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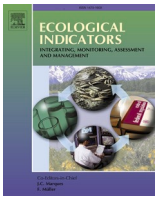

Improving ecological surveys for the detection of cryptic, fossorial snakes using eDNA on and under artificial cover objects

Laura Matthias [a], Michael J. Allison [b], Carrina Y. Maslovat [a], Jared Hobbs [c], Caren C. Helbing [b,*]

[a] Independent Consultant, Salt Spring Island, British Columbia, Canada
[b] Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada
[c] J Hobbs Ecological Consulting Ltd., Pender Island, British Columbia, Canada

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ABSTRACT

Performing ecological surveys for secretive, fossorial snakes is challenging. Traditional survey methods involve visual observation under artificial cover objects (ACOs); this is labor-intensive and requires multiple consistent surveys of suitable habitats. Detection of snake DNA deposited under ACOs represents an innovative method for species detection. However, for terrestrial species, common issues with soil-based methods include the challenges of adequately removing enzyme inhibitors that reduce environmental DNA (eDNA) detection and potential photodegradation of DNA taken from surface samples. These issues may be circumvented by obtaining swabs and soil samples directly from the underside of ACOs for eDNA analysis. We demonstrate the application of this method in surveys of sharp-tailed snake (*Contia tenuis*), an endangered species under the Canadian *Species at Risk Act*. We describe the design and validation of a new quantitative real-time polymerase chain reaction (qPCR)-based eDNA eCOTE3 assay with high specificity and sensitivity for sharp-tailed snake. We developed a practical and robust protocol for obtaining eDNA samples by swabbing the underside of ACOs and collecting soil samples under ACOs. Traditional surveys were conducted over two successive years (2018–19) on 220 paired ACOs at 110 sites monitored between 12 and 30 times each. Of the 6,060 ACO visits, only 24 resulted in sharp-tailed snake observations (0.4% success rate) illustrating the considerable difficulty in detecting these snakes. During this same time, 109 swabs were taken directly from the undersides of ACOs and 78 soil samples were collected from a subset of these ACOs. Of the 24 occurrences where sharp-tailed snakes were visually observed, 13 of 23 ACO swabs (57%) and nine of 20 soil samples (45%) tested positive for DNA. eDNA deposition is likely low because of the small size and behavior of this cryptic species, yet DNA was detected from soil exposed to captured snakes for only 10 min. Nevertheless, sharp-tailed snake eDNA was detected at eight sites (9%) from ACO swabs (n = 86) and seven sites (13%) from soil samples (n = 56) where snakes were not observed. This is an overall detection rate of 25% (14/56) for swab and soil samples testing positive in sites where both were tested, representing a substantial reduction in the effort required for detection of this species. Given the time-consuming nature of traditional surveys, eDNA holds great promise as a complementary survey tool for this terrestrial species. While further work is needed to delineate DNA deposition rates, this work represents a significant advance in monitoring a challenging species.

1. Introduction

World Wildlife Fund's *Living Planet Report* notes that about half of Canadian vertebrate species studied are in substantial decline with population decreases averaging 83% among species in Canada (World Wildlife Fund WWF Canada, 2018). Half of the world's ecosystems are already degraded or transformed (Pojar, 2010) and ongoing habitat loss is the greatest causal factor identified in species loss (World Wildlife Fund WWF Canada, 2018). Efforts to detect cryptic, rare species are often essential for guiding habitat conservation efforts and maintaining biodiversity.

The sharp-tailed snake (*Contia tenuis*) is a small (20–30 cm), slender, reddish-brown, non-venomous snake that is endemic to western North America, ranging from Canada continuing south through Washington, Oregon and California (Environment and Climate Change Canada, 2017). In Canada, the species is listed as Endangered under Schedule 1

* Corresponding author.
E-mail address: chelbing@uvic.ca (C.C. Helbing).

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of the federal *Species at Risk Act* (*SARA*) due to its restricted and discontinuous distribution in southwestern British Columbia, and due to ongoing threats from residential development and other human activities (Environment and Climate Change Canada, 2017).

Its secretive, primarily fossorial nature makes it difficult to study, resulting in significant data gaps in population biology, range, and habitat requirements (Environment and Climate Change Canada, 2017). Virtually nothing is known of their underground habitat use. Eggs have never been found in the wild in Canada and have only been observed once in the United States (Sharp-tailed Snake Recovery Team, 2008). Sharp-tailed snakes have restricted intra-seasonal movements (usually less than 55 m during their lifespan) and they do not migrate (Sharp-tailed Snake Recovery Team, 2008). A poor understanding of the extant distribution further challenges effective conservation and habitat protection for this species (Environment and Climate Change Canada, 2017).

Sharp-tailed snakes are found in woodland and forest openings on warm aspect slopes under loose talus, coarse woody debris and/or in fissures in rock (Environment and Climate Change Canada, 2017). Although some cryptic species are often more abundant than survey data might suggest, this pattern is highly unlikely for sharp-tailed snake given their highly specialized life history requirements and occupancy of an imperiled ecosystem that has suffered significant habitat loss in Canada. They require natural cover objects (NCOs) such as rocks or bark slabs that provide shelter yet NCOs need to be thin enough to warm up quickly and transfer heat efficiently to the snake (i.e., behavioural thermoregulation) (Environment and Climate Change Canada, 2017). Snakes will also use artificial cover objects (ACOs) such as small, asphalt shingles, to thermoregulate, when ACOs are placed on the surface of the ground in suitable habitat (Engelstoft and Ovaska, 1997; Sharp-tailed Snake Recovery Team, 2008). Surveyors can easily lift ACOs (optimal survey timing is during spring and fall) to detect snakes without disturbing their habitat (Engelstoft and Ovaska, 1997; Sharp-tailed Snake Recovery Team, 2008).

Although ACO observation is an important survey technique, it requires multiple consistent surveys for success, and it often takes years of monitoring before there is a detection (Sharp-tailed Snake Recovery Team, 2008; Environment and Climate Change Canada, 2017). As such, development of reliable and less time-intensive survey methods are required to more efficiently detect sharp-tailed snakes.

Recent technical advances in quantitative polymerase chain reaction (qPCR) have allowed detection of tiny amounts of species-specific biological material from environmental DNA (eDNA) (Jerde et al., 2011; Goldberg et al., 2016). The term "eDNA" refers to any trace fragment of exogenous DNA that is released by an organism into the environment (Ficetola et al., 2008). Aquatic eDNA sampling has been used successfully to survey for rare vertebrates (e.g., Goldberg et al., 2011; Thomsen et al., 2012; Sigsgaard et al., 2015; Fukumoto et al., 2015; Thomsen and Willerslev, 2015; Lacoursière-Roussel et al., 2016; Eiler et al., 2018; Helbing and Hobbs, 2019). Relatively few studies have explored detection of semi-aquatic snake eDNA from (primarily) water samples with mixed success in detecting snake DNA (Halstead et al., 2017; Jordan and Ratsch, 2018; Hunter et al., 2019; Rose et al., 2019; Baker et al., 2020; Ratsch et al., 2020). This may have been due to low rates of DNA release, sampling methods, or low sensitivity of the eDNA detection assays. Techniques have also been investigated for detecting terrestrial species using eDNA in soil, and several challenges have been encountered with this type of substrate, particularly the increased presence of enzyme inhibitors and an absence of DNA diffusion as may be the case in an aquatic environment (Anderson et al., 2012; Thomsen and Willerslev, 2015; Schwartz et al., 2017; Walker et al., 2017; Kucherenko et al., 2018; Mauvisseau et al., 2019; Leempoel et al., 2020; Baudry et al., 2021).

