that scat would provide an objective form of evidence to link predators to kill sites in my study. However, even fresh scat in the broader area of a kill is not necessarily attributable to the predator responsible for that kill in comparison to the more definitive methods of saliva swabbing and/or tracks. I ultimately did not include scat in analyses because I rarely observed scat that I could confidently link to the responsible predator for the kill site. While I often observed scat in the broader area (*e.g.*, >20 meters from the kill site), I anecdotally noted over time that it often belonged to a different predator than was indicated by predator sign (when present) at the kill site. Scat at or within a few meters of the kill was rarely present in this system. This may have been partly due to the reduction in handling time associated with the small body size of snowshoe hares. However, Mumma (2014) also did not find scat at most caribou calf kill sites, though there was no conflict between saliva and scat-based identifications when both were tested in that study. Among the few scats I found in the immediate vicinity of kill sites that were subject to genetic testing ($N = 4$), half of them identified a different predator species than the one identified as responsible for the kill using more definitive methods (tracks or saliva). Many additional scats were never collected – or were collected and never tested – because other evidence (*e.g.*, snow tracks) consistently demonstrated that predators (usually coyotes) may deposit scat in a general area without ever visiting the specific kill site. Thus, anecdotally and within my study area, predator presence in the vicinity as indicated by scat did not seem to provide strong evidence for predator responsibility for a kill.

Kill sites of small-bodied prey may be important to more than just smaller predators. When kill sites are found by monitoring GPS-point clusters from collared predators, for example, information about smaller prey may be lost because less time is spent at the kill site (*e.g.*, Cavalcanti and Gese 2010, Donoghue *et al.* 1998, Knopff *et al.* 2010, Martins *et al.* 2011,

Tambling *et al.* 2010, Webb *et al.* 2008). Many predators depend at least partly on smaller prey items and therefore cannot be linked to those kill sites by GPS cluster analysis (Bacon *et al.* 2011), which may underrepresent the importance of these prey to a predator's total biomass intake (Donoghue *et al.* 1998, Messier and Crête 1985, Neal *et al.* 1987, Ruth *et al.* 2010). This omission of smaller-prey data may be circumvented if the prey rather than the predator leads the researchers to the kill site, but only if the predator can still be identified, underscoring the value of the saliva-swabbing method for objective predator identification.

Factors Reducing Apparent Success Rates

My success rate does not approach that of studies on species with larger amounts of carcass remains such as congregated domestic sheep (*e.g.* Williams *et al.* 2003), ungulates (*e.g.*, Kilgo *et al.* 2012 and Mumma *et al.* 2014) or meso-carnivores that are less likely to be fully consumed (Wengert *et al.* 2014), yet it was much higher than expected given the lack of data available on the feasibility of success on limited remains or small prey, particularly at the outset of my study in 2010. For the purposes of my broader predation study, any objective predator identifications at kill sites were highly valuable, and even very low saliva-swabbing success rates would have been informative to supplement my season-dependent identifications from snow tracks.

Certain aspects inherent to a study on a small-bodied prey species may have reduced my apparent success rate. Visual evidence of raptors at several kill sites (*e.g.*, raptor excrement or a pellet and the lack of adjacent mammalian tracks in uncompacted snow) indicated that raptors are an important predator in this particular system. However, no raptor mtDNA was obtained using the saliva-swabbing technique, even from the known raptor kills not included in my analysis, possibly because raptors are known to have poorly developed salivary glands (Scanes

2014). Thus, a portion of the kill sites tested may simply not have retained detectable saliva. The rates of obtaining predator mtDNA from saliva in this study may have been higher if non-mammalian predators could have been fully excluded from testing.

Conclusions and Future Directions

I adopted the specific components of my saliva-swabbing protocol, not only for definitive predator identifications at kill sites with limited remains, but also in the hopes that I could (1) apply this method year-round, even when snow-tracking was not possible or substrate conditions were inferior; (2) maximize genetic yield by using a foam swab-type specifically designed to collect epithelial cells from saliva when combined with the double-swab method; and (3) eliminate dependence on a freezer during swab sample storage and/or transport to a lab from a remote field location by instead using lysis buffer for self-stable storage.

