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Research article

Evaluation of an odour-detection dog for non-invasive pregnancy diagnosis in polar bears *Ursus maritimus*: Considerations for training sniffer dogs for biomedical investigations in wildlife species

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Abstract
Odour-detection dogs are utilised by law enforcement to identify illegal substances, by conservation groups to locate scat from specific species, and more recently, in biomedical investigations such as cancer detection. Because dogs can detect target odours in samples that can be collected non-invasively, such as faeces, they may be useful in diagnostic evaluations of zoo animals; however, reports are scarce describing the application of odour-detection dogs in medical diagnoses of wildlife species. The objective of the current study was to investigate the reliability of a trained dog for pregnancy detection of polar bears *Ursus maritimus*, a species for which there is no means of non-invasive pregnancy diagnosis. Using over 300 faecal samples collected from zoo-housed polar bears, a 2-year old beagle was trained to discriminate samples originating from pregnant bears from samples collected from non-pregnant individuals. At training evaluation, the dog's sensitivity (true positive rate) and specificity (true negative rate) were 1.00. In two real-time tests performed during consecutive cubbing seasons, the dog evaluated samples collected from 16 female bears in the first year and 17 the subsequent year. The dog's sensitivity was 0.00 both years and specificity was 0.97 and 1.00 during the first and second year, respectively. The reduced sensitivity in testing versus training may be attributable to several causes, including failure to generalise the target odour to novel pregnancies. It is likely that a large number of unique cases of condition are required to sufficiently train an odour-detection dog, which may be prohibitive in wildlife studies when sample sizes are liable to be limited.

Introduction

The natural olfactory capabilities of domestic dogs *Canis familiaris* have been deployed by humans for diverse tasks, including detecting narcotics, explosives and accelerants, and for tracking human scent (Sommerville et al. 1993; Settle et al. 1994; Williams and Johnston 2002; Brown et al. 2006). In conservation efforts, odour-detection dogs have been trained to locate scat from species of interest and to track individual animals (as reviewed by Beebe et al. 2016). More recently, mounting evidence from biomedical research suggests that dogs can detect odours characteristic of various cancer types from numerous tissue sources (Pickel et al. 2004; Willis et al. 2004; McCulloch et al. 2006; Horvath et al. 2010; Cornu et al. 2011; Sonada et al. 2011) and even breath (Ehmann et al. 2012). Although the majority of biomedical studies focus on cancer research, several describe the use of odour-detection dogs for other physiological investigations, such as clostridium diagnoses (Bomers et al. 2014), hypoglycemia recognition in diabetic patients (Deblinger et al. 2013), and even patterns of SARS-CoV-2 from respiratory secretion samples of infected patients (Jendrny et al. 2020). Some veterinary studies evaluated giant African pouched rats *Cricetomys gambianus* in their ability to detect horse faeces inoculated with *Salmonella* spp. (Mahoney et al. 2014) and dogs trained for estrous detection in dairy cows (Fischer-Tenhagen et al. 2011); however, publications describing the application of sniffer dogs in veterinary investigations of wildlife species are non-existent.

In zoological settings, diagnostic tests performed on materials that can be obtained non-invasively, such as faeces and urine, are desirable because the requirements for animal restraint, anaesthesia, and/or significant changes to husbandry are eliminated; however, the information provided from these biological matrices can be complex, difficult to interpret, and restricted in scope. For most species in human care, faecal samples are relatively easy to obtain, as their collection requires little to no training or changes to husbandry protocols. Consequently, many non-invasive monitoring techniques are focused on the use of this sample type. Although faecal steroid metabolite analyses are valuable for monitoring select reproductive processes and stress responses (Schwarzenberger 2007), there remains a deficit in pregnancy detection methods for many wildlife species, especially those that experience pseudo-pregnancies, such as polar bears *Ursus maritimus*. Pseudo-pregnancy is characterised by increases in urinary and/or faecal progesterone metabolite concentrations which are indistinguishable from those of pregnant females (Schwarzenberger et al. 2004; Dehnhard et al. 2006; Stoops et al. 2012; Curry et al. 2017).