The present study explored the possibility of sampling eDNA directly from the underside of ACOs as an alternative substrate for the detection of cryptic, fossorial snake species. eDNA sampling protocols were developed and samples were collected during traditional surveys. Samples were analyzed at the University of Victoria (UVic) using a species-specific qPCR assay which was validated using fecal and cloacal swabs from sharp-tailed snakes captured and sampled in the field. eDNA samples were collected by swabbing the underside of ACOs, and by collecting soil beneath snakes, to determine if eDNA could be detected in these samples. ACO swabs and soil samples were also collected in locations where no sharp-tailed snakes had been observed (during surveys completed between 2018 and 2019) to further test the efficacy of the eDNA sampling tool.

2. Materials and methods

2.1. eDNA assay design and validation

2.1.1. Assay development

Quantitative real-time polymerase (qPCR) primers and probes were designed using mitochondrial gene sequences obtained from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov). All publically available sequences for the all native reptile species that occur in BC, including: sharp-tailed snake, rubber boa *(Charina bottae),* western painted turtle *(Chrysemys picta),* western yellow-bellied racer *(Coluber constrictor Mormon),* western rattlesnake *(Crotalus oreganus),* northern alligator lizard *(Elgaria coerulea),* western skink *(Eumeces skiltonianus),* desert nightsnake *(Hypsiglena torquata),* common wall lizard *(Podarcis muralis), Great Basin* gopher snake *(Pituophis catenifer deserticola),* terrestrial gartersnake *(Thamnophis elegans),* northwestern gartersnake *(T. ordinoides),* common gartersnake *(T. sirtalis),* plus corresponding genes for human and dog were assembled and aligned using ClustalW (http://www.genome.jp/tools-bin/clustalw). Candidate assay components were designed and chosen using BioEdit (Ibis Biosciences, Carlsbad, CA, USA) and Primer Premier Version 6 (Premier Biosoft, Palo Alto, CA, USA) based on regions of the genes that were unique to the sharp-tailed snake. Special care was taken to ensure the test would not amplify human DNA. Finally, assay candidate sequences were input into NCBI Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to ensure the sequences would not amplify any sympatric species that were not considered in the design phase.

2.1.2. Assay lab validation

Assay specificity was empirically determined using DNA isolated from voucher tissues and swabs collected from specimens housed at the Royal BC Museum and the University of Victoria laboratory under Wildlife Permit #NA18-286900. Human total DNA was obtained from a HEK293 cell line (American Type Culture Collection (ATCC) Manassas, VA; Catalog number CRL-1573). All primers and the probe containing a 5′FAM reporter dye and 3′ ZEN/Iowa Black FQ quencher were ordered from Integrated DNA Technologies (IDT; Coralville, IA, USA). The primers were first tested for specificity against total DNA from sharp-tailed snake and all potentially confounding (sympatric reptile) species (Table 1) with two technical replicates using SYBR green (Invitrogen, Carlsbad, CA, USA) qPCR assay and agarose gel visualization of the amplified product (amplicon).

Once the primers were confirmed to produce an amplicon of the desired length, they were tested in combination with their corresponding candidate Taqman hydrolysis probe in two technical replicates containing 5 μg/L (equivalent to 10 picograms per reaction) gDNA based on Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) A_{260} spectrophometry readings. This quantity of gDNA is a mixture of mitochondrial and nuclear DNA with varying proportions of each. Despite this, 10 picograms per reaction was sufficient to reliably expect 100% amplification of all technical replicates from the target DNA sample. We tested gDNA from seven individual sharp-tailed snake specimens plus the other eight species in Table 1, and a no-template control (NTC). If amplification was detected in a reaction within 50 cycles it was scored as

Table 1
Names and abbreviations of species used for sharp-tailed snake eDNA test validation. All species listed were initially validated with two technical replicates, and those in bold were validated with 25 technical replicates.

Species Name	Common Name	Species Abbreviation	Percent Detection by eCOTE3
Contia tenuis	**Sharp-tailed snake**	**COTE**	**100%**
Canis familiaris	Dog	CAFA	0%
Elgaria coerulea	**Northern Alligator Lizard**	**ELCO**	**0%**
Homo sapiens	**Human**	**HOSA**	**0%**
Lithobates (Rana) catesbeiana	American Bullfrog	LICA	0%
Podarcis muralis	**European Wall Lizard**	**POMU**	**0%**
Thamnophis elegans	**Western Terrestrial Garter Snake**	**THEL**	**0%**
Thamnophis ordinoides	**Northwestern Garter Snake**	**THOR**	**0%**
Thamnophis sirtalis	**Common Garter Snake**	**THSI**	**0%**
No Template Control	**No Template Control**	**NTC**	**0%**

a positive. The run conditions for each amplification reaction on a CFX96 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) were as follows: Two μL of DNA sample were run in a 15 μL total reaction volume consisting of 10 mM Tris-HCl (pH 8.3 at 20 °C), 50 mM KCl, 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 69.4 nM ROX (Life Technologies, Burlington, ON, Canada), 10.5 pmol of forward and reverse PCR primer, 1.5 pmol of TaqMan hydrolysis probe, 200 μM dNTPs (FroggaBio Inc., North York, ON, Canada), and one unit of Immolase DNA polymerase (FroggaBio). DNA amplification reactions were subject to the following thermocycle conditions: an initial activation step of 9 min at 95 °C followed by 50 cycles of 15 sec denaturation at 95 °C, 30 sec annealing at 64 °C, and 30 sec polymerization at 72 °C.

Sharp-tailed snake DNA isolated from voucher tissue was also tested in two replicates at five different concentrations between 0.008 and 5 μg/L based on Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) A_{260} spectrophometry readings to determine initial sensitivity (Supplementary Fig. 1). Candidate assays that exhibited specificity and sensitivity were further tested to bring total technical replicates for six of the species and all target specimen DNA concentrations to 25. The assay eCOTE3, which amplifies a 220 bp region of the NADH dehydrogenase subunit 4 gene, was selected based on its superior sensitivity and selectivity (Table 2).

As only part of the genomic DNA from a tissue sample is from the mitochondria and the number of mitochondria and mitochondrial DNA can vary widely from tissue to tissue and between individuals, we used synthetic DNA as a template as a reproducible way to create a standardized means for expressing eDNA assay performance (Klymus et al., 2017; Langlois et al., 2020). The eCOTE3 test efficiency was further empirically assessed using gBlocks® synthetic DNA from Integrated DNA Technologies (Coralville, Iowa, United States) using a 5-fold serial dilution according to protocols described in Hobbs et al. (2019). This step allows for a standardized indicator of assay performance. Briefly, a 10^7 copies/μL synthetic DNA stock was made containing 10 ng/μL tRNA as a stabilizer (Sigma-Aldrich Canada Co., Oakville, ON, Canada). One μL of this dilution was added to 31 μL of working tRNA solution to produce a temporary stock containing 312,500 copies/μL. This stock was then serially diluted five-fold with 10 ng/μL tRNA in UltraPure DNAse/RNAse-free distilled water (Thermo Fisher Scientific).

to produce a range of ten synthetic DNA concentrations from 31,250 copies/μL to 0.016 copies/μL. Two μL of each dilution were run in qPCR reactions with eight technical replicates. Therefore the final range tested per reaction was 0.032 to 62,500 copies per reaction. These data were used to calculate the limit of blank (LOB), limit of detection (LOD), and limit of quantitation (LOQ) as defined by Lesperance et al. (2021) and described in section 2.6.

Table 2
Nucleotide sequences for the qPCR-based eCOTE3 eDNA tool comprised of primers and a probe for *Contia tenuis* detection. The amplicon sequence for the creation of the synthetic DNA sequence is indicated.

