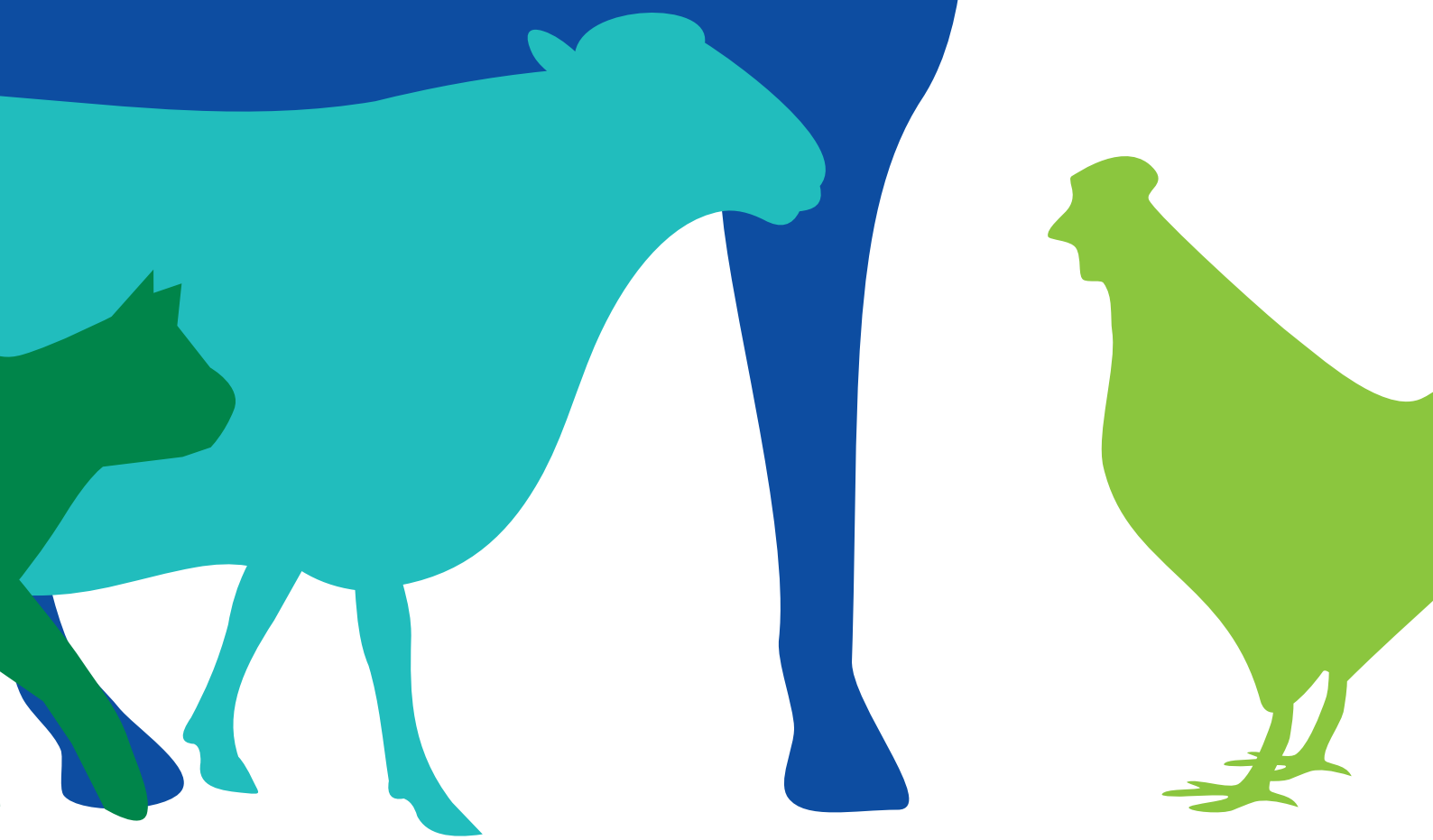
CANINE OLFACTORY DETECTION

EDITED BY: Cynthia M. Otto and Claire Marie Guest
PUBLISHED IN: Frontiers in Veterinary Science



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-634-1
DOI 10.3389/978-2-88963-634-1

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CANINE OLFACTORY DETECTION

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Citation: Otto, C. M., Guest, C. M., eds. (2020). Canine Olfactory Detection. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-634-1

Table of Contents

frontiers in Veterinary Science

EDITORIAL
published: 27 February 2020
doi: 10.3389/fvets.2020.00100

Editorial: Canine Olfactory Detection

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Keywords: olfaction, detection, behavior, scent, working dogs

Editorial on the Research Topic

Canine Olfactory Detection

Throughout history, dogs have fulfilled a whole range of different functions of which the number and diversity are continually expanding. Whilst traditionally dogs have been trained to hunt, herd, and guard, more recently canine roles have targeted olfactory and disease detection tasks. Although not the only species with excellent potential to harness the olfactory senses, dogs have remarkable olfactory acuity and their anatomy and physiology have evolved to support dogs in "seeing" the world through their noses. This Research Topic features original studies and reviews relevant to our theme of Canine Olfactory Detection and highlights the use of dogs in olfactory detection with several papers describing novel targets or capabilities of dogs. In addition, factors that influence canine olfactory detection, from availability of dogs, to training, behavior, medications/interventions, and disease round out the collection.

The expanding list of odors that dogs have been trained to identify raises new opportunities to improve human health and safety. Francis et al. describe a potential new application in narcotic detection to locate illegal synthetic cathinones or "bath salts." "Bath salts" have variable compositions, induce psycho-stimulant effects and are increasingly being abused (1). This is the first published study to explore whether "bath salts" can be detected by dogs. Certified narcotics detection dogs, which had never been exposed to the odor of "bath salts" were unable to reliably alert to the presence of cathinones, suggesting these compounds do not share volatiles with narcotics on which dogs are commonly trained. However, dogs "imprinted" on the odor reliably alerted to the presence of cathinone and generalized from the trained compound to other "bath salts." Head space analysis of different "bath salts" suggests some common volatile compounds (e.g., methylone) which may be candidates for the development of a synthetic training aid for "bath salts."

Medical detection is a growing field for canine olfaction and holds promise for new insights into disease etiology and diagnosis. Fischer-Tenhagen et al. in a proof of concept study, trained two dogs to distinguish breath samples from patients with lung cancer from those from healthy controls and then tested the dogs using synthetic air samples fortified with 1-butanol, 2-butanone, 2-pentanone, and hexanal (compounds increased in lung cancer sufferers) (2). The dogs alerted to three of the four synthetic samples tested. The low sample size limits the conclusions, but this study does suggest that dogs have the potential to verify biomarkers. This is an area of growing interest particularly in the field of medical detection where, long-term, diagnostics using dogs may be inappropriate or unlikely because of the number of tests required due to disease prevalence or complex etiology in the human population. Validation of biomarkers could assist in the rapid development of bio-electronic (E)-noses in the future. Trained dogs could assist in the identification of valid molecules or biomarkers associated with complex disease.

One of the greatest challenges in medical detection is the low concentration of disease associated volatiles, in a background of "normal" volatile compounds present in liquid samples (e.g., blood, urine, sputum). Efforts to determine the limits of canine olfactory detection may help identify dogs with superior potential for medical detection and may help monitor the day to day reliability of

Edited and reviewed by:
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Specialty section:
This article was submitted to Veterinary Humanities and Social Sciences, a section of the journal Frontiers in Veterinary Science

Received: *15 January 2020*
Accepted: *11 February 2020*
Published: *27 February 2020*

Citation:
Guest C and Otto CM (2020) Editorial: Canine Olfactory Detection. Front. Vet. Sci. 7:100. doi: 10.3389/fvets.2020.00100

the trained dogs. Concha et al. present the first published study to measure limits of detection of known odorants in the fluid phase. Although subject to individual variability, dogs can detect concentrations as low as 1.5 parts per trillion. This detection sensitivity is pertinent if dogs are to assist in the validation of molecules or markers of disease; the markers can be added to liquid background to simulate urine or other fluids for presentation and validation. Selected substances could even be used to "spike" healthy control samples. Dogs trained to assist in this validation task would need to be able to generalize confidently whilst maintaining a high sensitivity, as it is unlikely that the first molecules tested would represent the complete disease signature.

Using functional magnetic resonance imaging (fMRI) on awake unrestrained dogs, Ramaihgari et al. were able to show that zinc nanoparticles can enhance olfactory sensitivity, potentially upregulating both activity and connectivity (3). This provides an explanation for previously reported enhancement in the odor detection capability in the presence of zinc nanoparticles. Behavioral studies are now needed to confirm the findings but potentially these zinc nanoparticles could be used to improve detection capabilities, particularly in environments where very low concentrations of odorants might not otherwise be detected. This possible amplification of the olfactory signal is of great significance to future work and could assist in numerous complex detection tasks. However, further research is necessary to determine if amplification is beneficial or practical.

As applications of canine olfactory ability expand, it is imperative that the value of these dogs is objectively assessed, and their potential capabilities are optimized. We need to optimize accuracy, performance, and welfare in these working dogs. Olfactory performance relies on both the olfactory anatomy (including olfactory receptors, neurons, and olfactory bulb) and the behavior of the dog to communicate the information. Dogs are quick to learn patterns of behavior and past patterns will influence future performance. Typically, we use this to our advantage in training a dog to recognize and respond to an odor of interest; however sometimes the dogs try to "game the system." Some dogs find the process of searching to be the most valuable reward and if a particular cue leads them to anticipate that they will no longer be able to continue searching, their performance may suffer. Topoleski et al. discuss how these cues which signal the end of a training or testing session are often overlooked by handlers or trainers and may in fact negatively affect the performance of the detector dog. After a complete evaluation of the dog's performance and health, strategies to overcome learned associations may be a valuable tool to improve performance.

With increasing opportunities for employment of detection dogs, there is growing demand for dogs that possess the characteristics for the required tasks. In the United States, many agencies are experiencing a shortage of dogs with the necessary traits for successful olfactory detection (Leighton et al.). In order to identify dogs with the potential for different types of detection work, the working phenotype needs to be clearly and quantitatively defined. As such the selection of dogs with the optimal physical, genetic, and behavioral characteristics is imperative.

Whilst the knowledge of genetics increases, it is essential to address the importance of rearing and training environments. Two papers in this series explore behavioral differences in different types of detection dogs [i.e., search-and-rescue (SAR) and explosive detection dogs]. Behavioral traits such as trainability, fearlessness, and energy are often cited as required for dogs to succeed in search-and-rescue. Hare et al. used the Canine Behavioral Assessment and Research Questionnaire (CBARQ) tool to compare the reported behavior of SAR dogs with a breed-matched sample of pet dogs. SAR dog handlers rated their dogs to have significantly higher trainability and energy, and lower aggression and fear than pet dogs. This study, however, was unable to determine whether the reported differences were the result of underlying personality differences on the basis of which the SAR dogs were initially selected or were a result of rearing and training. It is also not known if the behavioral traits translate to measurable differences in performance. Prospective behavioral and performance studies of dogs specifically bred for detection work are essential to select for dogs with the greatest potential to address the working dog shortage (Leighton et al.).

Lazarowski et al. describe the behavioral evaluation of 146 dogs in an explosive detection dog breeding program. The authors define the phenotype of success in dogs trained to detect and alert to target odors in the aerodynamic wakes of moving persons ("Vapor Wake"). Subjective evaluation scores differed between the 63% of dogs which were successfully trained for detection of person born explosives (e.g., "Vapor Wake," hand carried, and body worn explosives), and the 17% that were deemed more suited to traditional explosives work or the 20% rejected for any type of detection work. Performance-related measures such as "hunt" and "focus" distinguished the "Vapor Wake" dogs. Environmental traits, often described as fearfulness, distinguished failures from successes but not between "Vapor Wake" and traditional explosives dogs. As shown in previous studies (4), fearfulness can be predicted early in life and has a strong genetic component, it is therefore imperative to select and breed against.

Future studies will be strengthened by more objective and validated measures of these traits. Development of common language and clear phenotype is necessary to increase the success and availability of detection dogs. A multi-disciplinary team of authors representing a variety of expert stakeholders suggest a solution to the current shortage of detection dogs in the USA (Leighton et al.). A "Detector Dogs Center of Excellence" would serve as a national resource for governmental, military and law enforcement working dog agencies to utilize as a data collection and genetic evaluation center. The research goals would be to define quantitative traits involved in odor detection, to better understand how these traits develop, and to evaluate methods to optimize breeding, raising and training detection dogs across all disciplines. This model demonstrates the potential value of a truly collaborative approach.

With an alternative view, perhaps addressing the problem of a shortage of detection dogs, Prada and Furton review the biology and natural olfactory capabilities of birds, suggesting that birds represent a plausible avenue of olfactory detection and urging the research community to consider their use. The use

of birds in forensic detection would take advantage of natural avian behaviors including use of odor gradients for navigation and food location. In addition, chickens have provided insight into chemosensory learning and early odor exposure. Although birds may not become common screening agents in airports, they should not be overlooked as a species that can contribute to our understanding of odor detection and may be employed in specific areas.

Despite dramatic progress in understanding areas where canine olfaction can be leveraged for enormous value, there are still many known and unknown factors that could impact canine detection performance. Jenkins et al. provide a much-needed review of the existing literature on the potential effects of health, management (including diet), and microbiota on olfactory performance of dogs. Although few studies have evaluated the effects in dogs, the authors highlight evidence from other species to identify factors that could impact canine olfactory performance. The influence of the microbiome is a growing and fascinating field which is becoming increasingly studied in many areas of biology, performance, and health. This is a fast-growing area which could have major significance for both the canine detector and, in relation to biological or medical detection, may affect the odor signature itself. It is now thought likely that the microbiome is altered by changes in health and wellness of an organism. The authors also highlight the potential beneficial effects of fasting. However, although investigating the timing of feeding and its effect on working ability is an important area of future research it will be vital that dog welfare is prioritized. Metronidazole is a common drug for the treatment of diarrhea; at high doses in some dogs, olfactory abilities are impaired (5). Understanding the potential chemical, environmental, and physiologic factors that could either enhance or impair performance is a critical next step to developing our knowledge of canine olfaction.

The studies in this Research Topic have touched on areas that are essential to further our understanding of the role and performance of working detection dogs. As commented earlier, as canine olfactory usage expands, it is imperative that the value of these dogs is objectively assessed and that their potential capabilities are optimized. The role of the dog as a detector may in the future have many applications, and research such as that published in this Research Topic will assist in ensuring that we use these abilities to further optimize accuracy, performance, and welfare in these working dogs.

AUTHOR CONTRIBUTIONS

CG and CO contributed to the content and style of the editorial.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 14 March 2018
doi: 10.3389/fvets.2018.00052

A Proof of Concept: Are Detection Dogs a Useful Tool to Verify Potential Biomarkers for Lung Cancer?

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Early and reliable diagnostic test is essential for effective therapy of lung cancer. Volatile organic compounds that are characteristic for cancer could serve as valuable biomarkers in cancer diagnosis. Both trace analytical and detection dog approaches give some evidence for the existence of such biomarkers. In this proof of concept, study dogs and trace analysis were implemented in combination to gain more information concerning cancer biomarkers. Two dogs were trained to distinguish between absorbed breath samples of lung cancer patients and healthy persons and succeeded with correct identification of patients with 9/9 and 8/9 and correct negative indications from of 8/10 and 4/10 samples from healthy individuals. A recent observational study found that breath samples from lung cancer patients showed an increase in 1-butanol, 2-butanone, 2-pentanone, and hexanal. Synthetic air samples were therefore fortified with these compounds and adsorbed to a fleece. Tested against breath samples from healthy probands, on presentation to the dogs these synthetic samples provoked an indication in three out of four samples. We were able to demonstrate that a combination of the natural nose of a dog and a trace analytic technique can be a valuable concept in the search for cancer biomarkers.

Keywords: detection dog, breath, sampling, biomarkers, synthetic air, lung cancer

OPEN ACCESS

Edited by:
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Reviewed by:
Joseph Wakshlag, Cornell University, United States
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Specialty section:
This article was submitted to Veterinary Humanities and Social Sciences, a section of the journal Frontiers in Veterinary Science

Received: *30 November 2017*
Accepted: *01 March 2018*
Published: *14 March 2018*

Citation:
Fischer-Tenhagen C, Johnen D, Nehls I and Becker R (2018) A Proof of Concept: Are Detection Dogs a Useful Tool to Verify Potential Biomarkers for Lung Cancer? Front. Vet. Sci. 5:52. doi: 10.3389/fvets.2018.00052

INTRODUCTION

Lung cancer is one of the most frequent cancer forms in Europe. In 2012, it was the leading type of cancer for males and the third most frequent type for females, causing 353.000 deaths in the 745 billion Europeans (1). Early and accurate diagnostic test is essential for improving the 5-year survival rate. There is science-based evidence that dogs are able to identify cancer specific odors in breath, blood, and urine samples of cancer patients (2–4). Although some studies reported promising sensitivities (71–99%) and specificities (78–98%) (5–8), other studies described discouraging test characteristics (sensitivities: 3–71% and specificities: 8–53%) and discussed this approach more critically (4, 9–12). These controversial results led to the question whether there are specific volatile organic compounds (VOCs) that are characteristic for a certain type of cancer cell or for metabolic processes in patients suffering from cancer.

Filipiak et al. (13) found that lung cancer cell lines and non-pathogenic cells cultured *in vitro* showed significant differences in the headspace in gas chromatography-mass spectrometry. However, in a study by Schallschmidt et al. (14) dogs and honey bees failed to discriminate between

air from *in vitro* cultured lung cancer cells and cell-free culture medium. Other approaches for biomarker identification used *in vivo* cancer models (15) and resected tumor tissue instead of cell cultures (7, 16, 17) which were analyzed with respect to differences in VOC profiles of pathogenic and non-pathogenic samples.

Volatile organic compounds in breath samples of patients suffering from lung cancer have additionally been investigated using gas chromatography. Aliphatic aldehydes were among the compounds repeatedly suggested to display increased levels in exhaled breath of patients (18–20). In addition, aliphatic aldehydes have been detected in urine (21) and blood (22) of lung cancer patients. Butanol (20, 23) and volatile 2-oxoalkanes (7, 20, 24) in breath have also repeatedly been associated with lung cancer.

The only attempt to combine detection dogs with instrumental VOC analysis to distinguish between healthy and diseased people was reported by Buszewski et al. (7). Dogs' indication of breath odor adsorbed to polypropylene tubes were compared with gas chromatography/mass selective detection data of VOC in breath samples taken with Tedlar bags from the same individuals.

Nevertheless, so far no single VOC or set of VOCs has reached clinical relevance for reliable disease recognition with sufficient sensitivity and specificity.

The objectives of this study were

(i) to set up a system that allows sampling and handling of breath for detection dog training, testing in an unbiased and reproducible way and the ability to spike samples with potential biomarkers;
(ii) to test if dogs can be trained using this set-up to distinguish between breath of lung cancer patients and healthy controls;
(iii) to test how these dogs react to synthetic air samples with potential volatile biomarkers observed in breath. These compounds were 1-butanol, 2-butanone, 2-pentanone, and hexanal, which have previously been associated with lung cancer (20).

MATERIALS AND METHODS

Material and Technological Developments

The main innovations were the improved fleece tubes for breath sampling and the cone-shaped sample-holder (**Figure 1**, patent DE 10 2013 109 901.7) that allowed placing and changing the fleece tube easily for detection dog training and breath sample testing. The design of the fleece tubes and the sample-holders enabled reproducible test runs, efficient cleaning of devices between runs and avoided contaminations of memory effects.