Polar bears exhibit a complex suite of reproductive phenomena, including reproductive seasonality, induced ovulation, embryonic diapause and an apparently obligate pseudo-pregnancy in the absence of true pregnancy (Stoops et al. 2012). Furthermore, in US zoos, only ~10% of females that breed produce cubs each year (Meyerson and Long 2016). The identification of the point or cause of reproductive failure is obfuscated by a deficiency of monitoring methods, including a lack of a proven non-invasive pregnancy test at any stage of gestation. The ability to differentiate pregnancy from pseudo-pregnancy would aid in the management of individual polar bears in human care and may be useful for monitoring reproduction in wild populations. Considering the substantial physiologic and metabolic changes associated with pregnancy, it is plausible that certain compounds are altered in the faeces of pregnant bears compared to pseudo-pregnant bears and may be detectable by the sensitive olfactory abilities of canids.

Odour-detection dogs are likely recognising volatile organic compounds (VOCs) in the airspace above the samples. Specific VOCs that coincide with oestrus have been identified in cow faeces (Sankar et al. 2008) and bear urine (Dehnhard et al. 2006) and there are increasing numbers of reports describing VOCs in the faecal matrix. Using gas chromatography/mass spectrometry (GC/MS) analysis, over 267 chromatographic peaks representing individual VOCs have been identified in human faeces (Dixon et al. 2011; Garner et al. 2007). Furthermore, volatile patterns from patients with gastrointestinal disease were altered when compared to healthy patients (Garner et al. 2007) and VOC profiles have been associated with cholera as well as other diseases (Shirasu et al. 2011), suggesting value of VOCs as diagnostic indicators of a physiological condition. Although no faecal VOC patterns specific to the pregnant state have been described in any species, dogs may be capable of discriminating target odours from faeces of pregnant polar bears from those of pseudo-pregnant bears. The aim of the current study was to investigate the reliability of a trained odour-detection dog for non-invasive pregnancy diagnosis of zoo-housed polar bears.

Materials and methods

Subject

A 2-year old, castrated male beagle dog was selected following evaluation by an experienced odour detection dog trainer. The dog had not been used in odour discrimination work previously and was handled by two professional trainers throughout training and during testing.

Table 1. Sample type and numbers used for training.

Sample type	# of samples used for training	# of individuals
Positive samples	92	6
Males	42	8
Juveniles	15	2
Contracepted females	30	4
Cycling females, non-breeding	30	4
Oestrus	83	10
Pseudo-pregnant females	27	5

IACUC statement

The polar bear faecal samples utilised for training and testing were collected as part of on-going reproductive monitoring studies conducted by scientists at the Center for Conservation and Research of Endangered Wildlife (CREW) at the Cincinnati Zoo & Botanical Garden. Research protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Cincinnati Zoo & Botanical Garden (Protocol number 17-142 'Fecal and/or urine collection for non-invasive characterization of reproductive and adrenal events') as well as by the IACUC and/or supervisory personnel at each collaborating institution.

Samples

Frozen-thawed faecal samples (n=319) collected from 27 zoo-housed polar bears at 15 North American zoological institutions were used for training. All samples were collected non-invasively from individuals of known reproductive status from 2008–2013 and were stored at −20°C in the same freezer at CREW. Positive samples (n=92) were those collected from pregnant bears between 46 and 33 days pre-partum and represented 13 pregnancies of six individuals. This date range was chosen from a polar bear's ~60-day placental pregnancy because a foetus and placenta would be present, and presumably, any potential changes in the maternal faecal VOC profile associated with pregnancy might be detectable during this time. Also, samples are oftentimes difficult to collect during the final month of pregnancy because the female is usually denning and left undisturbed. Negative controls ('proofing' samples; n=227) were included from males, juveniles, females receiving contraceptive treatment (either melengestrol acetate (n=2), or gonadotropin-releasing hormone agonist (n=2)), cycling females that were not housed with a male, females in oestrus, and pseudo-pregnant females (Table 1). Females classed as pseudo-pregnant exhibited an increase in faecal progesterone metabolites in the autumn similar to pregnant females, but did not have access to an intact male.