Sequence Type	Sequence
Forward Primer	5′CACATAGGCTTAGTCATTGC
Reverse Primer	5′TTATTAGGCTGGTTAGGAGTC
Probe	5′FAM- CTCCTCAGCACTCTTCTGCTTAGCCAACAC-ZEN/Iowa Black FQ
Amplicon	5′CACATAGGCTTAGTCATTGCCGCAATCATTATTCAAACACAAT GAAGCCTATCAGGGGCCATAGCCCTTATAATCGCTCACGGCTTC ACCTCCTCAGCACTCTTCTGCTTAGCCAACACCACCTACGAACG AACCACAACCCGAATTATAATTCTCACACGAGGTTTCCACAATA TCCTACCAATAACTACAGCCTGATGACTCCTAACCAGCCTAATAA

2.1.3. Assay field validation

Field validation was accomplished in two ways – by collecting swabs directly from animals and by taking DNA samples from ACOs where snakes were found. Cloacal DNA samples were collected from two sharp-tailed snakes found in the field in 2018. Cloacal swabs were taken by rotating a wet (water-moistened), long-stem sterile swab around the outside of the cloacal vent as described in Ford et al. (2017). An additional Q-swab was collected from fecal matter deposited on a surveyor's gloved hands by one of the snakes. A blank Q-swab was included as a negative control. Swabs were stored frozen at −20 °C until the DNA was isolated from them.

Two captured snakes were placed in *ex situ* soil to determine if short-term deposition of eDNA could be detected (adapted from Kucherenko et al., 2018). Each snake was placed in a separate small plastic 250 mL container with 100 mL of *ex situ* soil for 10 min before being gently placed back in the location where it was found. The soil was collected from forested habitat well outside the habitat for sharp-tailed snakes. A sample of forest soil that did not have a snake added was tested to assess potential for false positives.

All live animals were treated with due consideration to alleviate distress according to procedures and permits reviewed and approved by the Animal Care Committee at the University of Victoria for compliance under the Canadian Council on Animal Care guidelines (UVic Animal Care Committee (ACC) Protocol #2018-010; Species at Risk Act Permit #SARA-PYR-2018-0422; Wildlife Permit #NA18-286900).

2.2. Field surveys

The study location was on Salt Spring Island, British Columbia, one of 15 known sharp-tailed snake subpopulations in Canada (Environment and Climate Change Canada, 2017). Salt Spring Island has an area of 190 km^2 and is located 1 km east of Vancouver Island and 44 km west of Vancouver (Fig. 1). Sharp-tailed snake were previously detected at one site near the summit of a low mountain on the south end of Salt Spring Island, and at another discrete site next to the ocean (1.4 km away); the area between these two sites had not been surveyed.

The study site is located in a Garry Oak (*Quercus garryana*) ecosystem in the Coastal Douglas-fir (CDF) moist maritime biogeoclimatic zone (Meidinger and Pojar, 1991). This biogeoclimatic zone, including the Garry oak ecosystems within it, rank very high in conservation importance in British Columbia, supporting over 100 at-risk flora and fauna found nowhere else provincially or nationally (Fuchs, 2001). Garry oak ecosystems have <5% of their original area remaining in near-natural condition (Lea, 2006), and the CDF biogeoclimatic zone has less than 1% remaining old-growth (>250 years) forested habitat (Madrone Environmental Services, 2008).

In 2018, 126 (63 pairs of) ACOs were installed on three land tenures (Crown, Ecological Reserve, and Transport Canada) on the upper portion Mt. Tuam, Salt Spring Island, British Columbia, Canada. In 2018

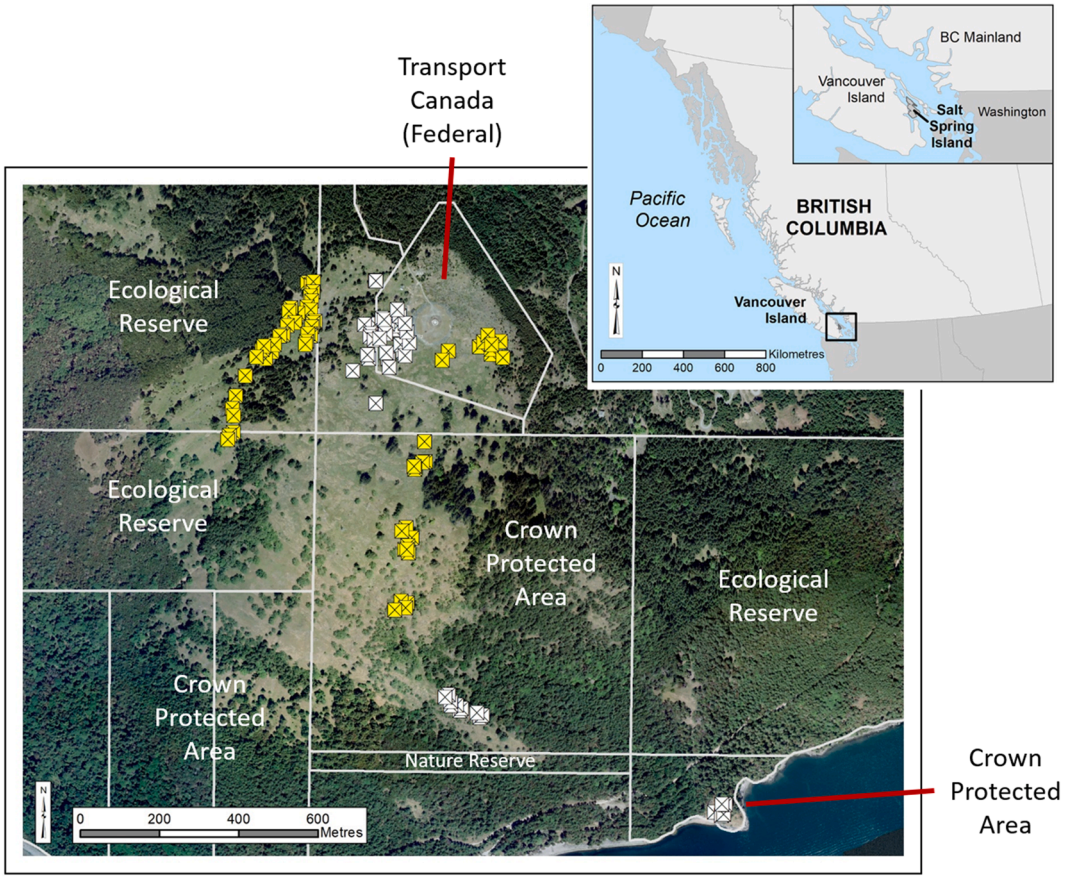

Fig. 1. Locations of Artificial Cover Objects (ACOs) monitored on Mt. Tuam, Salt Spring Island, British Columbia, Canada. ACOs are indicated as squares (installed prior to 2018, white; installed 2018–19, yellow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and 2019, these ACOs were regularly monitored along with 64 (32 pairs of) ACOs that were previously installed from 2008 to 2015 on two land tenures: Transport Canada land and private covenanted land (Fig. 1). In 2019, an additional 30 (15 pairs) ACOs installed in 2011 on Crown land (on the lower elevation sites on the same mountain) were monitored. Each ACO measured approximately 0.6 m × 0.6 m and was cut from a roll of asphalt roofing-shingle material. They were placed in areas of suitable sharp-tailed snake habitat, near rotting woody debris or loose talus, in areas with prolonged sun exposure (following protocols recommended in Engelstoft and Ovaska, 1997; Sharp-tailed Snake Recovery Team, 2008). The ACOs were secured in place with small rocks or pieces of woody debris and were visited and checked on warm sunny days (ambient temperatures between 10 and 22 °C). The ACOs on the higher area of the mountain were visited in the spring and fall during peak snake activity (mid-day) 12 times from May 31, 2018 to March 20, 2019 and in the following spring and fall 18 times from April 4-October 23, 2019. The ACOs on the lower area of the mountain were visited 12 times between May 4, 2019 and October 9, 2019.

When a snake was found its relative age (hatchling, juvenile, subadult, adult) and suspected sex was recorded. Individual snakes were also distinguished by their unique throat patterns and distinctive markings including ventral tail colouration, branching in their ventral patterns or other small spots or distinctive markings.