Genuine Breath Samples

Probands (patients $n = 30$ and healthy controls $n = 30$) participating in the study were enrolled in the cooperating clinic (Evangelische Lungenklinik, Berlin) following a jointly developed standard operating procedure for breath sampling, documentation of medical status, and ensuring patient anonymity. Breath samples of patients were taken on the first visit in the lung clinic for diagnosis, patients were not on any cancer treatment. Control persons were matched concerning age, sex and smoking habits. Patients and controls were not fasted prior to testing. The involvement of probands was approved by the Ethics Committee of the Charité, Berlin, Germany, and registered as clinical trial with proof-of-concept (EA1/207/13). An absorber technique using polypropylene fleece, developed on the basis of findings by Ehmann et al. (25) was chosen for breath sampling. The sampling tubes dedicated for dog training and testing consisted of a glass tube (length: 150 mm, inner diameter: 18 mm) with GL25 sockets on both sides (Gaßner Glastechnik, Munich, Germany). Each tube was filled with two 70 mm × 43 mm polypropylene fleeces (Asota GmbH, Linz, Austria) (**Figure 1**). One was hydrophilically (Asota® olefin hydrophilic) and the other was hydrophobically (Asota® olefin hydrophob) modified. Tubes were closed with silicone septa for GL25 sockets (neoLab Migge Laborbedarf-Vertriebs GmbH, Heidelberg, Germany) and with polybutylene-terephthalate screw caps (Bohlender GmbH, Grünsfeld, Germany) (26, 27). During each breath sampling the volume of the airflow was

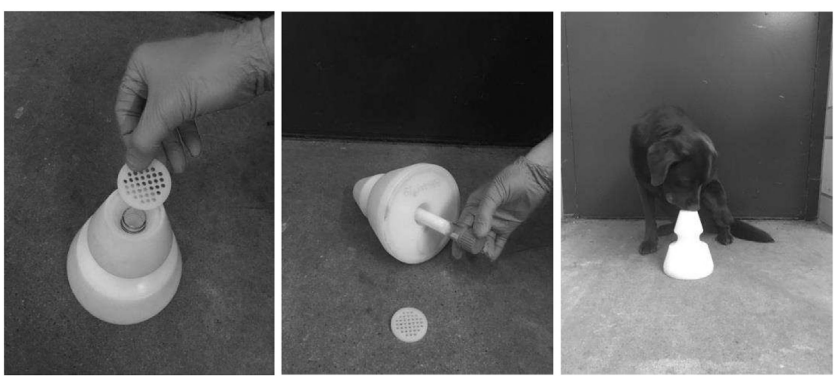

FIGURE 1 | Polyethylene cone for presenting glass tubes to dogs in training and testing. Left picture: perforated plate changed after every contact of a dog's nose; middle picture: glass tube with air sample attached to fleece is inserted into cone. Right picture: dog indicates a cone with a sitting response.

monitored with a spirometer (Ganshorn Medizin Medizin Electronic GmbH, Niederlauer, Germany). Each patient donated two fleece tube samples with a resting time of 10 min in between. Samples were stored refrigerated at 8°C until use. For dog training, 21 patient samples and 20 controls samples were used, 9 novel patient samples and 10 novel samples of controls were introduced for testing.

Synthetic Air Samples

Humidified synthetic air (80% N_2, 20% O_2; relative humidity: 84–89%) in a 1 L glass beaker with lateral septum as described in detail elsewhere (20) was spiked with 10 µL of a demineralized water solution containing 1-butanol (10.4 µg/L), 2-butanone (9.7 µg/L), 2-pentanone (3.2 µg/L), and hexanal (5.4 µg/L). The concentrations of the four compounds were chosen such that their concentrations in the glass bulb after injection through the septum were equal to the respective medians found in the breath of lung cancer patients in an observational study (20). After an equilibration period of 30 min, the air was transferred onto a fleece tube (see above) through a glass tube by means of an argon flow (160 mL/min for 30 min). Fleece tubes were closed tight and stored refrigerated at 8°C until use. A total of four fleece tubes loaded with spiked synthetic air were prepared.

Dog Training

All training was performed at the dog training and testing lab, Freie Universität Berlin. Four privately owned dogs were trained in this study, one 5-year-old spayed Labrador bitch, a 3-year-old intact female poodle, a 7-year-old female intact Dachshund, and an 8-year-old spayed Labrador Mix bitch. Selection was by convenience. Inclusion criteria were: dogs had to be clinically healthy, regularly available for training and familiar with training and testing of odor discrimination procedures (27).

In accordance with the European legislation (Dir. 2010/63/EU), no animal was exposed to harmful conditions throughout this study. During the study the dogs lived in their familiar home. The trainers had contributed to earlier odor detection studies with dogs (14, 28, 29). Training was conducted between June and December 2015–2016. The Labrador Mix and Dachshund did not make a considerable training progress in discriminating breath samples and were excluded from training after 3 months. Numbers of training days for the Labrador and the poodle were 73 and 82 days, respectively, with training trials (decision on a presented odor sample) ranging from 5 to 20 (average 15) per training day. Training took place only once a week, dogs were not trained at the same time or same day.

Training methods were based on positive reinforcement using a clicker as a secondary reinforcement and small food treats as reward and dogs were off leash during training and testing. Every dog was rewarded with its favorite food. In case of a wrong indication, a reward was not given and the dog had to pause for at least 1 min before repeating the trial. The dogs were familiar with the sound of the clicker as a predictor for food. A minimum of 80% correct indications were required in order to progress to the next training step. In brief, the training approaches included following steps:

In the first step, a cone with a fleece tube sampled from a lung cancer patient as positive sample was presented to the dog, and the dog was immediately rewarded for sniffing the holder. The cone was standing on the floor approximately 1 m away from the dog. Then, the dog was trained to indicate the cone by standing still and pointing or sitting next to it. In step two, a second, empty cone was introduced and placed approximately 50 cm away from the positive cone. The dog was required to identify and indicate the cone with the positive sample. In the third step, the second cone was loaded with a negative fleece tube sample (sampled from a healthy person) and the number of cones was increased to four (one positive, three negatives). Thus, the dog had to make a one-out-of-four decision (25% chance). After the dogs had performed a minimum of 80% correct indications, training was conducted in a double-blind manner. The dog handlers were not aware of the position of the positive sample and the experimenter was in a cubicle separated from the test room by a non-transparent curtain.

In the final training phase, number of positive cones per trial could vary from 0 to 4. When there was no positive in the trial, the dog was rewarded for returning to the handler after sniffing at all cones.

The perforated plate on top of a cone (positive or negative) was replaced whenever a dog's nose had contact to it. Plates were cleaned in an ultrasonic bath for 90 s. The cones were wiped with a wet cloth to minimize the risk of scent contamination. Fleece tube samples were used multiple times and stored refrigerated at 8°C in between training days.

Dog Testing

Dog testing took place in the same room as dog training. Two tests were performed in the scope of this study. The first test was to evaluate ability of the dogs' to distinguish between sampled breath from cancer patients and healthy controls. In the second test, we included synthetic air samples spiked with 1-butanol, 2-butanone, 2-pentanone, and hexanal. No sample used in training was used for the test.

For the first test, we used 9 cancer positive breath samples and 10 samples from healthy controls. The samples were used up to three times. Each dog was presented with a total of 40 samples. We documented the reaction of the dogs at the first contact of the sample to avoid studying a memory effect.

The samples were presented in trials. Each trial consisted of 2–4 cones presented to the dog. The cones were placed 40 cm apart from each other and at a distance of 2 m from the dog's starting point. The number of cones with positive samples in one trial was random and could vary from 0 to 4.

Randomization both for samples within a trial and over all trials occurred using the random number function of Excel (Microsoft Office, Microsoft Cooperation, Redmond, WA, USA).

A positive indication varied between dogs and consisted of the dog in question either sitting down at the cone or standing still, nose pointing at the cone, for a minimum of 3 s. After a negative indication the dog moved on to the next cone. The handler announced each indication to the experimenter.

In case of a correct positive indication, the dog was rewarded and the trial was finished. Cones the dogs had not sniffed remained in the lineup for the next trial, to make sure that dogs had to make decisions on every single cone. If the trial had no positive sample the dog was rewarded when it returned to the handler after sniffing all cones. Correct negative or false negative decisions were documented. Dog, handler, and any other person in the room were blinded to the position of the sample to avoid hidden clues.

In the second test, we wanted to observe the reaction of the dogs when they were presented with synthetic air samples. Therefore, 4 spiked synthetic air samples served as positive samples and all 10 healthy controls were included in the test. We presented 40 samples to the dogs. All synthetic air samples of the same composition, but different preparation days.

Statistical Data Analysis

The experimental set-up for breath samples with the random presentation of positive or negative samples led to the identification of any sample as true positive, true negative, false positive, or false negative. In the second test, only breath samples from healthy probands served as controls but no synthetic control samples. Therefore, true negative rate was not calculated.

RESULTS

In the first test, the Labrador had a correct identification rate at first presentation of nine out of nine and the Poodle of eight out of nine. True negative rate was 8 out of 10 for the Labrador and 4 out of 10 for the poodle. Results are summarized in **Tables 1** and **2**.

In the second test with synthetic air sample, both dogs indicated three out four synthetic samples as positive. Results for the first choice of the dog sniffing at the synthetic air samples are summarized in **Table 3**.

TABLE 1 | Indication (+ = correct; − = false) of dog at first contact with breath sample of patient suffering from lung cancer.

Sample ID	1024	1025	1026	5074	5075	5076	5077	5078	5079
Labrador	+	+	+	+	+	+	+	+	+
Poodle	+	+	+	−	+	+	+	+	+

TABLE 2 | Indication (+ = correct; − = false) of dog at first contact with breath sample of healthy probands.

Sample ID	1515	1516	1517	1518	1519	7515	7516	7517	7518	7519
Labrador	+	+	+	+	+	+	−	+	+	−
Poodle	−	−	−	+	−	−	−	+	+	+

TABLE 3 | Indication (+ = correct; − = false) of dog at first contact with synthetic air samples with potential biomarker for lung cancer.

Sample ID	1_DOT	2_DOT	3_DOT	4_DOT
Labrador	+	−	+	+
Poodle	+	+	+	−

DISCUSSION

In this study, two dogs were successfully trained to distinguish between breath samples from cancer patients and healthy controls. During the course of the study, four adult dogs undertook training. Due to insufficient training progress, two dogs were excluded after 3 months of training.

With regard to similar studies, Elliker et al. (12) reported that 7 out of 10 dogs in training were unable to reach the final stages of training. In the study of Gordon et al. (10), only 4 out of 10 dogs learnt to detect breast or prostate cancer in urine of human patients.

Due to the low number of dogs included in detection dog studies, it is difficult to prove an influence of the individual dog on the result statistically. We assume that some dogs have a higher ability to be trained for cancer detection than others. Further research is needed to identify selection criteria for the best possible cancer detection dog. Number of dogs in studies on cancer detection with dogs varies from 1 to 10 (28).

Unfortunately, only two dogs progressed to the final stage of training and could thus be included in the testing. While this is clearly an insufficient sample size, both dogs were able to indicate synthetic air samples as positive for cancer, which provides some proof of concept.

Our training duration of 16 months was long in comparison to shorter training periods described by other authors of 3 weeks (5), 7 months (9), or 12 months (6). Frequency of training in our study was once a week, which may have led to the longer total training period required. Number of sample of probands was limited, so we had to use same samples for multiple training sessions. This bears the danger of teaching dogs the individual odor of these probands instead of the specific odor of cancer (4). For the test, we used 19 samples (9 positives and 10 negatives) of probands the dogs had no contact with before. For this reason, we only documented the reaction of the dogs at first contact with the samples.

Ability for distinguishing the probands is within the range of previous studies conducted on lung cancer detection by detection dogs. With regard to all articles published so far on canine detection of lung cancer in humans on the basis of breath odor, the mean sensitivity was 78%, whereas the mean specificity was 71.5% (3). The results of the studies differ substantially. Whilst McCulloch et al. (5) found a sensitivity and specificity of 99% in detection of positive breath samples on lung cancer patients, the study by Amundsen et al. (11) revealed a mere 55.6% sensitivity and 8.3% specificity for small cell lung cancer. For a review of studies on lung cancer detection by detection dogs refer to Pirrone and Albertini (3).

The ability of the two dogs to discriminate breath samples was deemed satisfactory to continue with the project. The purpose of training the dogs to indicate breath samples of lung cancer patients was to test their response when they sniffed at synthetic air samples containing prospective VOCs that have previously been associated with lung cancer (20).

In the test to observe the reaction of dogs to synthetic air samples, both dogs indicated three out of four samples as cancer positive samples. Both dogs assigned the synthetic air samples to the cancer patients' group. Our results suggest that dogs have potential be used to verify potential biomarkers for lung cancer. As a future perspective, this preliminary finding needs to be reproduced with

control samples spiked with substance with no potential for biomarkers. As a next step genuine breath samples should be spiked with potential biomarkers and as controls with other substances found in breath samples not specific for cancer. Potential misleading of the dogs by unknown characteristics of involved genuine or spiked samples have to be ruled out by experimental design.

Lippi and Cervellin (30) suggested that, the "natural nose" of the animal might help to identify candidate biomarkers found by analytic technology. Buszewski et al. (7) found a tendency of greater concentrations of butanal, 2-butanone, ethyl acetate, ethyl benzene, 2-pentanone, 1-propanol, and 2-propanol in the breath of 44 lung cancer patients in comparison to 29 controls. In addition, they trained dogs to distinguish between both groups with a sensitivity of 82.2% and specificity of 82.4%. They found that ethyl acetate and 2-pentanone correlated positively with the dogs' positive indications. In a more recent study by this group with 108 lung cancer patients and 121 controls, including healthy probands and patients suffering from other lung diseases, a significant increase of concentrations of 1-propanol, 2-propanol, methyl acetate, hexanal, dimethyl sulfide, and carbon disulfide was found in the group of patients with lung cancer (31). With the help of Chi-squared automatic interaction detection, they were able to show that dimethyl sulfide is the main compound enabling differentiation between two groups: patients with cancer and healthy volunteers. They prepared synthetic samples on the basis of exhaled air of cancer patients. The indication of synthetic samples by the trained dogs was significantly worse (21%) than the indication of breath samples from cancer patients (86% correct positive). Unfortunately, the authors did not describe how the synthetic samples were prepared and which substances were included.

Based on the study by Schallschmidt et al. (20) we included 1-butanol, 2-butanone, 2-pentanone, and hexanal in the synthetic air samples.

Although methods in these studies were different 2-butanone, 2-pentanone, and hexanal were found throughout.

Currently available literature suggests that rather than there being one cancer-specific VOC, a combination of several VOCs display significantly higher concentrations in cancer patients (32). Buszewski et al. (7) stated that the signature odor of cancer that dogs use for differentiation between samples may be related to specific qualitative or quantitative olfactory impressions produced by a mixture of VOCs.

In this study, the two dogs discriminated the synthetic samples against healthy controls. In a more ideal test, it should be assessed if dogs discern between patients samples and synthetic samples, including VOCs potentially specific for cancer and VOCs not suspicious for cancer. This would underline the similarity between synthetic and patient samples.

Further research is warranted to test more combinations of potential biomarkers for lung cancer. We believe that specially trained detection dogs are a useful tool for finding the best possible biomarker for an effective diagnostic system for lung cancer.

ETHICS STATEMENT

The involvement of probands was approved by the Ethics Committee of the Charité, Berlin, Germany and registered as clinical trial with proof-of-concept (EA1/207/13).

AUTHOR CONTRIBUTIONS

CF-T and IN were involved in study design, dog training (as well as DJ), and writing the manuscript. RB contributed substantially to the manuscript.

ACKNOWLEDGMENTS

Authors are indebted to W. Heuwieser (FU Berlin) for the help with the manuscript and to H. Schmutz (Evangelische Lungenklinik, Berlin) for valuable support with sampling and to Christian Jung (BAM) for the preparation of spiked samples.

FUNDING

This study was not funded through external sources.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 20 March 2018
doi: 10.3389/fvets.2018.00050

Investigation of the Behavioral Characteristics of Dogs Purpose-Bred and Prepared to Perform *Vapor Wake*® Detection of Person-Borne Explosives

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Specialized detector dogs are increasingly being utilized for the detection of modern threats. The *Vapor Wake*® (VW) dog was developed to create a dog phenotype ideally suited for detecting hand-carried and body-worn explosives. VW dogs (VWDs) are trained to sample and alert to target odors in the aerodynamic wakes of moving persons, which entrains vapor and small particles from the person. The behavioral characteristics necessary for dogs to be successfully trained and employed for the application of VW are a distinct subset of the desired general characteristics of dogs used for detection tasks due to the dynamic nature of moving targets. The purpose of this study was to examine the behavioral characteristics of candidate detector dogs to determine the particular qualities that set apart VW-capable dogs from others. We assessed 146 candidate detector dogs from a VW breeding and training program. Dogs received identical puppy development and foundational odor training and underwent performance evaluations at 3, 6, 10, and 12 months old, after which they were sold for service. Dogs were categorized based on their final outcome of the training program, independently determined by private vendors, corresponding to three groups: dogs successfully sold for VW, dogs sold for standard explosives detection, and dogs that failed to be placed in any type of detector dog service (Washouts). Comparisons of behavioral evaluations between the groups were made across domains pertaining to search-related behaviors (Performance), reactions to novel stimuli (Environmental), and overall ease of learning new tasks (Trainability). Comparisons were also made at each evaluation to determine any early emergence of differences. VWDs scored significantly higher on Performance characteristics compared to standard explosives detection dogs (EDDs) and Washouts. However, Environmental characteristics did not differentiate VWDs from EDDs, though scores on these measures were significantly lower in the Washouts. Furthermore, differences between groups emerged as early as 3 and 6 months for select measures. We describe the behavioral characteristics targeted for selection in developing the VW phenotype and discuss the relative merit and degree of expression of those characteristics in the success of dogs bred and trained for the VW application.

Keywords: *Vapor Wake*®, detection dog, phenotype, behavior, selective breeding, working dogs, canine, person-borne explosives

OPEN ACCESS

Edited by:
Cynthia M. Otto, University of Pennsylvania, United States

Reviewed by:
Manja Zupan, University of Ljubljana, Slovenia
Nicola Jane Rooney, University of Bristol, United Kingdom

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Specialty section:
This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science

Received: *01 December 2017*
Accepted: *27 February 2018*
Published: *20 March 2018*

Citation:
Lazarowski L, Haney PS, Brock J, Fischer T, Rogers B, Angle C, Katz JS and Waggoner LP (2018) Investigation of the Behavioral Characteristics of Dogs Purpose-Bred and Prepared to Perform Vapor Wake® Detection of Person-Borne Explosives. Front. Vet. Sci. 5:50. doi: 10.3389/fvets.2018.00050

INTRODUCTION

Detector dog applications are becoming ever more technically specialized. Examples of such specialization include the following: military off-lead, directionally controlled down-range improvised explosive device detection; cargo inspection; evidence retrieval; concealed human detection; pest and agricultural pathogen detection; and air passenger screening. The required characteristics of dogs for traditional detection tasks are also being more narrowly defined as state-of-the-art for certain applications, e.g., refining urban vs. wilderness search and rescue (SAR), immediate vs. aged human trail tacking, and trace vs. bulk substance detection. Growing recognition of canine olfaction as the most capable tool for the majority of detection tasks and growing technical sophistication of detector dog practitioners have given rise to the expansion of the types and specialization of detector dog applications. Consequently, the numbers of dogs exhibiting suitable characteristics to perform contemporary detector dog tasks have declined. Moreover, despite the widespread recognition of the important role of detector dogs in security operations, systematic examinations of the characteristics of such specialty search dogs are scarce in the literature (1–3). Additionally, there is a lack of standardization and consistency in identifying and describing specific desired detection dog behavioral characteristics and screening processes (1).

A primary means by which detector dogs are sourced is the selection of dogs from populations bred for purposes other than security-related detection tasks. An example of this repurposing of dogs is the selection of sporting breed dogs purpose-bred for hunting and field trial activities to be trained to perform detection tasks. With few notable, but fairly exclusive, exceptions, such as the Norwegian People's Aid Global Training Centre for mine detection dogs selective breeding program (4), and the former Transportation Security Administration's Canine Breeding Program for detection dogs, there have been only small-scale and short-lived efforts to breed dogs for specific detector dog applications. There are scant examples of technical or scientific reporting of such efforts, thus, there exists little formal research or technical guidance to provide direction in selective breeding of detector dogs (2, 5).

It is becoming increasingly acknowledged that behavioral characteristics are greater determinants of detector dog success than sensory or morphological characteristics (6, 7). Thus, accurately evaluating behavioral characteristics for selection and prediction of successful working dogs is vital for the sustainability of working dog programs. Maejima et al. (8) reported a 30% success rate of 197 Labrador retriever dogs entering drug detection programs and Wilsson and Sundgren (9) reported a 4.9% composite success rate for search tasks from 2,107 candidate German shepherd and Labrador retriever dogs. Given the low levels of successful candidate detector dogs reported across working dog programs, identifying and selecting for traits related to success as a detector dog are clearly challenging. Without the ability to identify the key behavioral characteristics that are predictive of successful candidate working dogs, precision in mating selection is greatly reduced, impeding advancement of specific capabilities in working dog populations.

It can be argued that traditional means of producing and raising most detector dogs are inadequate to meet the growing demand for specialized applications. One such specialized application that has emerged in response to modern threats, such as person-borne improvised explosives devices, is the *Vapor Wake*® (VW) detection methodology (10, 11). VW detection dogs are trained to sample and alert to target odors in the aerodynamic wakes of moving persons, which entrains vapor and small particles from the person. The behavioral characteristics exhibited by dogs capable of performing VW detection differ from those of traditional standard explosive detector dogs (EDDs) that are trained to detect static odor sources. Vapor Wake dogs (VWDs) must independently and constantly sample the air making efficient use of air currents to interrogate the human aerodynamic wake for target odors (12, 13). VWDs must be highly vigilant in searching for target odors and resilient from distraction in high-stimulus environments, such as large event venues and mass transit stations, where they are most often utilized. Thus, the VW application requires dogs with a pronounced expression of what are generally considered desirable characteristics in all detection dogs, plus some distinct characteristics such as vigilance (i.e., sustained attention) in searching for and alerting to target odors and deference for engaging in such searching as compared to engaging in other activities, such as, particularly, social interaction with people. With the demand for VWDs rapidly growing due to increasing incidents of terrorism involving body-worn and hand-carried moving targets, identifying and characterizing these traits are critical to the successful application of VW technology.