The specific components of any technique must be reevaluated over time, however, and future studies might improve upon mine in numerous ways. Larger-scale studies might examine whether there is an asymptote at which taking additional swabs no longer increases the chances of collecting predator saliva. Different collection materials might also be explored. For example, Verdon *et al.* (2014) compared the effectiveness of different swab types at collecting human forensic DNA and found that a swab with a small-open-cell foam tip was the most effective at collecting both neat and dilute saliva, but it was less effective than cotton at collecting blood. Although my decision to use a foam swab designed specifically for buccal cell collection produced favorable results, variations on this swab type could be considered. For example, friction is more effective than pressure when transferring human saliva deposits from one substrate to another (Goray *et al.* 2010). Thorough swabbing of samples in wildlife forensic studies might produce sufficient friction, but a rougher swab-tip material (along with a strong

swab shaft) could be even more effective at increasing the rate of cell transfer from the surface of a radio-collar or carcass remains. Radio-collar modifications could also potentially increase predator saliva yield. The surface area of the collar portion of a radio-collar might be increased slightly, so long as it does not compromise the safety of the study species. Although the smooth and non-porous substrate of my radio-collars was unexpectedly effective at collecting saliva despite the small surface area, other substrate types could be considered, particularly by looking at human forensics (*e.g.*, Goray *et al.* 2010). Smoothness of the primary substrate for saliva deposition has rarely been examined separately from porosity, even in human forensics studies, but Verdon *et al.* (2014) did find that a smoother non-porous surface was a better substrate for collecting saliva with foam swabs than a less-smooth non-porous surface (pitted plastic). These and other findings from both wildlife and human forensics research can be used in tandem to inform saliva collection protocols at predator kill sites.

The significantly higher success of my radio-collar swabs at yielding saliva also has meaningful implications for future studies. Given that swabbing of the radio-collar facilitated predator identifications from saliva far beyond most traditional methods, perhaps other types of implements comprised of similarly non-porous material, such as lightweight non-tracking collars, could be deployed on smaller animals that do not require relocation using telemetry and/or are cannot withstand the weight of most radio-collars.

In summary, I found that a saliva-swabbing method can be used on limited remains and small prey, an endeavor that has rarely been explored. These results contribute to the small yet growing number of studies that demonstrate the utility of saliva DNA for forensic predator identification, thus providing an objective alternative to more traditional methods that require adequate tracking conditions or can only tenuously link the predator to the kill site. My findings

invite future studies that use knowledge gained from this swabbing protocol to identify diagnostic differences in carcass-handling characteristics of predator species, especially those that target smaller prey. Such diagnostic features of kill sites would be useful in cases where DNA is difficult to collect, corrupted, or unavailable.

Expanding saliva-swabbing methods to smaller prey and/or limited remains should provide wildlife ecologists and managers with new capacity to identify predators at kill sites and, as a result, reveal data on depredation of domestic animals, the presence and foraging habitat of elusive or rare predators, and the impacts of predators on prey species, among other applications. Moreover, by providing a detailed and reproducible field-collection and swabbing protocol, I hope to assist field-based biologists and wildlife managers who might otherwise assume that saliva testing of minimal remains and/or a radio-collar is futile.

Figure 4. Example photographs of snowshoe hare kill sites.



Figure 5. Collection of a remains sample during processing of a kill site.



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APPENDIX

Kill Site Processing Protocol

Materials

- Sterile, tight-fitting, disposable gloves (latex or nitrile only)
- Sterile, disposable, wooden chopsticks (still in their sleeves and in a waterproof bag)
- Metal long-handled tweezers (for collection of small items)
- Sterile alcohol wipes (packaged individually)
- Lighter (for sterilizing tweezers)
- Sterile 1-gallon slider Ziploc bags (several)
- Sterile 2.5 or 3-gallon slider Ziploc bags (2+; use more if working with larger prey remains)
- Sterile quart-size slider Ziploc bags (several)
- Sharpie (permanent marker; no ultra-fine-point pens because they tear the bags)

Kill Site Processing Steps

1. Mortality sites were approached with extreme caution prior to having a visual on the collar to avoid disturbing/contaminating any remains in the vicinity, such as small tufts of fur. Even after sighting the collar, all actions were conducted carefully to avoid touching any potential evidence. To date, no samples from this study have amplified human DNA, indicating that the level of caution implemented most likely avoided contamination by non-target predator DNA (*i.e.*, from previously visited site), which would be essentially impossible to recognize on its own. Contamination of the kill site was my foremost concern, so I abruptly slowed my hiking/snowshoeing pace when I

suspected I was near the vicinity of the collar. Proximity to the radio-collar was determined based on signal strength (*e.g.*, the signal began to “chirp” or the signal strength at the center of the arc intensified). As the signal became stronger, I visually verified the absence of remains/fur or tracks before taking additional steps forward.