2.3. eDNA sample collection protocols

eDNA samples were collected using specific protocols developed to prevent contamination as adapted from provincial aquatic eDNA sampling guidelines (Hobbs et al., 2017). New gloves were put on before each new sample was collected. One surveyor was responsible for handling snakes (if found) and a second surveyor collected the eDNA samples to avoid cross-contamination. Hands were cleaned with alcohol wipes after any snakes (regardless of species) were handled.

2.3.1. ACO swab collection

ACO swabs were collected from a subset of ACOs surveyed. In 2018, ACO swab kits were prepared in the laboratory at UVic using Whatman 3 filter paper cut to the size of a 42.5 mm Whatman 1 Filter as a guide, with a 5 mm tail to enable a firm grip on the filter while swabbing. Each newly cut, tailed filter was placed in a paper coin envelope and then put in a separate sealable plastic ziploc bag. This technique was modified in year two of the project as the filter paper often fell apart while collecting the swabs, thus complicating lab analysis. In 2019, a cotton finger cot, placed on the index finger over top of the disposable gloves, was used for swabbing: the finger cot increased the consistency of the area that was in contact with the ACO by providing a more focused area of contact between the surveyor's finger and the ACO (Fig. 2).

Swab samples were collected from the underside of an ACO that was in contact with the substrate. The surveyor put on sterile gloves and sprayed either the filter paper or finger cot with 70% isopropyl alcohol (Figs. 2D and 3A) until it was damp. The back of the finger cot (i.e., over the fingernail) was marked with an "x" using a permanent marker to later enable easy identification of the part of the finger cot that was in contact with the ACO (Fig. 3B). The following swab pattern (Fig. 3C) was used on each ACO: 1) small square midway between the edge of the ACO and the centre, starting at the top left; 2) an "X" pattern connecting each corner; 3) a "+" pattern; and 4) along the outside edges of the ACO. After swabbing, the filter paper samples were folded so the side that was in

Fig. 2. Field site photos illustrating the terrain, ACO placement, and field sampling technique. A-B) Examples of the diversity of terrain and placement of ACOs. C) Demonstration of physical survey method. D) Demonstration of moistening a finger cot with 70% isopropyl alcohol in preparation for taking an ACO swab for eDNA. E) ACO flipped so the underside is visible and the area covered is exposed to the left, F) Photo of a visual observation of a sharp-tailed snake under an ACO. The snake is indicated by an arrow. Photos credit: Laura Matthias.

contact with the ACO was inside the fold, the tail was torn off the filter and the filter was folded a second time and inserted into a paper coin envelope. Finger cots were placed into a coin envelope and then into a sealable plastic bag as they came off the glove (Fig. 3D–F). Field blanks consisted of the filter paper or finger cot being sprayed with 70% isopropyl alcohol and then placed into the paper coin envelope.

Each coin envelope was labelled with the ACO site information, location co-ordinates, date, surveyor name, and sample number. Self-indicating silica dessicant beads (approximately one tablespoon) were added to each sealable plastic bag and samples were handled as described in section 2.3.3 below.

2.3.2. Soil sample collection (in situ)

Soil samples were collected from under a subset of ACOs for comparison with the ACO swabs. Fresh gloves were put on prior to collecting each soil sample. The samples were collected by placing the open end of a sterile 100 mL vial at the surface of the soil and scraping the surface soil directly into the vial. Soil samples had variable mixes of small rocks (talus), decayed wood, and organic matter. Care was taken not to touch the soil, even with gloved hands, and to limit contact with the outside of the container while the collection was being made.

2.3.3. Sample storage

All swab and soil samples were kept in a cooler with frozen ice packs while in the field, then transferred after the field day and stored for up to one month in a non self-defrosting −20 °C freezer until transported to the University of Victoria. Once at the University of Victoria lab all samples were stored in the dark at 4 °C until processing.

2.4. DNA isolation

All samples were randomized and assigned sample processing numbers to reduce processing bias (Hobbs et al., 2019). The method of DNA extraction was chosen based upon the sample material collected.

2.4.1. Tissue, Q-swab and ACO swabs

In a laminar flow hood, total DNA was recovered from each filter, swab, or finger cot sample using the DNeasy Blood and Tissue Kit (Qiagen Inc., Mississauga, ON, Canada; Cat# 69506) using methods outlined in Hobbs et al. (2019). Tissue and swabs were extracted as per the manufacturer's protocol, and filter and finger cot samples were extracted using the same methods with the following modifications: 280 μL of Buffer ATL was used in the initial incubation, then following incubation both buffer and filter were transferred to a QIAshredder

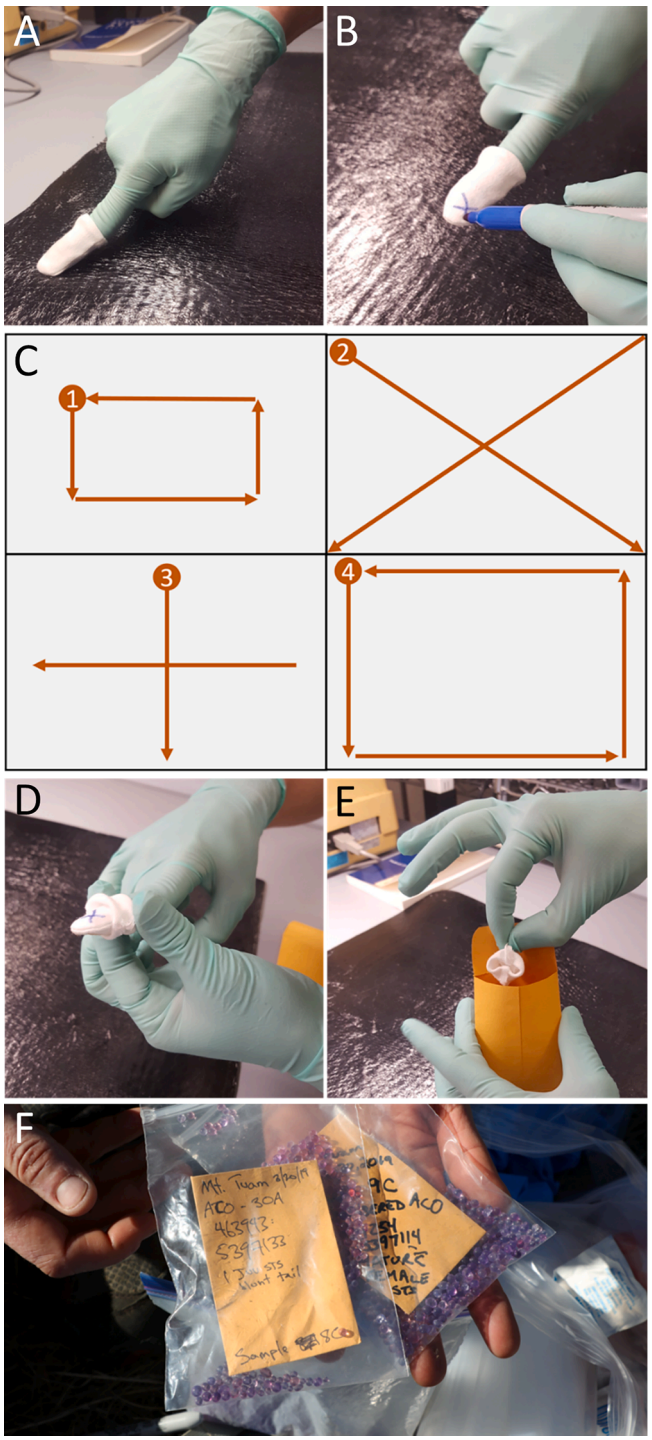

Fig. 3. Demonstration of the ACO swabbing technique and storage of the finger cot. A) A cotton finger cot that has been moistened with 70% isopropyl alcohol (see Fig. 3D) is run across the underside of an ACO. B) The top side of the finger cot is clearly labelled with a permanent marker to aid in later DNA isolation. C) The swabbing pattern used on the ACO follows the pattern indicated. D) The finger cot is removed and E) carefully placed into a labelled coin envelope. F) The coin envelope is placed into a plastic bag containing moisture indicating silica beads for storage prior to DNA processing.