To ensure the dog was not inadvertently trained to recognise an individual bear's scent, samples collected outside of pregnancy, including oestrus, were incorporated as proofing samples from females whose samples from pregnancy were used as positives. Nitrile gloves were worn when handling samples and human processing of the training samples (labeling containers, transferring faecal matter) prior to shipment for training was randomised across multiple technicians.

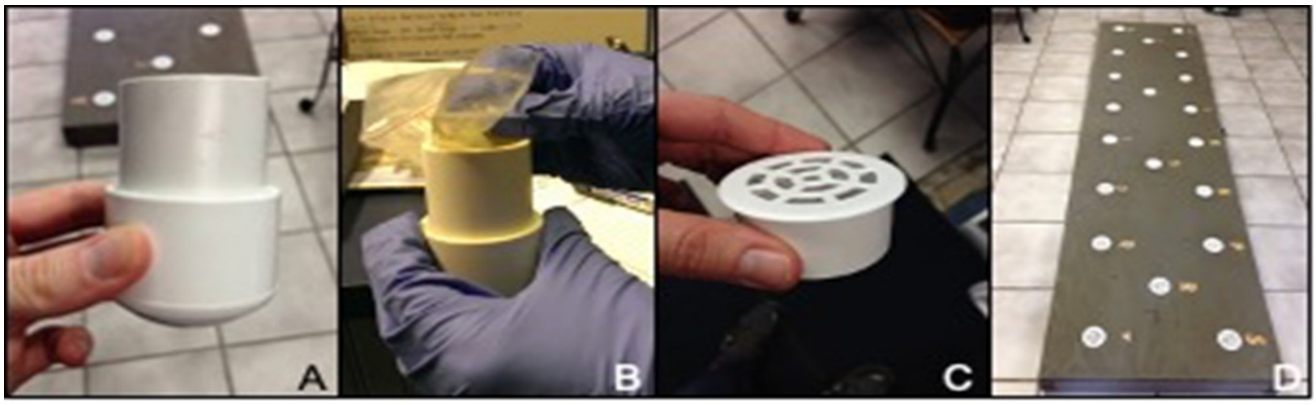

Figure 1. Scent tubes and board used for training and evaluation of detection dog. Panels A through C depict the PVC tubes in which the faecal samples were presented. Panel D shows the wooden array containing the recessed scent tubes.

Training

Faecal samples were warmed to room temperature prior to training sessions. Individual samples were presented in scent tubes manufactured from polyvinyl chloride (PVC) pipe with an inner diameter of ~3.8 cm and depth of ~7.5 cm (Figure 1). Containers were permanently capped at the bottom, the bag containing the faecal sample was inserted, and the tube then capped with a gridded PVC drain cover (Figure 1C). Scent tubes were recessed into either a 1 or 3-m long wooden array, with individual scent holes labelled alphabetically and spaced ~30 cm apart. The samples were not visible or accessible to the dog. Trainers wore nitrile gloves when handling samples and the scent tubes were washed thoroughly between training sessions.

Training was based on positive reinforcement with a food or toy reward, with a verbal bridge for correct responses. The dog was led on a leash, encouraged to sniff each grid, and conditioned to demonstrate positive identification with a specific behaviour, sitting (Figure 2). The dog initially was imprinted on positive samples from pregnant bears. When the dog correctly identified the target, the 'sit' command was given. This was repeated until the dog reflexively sat in front of the target after detection. Discrimination training was then performed by first introducing samples that likely were most different (e.g. samples from adult male bears) from the target odour, gradually phasing in samples from more similar groups, and lastly presenting samples collected from pseudo-pregnant females. Sample numbers presented

Figure 2. Training session. The dog smelled each scent tube (left) and indicated the presence of a positive sample by sitting (right).

Table 2. Number of responses per category, prevalence of pregnancy, sensitivity, specificity, and predictive test values. PPV=positive predictive value; NPV=negative predictive value.

