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Tools and Technology

Using DNA from Hairs Left at Depredated Greater Sage-grouse Nests to Detect Mammalian Nest Predators

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ABSTRACT Despite a multitude of studies on sage-grouse (*Centrocercus* spp.), there is still sparse information on the predator communities that influence sage-grouse productivity and how these predator communities may change when sagebrush habitats are altered by human activities. As a proof-of-concept, we used mammalian hairs collected at depredated greater sage-grouse (*C. urophasianus*) nests and mitochondrial DNA sequencing to identify mammalian species that deposited the hairs at the depredated nests. We monitored nests of radiomarked female greater sage-grouse in an oil and gas development area in the Powder River Basin, Wyoming, USA, from 2009 to 2011. We collected mammalian hair samples from 56 depredated nests. We detected 5 species: American badger (*Taxidea taxus*), bobcat (*Lynx rufus*), coyote (*Canis latrans*), red fox (*Vulpes vulpes*), and striped skunk (*Mephitis mephitis*). Red fox and striped skunk are considered exotic predators—species outside of their historical range—in our study area and represented 20% of our detections. This method could be improved by gathering and analyzing various types of DNA sources including predator saliva from egg shell fragments, predator scat, and even feathers left by avian predators. Our results suggest that this method has merit as a noninvasive tool to better understand the community of mammalian nest predators present within large study areas, and role of exotic predators in sagebrush habitats. © 2018 The Wildlife Society.

KEY WORDS *Centrocercus urophasianus*, depredated nest, DNA sequencing, hair-snares, mammalian predators, mitochondrial DNA, sage-grouse.

Nest predation is a leading cause of nest failure among most avian species (Martin 1993), including the greater sage-grouse (*Centrocercus urophasianus*; sage-grouse; Webb et al. 2012, Lockyer et al. 2013). Despite a multitude of studies on sage-grouse, there remains sparse information on the predator communities that influence sage-grouse nest success and productivity (Conover and Roberts 2016). In undisturbed habitats, where habitat quality has not been compromised by human activities, research has indicated that nest predation is rarely found to be a limiting factor for sage-grouse (Bergerud 1988, Hagen 2011). In disturbed habitats, however, human features and habitat fragmentation have been shown to negatively influence avian nest success, due to an increase in nest predation, in a variety of ecosystems including the sagebrush (*Artemisia* spp.)-steppe (Robinson et al. 1995, Chalfoun et al. 2002, Connelly et al. 2011*a*).

Avian predators of sage-grouse nests, such as common raven (*Corvus corax*) and black-billed magpie (*Pica hudsonia;* Hagen 2011), are conspicuous and their presence is usually detected without intensive surveys. Mammalian nest predators, however, are difficult to observe because of many factors including nocturnal activity and wariness of humans (Sargeant et al. 1998, Ruell and Crooks 2007). Researchers use a variety of invasive and noninvasive methods to confirm the presence of mammalian predators. Invasive methods include live-trapping and radiotelemetry. Noninvasive methods include hair-snaring, scat surveys, snow-tracking, camera surveys, and track-plates surveys (Ruell and Crooks 2007). These methods are usually designed to gather information on predator communities and not directly associated with predation events (e.g., depredation of nests). Applying these survey methods to large study areas is labor-intensive and, for many studies, extensive mammalian predator surveys are impractical because of costs and logistics. This is especially true for studies in which the goals of the study are not directly focused on predators.