The purpose of this study was to examine the degree of expression of behavioral characteristics traditionally associated with detector dogs capable of performing the VW task in comparison to dogs not capable of performing VW within the recent (i.e., since 2013) population of dogs produced by the Canine Performance Sciences (CPS) program within Auburn University's College of Veterinary Medicine (AUCVM). To do so, performance evaluations of the purpose-bred population of candidate VWDs, performed at 3, 6, 10, and 12 months by CPS senior trainers, were compared between groups of dogs categorized according to their final disposition, i.e., whether the dog was ultimately sold as VWD, as an EDD, or failed to be sold as either (Washout), as determined by third-party customer independent evaluations. Additionally, comparisons between groups at each evaluation timepoint were conducted to determine whether early differences emerged, for the purpose of improvements in early identification of successful or unsuccessful candidates. We hypothesized that due to the rigorous demands imposed by performing VW, dogs qualifying for VW roles would exceed others in their behavioral and task-related performance characteristics. This work represents the first examination of behavioral characteristics and their accentuation through selective breeding and controlled early experiences that are related to the success of dogs in performing dynamic (i.e., moving persons) person-borne improvised explosive device detection.

MATERIALS AND METHODS

Subjects

The original breeding stock from which dogs described in this paper have been bred came from Australian Customs Service in the year 2000. The initial goal of the CPS program with this original population was to breed high-quality detector dogs. Since then, American Field Trial, Hunt Test, and Upland Game dogs have been integrated into the breeding population to incorporate new genetics and diversify the population. Currently, CPS has bred a total of 121 litters of purpose-bred detection dogs. In 2013, CPS began a concerted effort using evaluation measures to specifically enhance traits that were thought to be particularly important for the VW application. Dogs selected as breeders must have superior detection and behavioral characteristics and no medical issues. Prior to the concerted effort in 2013, 70 + dogs had been previously sold as VWDs. Since 2013 until now, 38 litters of dogs have been purpose-bred for producing dogs capable of performing the VW application. This paper describes dogs ($n = 146$) bred and trained at the AUCVM CPS from the time this concerted effort began (September 2013) to September 2016. The sample consisted of 28 litters from 17 dams and 18 sires. A total of 9 dams and 8 sires were bred more than once. No sire and dam breeding matchings were repeated. Dogs were Labrador retrievers ($n = 119$) and Labrador retriever X German wirehaired pointer (GWP) crosses ($n = 27$; 11 of which were 50% GWP and the remaining 25% or less). The sample consisted of male ($n = 71$) and female ($n = 75$) dogs that remained intact until matriculation out of the breeding puppy development program. Dogs that were medically disqualified from service ($n = 11$) were not included in the analyzed sample (i.e., 146 dogs remained after removing 11 medically disqualified dogs). Medical disqualifications were due to orthopedic issues: hip dysplasia ($n = 4$), elbow dysplasia ($n = 3$), stifle issues ($n = 2$), hip and elbow dysplasia ($n = 1$), and an indeterminate biomechanical issue ($n = 1$). All dogs were born, reared, and housed in the same environment and participated in the same standard CPS development and training protocols, described below, from the time they were born until they were sold. Dog care and use activities were approved and monitored by the Auburn University Institutional Animal Care and Use Committee.

CPS Puppy Development Phases

All dogs participated in standard CPS training and development protocols, intended to produce VW-quality dogs. CPS production and puppy development consists of 6 phases. In Phase 1, sires and dams are selected through a screening process for medical soundness, low inbreeding coefficients, and superior performance and environmental behavioral characteristics. Phase 2 consists of the breeding, gestation, and partition periods. In Phase 3, puppies are group-housed in the nursery with their littermates and mother until 7 weeks of age. This period of early puppy development includes the introduction of new sights and sounds, reward value building, and obstacle navigations to enhance motor skills and problem-solving abilities. In Phase 4, intermediate puppy development occurs through extensive social, environmental, and performance conditioning in Auburn, AL, USA and the surrounding areas and lasts from 7 weeks to 6 months of age. Puppies are housed in indoor/outdoor kennels, first pair-housed until 13 weeks and then single-housed. Successive approximation of age-appropriate conditioning and exposures, progressing from simple to complex using positive reinforcement, is used to cultivate a strong foundation for detector dog training. Intermediate puppy development continues through Phase 5 when at 6 months puppies are placed in participating prisons for further socialization and development by specially trained inmates until 10 months of age. Inmates participating in the program are enrolled in a 1,150-h CPS-developed Performance Canine Care and Development course taught in the prisons by trained program managers. The prison program engages dogs in activities like basic odor discrimination games and exposes dogs to tighter living quarters simulating operational work in crowds of people. Phase 6, final puppy development, commences upon return from the prison program at 10 months of age until 12 months of age. During this 2-month period, dogs undergo evaluations for detection performance, physical fitness, environmental soundness (i.e., responsivity to environmental stimuli), and medical soundness. Dogs receive 16 days of VW foundational training, undergo final behavioral evaluations, and complete their puppy development cycle at CPS by final placement through sale as a VWD or EDD, retained for CPS breeding or research activities, or, infrequently, offered for adoption.

Behavioral Evaluations

Evaluations were conducted by expert observers when the dogs were 3, 6, 10, and 12 months old. Evaluations consisted of 14 measures across three domains: seven Performance measures, six Environmental measures, and one overall Trainability measure. Performance measures consisted of characteristics associated with detection and searching abilities. Behaviors underlying a dogs' motivation to search are commonly collectively referred to as a dogs' "drive," or a natural motivation to perform a particular action. Several types of drives important to detection dog success have been described in the literature, including play drive (a dogs' desire to entertain itself by engaging with others or objects), prey drive (desire to chase and kill), and hunt drive (dogs' desire to search for hidden prey using their nose) (1, 14). Environmental measures consisted of responses and reactions to unfamiliar stimuli in the environment. Sometimes referred to as "nerve strength," these measures largely focus on the dogs' ability to deal with and adapt to stress-inducing experiences, and include tactile, auditory, and visual stimuli (15). Finally, Trainability consisted of just one measure of a dogs' ease and speed of learning new tasks (1). **Table 1** contains detailed descriptions of each item within each domain. These domains and evaluated characteristics are commonly used in the assessment of candidate detector dogs in the working dog industry that, over time, CPS has tailored and operationally defined for use in assessing the potential of dogs for successfully performing VW detection. Each characteristic assessed has a defined "most desirable" expression that should engender a score of 5, on a 1–5 Likert scale. The most desired expression of some characteristics are multifaceted and not a unidirectional, less-to-more display of a particular response, but rather the extent to which the expression of a, sometimes complex,

TABLE 1 | Descriptions of measures assessed during performance evaluations, scored on a 1–5 scale from least to most desirable performance.

Domain	Measure	Definition
Performance	Retrieve	Dog will enthusiastically retrieve any reward every time with full sprints out and back
	Hunt	Dog constantly uses nose to search and investigate targets using closed-mouth search, not looking for handler guidance. Dog does not become over-excited when target odor is present and does not get discouraged when odor is not easily found
	Focus	Dog is able to focus on rewards/tasks. Dog notices environmental stimuli, but does not respond to distractions (i.e., urine, ambient noises)
	Physical possession	Dog holds reward in mouth, returns to handler holding reward, and looks for engagement with handler
	Independence	Dog is willing to work at a distance from handler and spends a minimum amount of time looking back for assistance
	Work effort	Dog will give 100% effort on every search/task every time. Dog is eager to find target to interact with handler
	Air scenting	Dog is constantly using nose to find air currents, while consistently and efficiently searching air. Dog is not looking at specific targets/objects
Environmental	Surfaces	Dog will transition across any and all kinds of surfaces without any hesitation
	People	Dog notices people, but does not try to interact. Dog may sniff people, but does not focus on people. Does not show fear, distraction, or excitement elicited by people
	Vehicles/urban clutter	Dog adapts to clutter and works normally without disruption in searching behavior. The urban clutter should elicit the dog's searching behavior
	Visual startle	Dog notices new, unusual, or sudden stimuli but quickly resumes working. Dog may react by noticing stimuli, but holds ground and recovers quickly and then goes forward to investigate area
	Acoustic startle	Dog will notice loud stimuli, but holds ground and recovers quickly and then goes forward to investigate area
	Excitability	Dog is very active, exited to work, but not erratic. Dog may run through odor, but can recover and return to scent cone without giving up on task
General	Trainability	Dog is easily trainable. Dog learns new tasks quickly and easily with few trials and little direction

The descriptions listed above reflect the standard VWD.

pattern of behavior in response to particular stimuli in a particular context has, in the program's experience, been indicative of success as a VWD.

Each evaluation was conducted over two consecutive days. All evaluations had portions consisting of both on- and off-lead tasks simulating real-world detection scenarios. Evaluations were tailored to be appropriate for each age level. Scoring used a subjective 5-point Likert scale with higher scorers indicating more desirable performance. Observers were senior canine instructors at CPS ranging from 8 to 35 years of experience in the handling and training of detector dogs in operational environments. At least one and up to three of the same three observers evaluated each dog; 68% of the observations had two or more evaluators. These instructors did not directly participate in the activities of raising and preparing the dogs for detection training from 0 to 10 months of age. At final training at 10 months, one or more may have been involved in the advanced detector dog training. The intent was to always have at least one evaluator that had not participated in the dogs' training, which was most often the case.

Final Disposition Categories

After completion of the CPS puppy development and training cycle, each dog was assigned a final disposition category based on its placement in service, which was determined independently by third-party customers. The goal of the CPS breeding program is that all dogs are placed in service as VWD; those not accepted for VW service are offered for service as an EDD, or, having been assessed as not suitable for service as either, retained for CPS research or prison teaching assistant dogs. Infrequently, dogs not suitable for sale were adopted out as a pet.

Aside from deciding which dogs to present to vendors as VWD/EDD candidates or withhold for presentation, CPS personnel were not involved in customers' assessment or purchase decisions. Trainers' filtering of which dogs to present to vendors is a practical matter of not presenting dogs that are demonstrably incapable of performing VW. There is strong program performance and financial motivation for CPS to present all dogs with even marginal chances of being selected for service to customers.

Upon initial presentation, the customer performs a series of performance and environmental tests in environments unfamiliar to the dogs to assess their potential for VW. At this point, a dog may be rejected as VW and downgraded to EDD or assessed as not suitable for detector dog work by the VW customer. Furthermore, the customer has a 30-day period in which they engage dogs in training in which to reject or accept the dog as VWD or EDD. Dogs returned to CPS by the customer within this window are further assessed by CPS for their potential to be sold as EDD to other, non-VW, customers. Dogs that CPS trainers assess as being demonstrably incapable of performing VW but may have potential as EDD are also presented to these other, non-VW customers. Dogs presented to those other customers, again, are subjected to independent assessment regimens of those programs and their final disposition is determined by whether those dogs are accepted (i.e., purchased) by those customers. Therefore, while the final dispositions of dogs in this study are not entirely independent due to trainer selection of which dogs to present to customers, there is significant practical pressure on CPS to present all dogs with the possibility of being sold as VWD or EDD to independent assessment for their operational detector dog capability, which determined their final disposition for the purpose of analysis in this study. This is a real-world scenario

that adds significant ecological validity to the final outcomes observed in this study. Thus, for purpose of analysis in this paper, a dog's final disposition was categorized as having been successfully placed in service as a VWD (and retained beyond the 30-day return window), EDD, or, if not selected for service, as a Washout. Dogs selected as breeders were also characterized as VWD (breeders are subsequently sold as VWD after completion of breeding, unless they are unable to be sold due to age). Washout dogs were further categorized as having failed due to inadequate performance, environmental soundness, or both. It is important to note that all dogs in the population were trained for the same goal of sale as a VWD, and to this end experienced the same training. Group categorizations as VWD, EDD, or Washout were made *post hoc* according to their sale status; dogs' training or other experiences prior to sale did not differ.

Data Analysis

Evaluators' scores for each item were averaged to create a single score for each measure for each dog. Average scores for each group were compared for each of the items at each evaluation timepoint. Additionally, timepoints were collapsed and items pertaining to the same domain were averaged in order to create composite Performance, Environmental, and Trainability scores for each dog. Some dogs were not available for all evaluations or at one or more timepoints and thus were not included in certain analyses that excluded missing cases. Additionally, some items were more recently developed and thus scores for earlier dogs were not available. Measures that did not include all dogs were: Retrieve, Focus, and Work Effort at 10 mo ($n = 141$) and Final ($n = 133$); Hunt and Independence at 10 mo ($n = 141$) and Final ($n = 132$); Possession at 3 mo ($n = 123$), 6 mo ($n = 137$), 10 mo ($n = 141$), and Final ($n = 132$); Air Scenting at 3 mo ($n = 110$), 6 mo ($n = 115$), 10 mo ($n = 126$), and Final ($n = 131$); Surfaces, People, and Vehicles at 6 mo ($n = 137$) and Final ($n = 136$); Visual Startle at 10 mo ($n = 136$) and Final ($n = 130$); Acoustic Startle at 10 mo ($n = 139$) and Final ($n = 130$); Trainability at 10 mo ($n = 144$) and Final ($n = 133$); and Excitability at 3 mo ($n = 92$), 6 mo ($n = 15$), 10 mo ($n = 128$), and Final ($n = 131$). All analyses used IBM SPSS Statistics version 23 and an alpha level of .05.

RESULTS

Final Disposition

The final dispositions of 146 CPS-produced dogs (after removal of 11 medical releases) were 63% VWD, 17% EDD, and 20% Washouts. Of the Washouts, 62.5% failed for insufficient environmental soundness and 37.5% failed for inadequate performance.

Behavioral Evaluations

Composite Performance, Environmental, and Trainability scores for each of the three final disposition groups (VWD, EDD, Washout) were calculated by averaging all component measures of the corresponding evaluative domain across the four timepoints (**Figure 1**). Separate one-way analysis of variance (ANOVA) for Group (VWD, EDD, Washout) were conducted for composite Performance, Environmental, and Trainability mean scores and all yielded a significant effect, Fs (2, 143) > 12, ps < 0.01. The VWD group outperformed the other groups in each domain except for Environmental where VWD and EDD were equivalent (see **Figure 1**), as confirmed by *post hoc t*-test comparisons.

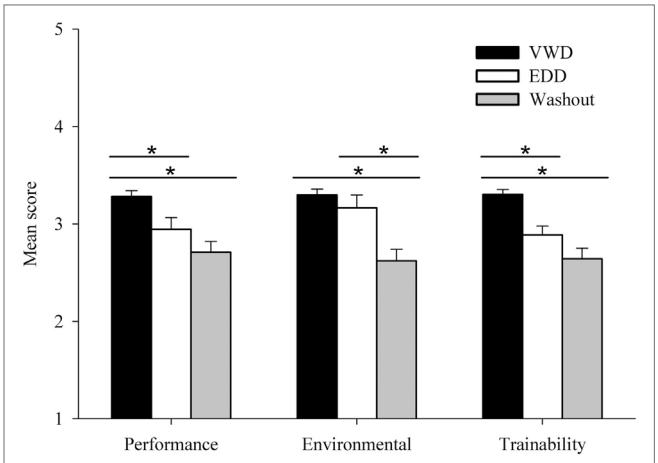

FIGURE 1 | Average scores for each group [VW dog (VWD), explosives detection dog (EDD), Washout] in the Performance, Environmental, and Trainability domains. Mean scores represent averages of submeasures corresponding to each domain and are collapsed across timepoints. Error bars represent standard errors of the means. *$p < 0.05$.

Performance Domain

Figure 2 (left panel) shows the composite mean score for all Performance measures across the four timepoints. A two-way repeated-measures ANOVA for Performance with Group (VWD, EDD, Washout) as the between-subjects variable and evaluation Timepoint (3 mo, 6 mo, 10 mo, Final Evaluation) as the within-subjects variable with adjusted Greenhouse–Geisser degrees of freedom revealed a significant effect of Group, $F(2, 128) = 9.423$, $p < 0.001$, Timepoint $F(2.4, 308.03) = 12.955$, $p < 0.001$, and the interaction, $F(4.81, 308.039) = 2.58$, $p = 0.028$. The interaction was due to all groups improving from 3 months to 6 months and the VWDs maintaining better performance than the other groups from 10 months to the Final Evaluation, as confirmed by the following follow-up analyses. *Post hoc t*-tests revealed that scores at the 3-month were lower than at the 6-month timepoint, $p < 0.001$, VWDs scored higher than Washouts across all timepoints, ps < 0.01, and no difference between EDDs and Washouts, ps > 0.293. The VWDs scored significantly higher than EDDs at 10 months, and Final Evaluation, ps < 0.05.

To explore each of the Performance measures, similar separate two-way repeated-measures ANOVAs were performed on the individual measures and yielded significant main effects of Group, ps < 0.001, for all of the Performance measures except *Retrieve* and *Air Scenting*. Of the measures that did result in significant group differences, pairwise comparisons revealed that VWDs scored significantly higher than both EDDs and Washouts on all of the measures, with no differences between EDDs and Washouts (**Table 2**). The Group × Timepoint interactions were significant, ps < 0.05, for *Focus*, *Hunt*, *Independence*, and *Possession*; these interactions are further interpreted in Section "Timepoints."

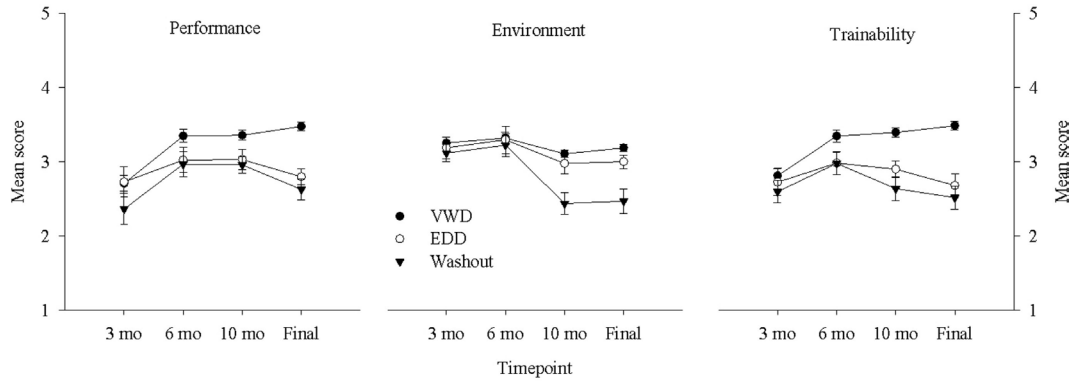

FIGURE 2 | Average scores for each group [VW dog (VWD), explosives detection dog (EDD), Washout] in the Performance (left panel), Environmental (middle panel), and Trainability (right panel) domains across each of the evaluation timepoints (3 mo, 6 mo, 10 mo, and Final Evaluation). Error bars represent standard errors of the means.

TABLE 2 | Mean (standard error) scores for each group by measure, collapsed across time points.

		VWD	EDD	Washout
Performance	Retrieve	3.07 (0.05)	3.03 (0.11)	2.84 (0.107)
	Hunt	3.31 (0.06)^{EDD,W}	2.93 (0.12)	2.87 (0.116)
	Focus	3.23 (0.06)^{EDD,W}	2.84 (0.12)	2.63 (0.121)
	Possession	3.03 (0.06)^{EDD,W}	2.62 (0.13)	2.67 (0.125)
	Independence	3.26 (0.06)^{EDD,W}	2.95 (0.12)	2.79 (0.12)
	Work effort	3.24 (0.06)^{EDD,W}	2.93 (0.115)	2.67 (0.118)
	Air scenting	3.06 (0.07)	2.79 (0.140)	2.99 (0.136)
Environmental	Surfaces	3.23 (0.05)^{W}	3.22 (0.09)	3.01 (0.08)
	People	3.28 (0.06)^{W}	3.16 (0.11)	2.86 (0.10)
	Vehicles	3.27 (0.05)^{W}	3.13 (0.11)	2.90 (0.09)
	Visual startle	2.96 (0.10)^{W}	2.98 (0.21)^{W}	2.17 (0.18)
	Acoustic startle	3.13 (0.09)^{W}	2.88 (0.19)^{W}	2.06 (0.16)
	Excitability	2.98 (0.04)	2.92 (0.07)	2.93 (0.07)
General	Trainability	3.26 (0.05)^{EDD,W}	2.82 (0.10)	2.69 (0.10)

^{EDD}Denotes that score was significantly higher than the explosives detection dog (EDD) group at the 0.05 level.