Column (Qiagen; Cat# 79654) using forceps that had been bleach-treated and rinsed thoroughly with deionized distilled water. The liquid collected from the QIAshredder was incubated with 300 µL Buffer AL for ten minutes at 56 °C, then vortexed and centrifuged with 300 µL of 100% Ethanol prior to spinning through the DNeasy spin column. DNA was eluted from the spin column with 150 µL of Buffer AE. DNA samples were stored at −20 °C prior to use in the eDNA qPCR assay.

2.4.2. Soil samples

Early attempts to selectively liberate DNA from soil samples of sufficient quality using DNeasy PowerSoil kits (Qiagen; Cat# 12888) were unsatisfactory as the kits could not adequately handle more than 0.25 g at a time. Additional limitations were identified; PowerSoil kits failed to produce positive target species detections, and phenol extraction methods lack the reliability of column-based kits (Deiner et al., 2015). Taking a subsample of the 100 mL soil samples was undesirable, so we elected to develop a method that suspended and concentrated the DNA prior to isolation.

All personnel in contact with the samples wore nitrile gloves and lab coats, and replaced or sterilized gloves between samples. All benchtops were wiped with a 10% bleach solution, followed by a 70% ethanol solution. Two paper towels were laid out on the benchtop, overlapping in the middle and soil samples were handled on these paper towels to reduce the amount of soil that touched the bench. A clean scoop was used to portion ~ 15 mL soil into a 50 mL Falcon tube. The Falcon tube was filled to the 45 mL line with UltraPure™ DNAse/RNAse-free distilled water (UltraPure-dH_2O) (Invitrogen, Waltham, MA, USA). The cap was secured and shaken vigorously. If needed, more Ultrapure-dH_2O was added to reach 45 mL line.

The tube was vortexed for 30 s, shaken in hand, then vortexed for another 30 s. The sample was allowed to settle at 4 °C for 24 h. Paper towels were changed and the previous steps repeated for the remaining samples. Between handling separate soil samples, gloves were wiped with ethanol or changed for a fresh pair. All implements were well immersed in a 50% (v/v) bleach solution, then well-rinsed with distilled water and wiped with a paper towel. The suspended material was filtered using sterile cheese cloth as a barrier to large particulate matter into a 250 mL single-use polypropylene filter funnel with a 0.45 µm pore size cellulose nitrate membrane as described in Hobbs et al. (2020). The DNA from one quarter of each filter was extracted with the DNeasy Blood and Tissue kit using the same protocol as was used for filter and swab extractions above.

Soil samples taken between September 28, 2019 and October 23, 2019 were isolated with a slightly modified protocol. Up to 100 mL of soil was added to a clean plastic 500 mL bottle, and autoclaved double-distilled water was added up to 450 mL. The sample was shaken vigorously for 30 s and allowed to settle at 4 °C for 24 h prior to filtration above.

2.5. qPCR analysis of field samples

Due to the high sensitivity of the eCOTE3 assay, extreme rigour was exercised in sample handling to reduce the possibility of cross contamination between samples. Nitrile gloves and lab coats were worn at every stage of sample analysis, and all surfaces were sterilized with a 10% (v/v) bleach solution and 70% ethanol. All qPCR reagents and DNA isolates were handled using sterile technique in a sterile laminar flow hood located in a designated room. Amplicons were kept in a separate room and personnel were required to apply separate lab coats and gloves to access them.

All samples were analyzed through a two-tiered targeted eDNA analysis approach (Veldhoen et al., 2016; Hobbs et al., 2019). Prior to eCOTE3 testing, the eDNA sample was first examined using the IntegritE-DNA™ test (Veldhoen et al., 2016; Hobbs et al., 2019) based upon the detection of an endogenous plant and algae chloroplast target to evaluate the integrity of the eDNA sample.

For this test, four technical replicates of the IntegritE-DNA™ qPCR assay were run as described in Veldhoen et al. (2016) and Hobbs et al. (2019) using the same run conditions stated in section 2.1.2. If the sample failed to amplify before 30 qPCR cycles, it was concluded that there was either too much inhibition or the integrity of the DNA was

compromised. A sample that failed initial IntegritE-DNA™ testing was cleaned in a Zymo OneStep PCR Inhibitor Removal Kit (Cedarlane, Burlington, ON, Canada; Cat# D6030S) and retested with the same assay. Samples that showed positive chloroplast amplification were tested for sharp-tailed snake DNA using the validated eCOTE3 assay with eight technical replicates as described in section 2.1.2. All qPCR tests included two positive DNA controls and eight NTCs per reaction plate.

2.6. Statistical analysis

The limit of blank (LOB), limit of detection (LOD), and limit of quantitation (LOQ) were determined using the eLowQuant R code (https://github.com/mlespera/eLowQuant) that uses a modified Binomial-Poisson distribution model (Lesperance et al., 2021). The default settings were used such that the false positive and false negative uncertainty was 0.05 and the coefficient of variation for the limit of quantification was 0.2.

Estimated copy numbers per sample were calculated as follows. If the qPCR reaction results produced 8/8 hits, then the equation in Fig. 4B was applied to determine estimated copies per reaction from each C_t value and the standard error of the mean was calculated from the eight technical replicates. If the qPCR reaction results produced less than 8/8 hits, then the estimated copy number with standard error for that sample were calculated using the eLowQuant script. Regardless of quantification method, swab reaction results were multiplied by a factor of 75 since 2 µL were run in a qPCR reaction from a total of 150 µL isolated DNA per swab. For soil samples, a quarter of the filter was used to isolate DNA from, so the multiplication factor was 4X75 = 300 (Supplementary Table 1).

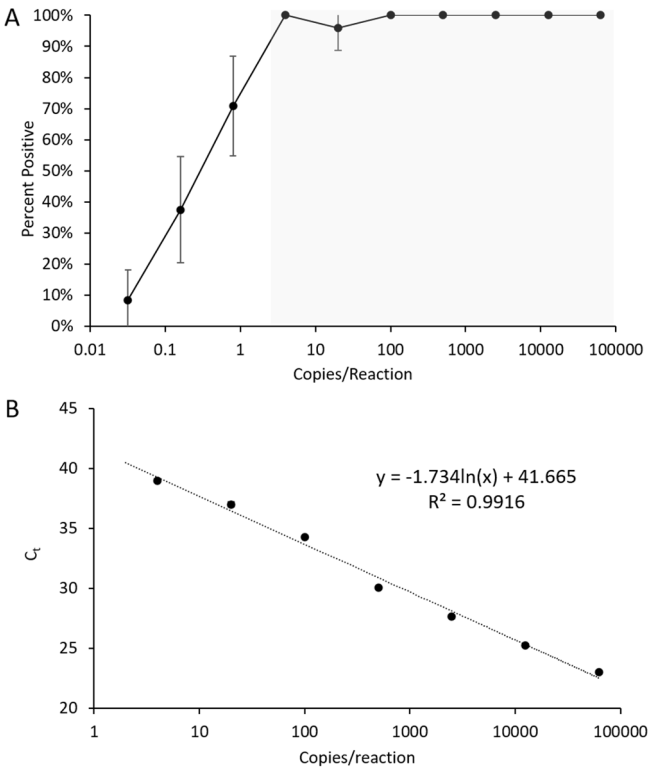

Fig. 4. (A) Percent of positive qPCR reactions containing known starting amounts of gBlocks® synthetic DNA. At four copies per reaction, 100% of technical replicates were positive so the C_t values obtained from these reactions and at higher concentrations (gray region) could be used to derive the standard curve in "B". Quantification of copy numbers below ~ 4 copies per reaction required the application of a Binomial-Poisson model in Fig. 5.