Response	Evaluation	Year 1	Year 2
# True positive (%)	4 (40)	0 (0)	0 (0)
# False positive (%)	0 (0)	1 (3)	0 (0)
# True negative (%)	6 (60)	29 (91)	32 (94)
# False negative (%)	0 (0)	2 (6)	2 (6)
Prevalence (95% CI)	0.40 (0.14–0.73)	0.06 (0.01–0.22)	0.06 (0.01–0.21)
Sensitivity (95% CI)	1.00 (0.40–1.00)	0.00 (0–0.80)	0.00 (0–0.80)
Specificity (95% CI)	1.00 (0.52–1.00)	0.97 (0.81–1.00)	1.00 (0.87–1.00)
PPV (95% CI)	1.00 (0.40–1.00)	0.00 (0–0.95)	n/a
NPV (95% CI)	1.00 (0.52–1.00)	0.94 (0.77–0.99)	0.94 (0.79–0.99)

varied per session, usually with at least one positive sample and five to 15 non-pregnant samples. When the dog signaled correctly on a target sample, he was rewarded, then re-oriented on the training board. The dog was rewarded only on the identification of known positive samples throughout the study. New samples were introduced throughout the training process to account for potential effects of repeated freeze-thaw cycles. After approximately four months of training, the dog was reported to exhibit an overall accuracy of ~97% in the identification of samples collected from pregnant bears. Following initial training in 2013 and between the two years of real-time testing in 2013 and 2014, training sessions were performed at approximately twice per month to maintain the behaviour, then frequency was ramped up to at least once per week in preparation for the second round of testing.

Training evaluation

To corroborate the dog's accuracy and to rule-out trainer bias, a double-blind trial was performed in which the trainer/handler was unaware of the sample types. Ten samples (four from pregnant bears, six from non-pregnant bears), to which the dog had never been exposed, were assessed. The pregnant samples originated from the same pregnancies on which the dog had been trained, although the dog had never been presented with these particular sample dates. The non-pregnant samples were from males (n=2) and pseudo-pregnant females (n=4). After testing these samples, the trainer reported the results to the principal investigator, who compared them with the true sample status.

Test 1

Approximately 2 weeks following the training evaluation in 2013, 32 unknown samples collected from 16 females residing at 13 zoological institutions were evaluated in real-time, with the true pregnancy status of the females unknown. Samples were collected between 14–23 October; these dates were selected to correspond to the pregnancy window to which the dog had been trained to recognise and ranged from 46 to 37 days prior to the average cubbing date of 29 November, as calculated from the North American polar bear studbook (Curry et al. 2015). Fourteen of the females (87.5%) were either observed breeding earlier in the year or had been artificially inseminated; two females were not housed with males but were included as negative controls. Two samples from each female were tested to verify outcomes between samples originating from the same individual. During testing, known positive samples were presented intermittently for calibration and to maintain the dog's motivation because he was not rewarded on unknown samples.

Test 2

One year later during the subsequent cubbing season in 2014, 34 unknown samples collected from 17 females at 14 zoological institutions were tested, adhering to similar procedures as the previous year. All females were housed in breeding pairs or had undergone an artificial insemination procedure.

Evaluation

Sensitivity, the probability that the dog would signal on a sample collected from a female that produced cubs, was calculated as: number of true positives / (number of true positives + number of false negatives). Specificity, the probability that the dog would ignore the sample collected from non-pregnant females, was calculated as: number of true negatives / (number of false positives + number of true negatives). Sensitivity and specificity values were calculated with 95% Confidence Intervals (CI); positive and negative prognostic values were calculated by using MedCalc Statistical software version 19.2.6 (MedCalc Software Ltd, Ostend, Belgium).

Results

Training evaluation

The dog signaled correctly on all four samples collected from pregnant bears and ignored the six samples collected from non-pregnant bears, resulting in both sensitivity and specificity of 1.00 (Table 2).