For studies on avian nests, the use of surveillance cameras placed at nest sites has become a common research tool and is a reliable method of identifying nest predators (Pietz and Granfors 2000, Lockyer et al. 2013). Although attractive as an easy tool, using cameras on nests is an invasive method that can have drawbacks because of the placement of camera equipment at active nests. Camera equipment is placed at

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Figure 1. Nesting greater sage-grouse under sagebrush shrubs, Powder River Basin, Wyoming, USA. Photo taken by Christopher Kirol, 5/20/2009.

sage-grouse nests during incubation and typically causes the female to flush off the nest, and nest abandonment risk has been shown to increase because of the placement of camera equipment at nest sites (Anthony et al. 2006, Coates et al. 2008). Some studies also suggest that the presence of camera equipment and investigator disturbance when placing equipment at nest sites may introduce its own biases by altering predator behavior and increasing nest predation by drawing predators to the nest to investigate these novel objects or human scent left at the nest site (Anthony et al. 2006, Richardson et al. 2009). Videography research can be time- and labor-intensive and, with the costs of the camera equipment, can often be cost-prohibitive, especially for large areas, such as with sage-grouse whose nests are widely dispersed over large landscapes.

Noninvasive genetic sampling, such as using hair-snares (often barbed wire) to collect samples for species identification using DNA analysis, have become an effective sampling method used for detecting and identifying animals (Schwartz et al. 2006, Zielinski et al. 2006). Genetic sampling methods have been used as an alternate to camera surveys (Onorato et al. 2006), but could also be used in conjunction with camera surveys. Hair-snares have been used in studies to identify large and small mammal species across large areas. Some of these species include the coyote (*Canis latrans*), red fox (*Vulpes vulpes*), striped skunk (*Mephitis mephitis*), American badger (*Taxidea taxus*), and weasels (*Mustela* spp.; Zielinski et al. 2006, Kendall and McKelvey 2008).

Female sage-grouse typically nest under sagebrush shrubs (Connelly et al. 2011*b*). Sagebrush is a woody, coarse, and multibranched shrub species (Beetle and Johnson 1982, Rosentreter 2005). Sagebrush shrubs used for nesting by sage-grouse typically have greater branching densities with more obstructing vegetation cover and have 1 or 2 openings in the shrub to permit the females to escape (Schroeder et al. 1999). These characteristics of the "nest shrub" create a natural hair-snare because hair often gets snagged on the woody branches of the plant when a predator crawls into the shrub to consume the eggs (Fig. 1).

While monitoring telemetry-marked sage-grouse for research related to oil and gas development and mitigation in the Powder River Basin, Wyoming, USA (Fedy et al. 2015, Kirol et al. 2015), we opportunistically observed mammalian hair on the nest shrub sheltering many of the depredated nests. In 2009, we began collecting hair samples by systematically searching the nest shrub of depredated sage-grouse nests, paying special attention to the escape opening(s) in the shrub for any snagged hairs. We collected the hair as a DNA source in an attempt to identify the mammalian species that left the hair. Native mammalian predators that likely occurred in our study area and known to depredate sage-grouse nests include American badger, coyote, bobcat (*Lynx rufus*), and long-tailed weasel (*M. frenata;* Conover and Roberts 2016). Further, anecdotal evidence suggested, in recent years, nonnative or exotic mammalian predators were expanding into sagebrush habitats in the Powder River Basin. Suspected species outside of their historical range in our study area include the red fox, stripped skunk, and common raccoon (*Procyon lotor;* Aldridge and Brigham 2003, Baxter et al. 2008, Hagen 2011). These suspected exotics are all known to be proficient nest predators (Vickery et al. 1992, Pasitschniak-Arts and Messier 1995, Conover and Roberts 2016).

We provide a proof-of-concept regarding the use of mammalian hair and mitochondrial DNA (mtDNA) sequencing as a noninvasive tool to identify potential mammalian nest predators. Specifically, our objectives were 1) to determine whether using mtDNA from mammalian hairs is a practical and effective approach to identify mammalian nest predators and detect their presence at depredated sage-grouse nests, and 2) use detections at depredated sage-grouse nests to describe the assemblage of the predator community.