3. Results

3.1. Validation of the eCOTE3 assay

After extensive validation, the eCOTE3 assay exhibits highly robust, sensitive and selective amplification of sharp-tailed snake DNA. Including qPCR from all validation phases and sample testing, over 700 NTCs were run with no amplification, setting the background false positive rate at zero with a high degree of confidence. Indeed, the calculated LOB = 0 (Lesperance et al., 2021). No off-target amplification was recorded for any sympatric or confounding species DNA at concentrations of 5 µg/L (Table 1). The gBlocks® sensitivity validation demonstrated that the assay is able to reliably amplify target total DNA at extremely low concentrations (Figs. 4 and 5) and the mean copy estimates and proportion detects were computed using the Binomial-Poisson maximum likelihood no intercept model (Lesperance et al., 2021). The calculated LOD was 0.2 copies/sample (95% confidence interval = 0.1–0.3) and LOQ of 0.7 copies/sample (95% confidence interval = 0.5–1.2). Using this model and derived standard errors, we were

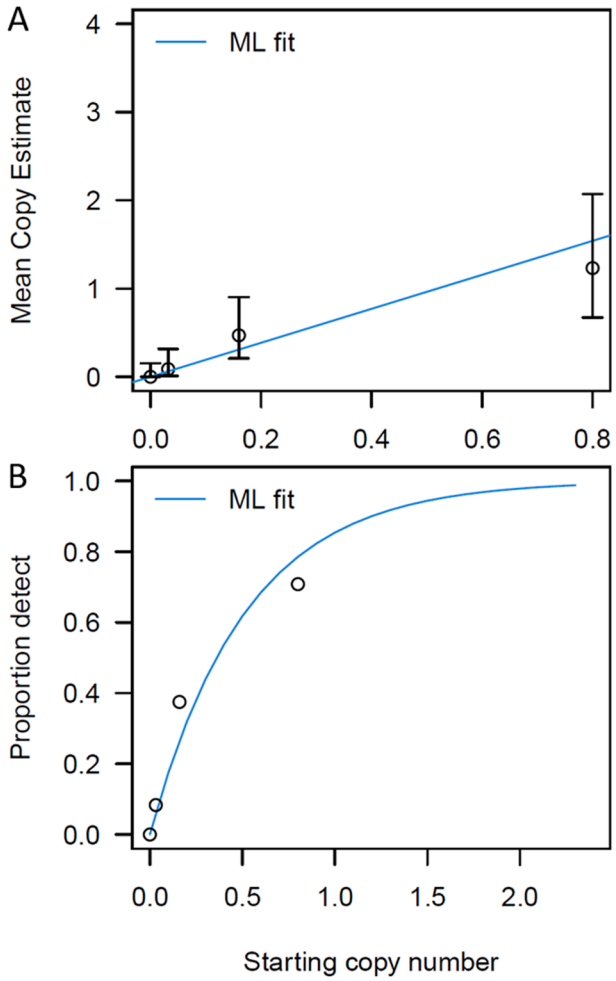

Fig. 5. Maximum Likelihood (ML) Binomial-Poisson model of gBlocks® synthetic DNA for the quantitation of low copy number DNA using eLowQuant. (A) Unconstrained binomial estimate of the Poisson mean copy number and transformation of exact 95% binomial confidence intervals versus starting copy number of target DNA per reaction. (B) The proportion of detected target and the ML fit on the probability scale relative to starting copy number of a sample. The ML model fit is depicted as a blue line and is used to calculate LOD, LOQ and LOB using eLowQuant (Lesperance et al., 2021). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Swab results from three individual snakes. All snakes were in Zone 10U.

Sample #	Date	Easting	Northing	Sample Type	Snake Found	Sex	IntegritE-DNA Frequency	Clean Up Required	COTE Frequency	Lab Call	Estimated Counts per Sample[b]
1	2018-09-28	464,058	5,397,321	Q-swab (cloacal)[a]	Juvenile	Female	4/4	N	8/8	Y	2,395 ± 385
2	"	"	"	Q-swab (fecal)[a]	"	"	4/4	N	8/8	Y	1,060 ± 113
3	2018-09-28	464,058	5,397,321	Q-swab (cloacal)	Hatchling	Female?	4/4	N	8/8	Y	49,678 ± 4,392
4	2019-03-18	464,059	5,397,283	Snake placed on forest soil	Juvenile	Male	4/4	Y	3/8	Y	72 ± 45
5	2019-03-18	464,019	5,397,253	Snake placed on forest soil	Subadult	Female	4/4	Y	0/8	False negative	0 ± 0
6	2018-09-26	–	–	Swab Blank	–	–	0/4	Y	0/8	N	0 ± 0
7	2019-03-18	463,324.8	5,399,082	Forest soil Blank	–	–	4/4	Y	0/8	N	0 ± 0

[a] A cloacal and fecal swab was taken from the first snake observed under this ACO.
[b] For 8/8 hits, the mean ± standard error of the mean. For less than 8/8 hits, the estimated copy number ± standard error as calculated by eLowQuant based upon binomial data is shown.

able to estimate the copy numbers per reaction when there were less than 100% hits out of eight technical replicates (Supplementary Table 1). These values were then converted to copy number with standard error per sample depending upon the sample type (Supplementary Table 1).

Initial field validation of the eCOTE3 eDNA assay was accomplished in two steps. First, two snakes were briefly handled and cloacal swabs taken. A fecal swab was taken from one snake. All three samples tested positive for DNA integrity using the IntegritE-DNA^{TM} assay (Table 3). No sample inhibition was detected and all three samples returned strong positive detection ranging from 1,060 ± 113 to 49,678 ± 4392 estimated copies of eDNA per sample (Table 3). The swab blank containing no DNA returned no hits for either assay, as expected (Table 3). Second, two different snakes were placed onto forest soil for 10 min and the soil was used to extract DNA for eDNA analysis. Both samples required inhibitor clean up to pass the IntegritE-DNA^{TM} assay (Table 3). One soil sample returned a comparatively weak positive signal (estimated 72 ± 45 eDNA copies; Table 3) while the other produced no detectable signal (0/8, false negative; Table 3). The swab blank from forest soil passed the IntegritE-DNA^{TM} test and returned no hits for the eCOTE3 assay, as expected (Table 3). The data suggest that while the eCOTE3 assay works on multiple snakes, the deposition of DNA over a short time is not extensive. Taken together, the eCOTE3 assay was validated to level 5 according to Thalinger et al. (2021).

3.2. Field sample analysis

Of the 109 ACO swab samples, 57 (52%) ACO swab-derived DNA samples required inhibitor clean-up as determined by the IntegritE-DNA^{TM} assay. After inhibitor clean up, 100 (92%) samples passed the IntegritE-DNA^{TM} assay (Table 4 and Supplementary Table 2) and the DNA from nine samples were deemed poor quality. Of the 78 soil samples that were paired with a subset of the ACO swab samples, 47 (60%) required inhibitor clean-up as determined by the IntegritE-DNA^{TM} assay. After inhibitor clean up, 69 (88%) samples passed the IntegritE-DNA^{TM} assay and the DNA from nine samples were deemed poor quality (Table 4 and Supplementary Table 2).

Table 4
Summary of the overall eDNA analysis results for sharp-tailed snake.