^{W}Denotes that score was significantly higher than the Washout group at the 0.05 level.

Environmental Domain

Figure 2 (middle panel) shows the composite mean score for all Environmental measures across the four timepoints. A two-way repeated-measures ANOVA for Environmental with Group (VWD, EDD, Washout) as the between-subjects variable and evaluation Timepoint (3 mo, 6 mo, 10 mo, Final Evaluation) as the within-subjects variable with adjusted Greenhouse–Geisser degrees of freedom revealed a significant effect of Group, $F(2, 131) = 8.251$, $p < 0.001$, Timepoint, $F(1.78, 233.33) = 15.30$, $p < 0.001$, and the interaction, $F(3.56, 233.33) = 4.022$, $p = 0.005$. The interaction was due to generally stable scores for all groups from 3 to 6 months, and Washouts dropping significantly lower than both VWD and EDD at 10 months and Final Evaluation, as confirmed by the following follow-up analyses. VWDs and EDDs scored significantly higher than Washouts at 10 months, ps < 0.01, and at Final Evaluation, ps < 0.01. VWDs were equivalent to EDDs at all timepoints, ps > 0.36.

To explore each of the Environmental measures, similar separate two-way repeated-measures ANOVAs were conducted on the individual measures within the Environmental domain and yielded a significant main effect of Group, ps < 0.001, for all of the Environmental measures except *Excitability*. Of the measures that resulted in significant group differences, pairwise comparisons revealed that VWDs scored significantly higher than Washouts on all measures, but did not differ from EDDs on any measure. EDDs scored significantly higher than Washouts only on *Visual* and *Acoustic Startle* (**Table 2**). The Group × Timepoint interactions were significant, ps < 0.05, for *People* and *Vehicles*. These interactions are further interpreted in Section "Timepoints."

Trainability

Figure 2 (right panel) shows the mean score for the Trainability measure across the four timepoints. A two-way repeated-measures ANOVA comparing group scores for Trainability across the four timepoints with adjusted Greenhouse–Geisser degrees of freedom revealed a significant effect of Group, $F(2, 130) = 17.218$, $p < 0.001$, Timepoint, $F(2.26, 294.57) = 6.381$, $p = 0.001$, and the interaction $F(4.532, 294.57) = 4.176$, $p = 0.002$. *Post hoc* tests revealed that the VWD group had a significantly higher Trainability score than both the EDD and Washout groups, ps < 0.002 (see **Table 2**). The interaction was due to the VWDs improving across time while EDDs and Washouts decreased from 6 months to Final Evaluation, as confirmed by the following follow-up analyses. VWDs scored higher than EDDs at 10 months and Final evaluation, ps < 0.005, and higher than Washouts at 6 months, 10 months, and Final Evaluation, ps < 0.01. EDDs and Washouts did not differ at any timepoint.

Timepoints

Independent sample *t*-tests with adjusted Levene's test degrees of freedom were performed for each of the individual measures to determine the earliest timepoints prior to the Final Evaluation in which significant differences between groups emerged. The only measures in which group differences emerged at the 3-month

timepoint were *Focus*, *Work Effort,* and *Surfaces,* with VWDs scoring significantly higher than Washouts on each, $ps < 0.05$.

At the 6-month timepoint, *Air Scenting* was the only measure in which VWD scored higher than EDD, $p = 0.39$, with no difference between EDDs and Washouts. VWD outperformed Washouts on *Hunt*, $p = 0.02$, *Focus*, $p = 0.005$, *Possession*, $p = 0.03$, *Work Effort*, $p < 0.001$, and Trainability, $p = 0.008$.

At 10 months, VWDs were significantly higher than EDDs on *Hunt*, $p = 0.013$, *Possession*, $p = 0.049$, *Independence*, $p = 0.026$, and *Trainability*, $p = 0.002$, and significantly higher than Washouts on every measure except *Possession*, *Excitability*, and *Air Scenting*.

Sex Effects

Significantly more VWDs were male (61%) than female (39%), as confirmed by a chi-squared test of independence, $X^2 (1, N = 92) = 4.35$, $p = 0.037$. Conversely, significantly more EDDs were female (80%) than male (20%), $X^2 (1, N = 25) = 0$, $p = 0.003$, and no sex differences were found for the Washout group.

Separate two-way repeated-measures ANOVAs with adjusted Greenhouse–Geisser degrees of freedom were performed for each measure to determine the effect of sex (male, female), timepoint (3 mo, 6 mo, 10 mo, Final Evaluation) and their interaction. A main effect of sex was found for *Hunt*, $F(1, 128) = 4.48$, $p = 0.036$, *Visual Startle*, $F(1, 121) = 8.86$, $p = 0.003$, and *Trainability* $F(1, 131) = 4.541$, $p = 0.035$, with males scoring higher than females. Additionally, significant interactions of Sex and Timepoint for *Hunt*, $F(2.67, 342.43) = 4.78$, $p = 0.005$, and *Trainability*, $F(2.216, 290.361) = 3.18$, $p = 0.038$, were found. Interactions between Sex and Timepoint, but no main effect of Sex, were found for *Focus*, $F(2.63, 339.63) = 3.01$, $p = 0.037$, *Possession*, $F(2.67, 235.47) = 4.07$, $p = 0.017$, *Air Scenting*, $F(2.48, 247.86) = 5.10$, $p = 0.004$, and *Excitability*, $F(2.44, 207.77) = 4.07$, $p = 0.013$.

DISCUSSION

The demand for dogs capable of performing increasingly specialized and challenging detection tasks is high. While dogs have been selectively bred for a variety of working tasks such as guarding, herding, and hunting for hundreds of years, the detector dog is a relatively modern development for which there has not been concerted and protracted selective breeding (3). The importance of canine detection technology in protecting against current and emerging threats establishes strong precedence for identifying, defining, and measuring behavioral characteristics in order to refine and advance canine detection capabilities.

In this study, we identified a number of behavioral characteristics that differentiate specialty VWDs suitable for detecting body-worn moving targets from standard EDDs and dogs unsuitable for service. The resulting analyses across multiple measures making up three evaluative domains, Performance, Environmental, and Trainability, provides a partial description of the VWD behavioral phenotype. Dogs were evaluated on 14 measures: seven Performance measures (characteristics related to detection and searching abilities); six Environmental measures (responses and reactions to novel and varying stimuli); and one overall Trainability measure.

Overall Findings

Our findings further confirm the importance of behavioral characteristics as important factors in working dog suitability (1, 2, 5–9, 16–18). Analyses of individual behavioral measures suggest that, compared to standard EDDs, a number of characteristics and the degree of their expression appear to define the VWD behavioral phenotype. Furthermore, differences in search-related performance characteristics appeared to be more important than differences in environmental soundness in differentiating between VWDs and EDDs.

The partial picture of the behavioral phenotype of a VWD that emerges from the analyses of the evaluations of CPS dogs includes the following characteristics: high expression in the Performance and Trainability domains but no aggregate difference in the Environmental domain as compared to EDDs. In particular, within the Performance domain, VWDs appear to express higher overall levels of *Hunt*, *Focus*, *Possession*, *Independence*, and *Work Effort*, but not *Retrieve* and *Air Scenting* as compared to EDDs. However, VWDs did exhibit higher levels of *Air Scenting* at an earlier age than EDDs. At 10 months, VWDs also appeared to have greater environmental soundness in response to *Surfaces*, *People*, *Vehicles & Urban Clutter* and *Acoustic* and *Visual Startle* than Washout dogs.

A notable pattern emerging from our findings was that the majority of the Performance-related measures differentiated the VWDs from both other groups, but EDDs did not differ from Washouts in this domain. Many performance characteristics, which predominantly relate to searching and hunting behaviors, have been described in the literature as important for detector dogs. For example, detector dog handlers surveyed on their opinions of important detector dog traits identified "acuity of sense of smell" and the "tendency to hunt by smell alone" among the most important (3). Not surprisingly, then, we found that VWDs scored significantly higher on *Hunt* than both EDDs and Washouts. Interestingly, *Hunt* did not differentiate EDDs from Washouts. A likely reason for the lack of difference between EDDs and Washouts on this and all Performance measures is that the majority of Washout dogs failed due to Environmental reasons, and thus may have exhibited adequate performance-related characteristics.

Our finding that *Focus* differentiated between VWDs and EDDs is also consistent with previous reports identifying "ease of distraction" and "tendency to be distracted" as undesirable traits for working dogs (3). Sinn et al. (7) described "object focus" as an underlying dimension of military working dogs' performance which included physical possession of objects, reflecting our finding regarding the importance of *Possession* for VWDs. Similarly, *Independence* differentiated between VWDs and EDDs, which has been commonly reported as a critical trait in a detector dog's ability to work autonomously and not be influenced by human cueing or biasing (1, 14, 19). Dogs that are less dependent on a familiar human have also been shown to be more successful and persistent in problem-solving scenarios (20).

Perhaps the trait most widely recognized as important for detector dogs relates to an overall desire for work and is often referred to as "drive" (1, 8, 21). For example, Maejima et al. (8) found that the principal factor "Desire for Work" was associated

with successful completion of training in candidate drug detection dogs. Rocznik et al. (2) also reported that operational detection dog handlers ranked search drive, the general drive to search for a hidden object, as one of the top performance characteristics for operational conditions. The incentive to search for objects out of sight is considered critical to dogs' motivation to continue searching in strenuous conditions and contexts where the rate of encountering targets is low, as is often the case in operational contexts (1, 3, 22, 23). Consistent with this literature, *Work Effort* was a determining factor between VWDs and EDDs in our population.

Our finding that *Retrieve* did not significantly differ between groups mirrors handler rankings of this trait among the least important (3). Rocznik et al. (2) found that "chase retrieve," the desire to pursue and pick up a thrown toy, to be marginally important to working dog handlers of different breeds. However, Slabbert and Odendaal (17) found retrieval to be an early predictor of police dog suitability. One possibility for this discrepancy may be due to breed. Dogs studied by Slabbert and Odendaal (17) were all German shepherds, whereas our study used retrievers. Given that retrievers have been bred for their propensity to retrieve objects, this trait may not vary considerably within the breed minimizing differences between individual dogs. However, our finding that a significant difference emerged at the final evaluation for *Retrieve* despite an overall effect suggests that puppy development and training may enhance this behavior in high-performing dogs.

A distinctly different pattern emerged for the Environmental domain in that VWDs did not differ from EDDs on any of these measures. While VWDs scored significantly higher than Washouts on most, EDDs only differed from Washouts on *Visual Startle* and *Acoustic Startle*. Notably, Washouts were more likely to have failed due to Environmental than Performance reasons. These findings are not surprising as fearful reactions, including reactivity to noise and novel stimuli, are widely considered undesirable traits for working dogs (24). The ability to appropriately react to, and cope with, stressful stimuli such as a variety of sights, sounds, smells, and textures, are critical for detection dogs who must work under varying conditions (1). Thus, it is likely that an environmental soundness capability threshold exists for dogs to become a detection dog of any kind, driving the lack of difference between VWDs and EDDs.

The only Environmental characteristic that did not differ between any of the groups was *Excitability*, which is found to have conflicting reports in the literature. Some instances ranked excitability lower for handler importance (3), while others rated it as one of the top measures for search team performance for operational conditions (2). Likely, the importance of excitability is operationally specific as multiple types of dog teams were evaluated in these studies. Also, as with all comparisons between such studies, definitions of the evaluative terms may differ.

Finally, Trainability scores significantly differed between VWDs and EDDs, but not between EDDs and Washouts. Trainability has been defined as the ability and speed of learning new tasks and is widely recognized as an important trait for detector dogs (1). The importance of this measure is obviously critical to a dog's ability to learn numerous odor discriminations, corresponding behavioral responses, search patterns, and certain operational skills in as few trials and with as little direction as possible. Highly trainable dogs will reduce time and costs of training programs to produce high-quality detection dogs.

Timepoints

VWDs were consistently highest across all four evaluation timepoints for all three domains. While VWDs showed a general increasing pattern across time in Performance and Trainability domains, EDDs and Washouts did not. Furthermore, VWDs exhibit a jump in scores for the three domains between 10 months and Final Evaluation, which coincides with the final training period, while EDDs and Washouts decrease during this time. This would suggest that the pressure imposed during final training may enhance the performance of the VWDs, while "breaking" less suitable dogs. Moreover, VWDs and EDDs Environmental scores appear generally stable over time, which likely indicates that these environmental characteristics may be more genetically determined and less influenced by experience. Washouts, however, appear to deteriorate over time on Environmental measures, with a sharp drop from 6 to 10 months. This period reflects the transition from the prison program back to CPS, which may represent a stressful event for less environmentally sound dogs. Alternatively, or perhaps in combination with, this may reflect a critical period of development which has been suggested to increase fear and awareness between 6 and 9 months (17). Evidently, service-capable dogs are better able to withstand transitions between locations. As described by Rooney et al. (24), some dogs are apparently more resilient while others are more susceptible to the same environmental disturbances.

Of significant interest to the working dog industry is the value of predicting dogs' performance from an early age (17). Therefore, we also determined the earliest evaluation timepoints in which individual behavioral measures were predictive of success. The only measures in which groups differed at the 3-month timepoint were *Focus*, *Surfaces*, and *Work Effort*, in which VWDs scored higher than Washouts. At 6 months, VWDs differed from EDDs only in *Air Scenting*, but scored higher than Washouts on several other measures. Though the predictive value of early puppy tests has been questioned due to the uncertainty of the extent of environmental influence (6), "drive" or desire for work has been regarded as an innate trait that is difficult to manipulate. The finding that VWDs differed from Washouts as early as 3 months in our study may suggest a genetic basis for these particular measures. The predictability of early puppy tests may therefore only be valuable for traits with a stronger genetic basis and low susceptibility to experience. Some studies have reported high heritability of particular traits including human-directed social behavior (25), which could affect a working dogs' focus and distractibility. Fearful behavior has also been reported to be heritable; however, without explicit genetic controls, the presence of a particular behavior cannot be determined to be inherited or environmentally influenced (24).

Few studies have reported reliable prediction of adult behavior from puppy tests and results have been mixed (26–28). Goddard and Beilharz (29) determined fearfulness was highly heritable among guide dogs and found that behavioral assessments as

early as 12 weeks predicted fearfulness, with predictability strengthening at 6 months. However, evaluations of acoustic and visual startle in our study were not performed at 3 and 6 months due to the risk of creating lasting negative associations during testing (14), and so we cannot determine whether these traits may have emerged earlier. By 10 months, VWDs were significantly higher than EDDs on *Hunt*, *Possession*, *Independence*, and *Trainability*. Whether our evaluations were not sensitive enough to capture differences at earlier ages, or differences emerge due to maturity, development, training, or some combination, is not presently clear.

Sex Differences

A sex difference was found in our population in which significantly more VWDs were male and significantly more EDDs were female. Though this may be partially attributed to a selection bias in the industry for males (1), further analyses of sex effects of individual traits revealed that overall, males scored higher than females on *Hunt*, *Visual Startle*, and *Trainability*, which may have contributed to overall performance. Although such differences could be affected by the bias of CPS evaluators, there is no evidence that fewer females than males were presented as candidate VWDs for sale and subject to the customer's independent assessment. There remains the possibility that CPS employees working with young dogs are biased in the ways in which they interact with male and female dogs. However, the difference in male and female dogs may be an inherent difference in the expression of characteristics related to success as a VWD similar to biologically based sex differences seen in the expression of certain traits, such as aggression and cooperative behavior, across many species (30, 31).

In an analysis of sex differences in behavioral characteristics, Hart and Hart (32) found that males scored higher in activity levels than females. One possibility is that general activity levels may drive differences in traits related to motor activity such as *Hunt*. On the other hand, the same study also found that females ranked higher in Trainability, which is opposite to our findings. Importantly, only gonadectomized dogs were included in their study, whereas dogs in our population were left intact until point of sale; thus, inconsistencies in sex effects may be due to neuter status, which is thought to alter behavioral characteristics (32). In fact, effects of neutering on trainability have been suggested for some breeds including working dogs, indicating potential hormonal influences on this particular trait (9, 33).

Other studies have also reported effects of sex on behavioral differences specific to working dogs, though findings have been inconsistent. For example, Wilsson and Sundgren (9) found that for Labrador retrievers, "defense drive" and "hardiness" scores were higher for males than females, but females scored higher on "ability to cooperate." Wilsson and Sundgren (28) also found increased motor activity and independence in female puppies than males. For some traits in our study, sex differences were dependent on timepoint with females scoring higher early on but lower toward the end of training, including *Focus, Possession, Air scenting,* and *Excitability*. Dogs in our population were still maturing throughout the duration of training, so males and females may have been differentially affected by developmental changes that coincided with evaluation timepoints. Another possibility reflects findings that female dogs score higher on human-directed social behavior and seek more physical contact from humans compared to male dogs, which may hinder female dogs' working performance due to handler dependency (25).

Overall Success of the Breeding Program

The overall success rate of the program, indicated by percentage of dogs sold as VWDs (63%) and EDDs (17%), exceeds previous reports of working dog program success rates of 30% or less (8, 9). One could argue that the overall program success rate is 80% (i.e., VW 63% + EDD 17%). VW is the standard to which dogs are bred in this program, but any dog born and raised in the program that had a final disposition of VWD or EDD can be considered a success. It should be noted that our reported success rate refers to dogs that were medically sound and does not reflect medical releases, though the number of dogs disqualified for health reasons was low ($n = 11$). Future discoveries in behavioral characterization, puppy development, and genomics may assist in elevating the success rate of detector dog breeding programs. These discoveries will help focus resources, increase the efficiency and economics of program operation, and produce adequate amounts of highly specialized dogs to detect specific targets.

Limitations and Future Directions

Though common practice in the working dog literature, the subjective nature of behavioral evaluations is a limitation of the current study. While the aim was to have at least two independent evaluators present at each observation, this was not always possible and for practical reasons the number of evaluators and their familiarity with the dogs may have been a limiting factor. Furthermore, progress in examining characteristics across larger populations of dogs is muted by discordant definitions and procedures for scoring commonly labeled characteristics (i.e., *hunt*, *possession*, *focus*, *trainability*, *acoustic startle*, etc.) between programs. Future research should be directed at developing more objective measures of behavioral traits in order to triangulate metrics. One promising area is the use of genomics to identify genetic markers associated with behavioral phenotypes of successful detector dogs (8). Another recently advancing technology that may shed light on the neural mechanisms of behavior is the neuroimaging of awake, unrestrained canines (34). For example, Berns et al. (35) recently demonstrated the use of fMRI for predicting suitability as a service dog, and investigations by Auburn University's multidisciplinary *Canine fMRI Team* have shown that canine brain response to trained odors (36) and brain connectivity patterns and their strengths are related to behavioral assessments of working dog performance (37).

Comparisons between VWD, EDD, and Washout dogs at each timepoint were conducted in order to determine whether differences between groups emerged at early ages. Early prediction of such differences would allow for the efficiency of redirecting dogs unlikely to be successful as detector dogs to other purposes at an early age. Although we found some measures to differentiate successful vs. unsuccessful candidates as early as 3 months, the relative contributions of inherited characteristics, maturation, and past experiences cannot be isolated.

It should also be acknowledged that our use of final disposition at point of sale as group determination may not necessarily be a reliable indication of continued long-term service. Though many studies have used program outcome as classification of success for working dogs, few have followed up to determine the longevity of such classifications. One study with guide dogs found low retention 1 year after program graduation, with significantly more dogs successfully completing their training program than were still working 1 year later (38). Though we did not obtain data on long-term success of dogs in our program, the 30-day post-sale window in which vendors were able to return dogs increases the validity of our endpoint, to some extent, compared to sale status at the completion of training alone. Future studies should track the continued success of working dogs well into their service.

We have recently begun collecting measurements of the following additional behavioral characteristics that we believe may help further refine the VWD phenotype (not included in the current sample). *Engagement*: Extent to which a dog is eager to please and involve the handler in its execution of a directed task, remaining involved in the game and returning rewards to handler to engage in play. This characteristic has been added because we have produced some dogs with an extreme propensity to search for odors but with very low interest in a reward object or interaction with a handler, which interferes with the handler's management of the working of the dog. *Hypervigilance*: Excessive attention to the environment due to apprehension of potential threats—exhibits anxiety/fear, repeatedly scans environment, overly responsive and cowers in response to mild-moderate visual and auditory stimuli. *Distractibility*: Extent to which ongoing searching is interrupted by attention and/or attraction (not fearful or anxious) to objects, people, or other activities occurring in the environment—execution of task easily or frequently interrupted by ancillary events in surroundings, differentiated from "focus," the measurement of which is mostly related to attending to reward or immediate presence of odor, by measurement during operational style searches. Additionally, age-appropriate acoustic and visual startle tests have been adapted for 3- and 6-month-old puppies in order to examine how such reactivity may predict the environmental soundness and/or success earlier than 10 months of age for VWDs and EDDs.