STUDY AREA

Our research occurred in the Powder River Basin, primarily in Johnson County, with the northern portion extending slightly into Sheridan County, Wyoming (106°20′2.538″W, 44°18′35.431″N). The study area encompassed 937-km^2, of which 61% was private land, 33% was public land administered by the Bureau of Land Management, and 6% was Wyoming state land. Cattle and sheep ranching were the primary agricultural uses and energy development, predominantly in the form of coal-bed natural gas, was the primary energy extraction activity occurring in the study area. The majority of the study area was shrub-steppe habitat dominated by Wyoming big sagebrush (*A. tridentata wyomingensis*).

METHODS

We captured female sage-grouse in spring (mid-Mar through late Apr) and in late summer (Sep) using a rocket-net (Giesen et al. 1982) and a CODA netlauncher (CODA Enterprises, Incorporated, Mesa, AZ, USA) mounted on a truck or all-terrain vehicle. We fitted very-high-frequency radiotransmitters (Model A4060; Advanced Telemetry Systems Inc., Isanti, MN, USA) to female grouse. Transmitters weighed 22 g (~1.4% of mean female sage-grouse body mass), had a battery life expectancy of 789 days, and were equipped with motion-sensors (radiotransmitter pulse rate increased in response to inactivity after 8 hr). We located radiomarked female sage-grouse on the ground using hand-held receivers and 3-element Yagi antennas during the nesting period (late Apr–Jun) in 2009, 2010, and 2011. Nesting was confirmed by 2 consecutive visits that identified the radiomarked grouse under the same shrub using triangulation or by visually observing the female on a nest with binoculars. After confirming a nest location, we monitored the status of the nest every 2–6 days until the nest hatched or failed. To minimize disturbance to the female, we confirmed that the female was still on the nest from a distance of >30 m by triangulating to the nest location using radiotelemetry. After recording or visiting a nest location, we retreated in a nonlinear and varying pattern each visit to prevent predators from following human scent to the vicinity of the nest. The fate of the nest (successful or unsuccessful) was determined by the condition of the eggshells and shell membranes and other diagnostic evidence (Wallestad and Pyrah 1974). We classified a nest as unsuccessful if it was depredated, naturally abandoned, or if the female was killed while incubating. If a nest was depredated, we collected any hair samples attached to the nest shrub and stored hairs in glass collection vials. Most often we would only find a few hairs that remained attached to a single nest shrub snare (i.e., branch). We noted diagnostic evidence at depredated nests such as nest bowl disturbance, eggshell remains, scat, or tracks and, if enough evidence was present, hypothesized the responsible predator (Sargeant et al. 1998, Thirgood et al. 1998). The Wyoming Game and Fish Department issued a Chapter 33 Permit for this research, and research adhered to regulations and guidelines for use of wild birds in research (2008; http://naturalhistory.si.edu/BIRDNET/guide/index.html).