Test 1

Of the 32 samples evaluated, the dog signaled on just one (3.1%) and ignored all others. When results were compared to the outcomes of the cubbing season, the dog signaled appropriately on 29 of 32 samples. The dog provided a false positive result on one of two samples collected from a female that did not produce cubs; he ignored the first sample from this female, but signaled positively on the second sample collected three days later. Because results for this individual were contradictory, four more samples from the same female were tested. He signaled on one of these four additional samples, which was collected on the same date as the first positive sample. The dog failed to alert on two samples collected from a female that produced cubs, resulting in two false negatives. The dog's sensitivity and specificity were 0.00 and 0.97, respectively (Table 2).

Test 2

The dog did not signal on any of the 34 samples presented. Again, one female produced cubs (a different female than the previous year), resulting in two false negatives. The dog's sensitivity and specificity were 0.00 and 1.00, respectively.

Discussion

Only one female had cubs each year of the study, which offered limited opportunities for accurate positive signaling and likely contributed to the poor sensitivity during testing. Despite this, the dog's overall specificity, in which he ignored samples originating from non-parturient females, was high in Tests 1 and 2 (0.97 and 1.00, respectively). During the training evaluation, performed just prior to Test 1, the dog's sensitivity and specificity both were 1.00, which was inconsistent with the low sensitivity attained during testing. Here, we offer several possible explanations for the discrepancies between training and testing.

During both years of testing, the dog failed to signal on samples collected from parturient females. The first year, the parturient female gave birth early in the cubbing season (9 November) and her samples were collected 25 and 19 days pre-partum, which were outside of the range used for training. The second year of testing, a different female produced cubs later in the cubbing season (20 December). Samples from this female were collected 67 and 61 days pre-partum, which also were outside the training range. These samples likely were collected around the time of embryo implantation, before a placenta or developing foetus were present. It is plausible that the compounds the dog learned to recognise are absent or present at concentrations too low for detection prior to day 67 or after day 25 pre-partum. Further investigations to characterise the window that pregnancy is detectable post-oestrus and pre-partum are warranted and future training endeavors should incorporate samples representing a wider range of time, especially for physiological conditions that may change temporally and, consequently, would yield shifting olfactory signatures.

The dog conveyed a positive signal on a sample collected from a female that was observed breeding earlier in the year but did not produce cubs. He ignored the first sample from this female, but signaled on a sample collected three days later. Because results were inconsistent, four additional samples from this female were tested. The dog signaled on one, which was collected on the same date as the previous sample on which he signaled. The false positive signal on these two samples collected on the same day may be an artifact of the bear's diet or other environmental factors; however, a sample collected on the same date from a different female residing at the same institution, presumably receiving the same diet, did not elicit a positive signal. This female had not produced cubs previously, so no samples collected from her had been used as positives during training, nor was she related to any other female in the study. A possibility is that the female actually was gravid and then lost the pregnancy, resulting in a transient target odour that the dog recognised on that date. More stringent criteria, such as two or more positive samples collected on different days, may be required to assign a pregnancy diagnosis. Ehmann et al. (2012) recommended a 'corporal dog decision analysis', in which three of four trained dogs were requisite to make the same decision on a sample. It is possible that additional dogs would increase the accuracy of the current study; therefore, the training and maintenance of multiple dogs for the same task should be considered in future endeavours.

During the training evaluation, the dog's sensitivity and specificity were 100% accurate in signaling on positive samples that had not been used in training. Although these samples were novel to the dog, they were collected from pregnancies which also were used in training. Because so few polar bears produce cubs each year, only 13 term pregnancies had occurred in the US during the 6 years prior to the onset of this study. Due to the low number of cases, samples were selected from all pregnancies to maximise positive sample numbers during training, which ultimately was a short-coming of the study. It is conceivable that the dog memorised the unique odour signatures of these 13 pregnancies on which he was trained and, despite the ability to recognise a novel sample from a learned pregnancy, as in the initial evaluation, he failed to generalise to new pregnancies. In an evaluation of odour-detection dogs trained to discriminate urine samples collected from men with prostate cancer, researchers reported that in a double-blind trial using novel samples collected from new patients, the dogs' ranges of sensitivity and specificity were only 0.13–0.25 and 0.71–0.75, respectively (Elliker et al. 2014). Despite training with urine samples representing 50 unique cases of prostate cancer, the authors postulated that the dogs may be capable of memorising odour signatures of large numbers of samples rather than generalising to an odour common to prostate cancer.