CONCLUSION

Search-related performance traits appear to be critical factors that elevate a detector dog from standard EDD suitability to VWD quality. On the other hand, certain traits related to environmental soundness appear to be important for a detector dog of any kind, differentiating both VWDs and EDDs from Washouts. Since 2013, CPS has produced 63% VWDs and an additional 17% EDDs from its breeding program suggesting that selective pressure has amplified behavioral characteristics that support VWD and EDD performance.

This work represents the first examination of the expression behavioral characteristics related to the success of *Vapor Wake*® detection, a specialized application for detecting body-worn or hand-carried explosives in settings with large volumes of moving persons, such as large event venues and mass transit. As such, this study is also one of the first to identify specific characteristics for any specialized detector dog application. The specialization and sophistication of detector dog applications is necessarily increasing to meet modern security and safety requirements. Identifying the characteristics associated with success in the performance of specialized detector dog applications will be critical to producing the necessary numbers and quality of dogs to fulfill future security and safety needs.

Identification, measurement, and validation of the contribution of particular behavioral characteristics to performing the VW task is vital to driving selective breeding and possible future genotyping for continual improvement of dogs for this task. Such phenotyping efforts support the tailored design of detector dogs for specialized applications, which are becoming more prevalent in response to the need for enhanced uses of dogs in security and safety operations. Although this present work is specific to VWDs, whether within or outside of the CPS breeding cohort, it is an example of a more general strategy to enhance the identification and production of dogs for specialized applications. If refined and practiced on a large scale, it could be envisioned that populations of purpose-bred dogs with highly defined behavioral phenotypes and identified genetic markers for particular characteristics might exist from which to build evermore technically competent detector dogs for specialized applications.

ETHICS STATEMENT

Dog care and use activities were approved and monitored by the Auburn University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

LL designed the study, analyzed data, and primarily prepared the manuscript. PH conducted selective breeding activities, collected data, and contributed to design of the study. PH, JB, TF, BR, and PW designed the evaluative instruments. JB, TF, and BR performed evaluations. PH and PW contributed to the preparation of the manuscript. CA, JK, and PW supervised the design of the study and preparation of the manuscript. JK supervised analyses of the data.

ACKNOWLEDGMENTS

We thank the CPS nursery attendant, Kristina Dattilo, CPS canine instructors for puppy development and training, Samantha Greene, Janie Woodyard, Laura Callaway, and Tanner Hough, and CPS kennel and dog health and welfare manager, Holli Thompson, for their excellent care and development of CPS dogs. We are very grateful to the inmates and program managers of the Auburn dog raising programs at our partnered correctional institutions for their skill and dedication in the

loving care and preparation of CPS dogs for detector dog training. We also thank all the veterinarians and clinicians at the AUCVM for their expertise and services related to canine medicine. Finally, Dr. Jim Floyd and Dr. Eldin Leighton provide significant leadership and technical expertise that support our breeding program.

FUNDING

The operation of the breeding program was supported by the sale of detector dogs and funding from the Auburn University College of Veterinary Medicine. The completion of the study was made possible by a gift from Walt and Ginger Woltosz.

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Conflict of Interest Statement: PW and JB are among the inventors of VW technology who receive a portion of the royalties collected by Auburn University upon the sale of VWDs by the licensee of VW technology. Otherwise, the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

REVIEW
published: 29 March 2018
doi: 10.3389/fvets.2018.00056

When the Nose Doesn't Know: Canine Olfactory Function Associated With Health, Management, and Potential Links to Microbiota

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The impact of health, management, and microbiota on olfactory function in canines has not been examined in review. The most important characteristic of the detection canine is its sense of smell. Olfactory receptors are primarily located on the ethmoturbinates of the nasal cavity. The vomeronasal organ is an additional site of odor detection that detects chemical signals that stimulate behavioral and/or physiological changes. Recent advances in the genetics of olfaction suggest that genetic changes, along with the unique anatomy and airflow of the canine nose, are responsible for the macrosmia of the species. Inflammation, alterations in blood flow and hydration, and systemic diseases alter olfaction and may impact working efficiency of detection canines. The scientific literature contains abundant information on the potential impact of pharmaceuticals on olfaction in humans, but only steroids, antibiotics, and anesthetic agents have been studied in the canine. Physical stressors including exercise, lack of conditioning, and high ambient temperature impact olfaction directly or indirectly in the canine. Dietary fat content, amount of food per meal, and timing of meals have been demonstrated to impact olfaction in mice and dogs. Gastrointestinal (GI) microbiota likely impacts olfaction via bidirectional communication between the GI tract and brain, and the microbiota is impacted by exercise, diet, and stress. The objective of this literature review is to discuss the specific effects of health, management, and microbiota shifts on olfactory performance in working canines.

Keywords: working canine, canine management, canine olfaction, canine performance, canine microbiota

OPEN ACCESS

Edited by:
Cynthia M. Otto, University of Pennsylvania, United States

Reviewed by:
Mitsuaki Ohta, Tokyo University of Agriculture, Japan
Federica Pirrone, Università degli Studi di Milano, Italy

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Specialty section:
This article was submitted to Veterinary Humanities and Social Sciences, a section of the journal Frontiers in Veterinary Science

Received: *28 November 2017*
Accepted: *05 March 2018*
Published: *29 March 2018*

Citation:
Jenkins EK, DeChant MT and Perry EB (2018) When the Nose Doesn't Know: Canine Olfactory Function Associated With Health, Management, and Potential Links to Microbiota. Front. Vet. Sci. 5:56. doi: 10.3389/fvets.2018.00056

INTRODUCTION

The extraordinary olfactory capability of the canine has long been used by humans for odor identification and discrimination (1). The canine's capacity for odor detection has been reported to be as much as 10,000–100,000 times that of the average human, and the canine lower limit of detectability for volatile organic compounds is one part per trillion (ppt) (2). This heightened sense gives canines the ability to detect a vast number of chemical compounds containing molecules that display subtle differences in stereoisomeric structures (3). This sensitivity, the unique capability to

detect a target odor among a myriad of odors in an operational environment (4), and the ability of the dog to learn by operant conditioning (5) has made the working canine an intrinsic component of law enforcement, military, search and rescue, medical and assistance/service functions worldwide. However, despite the critical nature of the service that our canine partners provide, evidence related to olfaction health and performance is underrepresented in the scientific literature. The objective of this review is to discuss the effects of management decisions related to diet and physical conditioning, medical care, and microbiota shifts on olfaction performance in working canines.

HEALTH AND DISEASE

Anatomy of Olfaction

To properly manage the health of the detection dog, one must understand the anatomy and physiology associated with olfaction. The major components of the olfactory system are the nasal cavity, olfactory epithelium and receptors, the vomeronasal organ (VNO), and the olfactory bulb. The nasal cavity is comprised of two chambers separated by the nasal septum, which are highly vascularized, primarily supplied by the sphenopalatine artery. Each nasal cavity chamber contains three turbinates (nasoturbinate, maxilloturbinate, and ethmoturbinate) (6) that contribute to increased mucosal surface area. However, total mucosal surface area may be heavily influenced by muzzle size and shape in the canine (7). Nasal turbinates project from the lateral chamber walls and contain a network of tortuous veins. Medial and dorsal to the turbinates is the olfactory cleft, where 5–15% of inhaled air is diverted, and multiple cranial nerves terminate. As inhalation occurs, air first reaches the maxilloturbinate where there are a small number of olfactory sensory neurons. Air continues to flow into the ethmoturbinates and paranasal sinuses and is then directed toward the pharynx (6). Engorgement of turbinates alters airflow into the olfactory cleft, affecting olfaction. Turbinate engorgement is reduced by exercise, hypercapnia, and increased sympathetic tone, whereas it is increased by cold air, chemical irritants, hypocapnia, and increased parasympathetic tone. Some airborne odorants/chemicals can stimulate trigeminal free nerve endings in the nasal mucosa, which cause sensations like warmth, coolness, sharpness, but not odor (8). The detection of odor occurs only through the olfactory epithelium and olfactory nerves.

The olfactory epithelium is comprised of neurepithelium lining the cribriform plate, dorsal septum, dorsal and middle turbinates, and pseudostratified columnar epithelium, with millions of olfactory receptor (OR) cells (ORC). Olfactory epithelium also contains supporting sustentacular cells that regulate the composition of nasal mucous, serve as insulators between ORCs, and protect the epithelium from damage from inhaled agents (9). The mucous layer of the nasal mucosa is derived from Bowman's glands embedded in the olfactory epithelium; this mucous layer maintains normal nasal humidity levels and traps odorants (10). Normal olfactory perception depends on this moist receptor area (9).

Olfactory receptor cells project directly to the olfactory bulb, with axons terminating in the glomeruli of the olfactory bulb (11). The ORCs have cilia that have surface odor receptors; human ORC have approximately 25 cilia per ORC, but dogs have hundreds of cilia per ORC, permitting the detection of significantly smaller concentrations of odorants in canines. There are more than 220 million ORs in the canine nasal cavity, which allow a vast number of odorants to bind (12). There is only one type of OR per ORC, and odor intensity is proportional to the number of ORC activated; ORC also have receptors for hormones and neurotransmitters. Olfactory neurons only live for 30–60 days, but unlike other mammalian sensory cells, ORCs constantly regenerate (13). The number and type of ORCs present in an individual dog are dictated by breed, genetics and training (7, 14–17); this concept will be explored later in the manuscript.

Embedded in the membrane of ORC cilia are extracellular portions which bind odorant, and intracellular portions coupled to G-protein. When an odorant binds the extracellular portion of the receptor, the G-protein A-subunit breaks away, activating adenyl cyclase, which subsequently converts ATP to cAMP. cAMP amplifies the incoming signal from the odorant by activating multiple sodium gated channels (11). The two-step opening of gated sodium channels causes depolarization, and the resultant action potential is transmitted through the olfactory bulb. Each odorant is recognized by a unique combination of activated ORs (18). The ability of the detection dog to properly recognize odors relies on this function.

The VNO lies along the ventrorostral aspect of the nasal septum, is bilaterally symmetrical, and acts as an additional site of odor detection (19). The VNO sensory neurons detect chemical signals that stimulate behavioral and/or physiological changes (20), provides alternate neuronal pathway to the hypothalamus, and is very slow to adapt to odors. The VNO contains both receptor epithelium and non-receptor epithelium, which differ structurally in the types of nerve fibers and types of embedded cells (21). The VNO functions in the detection of non-volatile odorants, especially pheromones, and is believed to play a role in social behavior and reproduction (21).

The olfactory bulb is a paired structure, which functions primarily as a relay station, and to filter sensory input (6). There are approximately 1,000 ORC axons per second-order neuron, resulting in significant amplification of the odor signal. The mitral cells of the olfactory bulb project one primary dendrite to one glomerulus, and one axon to the olfactory cortex. The olfactory bulb is located under the frontal lobes, above the cribriform plate in humans, but is located more rostrally in other mammals, which may play a role in improved smell in lower mammals (19). The olfactory cortex is located within the medial temporal lobes and communicates directly with cerebral cortex. The olfactory cortex functions to receive sensory input from the olfactory bulb, permit conscious awareness of odor, identification of odor, odor memory, and odor localization in lower mammals. The olfactory bulb has both a sensory role (initial processing of olfactory information) and a modulatory role in the forebrain, hypothalamus, and limbic system (22). The olfactory pathway of canines is demonstrated in **Figures 1** and **2**.

The olfactory cerebral areas of the brain are divided into two functional categories: the neocortical (e.g., orbitofrontal complex)

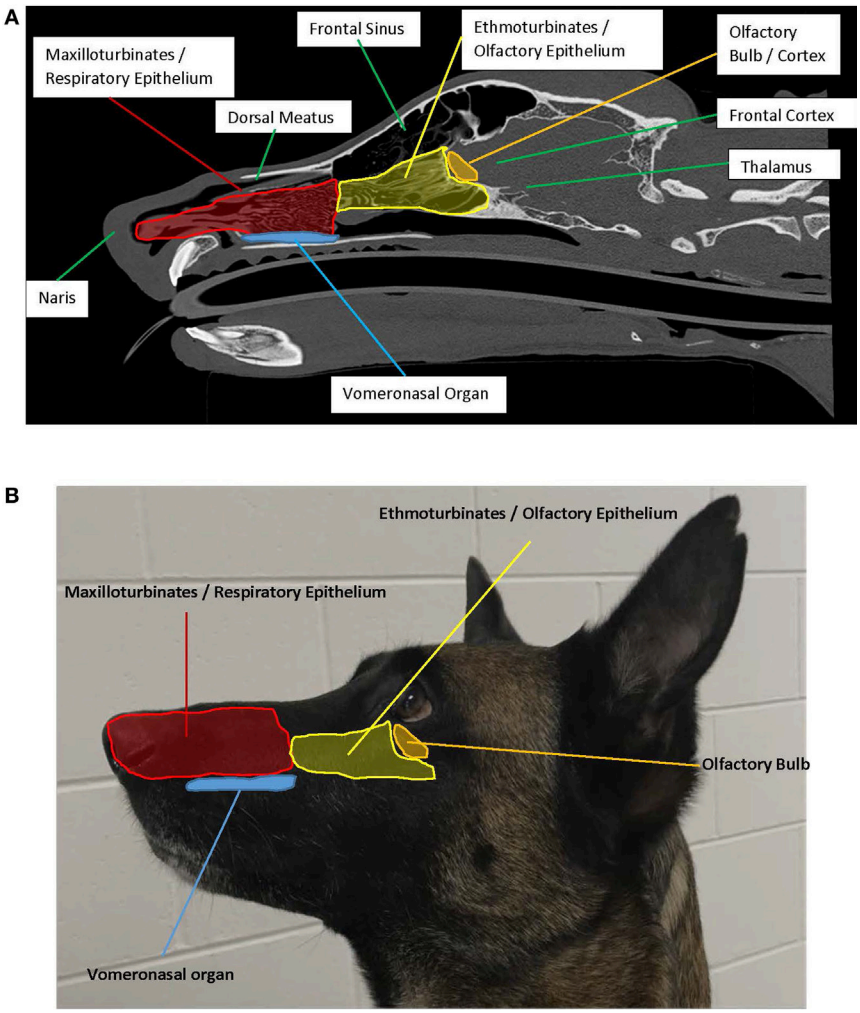

FIGURE 1 | **(A)** Left sagittal plane highlighting the anatomy associated with olfaction. Photo credit: Adrien-Maxence Hespel, University of Tennessee. **(B)** Left exterior view demonstrating placement of interior structures associated with olfaction.

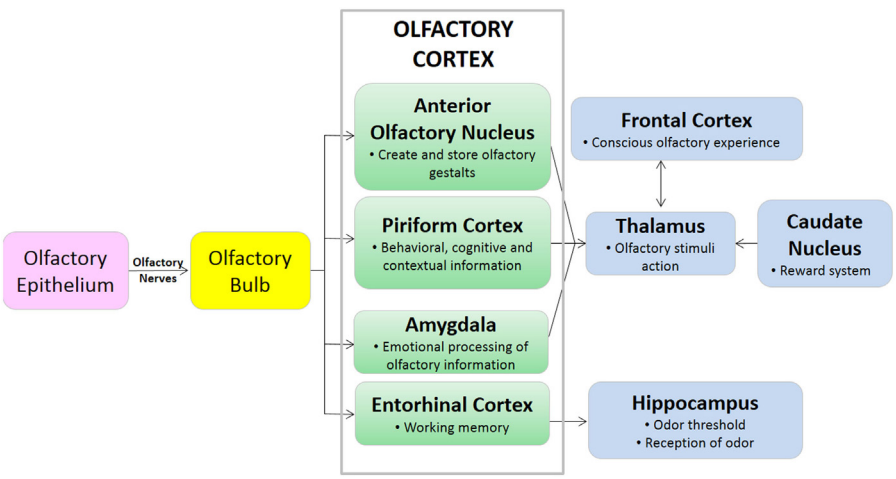

FIGURE 2 | Diagram of pathway demonstrating olfactory signaling process.

which regulates conscious odor perception, and the limbic (23). The limbic system is a collection of brain structures that collectively regulate olfaction, memory, behavior, and motivation. Components of the limbic system include the olfactory bulb, hippocampus, amygdala, and entorhinal complex, among others. The size and function of the limbic system varies across mammalian species, but in all species the limbic system has olfactory and non-olfactory components (24). The isocortex of the brain regulates higher-order functions such as sensory perception and cognition. While primates including humans have an inverse relationship between isocortex and limbic system volume, terrestrial carnivores including canines have high relative volumes of both the isocortex and limbic systems (24). These anatomical differences in brain component volumes may be partially responsible for the differences in olfactory capability between humans and canines.

Physiology of Olfaction

Compared to humans, dogs have significantly larger surface area of olfactory epithelium, with approximately 30% more ORs that can recognize a much larger variety of odorants. Dogs also have the capability for excellent odor localization, even in presence of significant background odor, likely due to the larger nasal cavity size as compared to other species (25) and the unique airflow patterns created by sniffing (26). The ability to find the source of the scent, even in the presence of competing odors, makes the detection dog a critical partner in many military, law enforcement, and search and rescue operations.

During inspiration, 12–13% of air flow travels to the olfactory portion of the nose, and the remaining airflow is directed toward the nasopharynx where it exits the nasal cavity (26). Dogs have improved airflow sampling and odorant collection via active sniffing, which is the production of short, sharp breaths at 4–7 Hz, independent of canine body size (26). The average dog inhales 30 ml of air per nostril per sniff (19), and air is inhaled from the front and exhaled to the side as seen in **Figure 3**; this permits more efficient sampling of odorants. When a canine is sniffing, air within approximately 1 cm of the nostril is drawn toward the naris (26), and the high velocity air flow is transported to the dorsal nasal cavity where it turns 180° and flows back over the ethmoturbinates. Each nostril samples air separately, yielding bilateral odor samples that assist in odor source localization (26). In contrast to humans and other microsmotic species, air does not enter or exit the olfactory recess of the dog during expiration, resulting in prolonged exposure of inspired air to the chemoreceptors of the olfactory epithelium and continued olfactory stimulation throughout the respiratory cycle (26). For the working canine, active sniffing during "nose down, tail up" searching (see **Figure 4**) and efficient localization of odor source are critical to completion of the mission.

Environmental conditions, such as relative humidity and barometric pressure can have direct impacts on olfaction, in addition to the impacts those factors have on the generation and movement of odor itself. Philpott et al. (27) reported that olfactory thresholds in humans were independent of room temperature, peak humidity and peak inspiratory nasal flow. A subsequent, larger study reported by Kuehn et al. (28) subsequently determined that olfactory threshold level was impaired in hypobaric conditions, and olfactory thresholds were lower (sense of smell improved) in a humid environment. Search and rescue dogs perform better when relative humidity is high (29), potentially due to improved nasal humidity and odorant trapping. Humidity, but not rain, increased the efficiency of dogs in tracking and searching tasks by increasing odor intensity (30), and improved olfactory detection of pheromones, leading to increased mating activity during monsoon season (31).

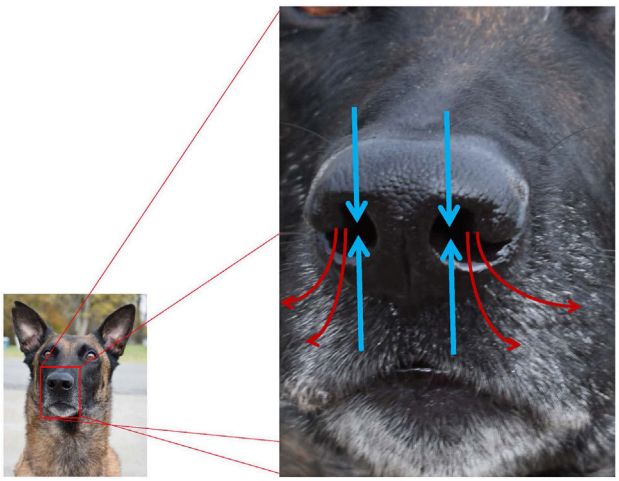

FIGURE 3 | Pathways for inhalation and exhalation and airflow associated with olfaction (blue arrows = inhaled; red arrows = exhaled). Canines preferentially use the right nostril to sniff conspecific arousal odors and novel odors, and the left nostril to sniff familiar odors, non-aversive stimuli, and heterospecific arousal odors.