2. Upon discovery of the kill, I assessed the proximate cause of mortality to determine if the hare had died of predation. Predation was determined based on tracks, signs of a struggle, sub-dermal hemorrhaging, and amount of carcass/fur remaining.
3. Evidence (carcass remains, fur, collar, entrails, tracks, etc.) was photographically documented.
4. Kill site and environs were thoroughly searched for carcass remains and other evidence that could indicate the predator species responsible for the kill.
5. Evidence was handled as minimally as possible while in the field, using new (disposable) chopsticks, sterile disposable gloves, sterilized tweezers, or inverted sterile Ziploc bags. Occasionally, branches were carefully cut from trees if they were small and high enough to preclude the likelihood of pre-existing predator DNA.
6. The radio-collar, remains, and other evidence (such as predator hairs or a raptor feather) were separated into Ziploc bags by evidence type and location, noting on the bag if cross-contamination may have occurred.
7. The specific location of the kill site was determined based on the location of the collar and other evidence (*e.g.*, signs of a struggle, evidenced by a light layer of fur), marked with a handheld GPS, and flagged for later vegetation plots.

8. Seasonal conditions at the time of the kill were then documented by taking photographs from the center of the kill site in four cardinal directions and directly above (canopy).
9. Evidence bags were placed on ice packs in coolers immediately upon returning to the vehicle, except for during winter when ambient temperatures were sub-freezing.
10. Remains and collars were either swabbed immediately or promptly stored in a freezer upon return from the field to be swabbed later.
11. A written Mortality Report was created at the kill site or upon return from the field.

Appendix Table 1. Materials used to swab for predator saliva on snowshoe hare carcasses and radio-collars at base camp.

Materials (in general order used during swabbing session)	Quantity
Paper towels, clean and unexposed	1 roll
Sterile, tight-fitting, disposable gloves (latex or nitrile only)	1 box in each appropriate size (several pairs used per session)
Sterile gallon Ziploc bags (for supply storage)	Several
Fine-tipped permanent marker (<i>e.g.</i> , Sharpie)	1
Sterile alcohol wipes/swabs (packaged individually)	2+ per sample*
30 mL HDPE bottles, sterile (HDPE = High Density Polyethylene or Polypropylene; <i>e.g.</i> , Nalgene brand)	2+ per session
Epibio “Catch-All” Collection foam swabs	2 per sample*
8mL amber glass vial with cap attached (<i>e.g.</i> , Wheaton 224984 Borosilicate Amber Glass Vial with Phenolic 14B Rubber Lined Cap Attached, 8mL)	1 per sample*
Phosphate Buffered Saline (PBS), 1%	~150-200 μ L per session, transferred to a HDPE bottle
Qiagen Buffer ATL	~150-200 μ L transferred to a HDPE bottle (<i>extra needed to extract 100 μL per sample* with syringe</i>)
Sterile needleless syringes, 1cc/1mL (<i>e.g.</i> , MonoJect Syringe, 1cc)	1+ per sample*
Adhesive labels	1+ per sample*
Ultra fine-tipped permanent marker or pen	1
Clear tape (to secure label on glass and protect ink from moisture/spills)	1 roll
Silver paint pen (or other light-colored paint-based pen)	1

**Each “sample” consisted of 2 paired swabs stored in a single vial. Number of samples needed per kill site may vary; multiply amounts as needed.*

***Note: needled syringes may be used instead, but these are more prone to clogging when used in cooler temperatures, when lysis buffer is prone to forming solid precipitate. Precipitate may be reversed by a warm water bath.*

Saliva-Swabbing Protocol Steps

1. Wash hands thoroughly.
2. Immediately upon opening the box of gloves, put on a pair and then carefully transfer all of the gloves from the box into a large gallon-size Ziploc bag. Discard any gloves that touch anything other than the inside of the box or the bag. Label this bag with a sharpie to avoid confusing it with glove bags for which cross-contamination is not a concern.
3. Clean and sanitize a work area before laying down clean paper towels (inner side up), or other clean, unused, disposable material, on a clean table.
4. Fill a clean, small HDPE bottle with a small amount (~125 μ L) of Phosphate Buffered Saline (PBS). This serves as a safe-guard against contamination of the larger source bottle. Never use or reuse the small bottle if anything (including the swab or your glove) has touched the lip or the inside of the cap or bottle. Never use the small bottle for more than one swabbing session. Label bottle with “PBS” and date using sharpie.
5. Fill another small HDPE bottle with ~150 μ L Buffer ATL or as needed, following the steps listed above for PBS.
6. Label the vials with the Mortality #, date, and brief description of what is being swabbed (*e.g.*, “Hind Foot #1, 0.5 m from collar”) using an ultra-fine-tipped marker. Cover with clear tape that overlaps itself in the back.
7. Prepare all of your supplies by opening one end of each package without touching the contents; open as many as you will need for swabbing the sample in front of you.