We extracted genomic DNA from hair samples using the QIAGEN Dneasy Blood and Tissue kit according to manufacturer's instructions for tissue and using modifications for hair samples from Mills et al. (2000). Up to 10 hairs were used in the DNA extraction from clumps of hair collected from a snare, and for samples containing <10 hairs, we used all hair available for extraction. We extracted DNA from hair samples in a dedicated laboratory used for samples with low-quantity or low-quality DNA; Rocky Mountain Research Station Missoula, Montana, USA. We included a negative control when performing DNA extractions to identify contamination. The control region of mtDNA was amplified using conserved, universal primers L15926 and H16498 (Kocher et al. 1989, Shields and Kocher 1991). This region has been shown to successfully amplify DNA from noninvasively collected hair samples from a wide variety of mammals (Foran et al. 1997, Mills et al. 2000, Onorato et al. 2006, Broquet et al. 2007) and has successfully amplified DNA from hair from all the candidate mammalian predators in the study area (K. Pilgrim, personal communication). The candidate mammalian predators provided for our study area included American badger, American mink (*Neovison vison*), bobcat, bushy-tailed woodrat (*Neotoma cinerea*), common raccoon, coyote, domestic cat (*Felis catus*), domestic dog (*Canis lupus familiaris*), long-tailed weasel, red fox, striped skunk, swift fox (*Vulpes velox*), and thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*). We included unlikely mammalian predators in the candidate list to ensure the list was comprehensive. Reaction volumes of 50 μL contained 50–100 ng DNA, 1× reaction buffer (Applied Biosystems, Waltham, MA, USA), 2.5 mM MgCl2, 200 μM each dNTP, 1 μM each primer, 1 U AmpliTaq Gold polymerase (Life-Technologies [Thermo Fisher Scientific, Waltham, MA, USA]). Samples were amplified in 2 polymerase chain reaction (PCR) reactions and a positive and negative control were used with each PCR reaction. The PCR program was 94° C/5 minutes, [94° C/1 min, 55° C/1 min, 72° C/1 min 30 sec] × 34 cycles, 72° C/5 minutes. Polymerase chain reaction amplicons were run on a 1.6% agarose gel electrophoresis. Polymerase chain reaction products were purified using ExoSap-IT (Affymetrix-USB Corporation, Cleveland, OH, USA) according to manufacturer's instructions. DNA sequence data were obtained using the Big Dye kit and the 3700 DNA Analyzer (High Throughput Genomics Unit; ABI, Seattle, WA, USA). DNA sequence data were viewed and aligned with Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences obtained were compared with sequences from known species available in the National Center for Biotechnology Information Genbank database (https://www.ncbi.nlm.nih.gov/genbank/) as well as internal reference sequence databases to identify species.

RESULTS

We monitored 228 sage-grouse nests ($n = 76$ in 2009, $n = 84$ in 2010, and $n = 68$ in 2011) over the study period. All of the sage-grouse nests in our study were under sagebrush plants. Of the nests monitored, 46.5% ($n = 106$) were unsuccessful. The bulk of unsuccessful nests were lost to predation (95.3%); however, 4.7% ($n = 5$) were abandoned for unknown reasons. One nest was unsuccessful because the female was killed on the nest, but the eggs were not consumed. On the basis of diagnostic evidence observed at the nest site, we suspected that 62.0% of depredations were caused by mammals and 7.0% depredations were caused by corvid (i.e., black-billed magpie). There was not enough diagnostic evidence to allow for inference on predator type for 31.0% of the depredated nests. As expected, all depredated nests in which mammal hair was found were suspected to have been caused by a mammal.

We collected hair samples at 56 depredated nests over the duration of the study; thus, we found hair at 52.8% of the depredated nests. The number of hairs snared and collected

at depredated nests varied from 1 to 20 (i.e., clumps of hair). However, we only found a few hairs attached to a single snare (branch of the nest shrub) in approximately 70% of the instances. When we found clumps of hair, these were snagged on a single snare. We did not have any instances when we found multiple clumps of hair snagged on different snares. Successful amplifications and sequencing data were obtained for 30 of the hair samples (53.6% of hair samples); all samples that amplified via PCR were able to be identified to species using DNA sequence data. Of the 101 nests that were depredated, we found hair and successfully amplified DNA for 29.7% of them. We did not observe contamination for either the DNA extraction or PCR negative controls. Generally, samples with more hair amplified better than samples containing <10 hairs. Of the 30 hair samples that contained sufficient DNA, 16 were identified as coyote, 7 were identified as American badger, 3 as red fox, 3 as striped skunk, and 1 as a bobcat. Twenty percent of our detections were predators considered exotic predators (red fox and striped skunk). We detected a coyote at the nest that failed because the incubating sage-grouse was killed on the nest. We did not detect long-tailed weasel or raccoon hair at any of the depredated nests. We did not find evidence that we were collecting hair, at a single depredated nest site, from more than one mammalian species at any time during the study. By direct observation, we confirmed a bullsnake (*Pituophis catenifer*) depredating eggs from one nest (Supporting Information online, Fig. S1).