Sniffing is advantageous compared to normal inhalation because it produces unidirectional laminar flow to the dorsal meatus and sensory epithelium of the ethmoturbinates (26, 32), increases the sensitivity to odors (32), drives activity in the olfactory cortex, and affects odorant intensity and identification (33). Nasal airflow patterns as described by Craven et al. (26) enhance olfactory acuity in the dog, but do not fully explain macrosmia, the enhanced ability to smell, in the canine. Lawson et al. (34) described the transport of specific types of odorants and the subsequent impact on olfaction. Odorant deposition patterns correspond to the anatomical organization of OR neurons: highly soluble odorants are deposited in the front of the olfactory cleft (dorsal meatus and nasal septum), whereas moderately soluble or insoluble odorants are deposited throughout the entire olfactory cleft (34). This combination of anatomical organization of OR neurons and airflow patterns induced during sniffing are likely responsible for the macrosmia widely demonstrated in working canines. Canines move more slowly and the period of sniffing lasts three times longer during the deciding phase of olfactory tracking (the "find"), as compared to the initial search phase and tracking phases (35). Concha et al. (36) demonstrated that sniffing patterns in working canines can be used to differentiate true negative from false negative responses. Trained scent detection dogs spent significantly less

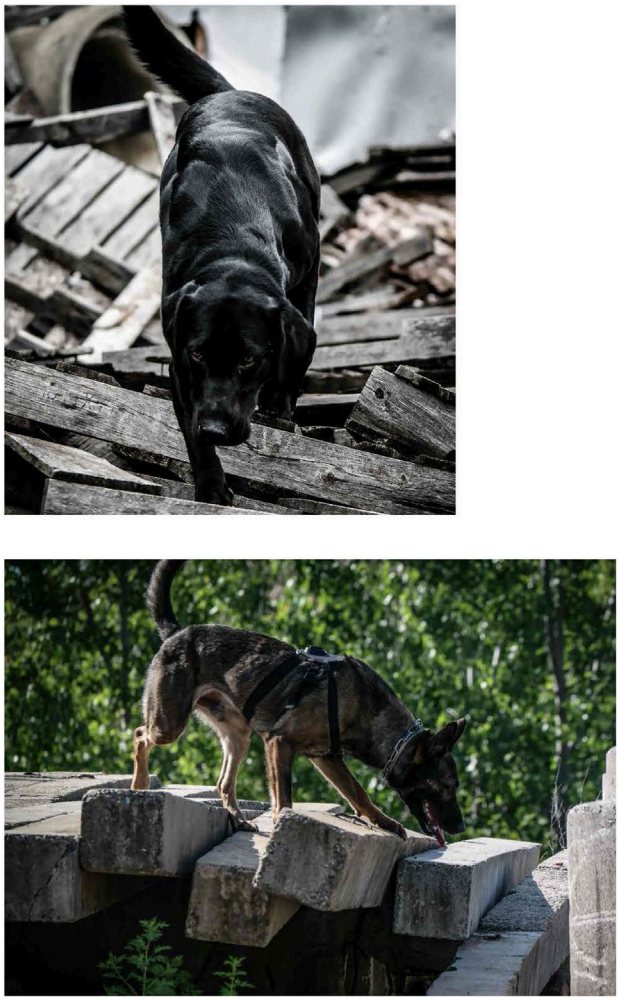

FIGURE 4 | Disaster canines performing search work displaying the typical "nose down, tail up" posture associated with active olfaction. Photo credit to Tracy Darling.

time sniffing true negative samples (no odor; no alert response), with only one sniffing episode for true negative samples (36). For detection dogs, these sniffing characteristics may result in more efficient detection work during a lengthy work cycle.

Dogs have repeatedly demonstrated "hemispheric specialization," that is hemisphere specific brain processing of emotional, acoustic, and olfactory stimuli (37, 38). Unlike other senses, olfactory pathways ascend from the point of detection (nasal cavity) to the point of perception in the brain (olfactory cortex) ipsilaterally: right nostril sensory input is delivered to the right brain hemisphere, and left nostril sensory input is delivered to the left hemisphere (37). Canines preferentially use the right nostril to sniff conspecific arousal odors and novel odors, delivering sensory input to the right brain hemisphere, which processes threatening and alarming stimuli. Canines preferentially use the left nostril to sniff familiar odors and non-aversive stimuli such as food, as well as heterospecific arousal odors (such as human fear-induced sweat samples) (37). D'Aniello et al. demonstrated that hemispheric specialization and chemosignaling enhances communication of emotional states (including stress) between dogs and humans (39). For detection dogs, this likely means that target odors are being processed through the left nostril.

Anatomical connections between the olfactory pathways of the amygdala and piriform cortex and the limbic system underlie the interconnection between olfaction and memory (9). Olfaction and other forms of learning/memory are regulated by the same neurobiological rules (40). In working canines, memory of smell is of critical importance: when does odor memory begin? How many odors can canines remember? How long do canines remember trained odors? How long can a dog maintain olfactory performance without training? Canines learn odor starting in the prenatal period, due to the influence of maternal diet on the composition of the amniotic fluid (41), but the learned odor memory appears to dissipate by 10 weeks of age (42). Olfaction and cognition have both been demonstrated to deteriorate with age in the canine, but no specific age exists at which the dog ceases to learn (40). Williams and Johnston (43) determined that canines could readily learn and subsequently identify 10 odors in a search task. Given that domestic canines have demonstrated the ability to learn and remember more than 200 words (44) and the names of more than 2,000 toys (45), it is likely that working canines can remember far more than 10 odors. The durability of memory on trained odors has not been extensively studied, but Johnston (46) demonstrated that in explosive detection canines there was no systematic deterioration in detection performance for up to 4 months. Training methods can impact durability of odor memory, or at least the signaling from canine to handler when a specific odor is detected. If alerts are not reinforced, or if the canine conducts several searches without detecting a trained odor, the alert or search behavior can be extinguished (47). It is unclear, however, if memory is maintained when alert or search behavior is extinguished or what the maximum duration of time is that a canine can maintain odor memory without training (47).

Genetics are increasingly recognized as a critical component of olfaction in canines, with a comprehensive review published elsewhere (7). There are four types of receptors involved with olfaction and chemosensation in the dog: OR, vomeronasal receptors, trace amine-associated receptors, and formyl peptide receptor-like proteins. Most research on the genetics canine olfaction has been focused on OR genes. The canine OR repertoire is composed of 1,094 genes, approximately three times more than a human. This large genetic repertoire is believed to be related to the macrosmia evident in canines, producing an expansive array of ORs that permit the detection of broad ranges of odorant (48). In the canine, approximately 20% of OR genes are functionally inactive pseudogenes, but the percentage of pseudogenes varies by breed, and is significantly lower than microsmotic species like humans, in which as much as 50% of olfactory genes are pseudogenes (49). Polymorphism of OR genes may also impact olfactory capability and sensitivity in breeds and individuals. Tacher et al. (15) reported that both the percentage of pseudogenes and the frequency of specific gene polymorphism varied by breed, and speculated that genetic changes may contribute to differences in

olfactory capabilities between breeds and individuals. This may offer some insight into the "working lines" within some breeds that tend to produce higher frequencies of successful detection canines than others.

The current literature contains conflicting information about breed-specific olfactory capacity. Jezierski et al. (50) demonstrated that German Shepherds were significantly better at detecting narcotics than Labradors and Terriers. In contrast, Hall et al. (5) reported that Pugs consistently outperformed German Shepherds and Greyhounds in olfactory acquisition and discrimination tasks. Polgar et al. (51) reported that "scent-group" dogs (e.g., basset hound, German pointer, etc.) performed better on a natural detection task than "non-scent" dogs (e.g., English greyhound, Afghan hound, etc.) and "short-nosed" dogs (e.g., Cavalier King Charles spaniel, Boston terrier, etc.). Additional research is needed to determine if breed specific olfactory capabilities are correlated with genetic polymorphism or if olfactory performance is more a function of behavioral attributes like inherent motivation (i.e., drive) and trainability.

Hyposmia: Disease and Medication

Hyposmia, defined as decreased sensation of smell, is characterized as type I, II, or III. Type I hyposmia is the inability to recognize odorants correctly. Type II hyposmia is a quantitative decrease in the ability to recognize odorants, recognized in working canines as change in threshold or persistent failure to alert to previously trained odorants. Type III hyposmia is a decrease in estimation of the magnitude of odors; this type of hyposmia is only recognizable in humans. The causes of hyposmia can be broadly categorized as conductive disorders, sensory losses, or neural causes (52). Conductive hyposmia results from the failure of odorants to reach the olfactory mucosa, e.g., nasal inflammation, excess mucous production, and physical obstruction by space-occupying masses (polyps, neoplasia, etc.) (53). Sensory hyposmia is caused by damage to the olfactory mucosa, e.g., viruses, toxic chemical or industrial agent exposure, and neoplasia (54–56). Neural hyposmia is caused by lesions of the central or peripheral nervous system, specifically the olfactory cortex, olfactory bulb, and cranial nerves I (olfactory) and V (trigeminal), e.g., head injury (57).

Disease

The impact of disease on olfaction has been widely documented in human medicine. In fact, "degradation in the sense of smell is a sentinel condition, particularly for neurodegenerative diseases such as Alzheimer's" (58). Conditions associated with hyposmia or anosmia in humans include congenital disorders (e.g., Kallaman's syndrome), endocrine or metabolic disorders, infections, inflammation, neurologic disorders including head trauma, local processes, occupational exposure to dust and toxic chemicals and materials, advanced age, and uremia (8, 57, 59–61). Hyposmia and anosmia are frequently self-reported in human medicine, but self-reporting may overrepresent the actual prevalence of hyposmia in humans. The prevalence of hyposmia in canines is unknown, but hyposmia in detection canines could be catastrophic.

When a canine is exposed to prolonged, increased body temperatures due to environmental or exertional extremes without the ability to acclimate properly, thermal injury may occur (62). Thermoregulation is compromised during heat stress; the canine increases respiratory rate (i.e., panting) and heart rate to compensate and cool the body. Panting decreases olfactory efficiency in canines and may lead to relevant hyposmia. Exogenous factors that may contribute to thermal stress and increase the likelihood of compromising olfaction include lack of acclimation to a novel environment, elevated humidity, lack of access to water, and poor ventilation (63). Heat injury likely leads to olfactory compromise, but the magnitude and duration of altered olfaction is unknown; further study is indicated.

As in humans, disease may impair olfaction in canines. Although there is limited canine research available, viral infection with canine distemper (64) and canine parainfluenza virus infections (65) have caused alterations in olfaction. Canine parainfluenza virus increased nasal inflammation and mucous secretions, causing a conductive hyposmia by reducing the contact between odorants and olfactory or trigeminal receptors in the nasal cavity. In addition, nasal inflammation, like that caused by canine distemper or parainfluenza viruses lead to vascular congestion in the respiratory mucosa, altering air flow patterns in the nasal cavity (65). Endocrine disease (e.g., hyperadrenocorticism, diabetes mellitus, and hypothyroidism) and neurologic disease (e.g., granulomatous meningoencephalitis and nasal tumors) have also been reported to cause hyposmia in canines (55); the exact mechanism of hyposmia in these disease states is not known but is likely neural. Recently, vomeronasalitis was associated with intraspecific aggression in cats (66). Asproni theorized that the inflammation present in the VNO and nasal cavity impaired sensory epithelium function and intraspecific communication but did not examine olfactory function in the studied cats. Given our understanding of the VNO and nasal physiology, it is likely that vomeronasalitis causes both sensory hyposmia and disrupted intraspecific communication in cats, and possibly in dogs. Trauma is a well-documented cause of neural hyposmia in people, but the impact of head trauma on olfaction in dogs has not yet been studied. If a detection canine experiences head trauma associated with lack of consciousness, evaluation of olfaction is indicated (67). Olfactory function diminishes with age in humans through a variety of mechanisms including altered nasal engorgement, cumulative damage to the olfactory epithelium, decreased mucosal enzymes, loss of selectivity of ORCs and neurodegenerative disease (68). Similar age-related changes were found in the olfactory system of dogs older than 14 years and were prominent in dogs over the age of 17 years (69). The older dogs had a decrease in number of ORCs and the number of cilia on ORCs. Interestingly, the older dogs demonstrated senile brain changes such as cerebrovascular amyloidosis in the olfactory bulb, but not in the olfactory mucosa. Disease-induced, but not age-induced, hyposmia in humans is generally reversible, possibly because olfactory neurons regenerate readily, but the duration of hyposmia and normalization of function cannot be predicted (54, 68); this is also likely true in canines.

Pharmaceuticals

Type II hyposmia is common in humans during or after pharmaceutical therapy (70); the hyposmia is usually bilateral and temporary. The list of pharmaceuticals known to induce hyposmia in humans is long, including: anesthetics, antiarrhythmics, antihistamines, antimicrobials, antiproliferative and immunosuppressive drugs, endocrine drugs, gastrointestinal (GI) drugs, neurologic drugs, and NSAIDs (8, 57, 59). Pharmaceuticals frequently cause hyposmia through impairment of odorant binding to the OR or injury to the OR (sensory hyposmia), or through neurologic impairment (neural hyposmia).

Most relevant information on pharmaceuticals impacting canine olfaction is extrapolated from human medicine. Zinc metabolism is directly related to olfaction function in both humans and laboratory animals. Zinc nanoparticles, when added to explosives, enhanced the odorant response in trained canines in a dose-dependent manner (70). Zinc chelation, however, causes sensory hyposmia at the OR level. Some cardiovascular drugs such as angiotensin-converting enzyme inhibitors (ACE-I) (e.g., captopril) chelate zinc and cause hyposmia in humans (8); this effect has not been studied in canines. Anesthetics are documented to cause hyposmia in humans; the impact on olfaction in canines is presently being researched at Auburn University.

Antimicrobials such as metronidazole and doxycycline are commonly prescribed to working canines to treat diarrhea and vector-borne diseases, respectively. Metronidazole has been reported to cause hyposmia in humans (8) and to decrease olfaction performance in detection canines (71). Doxycycline has been reported to cause hyposmia in humans (60) but does not cause hyposmia in detection canines (71). Jenkins et al. noted that 50% of trained explosive detection dogs demonstrated an elevation in olfaction threshold when administered high-dose metronidazole for 10 days, but doxycycline administration at standard doses for 10 days did not impact olfaction. Metronidazole-induced hyposmia could not be predicted based on male or female sex, neuter status, or age but hyposmia was temporary, as olfaction threshold returned to normal within 10 days of discontinuation of metronidazole. Alternative medical interventions should be considered when appropriate prior to the use of metronidazole for detection dogs; if metronidazole must be used, it should be used at the lowest efficacious dose for the shortest duration possible.

Steroids can cause hyposmia in humans (8) and in canines (72). Ezeh administered high doses of dexamethasone or hydrocortisone combined with deoxycorticosterone to laboratory dogs and noted hyposmia without apparent clinical signs after 7 and 18 days of treatment, respectively. The noted steroid-induced hyposmia in dogs was attributed to elevation in the olfactory detection threshold. However, studies of humans with nasal inflammation demonstrated that the administration of oral and/or intranasal steroids sometimes improved olfaction, likely due to the resolution of nasal inflammation (73–75). Thus, veterinarians and canine handlers should carefully weigh the clinical need for steroids against the potential effects on olfaction. The mechanisms of pharmaceutical-induced hyposmia include impairment of odorant binding through altered mucus quantity or quality (e.g., antihistamines), inhibition of normal turnover/regeneration of olfactory neurons (e.g., steroids and chemotherapeutics), nasal vasoconstriction (e.g., decongestants), enzyme-associated effects of drugs (ACE-I), altered levels of cyclic GMP (phosphodiesterase blockers), and zinc chelation (cardiac medications) (76).

Given the paucity of research on pharmaceutical-induced hyposmia in canines, handlers, trainers and veterinarians caring for detection dogs should exercise caution with pharmaceuticals known to cause hyposmia in humans. It is also important to consider which medications may be biotransformed by the GI microbiota when discussing medical care for working canines. Information on reduction, hydrolytic and other chemical reactions for commonly prescribed medications and their associated impacts on microbiota and olfaction should be considered. Olfaction threshold and discrimination ability should be tested in any detection dog that has been treated with hyposmia-inducing pharmaceuticals prior to return to work.

MANAGEMENT

There is a myriad of factors that can improve or compromise the performance of working canines. Frequency, intensity, and duration of work cycles should be considered prior to making management decisions particularly as pertains to olfactory acuity.

TABLE 1 | Categories of working canines and typical disciplines associated with each.

Sport	Detection	Service
Nose work[a]	Explosives[a]	Guide
Field trial/hunt test[a]	Narcotics[a]	Hearing
Agility	Search and rescue[a]	Mobility assistance
Flyball	Medical[a] (cancer, research)	Emotional support
Rally	Pest[a]	PTSD
Barn hunt[a]	Arson[a]	Allergen detection[a]
Sled dogs	Conservation[a]	Medical[a] (diabetes, seizure)
Obedience[a]	Invasive species[a]	Therapy
Conformation	Agriculture[a]	
Dock jumping	Patrol/apprehension[a]	
Lure coursing	Currency[a]	
Protection sports[a]	Prison (mobile phone)[a]	
Rally	Tracking/trailing[a]	
Herding sports	Firearm[a]	
Tracking[a]		
Weight pulling		

Factors to consider in the management of working canines	
Duration	Length of work cycle—# of hours spent performing work Example: agility course takes minutes to complete vs. guide dog working during all waking hours
Frequency	Incidence of work—# of times called to perform work Example: daily missions (law enforcement) vs. "on call as needed" (disaster)
Intensity	Energy exerted performing work—this should include physical as well as mental energy needed complete assigned task Example: patrol dog released to apprehend suspect vs. border patrol dog screening vehicles as they move through checkpoint

[a]*An olfactory component associated with job function.*

Detection dogs (explosives, narcotics, search, and rescue) are different than sport dogs (agility, hunting, sled) and are measured with very different performance criteria (see **Table 1**).

Conditions that can alter a dog's working potential include breeding and selection, regular fitness and conditioning, and the development of a dietary regimen that meets the nutrient requirements and utilizes quality ingredients. Maximizing olfactory function should be paramount in decisions regarding detection dogs. A summary of selected publications associated with working canine performance is presented in **Table 2**.

Conditioning and Training

As one might expect, training and physiological conditioning significantly impact olfactory performance. Decreased find rates using certified detection dogs on scent wheels have been reported following exercise (78); this is likely explained by the increased panting that typically occurs following exercise. Canines that were physically conditioned maintained greater olfactory acuity compared to canines that were not physically conditioned when both groups were challenged with exercise. Non-conditioned canines displayed a 63.6% decrease in olfactory sensitivity following exercise (78). Physically conditioned canines have a lower exercising heart rate compared to their non-conditioned counterparts and this improved cardiovascular condition may contribute to better thermoregulatory performance and subsequently decrease the need for panting (32). Other supporting work has shown that a rigorous training program leads to high frequencies of correct target alerts (32). Immediately following extreme physical exercise, there is a reduction in the sniffing rate and increased panting rate which result in reduced olfaction performance (32). This may be explained by the fact that non-conditioned canines pant harder during intense exercise instead of breathing through their nose, which decreases the quantity of odorants passing over olfactory epithelium in the nasal cavity (77). It seems clear that physical conditioning (specifically as pertains to minimizing panting) may support improved olfaction in the detection dog.

TABLE 2 | Summary of selected studies reporting effects on olfaction/performance associated with management or medical care.

Citation	Treatment	Classification	Duration	Olfaction response
(77)	Exercise and fat supplement	*n* = 18 Hunting	12 weeks	Coconut oil decreased olfactory acuity in non-conditioned dogs Exercise decreased olfactory acuity in non-conditioned dogs
(78)	Exercise and fat supplement	*n* = 17 Explosive detection	12 weeks	Corn oil increased olfactory acuity Exercise decreased olfactory acuity
(79)	Quail hunting and dietary protein	*n* = 23 Hunting	11 months	Animal-based protein increased olfactory acuity
(80)	Hunting and dietary fatty acids	*n* = 23 Hunting	12 months	EPA, DPA, DHA increased olfactory acuity
(72)	Steroids	*n* = 24; companion	28 days	Dexamethasone or hydrocortisone + DOCA decreased olfactory acuity
(32)	Exercise and panting	*n* = 6 Explosive detection	20 min treadmill	Olfaction and panting display inverse relationship
(47)	Conditioned odorant	*n* = 10 Companion	Odor condition 7 days	Conditioned odorant increased olfaction sensitivity
(81)	Handler–canine interaction	*n* = 60 Companion	3 months	No handler influence
(71)	Metronidazole	*n* = 18 Explosive detection	10 days	Degradation of detection threshold for 9 canines
(50)	Odor detection scenarios; novel environment; training	*n* = 164 Narcotics detection	Unknown	Final stage of training decrease olfactory acuity Known and novel environment similar olfactory acuity
(82)	Scent detection (live find and human remains)	*n* = 11 live find *n* = 12 cross-trained	Unknown	Cross trained canines compromised on alerting live scent when cadaver scent present
(1)	High intensity training	*n* = 13 Shepherd breeds	5 days per week; 18–20 months	High olfaction sensitivity and specificity
(65)	Canine parainfluenza virus (CPI virus)	*n* = 10 Companion	3 weeks	CPI virus prevented contact of odoriferous substances with olfactory receptors
(83)	Helicopter travel	*n* = 9 FEMA search and rescue	30 min helicopter travel	No effect on search performance or gut microbiota
(84)	Novel and known odorants	*n* = 21 Explosive detection	6 weeks	Decreased target performance with no exposure prior to scenario
(85)	Commercial air travel	*n* = 6 FEMA search and rescue	2.5 h air travel	No effect on search performance in spite of change to gut microbiota and fecal scores
(86)	Handler–canine interaction	*n* = 5 Military	10 days	Elevated handler anxiety improved canine target detection

Scent detection training techniques can also directly impact olfaction. Wang et al. (16) and Youngentob and Kent (17) demonstrated that dogs develop more ORs for odorants on which they are regularly trained. Gerritsen and Hank (14) also reported that ORC cell turnover is not static: new replacement ORC type is triggered by familiar scents. Simple odorants and complex odorants induce different neural responses in scent detection dogs. Wilson and Stevenson (87) theorized that cortical synaptic plasticity is enhanced by experience with odorants (simple or complex) in a variety of conditions. Gerritsen and Hank (14) further suggested that dogs will learn complex odors more rapidly if they are first trained on individual components of the odor, but results vary across studies. Fischer-Tenhagen et al. (88) found that detection dogs trained with mixtures of odor containing the target odor had more correct indications when the target odor was tested in a new context, than dogs trained on a pure reference odor. These data provide scientific evidence for the traditional training concept of "proofing" detection dogs with the use of distraction items. Functional MRI of the olfactory system in trained scent dogs indicated that odor concentration impacts brain activation: low odor concentration resulted in unilateral brain activation, whereas high odor concentration resulted in bilateral brain activation (58). In addition to odor type and frequency, training techniques impact olfaction sensitivity and discrimination. Pavlovian conditioning significantly improved odor acquisition (89) and improved resistance to disruptors (90). Continuous reward systems worked best for acquiring a behavior such as learning to discriminate a specific odor, and intermittent rewards worked best for maintaining a learned behavior (40). More research is needed to determine the impact of training simple versus complex odor, the impact of odor concentration on learning, and the interaction of genetics and training on performance in detection dogs.