(Prepping in advance prevents you from having to touch the outside packaging after putting on sterile gloves.) Count out the number you will need of the following, and open the end of each package:

- a. Alcohol wipes
 - b. EpiBio swabs
 - c. Syringes
 - d. Glass vials (place lid upside down on paper towel)
 - e. Small bottles of Buffer ATL and PBS (lids removed and placed upside down on paper towel)
 - f. Freezer bags containing the items to be swabbed (remains, collar, fur, etc.)
8. Put on a new pair of gloves. Wipe vigorously with alcohol after both gloves are on.
 9. Dip a swab in the small bottle of PBS.
 10. Rub swab firmly over bite wound, saliva-matted fur, or radio-collar, keeping the sample contents inside the open storage bag. In order to stabilize the item you are sampling, you will have to contaminate one hand (usually your left) by touching the outside of the bag. Do not forget that this is your contaminated hand. Only a new swab or a sterilized glove should ever touch the sample, the inside of the sample bag, or the inside edge of the vial (lip or lid).
 11. Break the tip of the swab against the inside wall of the prepared vial (carefully use the unattached lid for leverage if needed).

12. Swab the sample again with a dry swab (no PBS) and break the tip off inside the same vial as the first swab.
13. Using a new syringe, deposit 100 μ L of lysis buffer into the vial with the paired swabs. Avoid depositing buffer in the hollow stem of the swabs.
14. Repeat steps #6-13 for all remaining bags of evidence.
15. Discard the used HDPE bottles (even if they have remaining PBS/ATL), syringes, gloves, and other refuse.

Supplemental Information

Note on Swabbing Protocol

I initially utilized multiple storage and stabilization methods in the hopes of eventually comparing them, but this effort was abandoned due to small sample sizes and inefficiency. One alternative method I utilized was to insert a silica desiccant packet into the top of a small vial such that it was suspended above the swab tip below; this was intended to reduce issues of desiccant rubbing cells off during contact with the swab. However, I ceased using this method because it reduced efficiency during laboratory analysis.

Field Data Collection Issues

Several potential mortality sites were revealed to be the result of slipped collars from live hares. The zip-ties used to attach the collar transmitters turned out to be defective and could slip off of a live hare of their own accord, but this was not verified until hares that had been presumed dead were later recaptured. Thus, if a potential mortality site was found to have a

broken collar, and no evidence of a kill was found after thorough searching, it was eliminated from the analysis.

Age of Kill Sites

To determine a threshold for “fresh kills,” I binned for sample-size evenness by age of kill before running an ANOVA and *post hoc* Tukey’s HSD tests (Appendix Table 2). I could not split the data within any single-day age category; thus, the two categories with a fixed sample size (1 and 2 days) determined the target sample sizes for the remaining age categories.

However, alternative binning combinations resulted in the same differences detected between groups. In addition to the extremely low numbers of kill sites in the middle range of maximum time elapsed (see Fig. 1 in main text), the likelihood of scavenging by competing predators was also expected to be lowest (or nonexistent) for kill sites ≤ 10 days old; for example, Boutin *et al.* (1986) placed snowshoe hare carcasses on the landscape and observed that all of the carcasses remained intact for at least 10 days.

Older kill sites in this study resulted from collection delays caused by access issues, particularly during the spring hiatus, as well as intermittent radio-collar malfunctions that impeded continuous monitoring of some hares. Although I found the rate of successful predator identification at older kill sites ($N = 3/25$; Appendix Fig. 1) too low to warrant the overall processing time and effort, they did reveal some noteworthy information. The few successful identifications obtained from saliva at old kill sites were almost certainly not from a scavenging predator. Two of these older kills were attributable to felids (one lynx and one bobcat), which very rarely scavenge small prey (Squires and Ruggiero 2007, O’Donoghue *et al.* 2010, Svoboda *et al.* 2013). The third kill, which was attributable to a pine marten, had sufficient other evidence

buried under snow to eliminate the possibility of another predator's involvement. Still, for future projects, processing old kill sites would likely only be worthwhile if researchers are willing to accept the lower success rates, are not heavily time-limited, and/or can adopt a more streamlined/less-thorough processing protocol than is recommended here.