DISCUSSION

We identified several mammalian predators present at depredated sage-grouse nests based on hairs that remained on the nest shrub using DNA sequencing. We identified 5 mammalian predators including the American badger, bobcat, coyote, red fox, and striped skunk. Three of these species are known sage-grouse nest predators and native to the majority of the sagebrush ecosystem (Hagen 2011). Using videography, coyotes were recorded depredating sage-grouse nests by Bell (2011), Lockyer et al. (2013), and Taylor et al. (2017); American badgers were recorded depredating sage-grouse nests by Holloran and Anderson (2003), Coates et al. (2008), Bell (2011), Lockyer et al. (2013), and Taylor et al. (2017); and bobcats were recorded depredating sage-grouse nests by Lockyer et al. (2013). Prior to our study, both the striped skunk and red fox have only been detected once at depredated sage-grouse nests (Taylor et al. 2017). The striped skunk and red fox are known to be proficient predators of avian ground nests in other habitats such as grasslands (Vickery et al. 1992, Phillips et al. 2003).

Our results confirm that red fox and striped skunk, both exotic predators, are foraging in sagebrush habitats in the Powder River Basin. The red fox and striped skunk demonstrate selection for human-altered habitats with a greater amount of edge, and are exotics that benefit from human subsidies (Larivière and Messier 1998, Phillips et al. 2003, Frey and Conover 2006, Conover and Roberts 2016). As an example of the utility of this method to better understand differences in nest survival rates of sage-grouse nesting in disturbed versus undisturbed habitats (Connelly et al. 2011*a*, Kirol et al. 2015), we conducted a *post hoc* spatial comparison in a Geographic Information System framework, reported here with 95% confidence intervals (±). Detections of exotic predators (red fox and stripped skunk) averaged 0.36 ± 0.09 km (range = 0.15–0.75 km) from the nearest oil and gas development area while native mammalian predator detections averaged 1.18 ± 0.26 km (range = 0.19–5.82 km). Although these sample sizes are too small ($n = 6$ exotic predator detections) to draw any definitive conclusions, exotic predators appear to be more closely associated with development areas in our study area. Using the described method researchers could expand upon our preliminary analysis and collect a robust sample of detections across a large study area. A larger sample of DNA detections well-distributed across a landscape could provide for a rigorous spatial analysis allowing for a better understanding of relationships between human infrastructure and relationships with mammalian nest predators.

Our described method may be more effective at detecting larger predators because the size of the nest shrub opening relative to the predator size allows smaller nest predators to enter the nest shrub without snaring hair. Zielinski et al. (2006), monitoring populations of mesocarnivores in California, USA, showed that wire-snares were most effective at snaring hair from larger species (e.g., gray foxes [*Urocyon cinereoargenteus*]), but less effective at snaring hair from smaller species such as pine martens (*Martes americana*). They attributed this to the spacing between barbs relative to the predator's body size. Size of the nest shrub opening relative to the size of the mammalian predator may explain why smaller sage-grouse nests predators such as the long-tailed weasel were not detected at any of the depredated nests in our study (Lockyer et al. 2013).

Monitoring nests with cameras allows for unequivocal identification of responsible nest predators and can provide other insights into predator and prey behavior. The cost of videography equipment can often be prohibitive, especially if a large sample size is needed to address research questions. Camera equipment of varying quality can cost from US$200 to $650/unit. Thus, if a project places camera equipment at 60 nests, cost for just the equipment would range from approximately US$12,000 to $39,000. A large portion of the nests with the camera equipment installed will not be depredated (e.g., 50%); therefore, at best this investment might result in 30 predator detections. It is important to note that once purchased, camera equipment can be used repeatedly within a season or between years and videography provides additional information such as behavior of the nesting species while attending the nest and behavior of the predator species during a nest predation event (Lockyer et al. 2013). Two of the most rigorous videography studies on sage-grouse nests placed cameras at 55 nests and recorded 16 depredation events (Coates et al. 2008), and placed cameras at 39 nests and recorded 17 depredation events (Lockyer et al. 2013). Cost differences using DNA sequencing and videography are substantial. Using the method described in this paper, we detected mammalian

predators at 30 depredated nests at a total cost of US $1,400, or US$25/sample ($n = 56$ samples). Detection information could be gathered over large areas because of the low cost associated with this method, thus providing a better understanding of the assemblage of mammalian nest predators and patterns of nest predation across a landscape.