Hydration

Management of the detection dog in the field may often involve mitigation of dehydration and fatigue. Dehydration of the nasal mucosal membrane results in decreased enzyme activity and decreased membrane fluidity, altering neurosignal transduction and odorant receptor function. A combination of decreased airflow and dehydration of the mucosal layer can significantly decrease odor detection capabilities in the working canine (77). Dehydration in search-and-rescue canines was reported to occur in dogs working after the terrorist attacks on 9/11 (91, 92), the Haiti earthquake (93), and the Washington landslide (94). One recent study examined three intervention strategies for hydration of canines (95). Border patrol vehicle inspection canines were utilized (high frequency, low intensity searches) to investigate the benefits of water, oral electrolyte solution, or subcutaneous fluids for rehydration of canines working in hot conditions. The authors reported no clear benefits for any of the strategies examined but did note that voluntary consumption of the flavored oral electrolyte solution was higher as compared to water alone. Increased voluntary fluid consumption contributed to improved hydration. No benefits associated with the use of subcutaneous fluids were noted. On the contrary, hydration with subcutaneous fluids was associated with an increase in creatinine that was noted to indicate either dehydration or potential muscle damage. No information on dietary regimens was provided by the authors and behaviors recorded were not affected by hydration strategy. Olfaction as a measure of performance could not be quantified; standardized olfaction testing was not possible because of the operational nature of the field study. This study demonstrates that dehydration in the field is a concern which warrants more investigation especially when considered in relation to potential olfactory challenges.

Thermal recovery was enhanced when using a low protein diet top dressed with corn oil in Labradors exercised on treadmills (96). The authors reported lower core body temperatures 10 and 20 min following exercise and lower rectal temperatures in dogs fed a maintenance diet topped with corn oil as compared to dogs consuming the performance ration without corn oil. Olfaction acuity was not measured in this study. Conversely, hunting find rates in English Pointers improved in dogs fed a higher protein, higher fat (31:21%) diet, as compared to a diet containing lower protein and fat (26:17%) (79). Thermal recovery was not investigated. Factors associated with fatigue were not reported in either study. Extrapolation across studies is challenging due to the difference in methods, ingredients and parameters measured but thermal recovery and olfactory impact should be weighed heavily in decisions regarding diets for detection dogs.

Nutrient Content

The nutritional requirements for canine athletes have previously been examined in review (97). Mullis et al. (98) examined the maintenance energy requirements specific to detection dogs and reported that they were approximately twice the known resting energy requirement (RER = 70 kcal × $BW^{0.75\ kg}$). The authors noted no differences in energy requirements across breed, age, or gender, but did report a significant effect associated with number of searches performed. This is particularly interesting because the work performed by these dogs simply required that they be active and alert; it was not reported as physically strenuous. Findings in these dogs suggest that there may be an unexplained energy requirement associated with the mental focus/attention required by working canines. Duration, frequency, and intensity of work likely all impact energy requirements for the working canine. The impact of surgical sterilization on olfaction is unknown, but spaying of racing Greyhound bitches produced no change in overall performance, motivation, or racing speed (99).

Exercise and diet seem to be inextricably linked to canine performance, but there are few studies examining the relationship between these elements of detection dog management. English Pointers withheld from exercise and fed a diet supplemented with coconut oil appeared to experience compromised olfaction, but exercised dogs maintained olfactory acuity (77). The authors reported greater olfactory sensitivity for all exercised dogs regardless of dietary fat source (beef tallow; beef tallow + corn oil; beef tallow + coconut oil). Angle et al. (78) demonstrated benefits to olfaction when using corn oil supplemented diets and exercise.

The improved olfaction observed with increased polyunsaturated fatty acid (PUFA) content in the diet has also been reported in rodent studies (100). Rodent studies have also been used to measure olfactory sensitivity associated with nutritional status and have reported improved olfaction associated with fasting

(101, 102) and compromised olfaction as a result of satiety (101, 103). These findings are believed to be linked to the appetite inducing hormone ghrelin, which contributes to exploratory and sniffing behavior and improves olfactory sensitivity (104). This critically important work demonstrates a potential link between fasting and improved performance in the detection dogs. Anecdotal reports from seasoned trainers have often included recommendations for letting the dogs work hungry; these data may provide evidence for this long-standing canine training technique. Food has been documented to be a more effective reward than praise or petting but has not been compared for effectiveness against toys (105). Hall et al. (90) reported inconsistent responses for dogs offered presession feeding when odor discrimination tests were conducted. For detection disciplines requiring dogs to work independent of the handler (disaster, explosives), use of fasting to improve exploratory and sniffing behavior may be a useful training tool to examine. Further study is needed to determine the appropriate diet titration to maximize olfaction, the length of fasting time necessary, and the potential impacts on olfaction performance.

Diet and Behavior

The relationship between diet and behavior has been well studied in other species, but few studies have examined the relationship between diet and behavior in canines (106). Docosahexaenoic acid (DHA) is necessary for optimal neurological development in puppies, and lower concentrations of DHA have been associated with aggression in German Shepherd dogs (40). PUFAs are essential to membrane function and control of oxidative stress, especially in the hippocampus of the brain, the area responsible for associative learning (40). Hennessy et al. (107) reported a reduction in adrenocorticotropic hormone upon exposure to novel stimulus for those fed a premium (44% animal-based protein) diet as compared to those fed a maintenance diet (17% animal-based protein). Other studies have shown a reduction in territorial aggression in client-owned dogs fed a lower protein diet (106–109). DeNapoli et al. (110) reported that low protein diets with supplementary tryptophan reduced aggression in dogs. Sechi et al. (111) utilized a dietary intervention strategy of nutraceutical supplementation (including tryptophan) in dogs with behavior disorders. They reported a subsequent increase in serotonin, dopamine, and β-endorphins indicating reduced aggression, and reduced plasma cortisol and noradrenaline indicating reduced markers of stress. These studies offer a glimpse into the potential application of dietary manipulation for stress and aggression management. The need for working canines to operate without aggression in stressful environments warrants further research in this area. However, reduction of dietary protein could be a dangerous undertaking and this topic would require extensive research prior to the use of this mitigation strategy for dogs in the field.

MICROBIOTA

Understanding the Microbes

The GI microbial community is a complex ecosystem containing bacteria, fungi, archea, and protozoa. Improvements in molecular techniques such as next generation sequencing have increased our study and subsequently our understanding of both the composition and function of the GI microflora. However, there remains a great many unanswered questions regarding the impacts associated with changes in the microbiota on the overall health and performance of the working canine.

As more studies are published highlighting changes in the GI microbiota, it is increasingly important to understand how those changes are measured and how that data is presented (112–115). Microflora, microbiota, and microbiome are all words that seem to permeate the discussion in many scientific communities. Microflora is a term that refers to the collective community (fungi, archaea, protozoa, bacteria) in question. Bacteria are referred to as the "microbiota." Studies referencing the term microbiome are generally describing the genome of the microbiota and typically include information about by-products of fermentation (VFA's, pH, etc.) as well as genetic information about the community constituents (116).

Microbiota studies are typically visually presented to answer taxonomy-related questions such as (1) How many and which microbial communities are present? (2) What is the diversity of the population? Taxonomic diversity is generally represented using alpha and beta diversity. Alpha diversity (diversity within a given sample) is typically represented as a rarefaction curve and describes evenness and richness of a given sample (117). Rare microbial species are more likely to be missing from small samples, therefore, richness is an important factor to consider for small data sets. Alternatively, beta diversity (diversity between samples) is used to measure taxonomic similarity based on phylogenetic distance (118). Beta diversity also provides a visual assessment of the abundance (weighted) or presence (unweighted) of given taxa and is represented using a PCoA plot. Other techniques for visual depiction of data include heat maps or hierarchical cluster analysis.

Although a comprehensive discussion on the procedures associated with microbial sequencing is beyond the scope of this work, it is important to understand that primer selection and target region of the 16s RNA gene are critical (119, 120) and can cause significant variation in the results and subsequent interpretation of data generated. These techniques are culture-independent and have allowed researchers to greatly improve our understanding of GI microbiology. Data are highly impacted by several factors including sequencing techniques, primers, selection of correct hypervariable region and others. Inconsistent approaches used in many studies published have made it extremely difficult to make comparisons across data sets and continue to challenge interpretation.

Traditional culture-dependent techniques (i.e., Sanger sequencing) have allowed researchers to investigate the presence of specific pathogens and are useful to identify species commonly associated with GI disease such as *Salmonella, Campylobacter jejuni*, or *Clostridium perfringens*. However, these techniques are limited in their applicability as compared to currently molecular methods (i.e., next generation sequencing) that make taxonomic identification and metagenomics applications easier (121). The comprehensive characterization and community identification required for microbial profiling of the GI tract requires the more

sensitive techniques associated with next generation sequencing and has become the accepted standard for microbial studies.

Microbial Balance

The GI microbial ecosystem harbors significantly different communities within each compartment (122, 123). Predominant phyla in working canines are similar to other monogastric species and are typically dominated by Firmicutes and Bacteroidetes. The characterization of the collective GI microbial community and associated function is beyond the scope of this work and has been previously reported elsewhere (114, 122, 123). Resident bacterial groups within the GI tract play an intrinsic part in the regulation of homeostasis; their role in the regulation of the host innate immunity has been well described (124–126). The microbiota comprises part of the intestinal lumen barrier, contributing to the protection of the GI ecosystem via competition for nutrients and adhesion sites and by secreting compounds thought to inhibit the colonization of non-resident microbes (127). This may explain why puppies are generally more at risk for GI disease associated with pathogens such as *C. jejuni* as their bacterial profile may not yet be fully mature enough to provide sufficient protection or deterrence (128).

Microbial community structure variation between individuals is consistently present (129). Age, breed, and gender have all been shown to affect the microbial profile across multiple species (130–132). Cohabitation of humans and dogs has also been shown to impact the microbial community (133). The authors concluded that the factors affecting microbial homeostasis are not the same for the oral and GI communities as compared to the skin community. These data suggest that GI changes are related to other, heretofore, unknown factors. These reported variations must be considered when evaluating published microbial data. Studies including dogs across several age groups, breeds, and with both genders should be analyzed accordingly to account for the variation associated with those factors.

Microbial Imbalance

While it is relatively easy to predict the factors that will affect the microbiota (age, gender, breed, antibiotic use, travel), it is slightly more difficult to predict the associated impacts to the dog. Current evidence suggests that alterations in the GI microbial community can fundamentally alter the structure and function of the GI lumen; this has been termed "leaky gut syndrome" with prior review elsewhere (134). This condition describes the physical changes to the intestinal lumen associated with changes in the microbial profile and is particularly concerning because of the potential for immunological disruption and bacterial translocation resulting in endotoxemia. By-products of healthy microbial fermentation, specifically short chain fatty acids (SCFA's), are thought to provide energy for the host and contribute to the mediation between the microbial ecosystem and activation of the immune system (135).

High levels of bacterial diversity are generally associated with good health; diminishing diversity has consistently been reported with negative health outcomes in humans such as obesity, diabetes, and GI disease (136). Reductions in the phyla Firmicutes and Bacteroidetes, which are typically dominant, along with concomitant increases in Proteobacteria have been reported in dogs diagnosed with chronic GI inflammatory disease (137). Minamoto et al. (138) demonstrated slightly different microbial impacts but that may be due to the variation inherent with different techniques, breeds and ages of dogs sampled. Development of a dysbiosis index (DI) has offered a diagnostic tool to categorize microbial data into a simple ratio reflecting normal microbiota ($DI < 0$) or microbiota indicative of chronic enteropathies ($DI > 0$) (139). Unfortunately, the use of this index requires laboratory testing and is limited by its very small initial data set. However, the concept provides an important step in the direction of assessing fecal samples diagnostically with recommendations for treatment and dietary interventions.

The bidirectional communication that occurs between the brain and gut (microbiota–gut–brain axis) provides some insight into the dysbiosis that has been reported as a result of environmental stress (140). Stress associated with travel, change in environment, and physical exertion are common in the working canine. Changes in the fecal microbiota of working canines following in-cabin transport via commercial airline resulted in an impact on both abundance and type of bacteria and were accompanied by a poorer fecal score (85). Conversely, when researchers examined the effects of helicopter travel stress in working canines the relatively short nature of the stressor (hot-loading and 30 min of flight) did not result in any effect on the microbiota (83). Notably, both studies reported no effect on performance as determined by total search time or previously identified stress behaviors. The duration and type of travel required to induce microbial dysbiosis has not been examined in working canines.

Dietary Modification of the Microbiota

While studies in dogs are limited, some data have shown promising results for microbial manipulation through the use of different fiber supplements on microbial community and resulting SCFA production (112, 113, 132, 141–144).

Researchers examining the use of fructooligosaccharides reported improved production of butyrate, a volatile fatty acid beneficial to colonocyte and epithelial cell repair, as well as reductions in *C. perfringens,* a potentially pathogenic microbe. A second study yielded similar results along with increased numbers of bifidobacteria, a potentially beneficial microbe (144). Other work in sled dogs fed a synbiotic (combined pre- and probiotic) reported decreasing incidences of diarrhea (141). If researchers can develop dietary mitigation strategies that consistently reduce or prevent GI distress, this may benefit dogs working in field scenarios with limited access to veterinary intervention. The use of dietary supplements that may mitigate or prevent the onset of GI distress warrants further study.

Diet has long been identified as the dominant factor impacting microbial community structure (112, 113, 144–148). What we don't know is what impact meal size and frequency has on the GI microbiota. Handlers frequently must adjust meal times and sizes for detection dogs throughout the course of a mission. Data in horses has demonstrated an effect on the GI microbiota associated with meal frequency and size (148); it is not known if a similar impact would be observed in the monogastric canine.

Information elucidating potential impacts on the microbiota would be helpful in managing concerns associated with diarrhea in the field.

Microflora and Olfaction

The densely populated microbial niche in the GI tract has been reported to play a key role in the regulation of behavior and brain function. The microbiota–gut–brain axis influences neurotransmission and behavior. It therefore might be the key in nutritional interventions for maintaining brain and olfaction health (149), with early microbial modulation resulting in long-term impacts on stress-related physiology and behavior (150). Given the relatively unexplored nature of the communication occurring between the gut microbiota and the stress response system of the brain, it seems reasonable to question whether alterations of the gut microbiota could play a role in stress reduction as evidenced by the display of stress behaviors in the dog.

The olfactory epithelium has been generally overlooked regarding the potential role of microorganisms on the development and efficiency of odorant transduction. ORs are formed by many G-protein coupled receptor proteins that identify volatile odorant molecules (151). Originally it was thought ORs were only located in the olfactory epithelium. In the GI tract, ORs have been identified in enterochromaffin cells; these receptors can affect the secretion of serotonin in response to fragrant molecules with subsequent effects on GI motility (152). Serotonin also plays a critical role for olfactory information processing as the olfactory bulb is comprised of serotonergic fibers and was recently shown to effectively regulate the flow of olfactory processing in mice (153). Given the link between GI microbiota and serotonin regulation, it seems likely that a relationship exists between the GI microbiota and odorant detection although as yet it is unknown (154).

Nasal microbiota community structure has been linked to olfactory function (155). Human subjects demonstrated differences in microbiota of people assessed for olfactory function with deficiencies related to the presence of butyric-acid producing microbes (155). These findings suggest that the microbial composition of the nasal passage can potentially shape or alter olfactory performance. The implications of altered olfactory performance associated with bacterial fluctuations in the nose are significant. The nasal microbiota of dogs with chronic rhinitis and nasal neoplasia was reported to differ in community structure when compared to healthy dogs (156). Isaiah et al. (157) identified an effect associated with job type on canine nasal microbiota. Even though all dogs were housed in a single facility and fed a single diet, researchers reported differences in alpha diversity for canines that was related to job type (vapor wake, patrol and narcotics, explosives). No differences were reported in beta diversity suggesting that species richness but not bacterial community structure was affected by the work done by dogs in each group (157).

One specific OR (OR51E1) has been detected in pigs along the entire GI tract from the gastric cardia to the rectum (152). OR51E1 colocalizes with an enteroendocrine cell marker all along the GI tract and was expressed in the greatest density in the duodenum. Duodenal enteroendocrine cells are the primary source of gastric inhibitory peptide and cholecystokinin. Duodenal enteroendocrine cells are equipped with multiple receptors connected to sweet and bitter tastes. OR51E1 gene expression in olfactory bulbs has demonstrated feedback mechanisms, differential activation of transcription factors, and epigenetic regulation. Circulating hormones that control food intake and energy balance modulate olfactory epithelium, and the ablation of olfactory sensory neurons in mice protected them from diet-induced obesity (158). There are several factors like age and diet that impact gut luminal microenvironment and the intestinal microbiota modulate OR51E1 gene expression in GI tract tissues (152).

FUTURE DIRECTIONS AND UNANSWERED QUESTIONS

We lack evidence-based data conducted in working canines that will allow us to fully investigate the links between microbiota shifts and any possible performance (i.e., olfaction related) or health sequalae. We know that diet can both change the microbiota and impact olfaction in other species. What we do not yet know is what mechanism (if any) exists that links olfaction with the microbiota. When one considers the unique microbial community harbored by the individual dog, does that explain why olfaction was only compromised in 50% of the dogs who were received metronidazole (71)? Is it possible that the reduction in Firmicutes experienced by dogs receiving metronidazole provides the key to the olfactory challenge they experienced (114)? If olfaction is enhanced as a result of fasting (102) and satiety reduces olfactory performance (103), should we be rethinking the timing of our feeding programs? What impacts will that fasting have on the microbiota of the working canine? The critical impact of the work conducted by these canines requires much deeper understanding of all things that could hinder their job performance. A more thorough investigation of factors associated with microbial changes and associated impacts on job performance (i.e., olfaction) is vital.

AUTHOR CONTRIBUTIONS

MD developed the concept and wrote the first draft of the manuscript. EJ and EP wrote major sections of the document in its current form. All the authors read, edited, and approved the final manuscript.

ACKNOWLEDGMENTS

The authors thank Tracy Darling, RVT, VTS (SAIM), Adrien-Maxence Hespel, DVM, MS, DACVR, Nicholas Jenkins, MAS, MEd, and Kathleen Kelsey, MS, MBA, for assistance in developing figures and tables.