Raptors

Raptor saliva was not amplified from any swabs despite multiple kill sites being likely raptor-attributable, although this result was not unexpected, given the lack of saliva that raptors produce. Kill sites irrefutably attributable to a raptor based on visual evidence ($N = 4$) were therefore removed from analyses because saliva was apparently unobtainable from raptor kill sites using this method. However, there were several other kill sites where raptor was the likely predator and saliva would not have been unobtainable, likely reducing my apparent success rate.

Scavenging by small animals

Documenting scavenging by small animals was not a goal of my study and was therefore not consistently recorded in my notes, particularly because it became clear that these frequent disruptions of the evidence at kill site were attributable to small-bodied animals, not actual predators of snowshoe hares. These occurrences were ascertained from tracks (red squirrels, red-backed voles, and gray jays), diagnostic handling of the carcass or collar (usually red squirrels and occasionally red-backed voles), direct observations (gray jays), and saliva results (one red squirrel and one red-backed vole). Scavenging by gray jays was usually focused on the entrails. I also observed handling characteristics of the remains and/or collar that would have been impossible for a species large enough to predate a snowshoe hare, *e.g.*, caching of the collar or

small pieces of the carcass in a very small and tight hole in the soil or duff with no disruption of the surrounding material (Wall 1990), or behavior diagnostic of a particular species, *e.g.*, a foot or collar securely wedged in the crook between two small branches or placed neatly on the horizontal tableau of a subalpine fir branch (Appendix Fig. 3) with no trace of adjacent hare fur. I eventually realized that these occurrences could not be due to a predator of snowshoe hares because (1) the foot or radio-collar was found in younger trees with branches that were too weak or flexible to support a great horned owl (*Bubo virginianus*) or goshawk (*Accipiter gentilis*), the only two raptors in study area expected to depredate snowshoe hares (2) the handling appeared to be very neat and deliberately secure in its placement, lacking the evidence typically found at a hare kill site such as a light layer of fur and/or fur tufts, and (3) I commonly observed large mushrooms that had been placed in trees in the exact same manner. The latter is a behavior regularly observed in red squirrels (Elbroch 2003, Stephenson 2010), the only species in my study region known to do this. Red squirrels are notorious hoarders of a variety of items, including bird nestlings (Klugh 1927), antler pieces (Hatt 1943) and inedible mushrooms (Hardy 1949). The caching and/or “tree drying” of a bony snowshoe hare foot or a hard-plastic radio-collar may be a behavioral carryover of this general hoarding tendency.

Appendix Table 2. Previously reported field-based studies that tested for predator saliva DNA on >5 kill sites.

Prey Species	Weight (kg)	Study
Domestic sheep (<i>Ovis aries</i>)	5-45 ^{b, c}	Blejwas <i>et al.</i> 2006 Williams <i>et al.</i> 2003
Caribou (<i>Rangifer tarandus</i>) calves	8.4-8.7 ^b	Mumma <i>et al.</i> 2014
White tailed deer neonates (<i>Odocoileus virginianus</i>)	2.63 ^a	Kilgo <i>et al.</i> 2012
Domestic sheep and (1) horse (<i>Equus ferus caballus</i>)	5-45 ^c	Caniglia <i>et al.</i> 2013
Fisher (<i>Martes pennanti</i>)	1.55-4.5 ^a	Wengert <i>et al.</i> 2013 and 2014
Black-fronted tern eggshells and carcasses (<i>Chlidonias albostratus</i>)	0.094 (terns only) ^b	Steffens <i>et al.</i> 2012
Woylies (<i>Bettongia penicillata</i>)	1.1 ^a (<i>note: study included predator-baiting</i>)	Marlow <i>et al.</i> 2015