In some ecosystems, research has suggested that videography may introduce bias in nest survival estimates and predator identification (Séquin et al. 2003, Anthony et al. 2006, Richardson et al. 2009). For instance, Richardson et al. (2009) suggested that wariness of human scent and novel objects (e.g., camera equipment) exhibited by some predators contributes to a detection bias. Thus, another benefit of this method is a robust sample of detections at depredated nests without the need for placement of novel objects at active sage-grouse nests and human visitation to place the camera equipment at the nests under study.

We believe this method could be improved by expanding investigations of depredated nests to include other biological remains that provide DNA sources. Predator saliva from egg shell fragments and mammalian scat remaining around the nest are other biological remains that could provide DNA for identification (e.g., Onorato et al. 2006, Wheat et al. 2016). Based on diagnostic evidence at depredated nests, we suspected that approximately 7% of our nest depredations were avian-caused. This method could be expanded beyond mammalian predators by incorporating biological remains left by avian predators that could also be identified to species with DNA sequencing. Similar to hair, on several occasions during field work, feathers and scat of suspected avian nest predators, such as the black-billed magpie, were found at the depredated nest sites. Gathering and analyzing various types of DNA sources would considerably increase detection rates, likely increase detections of smaller mammalian nest predators (e.g., long-tailed weasel could be detected by its saliva or scat rather than hair), and allow for avian predator detection as well. Almost half of our hair samples did not contain enough DNA for identification because of DNA degradation. Biological samples analyzed more regularly (e.g., immediately after collection) would likely result in less identification failures due to DNA degradation.

Unlike videography, this method does not allow for unequivocal identification of the predator responsible for the depredation event. It is possible that we could have detected olfactory mammals that visited a nest soon after the initial depredation event to scavenge the nest remains and therefore, the predator we detected was not the initial nest predator. Nests were monitored every 2–6 days, so we believe that the majority of the mammalian detections from hair samples were likely the initial predator. Being certain that the predator detected at a nest was responsible for the nest predation was not critical to our research because we were using depredated nests to gather information on the presence and community assemblage of mammalian predators across our study area.

When used independently of each other, there are potential drawbacks to videography and limitations of the DNA method; therefore, the greatest amount of information may come from a combination of the 2 approaches. In conjunction, these methods would provide a suite of information on the community of predators, including avian predators, responsible for sage-grouse nest predations, and provide other insights into patterns of nest predation. For example, by simultaneously using cameras, placed at a subsample of the nests, and genetic sampling, one could obtain information on how frequently nest predators depredated sage-grouse nests without leaving behind any biological remains (e.g., hair and saliva) that allow for DNA identification and how often nest scavenging by secondary predators occurs.

By using biological remains and DNA sequencing as a noninvasive detection tool, we were able to identify presence of 3 native and 2 exotic predator species at depredated sage-grouse nests in a large study area (937 km^2) influenced by oil and gas development. With our suggested improvements, use of DNA to detect nest predators 1) allows for a robust sample of detections across large study areas with minimal time and cost investment; 2) minimizes human-induced collection bias; and 3) because of minimal cost and time investment, can be used concurrent with other research goals to provide ancillary information and help inform research findings. DNA sequencing from biological remains left at depredated nests proved to be an effective tool that, by itself or in conjunction with other predator identification techniques, could help us better understand patterns of nest predation and the role of exotic predators over large areas and across gradients of human disturbance.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site. Image of greater sage-grouse nest being depredated by a bullsnake (*Pituophis catenifer*) in the Powder River Basin, Wyoming, USA.