It would be impossible to train a detection dog to recognise every variation and alteration of a target odour under different conditions, so dogs must learn to categorise similar stimuli and respond appropriately when they encounter new variants of a learned odour (Moser et al. 2019). The number of unique cases required for sufficient training likely would vary depending on the target odour, amount of variation among samples, and by aptitude of the individual dogs. A large population of unique individuals affected by a specific condition of interest may be challenging to obtain for most wildlife studies, thus avenues to overcome small sample sizes should be explored. For example, researchers may consider pooling samples (Moser et al. 2019) to fabricate additional odour signatures. Alternatively, augmenting training with samples collected from individuals of closely-related species experiencing the same condition of interest may warrant consideration. Polar bears exhibit similar reproductive processes to other species of the Ursidae family as well as to most Mustelidae; therefore, the inclusion of additional samples collected from pregnant brown bears *Ursus arctos*, North American river otters *Lontra canadensis*, mink *Neovison vison*, and red pandas *Ailurus fulgens* potentially could bolster sample size so that the dog might generalise target odours to the pregnant state.

Despite the omission of novel cases in the training evaluation, the assessment ruled out handler bias as impacting the dog's likelihood to signal. It has been shown that trainer beliefs affect the accuracy of scent detection dogs (Lit et al. 2011) and that dogs were more likely to alert if the handlers had been led to believe that the target was present. In the current study, the trainer was unaware of the sample type, hence the dog's performance in successful target identification was not attributable to unintended

cues from the trainer and instead due to recognition of a target odour.

Edwards et al. (2017) performed a systematic review of literature describing the training and evaluation of odour-detection dogs in human diseases and found that canine accuracy was reported to vary widely. In a separate study, dogs were trained to detect oestrus in the vaginal fluid and urine of dairy cows with reported accuracy ranges of 58–100% depending on the dog (Fischer-Tenhagen et al. 2011). However, samples were taken from a small number of cows (n=12), positive and negative samples were held in different freezers, which may have permitted the dog to discriminate between sample types, and the same samples were used for both training and evaluation, so the dogs may have memorised specific samples. There currently are no standards for training or evaluating medical detection dogs (Walczak et al. 2012; Jezierski et al. 2015; Oh et al. 2015). The generation and implementation of standardised protocols and testing procedures would facilitate improved training methods and enable direct comparisons among studies.

In this analysis, the samples used for training were collected from a heterogenous population of polar bears residing at different zoological institutions. They originated from individuals of different ages with disparate medical histories, diets and undoubtedly gut microbiota, and sample retrieval time relative to defecation as well as temporary storage conditions probably varied by institution. Regardless, they are representative of the sample population on which the dog was tested. A possible drawback to the samples used for training is the age of the samples, as they were collected over the course of five years, and were handled for other projects; consequently, the target compounds were exposed to varying degrees of degradation and potential contamination. Conversely, the samples evaluated during testing were collected only weeks prior and were not subjected to long-term storage prior to evaluation by the dog. Odours indubitably change over time (Goss 2019), even when samples are frozen (Forbes et al. 2014); therefore, it is plausible that the target odour was not analogous or recognisable in the recently collected samples.

In conclusion, whereas the initial training outcomes in this study suggested that the dog would provide a rapid, non-invasive method for detecting pregnancy in polar bears, false negative results on two different parturient females indicated that the dog had not learned to generalise the target scent to novel pregnancies. The decreased sensitivity in testing compared to training may have been attributable to several causes: some of the samples tested did not fall within the range of pregnancy used for training; failure to generalise learned target odours to novel pregnancies; or, degradation of target odour signatures due to sample age or storage conditions. It is believed that the results of this investigation do not exclude the possibility that dogs could be trained to detect pregnancy using faecal samples; however, the large number of unique cases required to accurately train an odour-detection dog may be prohibitive in wildlife studies when sample size may be limited.

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