^a reported weights from study

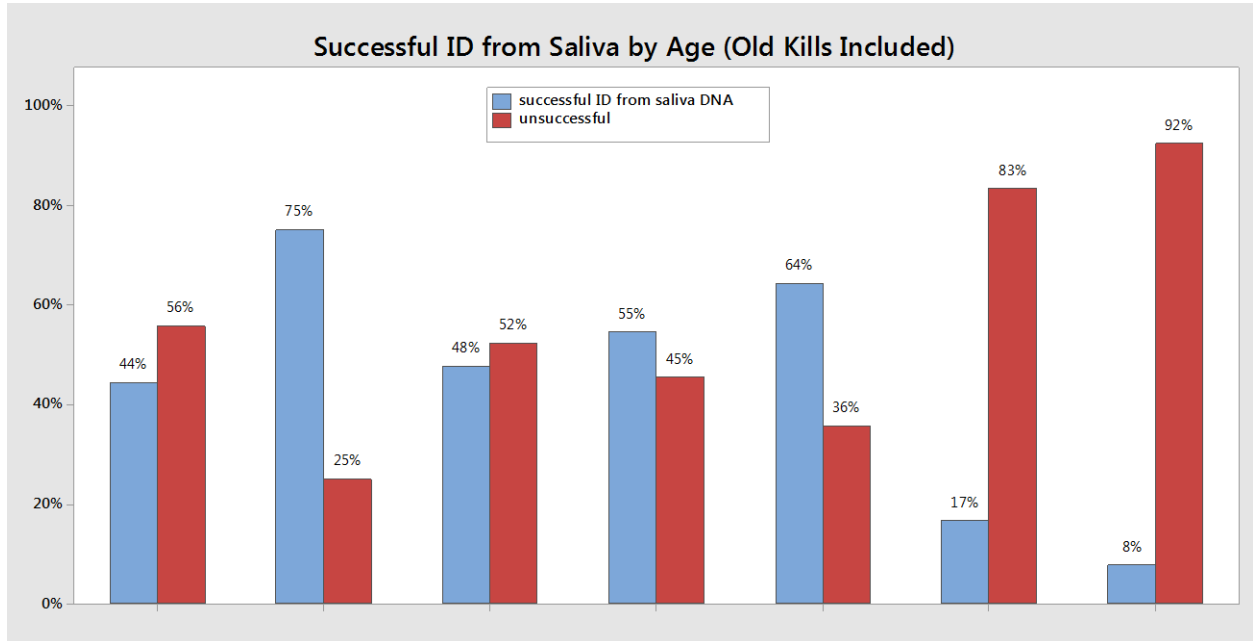
^b personal communication with the authors

^c inferred weights based on general knowledge of this species

Appendix Table 3. Significance of differences in saliva-swabbing success between kill-site age categories, including old kills not retained in the main dataset, using Tukey’s HSD, with *p*-values <0.10 in bold.

Max Age of Kill	1 day	2 days	3 days	4-10 days	20-110 days	112-167 days
1 day (<i>N</i> = 8)		0.6130	0.9255	0.9983	0.0663	0.0184
2 days (<i>N</i> = 20)			0.9932	0.7274	0.5289	0.2041
3 days (<i>N</i> = 11)				0.9843	0.3519	0.1315
4-10 days (<i>N</i> = 15)					0.0613	0.0123
20-110 days (<i>N</i> = 12)						0.9962
112-167 days (<i>N</i> = 13)						

Appendix Figure 1. Proportion of swabs at each kill site that were successful or unsuccessful at yielding predator DNA from saliva, plotted by maximum age of the kill (time since hare was last known to be alive), including old kills that were not retained in the main dataset ($N = 78$).



Appendix Figure 2. Visualization of the sample types (Collar swabs, Remains swabs, or both) that contributed at least one saliva swab with identifiable predator mtDNA at a kill site. Each cell represents one kill site ($N_{\text{successful sites}} = 31/53$, $n_{\text{swabs}} 45/109$). This figure shows identifications not included in the sample-type analysis because only one sample type was taken.

Collar and Remains	Collar and Remains	Collar and Remains	Collar and Remains
Collar and Remains	Collar and Remains	Collar and Remains	Collar and Remains
Remains	Collar and Remains	Collar and Remains	Collar
Remains	Remains	Collar	Collar
Remains	Remains	Collar	Collar
Remains	Remains	Collar	Collar
	Remains (<i>only sample type tested</i>)	Collar	Collar
		Collar	Collar (<i>only sample type tested</i>)
		Collar (<i>only sample type tested</i>)	Collar (<i>only sample type tested</i>)

Appendix Figure 3. Evidence of kill-site tampering by small animals presented itself in many forms, such as this snowshoe hare foot wedged into a small tree branch by a red squirrel (*Tamiasciurus hudsonicus*).