	ACO Swab	Soil
Total # of eDNA samples	109	78
# eDNA samples requiring inhibitor clean-up	57 (52%)	47 (60%)
# of sufficient quality samples after clean-up	100 (92%)	69 (88%)
# of eDNA detections	21 (21%)	16 (23%)

Overall, 21 ACO swab and 16 soil samples had detectable sharp-tailed snake eDNA for an overall detection rate of 21% for ACO swabs (n = 100) and 23% for soil samples (n = 69; Table 4 and Fig. 6). When both swab and soil samples were taken at an ACO on the same day and the DNA was of sufficient quality (n = 66), only five sites (8%) had positive detections (Supplementary Table 2). However, when swabs and soil samples are considered together independent of ACO and sampling event, the overall detection rate was 25% (14/56; Supplementary Table 2). Locations of positive eDNA detections and where snakes were found are indicated in Fig. 6. DNA extracted from soil using up to 100 mL of the sample exhibited roughly the same overall positive rate as that extracted using 15 mL (19 and 21%, respectively), but if sites with no snake observations are excluded, this comparison changes dramatically to 19% for larger volumes (n = 36) and 0% for smaller volumes (n = 19) attesting to an advantage of using greater amounts of soil for analysis to ensure target DNA detection, if present.

There were 24 snake sightings under 17 different ACOs that included repeat observations of two individual snakes on separate days, and one occurrence with two snakes under the same ACO at the same time (Fig. 6 and Supplementary Table 2). This enabled determination of

false negative rates for the ACO swab and soil samples. Of the eDNA samples taken where sharp-tailed snakes were visually observed during the surveys, 13 out of 23 ACO swabs (57%) and 9 out of 22 soil samples (41%) tested positive. As all ACO swabs related to snake sightings were of sufficient quality, the true positive rate for the 23 samples was 57% (13/23) (Table 5). Twenty-two corresponding soil samples were taken (Supplementary Table 2). Of these, two soil samples were of poor quality. Of the remaining 20 samples, nine (45%) were positive for sharp-tailed snake eDNA (Table 5 and Supplementary Table 2). Therefore the false negative level was 43% and 55% for swab and soil samples, respectively (Table 5).

For sites tested for eDNA at which no sharp-tailed snake obervation was recorded during the simultaneous physical surveying, sharp-tailed snake eDNA was detected at eight sites for ACO swab (n = 86) (9%) and seven sites for soil samples (n = 56) (13%) (Table 5 and Fig. 6). All field blanks were negative for sharp-tailed snake.

Examination of the relative amounts of estimated target snake eDNA detected revealed a considerable range over three orders of magnitude (5 ± 5 to 5,607 ± 348 copies from ACO swabs and 21 ± 21 to 29,235 ± 1,793 copies from soil samples; Supplementary Table 2). No clear trend in eDNA accumulation was evident in the limited cases where multiple snakes were observed under the same ACO together or on different sampling dates (Supplementary Table 2).

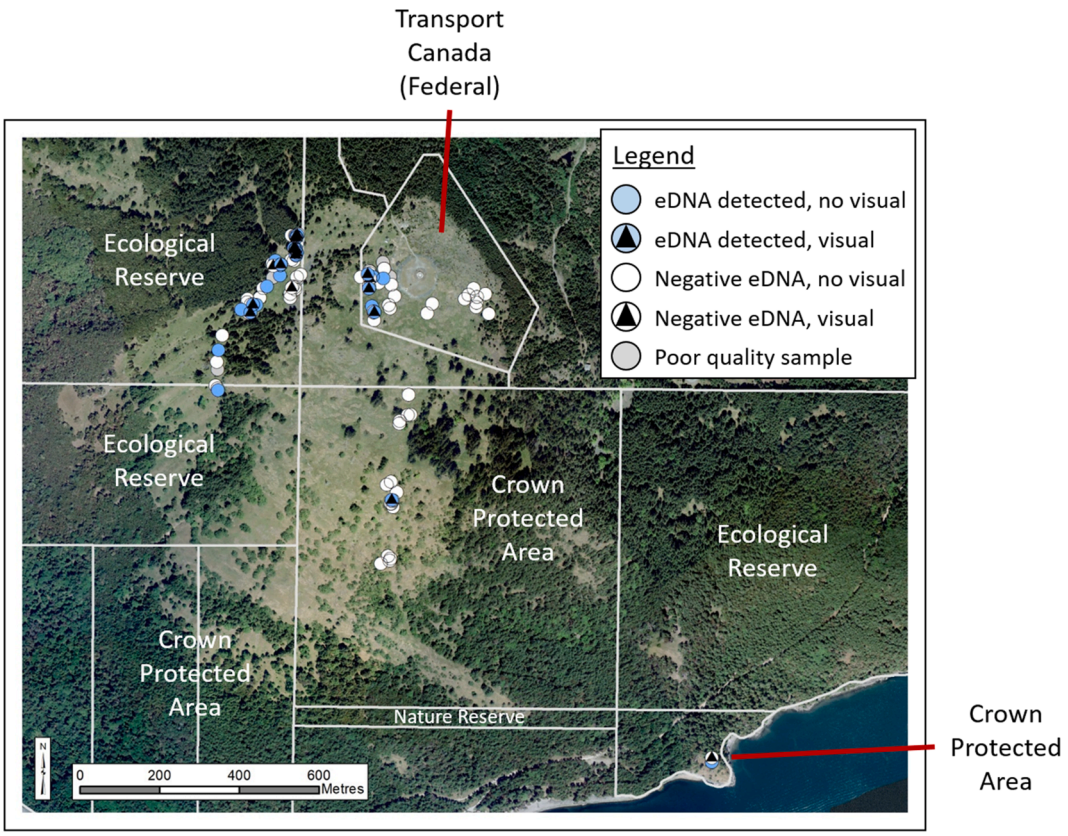

Fig. 6. Map showing the eDNA results and where sharp-tailed snakes were found. A positive eDNA detection is indicated by the blue circle and a negative eDNA detection is indicated by a white circle. A poor quality sample is indicated in grey. A black triangle inset into the circle indicates where a visual snake observation was made at the time of sampling. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Breakdown of eDNA analysis results for sharp-tailed snake according to success of conventional surveying method (visual observation).

	ACO Swab	Soil
Total # of visual detections during sampling[a]	23	20[b]
# Positive eDNA hits (True positives)	13 (57%)	9 (45%)
Estimated false negative rate	43%	54%
# ACOs where snakes were not observed during the time of sampling	86	56
# Positive eDNA hits	8 (9%)	7 (13%)

[a]Two snakes observed under one ACO at the same time were counted as one visual detection event.
[b]Two eDNA samples remained of poor quality and were not included in the tally.

4. Discussion

Surveys using eDNA are increasingly popular, especially when applied to cryptic species that are difficult to confirm using traditional survey methods. The development of a qPCR-based assay capable of detection of sharp-tailed snake is an important new tool that can improve the ability to confirm the distribution and range of this elusive fossorial species.

In comparison with eDNA analysis of more typical aquatic samples, soil and swab samples are much more likely to require a cleanup step. While purification columns are effective at removing humic acids and other qPCR inhibitors, the DNA concentration is still adversely affected by these protocols (McKee et al., 2015). Optimization of soil and sediment sample extraction methods is therefore a crucial step toward reliable eDNA analysis in terrestrial environments. Sample extractions using the DNeasy Blood and Tissue kit yielded better eukaryotic DNA signals than did those extracted with the PowerSoil kit, despite more often requiring a cleanup step. Similar findings were discussed by Goldberg et al. (2011) regarding the UltraClean Soil DNA isolation kit (MoBio Laboratories, Inc.), that may be due to the soil-based kits which are developed primarily to target microbial organisms.

Although eDNA survey methods cannot currently determine abundance or identify individuals, they potentially provide an alternative more efficient method relative to the application of conventional methods when applied to survey of this cryptic species. On Mt. Tuam, where 220 ACOs were monitored between 12 and 30 times resulting in 6,060 sample events, only 24 snakes were visually detected using traditional ACO survey methods. This demonstrates a positive detection rate of only 0.4%. By comparison, eDNA sampling had a much higher overall detection rate of over 20%. However, eDNA was not consistently detected when snakes were visually detected. The false negative rate was lower for ACO swab samples compared to soil samples likely due to the nature of the inhibitors present. It is currently unclear why the known-positive detection rate was still only 57% for the ACO swab samples.

There are several factors that could affect eDNA detection rates. Snakes under ACOs are likely sedentary (when coiled, juveniles can be the size of a 25 cent US or Canadian coin), and the snake may have come up from the substrate and not have been in contact with the ACO in the area targeted by the swabbing pattern. Similarly, it may not have even deposited DNA in the surrounding soils. The snake may have only been under the ACO momentarily before it was detected, which may not have been long enough for eDNA deposition to occur. In future surveys we will attempt to improve eDNA detection rates by swabbing the entire underside of ACOs with the more robust finger cots.

Detecting new occurrences of sharp-tailed snakes using eDNA will

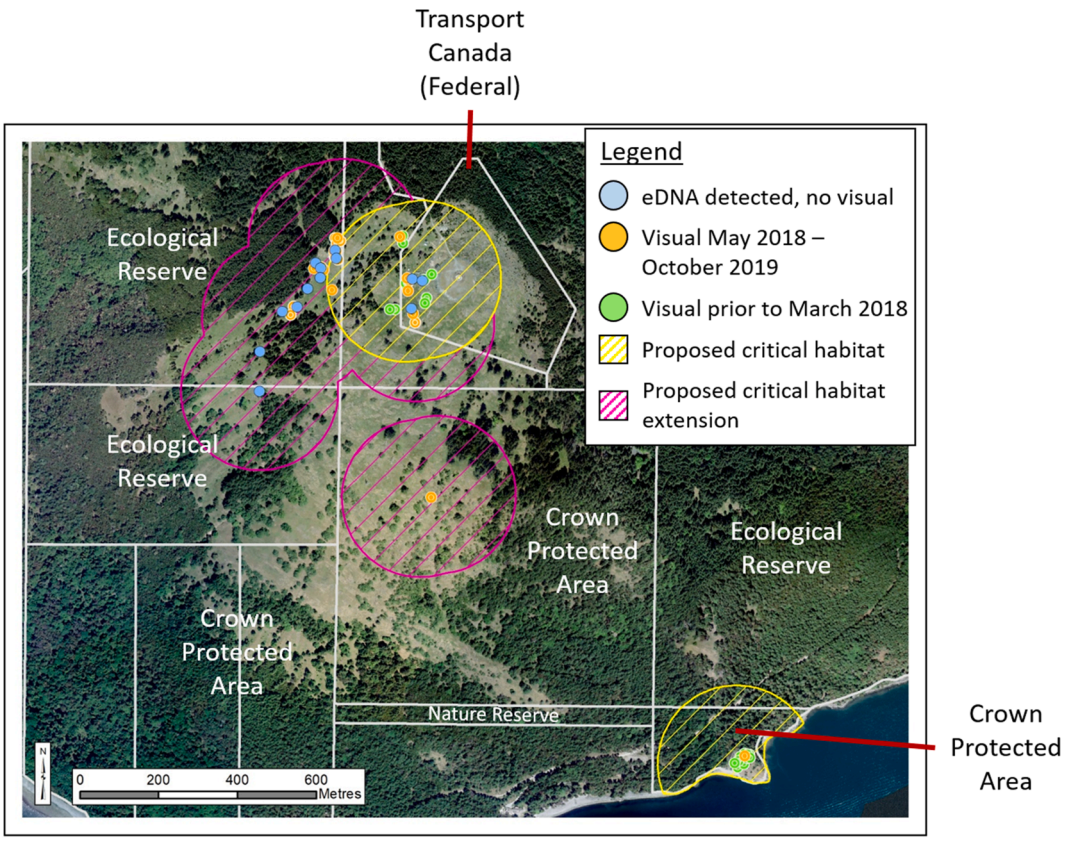

Fig. 7. Compilation of eDNA (blue circles) and visual sharp-tailed snake detections (prior to March 2018, green; between 2018 and 19, orange) and indicated proposed critical habitat (yellow hatched area) and extension (pink hatched area). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enable researchers and landowners to more efficiently identify sites where they are present. This may encourage conservation effort for the species. Increased detection will also provide insight into this species' habitat needs. These sites can be afforded legal protection under the federal *Species at Risk Act* via designation of Critical Habitat and identification of residence locations (Fig. 7). Habitat protection may also benefit over 100 rare species that occur in Garry Oak and associated ecosystems (Fuchs, 2001).

The present work indicates that eDNA deposition rates for sharp-tailed snake are relatively fast (within 10 min). Receiving even one positive detection in the *ex situ* samples is noteworthy since it suggests much faster deposition rates than those found by Kucherenko et al. (2018), who detected eDNA from the much larger Corn Snakes (*Pantherophis guttatus*) placed on *ex situ* soil at the first sampling interval of five hours. However, handling of the snakes may have made them prone to defecation and may not be indicative of deposition under natural conditions.

Future research is required to determine eDNA deposition rates and persistence under a range of environmental conditions (e.g., seasonality, thermal conditions, etc.). While many studies have sought to identify the relative impacts of variables that contribute to DNA degradation between eDNA deposition and analysis (Strickler et al., 2015; Thomsen and Willerslev, 2015; Andruszkiewicz et al., 2017; Walker et al., 2017; Kucherenko et al., 2018), most of these experiments have been focused on aquatic environments, and isolation of the variables for independent analysis is rare. Since the sampling and extraction methods were optimized throughout the course of the present study, separate experiments are required to properly investigate the conditions surrounding deposition and degradation of sharp-tailed snake eDNA in natural terrestrial ecosystems. Further research is also required to explore best methods for collection of swabs and soil samples under natural cover objects (NCOs) in situ, such as rock and bark.

Successful detection of reptiles using eDNA methods has shown variability in the literature and appears highly dependent on context and methods. In a study with a similar approach to the present study, Ratsch et al. (2020) reported greater success of Kirtland's snake detection with tradional methods, but suggested that eDNA reliability was likely hampered by assay design issues. Rose et al. (2019) found that their eDNA approach underperformed compared to traditional trapping methods, though the majority of their eDNA samples were tested using only one qPCR replicate due to budget constraints, which substantially limits detection success and statistical confidence (Lesperance et al., 2021). In contrast, Hunter et al. (2019) used digital droplet PCR to determine Burmese python presence in Florida wetlands, which vastly improved upon the success of traditional monitoring methods despite high concentrations of enzyme inhibitor present in their environmental samples. Determination of a method's efficacy in the context of species monitoring should take into account the reliability, efficiency, cost, timeliness, and degree of invasiveness associated with the method. In these terms, we credit the success of our approach to the collaborative effort in targeting the specific life history of sharp-tailed snakes, as well as both field and laboratory methods that evolved over the course of the present study to improve detection reliability.

There are numerous at-risk species, including sharp-tailed snake, that occur in Garry oak ecosystems; all face many threats (Environment and Climate Change Canada, 2017). The biggest threat is direct loss of habitat to land conversion associated with land or resource development. In addition, these ecosystems are adapted to frequent fires as fire suppression leads to increased conifer and shrub encroachment. Woody plant infilling by both native and non-native flora also degrades Garry oak ecosystems by shading habitat particularly for species that require open, sunny sites for behavioral thermoregulation and egg deposition.

Finally, the impacts of future climate change on sharp-tailed snakes is not understood. Identifying sharp-tailed snake occurrence locations is a necessary step towards conservation of their habitat. While there are obstacles to reliable detection of reptilian DNA in a terrestrial environment, this study demonstrates that eDNA techniques have promising and beneficial use for monitoring cryptic and endangered fossorial species.

CRediT authorship contribution statement

Laura Matthias: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing - review & editing. **Michael J. Allison:** Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing - review & editing. **Carrina Y. Maslovat:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing - review & editing. **Jared Hobbs:** Conceptualization, Methodology, Writing - review & editing. **Caren C. Helbing:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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