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Conflict of Interest Statement: This review was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 05 June 2018
doi: 10.3389/fvets.2018.00118

Behavior Differences Between Search-and-Rescue and Pet Dogs

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Behavioral traits such as trainability, fearlessness, and energy are required for dogs to succeed as search-and-rescue (SAR) dogs. Certification by the Federal Emergency Management Agency (FEMA) ensures that dogs and handlers have extensive training and have demonstrated specific skills in the field. To determine whether behavioral differences exist between SAR and pet dogs, and between FEMA-certified USAR and non-FEMA-certified SAR dogs, the Canine Behavioral Assessment and Research Questionnaire (C-BARQ) was administered to 129 SAR dogs participating in the post-9/11 medical surveillance study and a breed-matched sample of 2,131 pet dogs. Non-parametric mixed models were fit for each C-BARQ subscale with explanatory variables SAR/non-SAR status, FEMA certification status, breed, sex, neuter status, and age. SAR dogs had higher scores for trainability ($P < 0.001$) and energy ($P < 0.001$), and lower scores for aggression toward strangers ($P < 0.01$), aggression and fear toward dogs ($P < 0.01$), fear of dogs ($P < 0.001$), chasing ($P < 0.001$), fear of strangers ($P < 0.001$), and non-social fear ($P < 0.001$) than pet dogs. FEMA-certification was associated with lower fear of dogs ($P < 0.05$) and separation-related issues ($P < 0.01$) than non-FEMA certified SAR dogs. The traits identified in this study could provide guidance for more efficient selection of candidate SAR dogs and breeding stock.

Keywords: dog, behavior, questionnaire, working dog, trainability, fear, aggression, search and rescue

OPEN ACCESS

Edited by:
Lynette Arnason Hart, University of California, Davis, United States

Reviewed by:
Nathaniel James Hall, Texas Tech University, United States
Erik Wilsson, Swedish Armed Forces, Sweden

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Specialty section:
This article was submitted to Veterinary Humanities and Social Sciences, a section of the journal Frontiers in Veterinary Science

Received: *27 March 2018*
Accepted: *17 May 2018*
Published: *05 June 2018*

Citation:
Hare E, Kelsey KM, Serpell JA and Otto CM (2018) Behavior Differences Between Search-and-Rescue and Pet Dogs. Front. Vet. Sci. 5:118. doi: 10.3389/fvets.2018.00118

INTRODUCTION

Search-and-rescue (SAR) and human remains detector (HRD) dogs are selected and trained for behaviors correlated with success in the field. The United States Federal Emergency Management Agency (FEMA) certification includes "proper command control, agility skills, a focused bark alert to indicate a live find, and a willingness to persist to search for live victims in spite of possible extreme temperatures and animal, food and noise distractions. The canine must also be confident enough to search independently and must be able to negotiate slippery surfaces, balance wobbly objects underneath his feet and go through dark tunnels[1]." A survey of search dog handlers in the UK identified seven priority behaviors: acuity of sense of smell, incentive to find a hidden object, tendency to hunt by smell alone, ability to learn from being rewarded, tendency not to be distracted when searching, consistency of behavior from day to day, and motivation to chase an object (1). In the US, behaviors

[1]https://www.fema.gov/canine-handler-certification

thought to be associated with successful search work are prey drive, hunt drive, and ball drive (2).

Research using behavior questionnaires to study working dogs has been primarily focused on guide and service dogs. The C-BARQ (Canine Behavioral Assessment and Research Questionnaire) is a questionnaire completed by a dog's owner or caretaker. Most of the individual items are grouped into subscales describing a more broad behavioral trait, such as trainability, owner-directed aggression, stranger-directed aggression, rivalry or chasing. A prototype of the C-BARQ was validated in a population of 1,067 Seeing Eye dogs. Puppy-raiser evaluations on the behavior subscales: stranger fear, stranger aggression, non-social fear, owner aggression, dog fear/aggression, and trainability at 12 months of age were predictive of behavioral reasons dogs were released from the training program several months later (3). In a larger, related study of 7,696 dogs from five guide and service dog programs, C-BARQ scores at 6 and 12 months of age for 27 out of a possible 36 temperament traits were significantly different between dogs who successfully completed training and those released for behavioral reasons (4). In a sample of potential military working dogs, high scores on C-BARQ for trainability at 12 months were associated with better performance on standardized behavior test at 17 months, and negatively associated with non-social and stranger-directed fear (5).

After the terrorist attacks of September 11, 2001, between 250 and 300 dogs deployed to the World Trade Center, Fresh Kills Landfill, and the Pentagon (6). The health and behavior of these dogs was under surveillance until 2016, when the final dog responding to the attacks died (6–8). The handlers of the dogs that deployed after these attacks, along with handlers of SAR or HRD dogs who did not deploy to that event completed the C-BARQ. For the present study, C-BARQ test items and scores from pet dogs were also analyzed. The goal of the study was to determine whether behavior differences exist between SAR dogs whose handlers completed the C-BARQ within 1 year after the 9/11 deployment and pet dogs whose owners completed the C-BARQ from May 2005 through May 2010. A secondary goal was to ask whether SAR dogs who had completed training and FEMA certification had different behavior scores than SAR dogs who had not completed FEMA certification.

MATERIALS AND METHODS

Participants

The C-BARQ questionnaire (4, 9) was administered annually to the handlers of 129 SAR dogs as part of their participation in a medical surveillance study of SAR dogs who were either deployed to the World Trade Center, Pentagon, or Staten Island Landfill or served as control SAR dogs (not deployed after the attacks) in the study (6–8). The study year 1 (from September 11, 2001 to September 10, 2002) questionnaire results were utilized in this study.

The C-BARQ questionnaire was also administered to owners of 2,131 pet dogs. Pet dogs were solicited through one of two methods. They either received a mailing because they were clients of the Veterinary Hospital of the University of Pennsylvania or the completed the questionnaire via an online survey that was advertised via an article in the newsmagazine of the Veterinary Hospital of the University of Pennsylvania, USA (http://www.vet.upenn.edu/bellwether/v64/article10.shtml) and by notices sent to Philadelphia-area veterinary clinics and the top 20 USA breed clubs based on AKC registrations. Availability of the survey then spread via word of mouth. Pet dogs were included if their breed was represented in the sample of SAR dogs (**Table 1**). The entire population eligible to be included in this study consisted of 1,179 males (938 neutered and 241 intact) and 1,081 females (916 neutered and 165 intact). C-BARQs were completed between May 2005 and May 2010.

TABLE 1 | Breed distribution of dogs in 9/11 surveillance study and pet dogs.

Breed	SAR dogs	Pet dogs
Airedale Terrier	2	39
Australian Cattle Dog	2	105
Australian Shepherd	4	151
Belgian Malinois	1	26
Border Collie	8	152
Doberman Pinscher	1	135
English Springer Spaniel	1	53
German Shepherd Dog	53	381
German Shorthaired Pointer	1	26
Golden Retriever	13	285
Labrador Retriever	40	612
Rottweiler	3	166

For the SAR dog population, 129 completed CBARQs were included. The majority of dogs were deployed to 9/11 ($n = 86$) whereas 46 SAR dogs were not deployed to 9/11. Eighty-one SAR dogs were FEMA certified or eligible (USAR) and 48 SAR dogs were not affiliated with FEMA. There were 74 male dogs, of which 74% were neutered and 55 female dogs of which 93% were neutered. The median age for SAR dogs was 4 years with a range from 1 to 11. Age was rounded to the nearest whole number in years for further calculations. The entire pet and SAR population consisted of 1,179 males of which 80% were neutered and 1,081 females of which 85% were neutered. The median age was 3 years with a range from 1 to 20 years for both the pet and SAR dogs.

The CBARQ study was approved by the University of Pennsylvania Institutional Animal Care and Use Committee and was exempt from Institutional Review Board approval because personal information was not collected about the dog owners.

Statistical Analysis

Behavior subscales were computed as described in Hsu and Serpell (9). Descriptive statistics are shown in **Table 2**. Cronbach's alpha, a measure of the agreement of the items within each subscale, was computed using the Cronbach function in the "psy" package (10) in the R statistical software package [(11); open source software available at https://www.r-project.org]. Alpha varied from 0.48 to 0.87, with most subscales above 0.70, indicating good agreement between items. The distributions of all subscales failed the Shapiro-Wilk test for normality. Several transformation functions were attempted, however, all the scores except for trainability had positive skewness with many values

TABLE 2 | Descriptive statistics for C-BARQ subscales.

	All Sar dogs					Non-FEMA SAR dogs		FEMA SAR dogs	
Subscale	**Mean**	***SD***	**Skewness**	**Cronbach's Alpha**	**Number of Items**	**Mean**	***SD***	**Mean**	***SD***
Trainability	3.28	0.34	−0.42	0.48	8	3.26	0.35	3.29	0.33
Aggression toward strangers	0.35	0.39	1.72	0.83	10	0.50	0.49	0.27	0.30
Aggression toward owner	0.04	0.16	4.64	0.78	8	0.08	0.25	0.02	0.07
Fear and aggression toward dogs	0.64	0.53	1.31	0.81	8	0.74	0.62	0.59	0.47
Aggression toward dogs	0.91	0.79	1.12	0.87	4	1.04	0.90	0.85	0.71
Fear of dogs	0.39	0.59	1.63	0.87	4	0.51	0.71	0.32	0.50
Dog rivalry	0.53	0.57	2.10	0.74	4	0.70	0.68	0.34	0.47
Chasing	1.25	1.00	−0.21	0.84	4	1.49		1.11	0.89
Fear of strangers	0.12	0.30	2.01	0.82	4	0.18	0.36	0.08	0.25
Non-social fear	0.32	0.38	1.47	0.65	6	0.44	0.47	0.25	0.29
Separation problems	0.30	0.43	1.71	0.78	8	0.43	0.51	0.22	0.36
Touch sensitivity	0.47	0.59	1.61	0.49	4	0.59	0.65	0.40	0.55
Excitability	2.08	0.68	0.003	0.78	6	2.14	0.70	2.04	0.68
Attachment/Attention-seeking	2.00	0.63	0.14	0.65	6	2.23	0.69	1.86	0.55
Energy	2.61	0.81	−0.18	0.74	2	2.55	0.81	2.65	0.81

near 0 and few high values and there was no transformation that made the distributions more normal.

In order to determine whether SAR dogs differed from pet dogs on behavior subscales, and whether there were further differences associated with FEMA certification, non-parametric wmodels were fit to each subscale using the "np" package (12) in R. Non-parametric methods are used when a dependent variable is not normally distributed, and this R package fits models to ordinal dependent variables such as C-BARQ subscales. In addition to SAR and FEMA status, explanatory variables included breed, sex, neuter status, and age. Models were fit using a backward elimination strategy using the "drop1" R function. The first, full model for each subscale contained all explanatory variables. Subsequent refined models contained only variables that were significant at the $P < 0.05$ level. This process resulted in two steps and models for most of the subscales except fear of dogs and separation problems, which required three models. Because differences between means cannot be tested directly using non-parametric models, partial regressions were carried out using the "np" package's "npplot" function to determine the estimated mean values for each category when SAR status and/or FEMA status was found to be a significant factor.

RESULTS

The final model for each C-BARQ subscale is presented in **Table 3**. *P*-values are given for any explanatory variable that was significant at the 0.05 level. Means for SAR and pets, as well as FEMA and non-FEMA certified SAR dogs are provided. SAR dogs had higher scores for trainability ($P < 0.001$) and energy ($P < 0.001$), and lower scores for aggression toward strangers ($P < 0.01$), aggression and fear toward dogs ($P < 0.01$), fear of dogs ($P < 0.001$), chasing ($P < 0.001$), fear of strangers ($P < 0.001$), and non-social fear ($P < 0.001$) than pet dogs. FEMA-certification was associated with lower fear of dogs ($P < 0.05$) and separation-related problems ($P < 0.01$) than non-FEMA certified SAR dogs.

DISCUSSION

This is the first study comparing behavior traits measured by the C-BARQ in working SAR dogs and pet dogs. There have been analyses of behavior in puppies with the goal of using behavior measures to select dogs for work early in life. In a study of Swedish military working German Shepherd Dogs comparing C-BARQ scores with the outcome of a temperament test for acceptance into the program, trainability was significantly higher in dogs that passed the test, and stranger-directed aggression, stranger-directed fear, and non-social fear were significantly lower in dogs who passed the screening test (5).

In a study of guide and service dog puppies, using a logistic regression model with successful training as the dependent variable and C-BARQ scores at 6 months as explanatory variables, 27 of the C-BARQ traits explained significant proportions of the variation in success (4). Many of these traits from a 6-month C-BARQ were the same as those associated with working dog status in the present study, including trainability, stranger-directed aggression, owner-directed aggression, dog-directed aggression, non-social fear, stranger-directed fear, and chasing. The present study did not find differences in touch sensitivity, separation problems, or excitability between SAR and pet dogs. These traits might be more important for guide dog work than for SAR work since guide dogs work in closer proximity to humans where touch sensitivity is more problematic, and guide dogs in training are not required to be alone frequently. The guide and service dog study found significant negative associations with success for dog-directed aggression, rivalry, and attachment/attention-seeking, while the present study does not. The same model was fitted with C-BARQ scores from puppies at 12 months of age. Trainability

TABLE 3 | Final models for C-BARQ subscales (*NS* = not significant at $P < 0.05$ in previous model in backward elimination).

Trait	Model R^2	SAR	SAR mean	non-SAR Mean	FEMA	FEMA mean	non-FEMA mean	Breed	Age	Sex	Neutered
Trainability	0.08	<2.2e-16	3.16	2.64	NS	NA	NA	0.003	<2.2e-16	NS	NS
Aggression toward strangers	0.12	0.01	0.41	0.47	NS	NA	NA	<2.2e-16	0.003	NS	NS
Aggression toward owner	0.02	NS	NA	NA	NS	NS	NA	<2.2e-16	NS	NS	NS
Aggression and Fear of dogs	0.10	0.01	0.69	0.76	NS	NA	NA	<2e-16	<2e-16	NS	NS
Aggression toward dogs	0.13	NS	NA	NA	NS	NA	NS	<2.2e-16	<2.2e-16	NS	NS
Fear of dogs	0.02	<2.2e-16	0.53	0.63	0.04	0.62	0.63	0.03	NS	0.08	NS
Do rivalry	0.06	NS	NA	NA	NS	NA	NA	<2.2e-16	0.003	NA	NS
Chasing	0.15	<2e-16	1.24	1.96	NS	NA	NA	<2e-16	0.04	<2e-16	NS
Fear of strangers	0.09	<2e-16	0.29	0.36	NS	NA	NA	<2e-16	0.03	<2e-16	NS
Non-social fear	0.05	<2e-16	0.39	0.73	NS	NA	NA	0.01	NS	NS	0.02
Separation problems	0.02	NS	NA	NA	0.005	0.3	0.49	NS	<2e-16	NS	<2.2e-16
Touch sensitivity	0.04	NS	NA	NA	NS	NA	NA	NS	<2.2e-16	0.005	0.02
Excitability	0.03	NS	NA	NA	NS	NA	NA	NS	<2.2e-16	NS	NS
Attachment/Attention-seeking	0.05	NS	NA	NA	NS	NA	NA	<2e-16	0.01	NS	NS
Energy	0.17	<2.2e-16	2.79	2.23	NS	NA	NA	NS	<2.2e-16	NS	NS

was significantly higher in successful dogs, and all other behavior characteristics measured in the present study had negative relationships with success. Characteristics that did not distinguish pets from SAR dogs in the present study but did have a relationship with success in the guide and service dog study were dog-directed aggression, dog rivalry, and attachment/attention-seeking. The relationships between C-BARQ behavior traits and successful training as a service dog were similar at both ages, suggesting that it may be possible to use some C-BARQ subscales to screen and select dogs for SAR work as early as 6 months.

Boldness was found to be associated with high performance in working dog tests in Swedish female German Shepherd Dogs and Belgian Tervurens (13). The Dog Mentality Assessment is a broad-ranging test of a dog's aptitudes and differs substantially from the C-BARQ in that is not a questionnaire completed by owners but a behavior test scored by a judge. However, Svartberg (14) found correlations between the Dog Mentality Assessment boldness measures and C-BARQ fear subscales. High performing dogs had higher boldness scores then low performing dogs in agreement with the present findings that several types of fear (fear of dogs, fear of strangers, and non-social fear) are negatively associated with working dog status.

The only behavior differences between FEMA-certified USAR dogs and uncertified SAR dogs were lower fear of dogs and separation-related problems. This could be related to a general lack of fear that seems to be associated with successful working dogs, and could be a result of training. SAR training involves frequent travel to training events with other dogs, and dogs are required to work at a greater distance from their handlers than guide or service dogs.

The present study differs from the other behavior studies discussed here because it utilized two different populations of people to respond to the C-BARQ. It is unknown whether and how the increased knowledge of canine behavior possessed by working dog handlers relative to pet dog owners affects their understanding of the terminology of the C-BARQ or their ability to assess their dogs. Thus, the differences in subscales reported here could be biased upward or downward.

It is not clear whether the behavior differences found in the present study are due to selection of dogs with these traits or whether they result from training. Future research at a facility such as the Penn Vet Working Dog Center where puppy behavior is tracked during development could provide a means of observing changes in behavior during development and comparing dogs with different levels of success in SAR work. Future work should be aimed at developing questionnaires that focus on the specific requirements for SAR dogs such as the ability to work independently at a distance from the handler, persistence on odor, and ability to learn odors. A specialized temperament test involving such traits would facilitate the identification of individual dogs with potential to be trained as odor detection dogs.

Our results can be used to inform the selection of puppies and juvenile dogs for training as SAR dogs. More efficient selection would result in reduced costs associated with the purchase and training of dogs that are less likely to successfully complete FEMA certification. Some of the C-BARQ subscales for fear and aggression have been associated with specific genomic regions (15) and others such as trainability and aggression have been found to be heritable (16), so these findings can also be applied in selective breeding programs to produce future SAR dogs.

AUTHOR CONTRIBUTIONS

EH conducted statistical analysis. KK collected SAR dog data. JS provided interpretation of C-BARQ findings and petdog data,

and CO directed the research. All authors reviewed and edited the manuscript.

FUNDING

This research was supported by American Kennel Club Canine Health Foundation grant 2336.

ACKNOWLEDGMENTS

The authors would like to thank the dogs and handlers that responded to the September 11, 2001 attacks for their participation in an ongoing Penn Vet study of their health, as well as the pet dogs and owners who responded to the C-BARQ questionnaire.

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Conflict of Interest Statement: EH is the sole proprietor of Dog Genetics LLC, which provides statistical and genetic analysis for working dog organizations.

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 02 July 2018
doi: 10.3389/fvets.2018.00127

Zinc Nanoparticles Enhance Brain Connectivity in the Canine Olfactory Network: Evidence From an fMRI Study in Unrestrained Awake Dogs

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OPEN ACCESS

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Reviewed by:
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Specialty section:
This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science

Received: 19 August 2017
Accepted: 23 May 2018
Published: 02 July 2018

Citation:
Ramaihgari B, Pustovyy OM, Waggoner P, Beyers RJ, Wildey C, Morrison E, Salibi N, Katz JS, Denney TS, Vodyanoy VJ and Deshpande G (2018) Zinc Nanoparticles Enhance Brain Connectivity in the Canine Olfactory Network: Evidence From an fMRI Study in Unrestrained Awake Dogs. Front. Vet. Sci. 5:127. doi: 10.3389/fvets.2018.00127

Prior functional Magnetic Resonance Imaging (fMRI) studies have indicated increased neural activation when zinc nanoparticles are added to odorants in canines. Here we demonstrate that zinc nanoparticles up-regulate directional brain connectivity in parts of the canine olfactory network. This provides an explanation for previously reported enhancement in the odor detection capability of the dogs in the presence of zinc nanoparticles. In this study, we obtained fMRI data from awake and unrestrained dogs while they were being exposed to odorants with and without zinc nanoparticles, zinc nanoparticles suspended in water vapor, as well as just water vapor alone. We obtained directional connectivity between the brain regions of the olfactory network that were significantly stronger for the condition of odorant + zinc nanoparticles compared to just odorants, water vapor + zinc nanoparticles and water vapor alone. We observed significant strengthening of the paths of the canine olfactory network in the presence of zinc nanoparticles. This result indicates that zinc nanoparticles could potentially be used to increase canine detection capabilities in the environments of very low concentrations of the odorants, which would have otherwise been undetected.

Keywords: zinc nanoparticles, fMRI, canine, dog, brain connectivity, olfactory system

INTRODUCTION

Olfactory capability in canines is far superior to most known animals including human beings. This is in part due to the anatomical features responsible for the initial events in olfaction (1–3). The area occupied by the olfactory epithelium in human is ~3 cm^2, while the dog (German Shepherd) has a more than 50 times larger olfactory epithelium of 170 cm^2 (4–6). Humans have 50 million olfactory receptor neurons (ORNs), but dogs have 2 billion olfactory neurons, and dogs sniff 10 times faster than humans (7–9).

Utilization of dogs for detecting different materials in the environment is owed to this long established fact. Human society has successfully detected and evaded dangers in war zones, airports and terrorist targeted public places because dogs have been helping us with detecting explosives (10). Apart from this they have also helped us control drug/narcotics trafficking, tracking people (11). Other detection methods for explosives (12) also exist and have been proved to be effective in controlled lab environments, but sniffer dogs still have been the most effective method for this purpose outside the laboratory (10, 13–16). However, one should note that though sniffer dogs are an effective solution, they are not without stumbling blocks. One of the main hindrances is the concentration of the odorant (17) in the environment.

The process of olfaction starts with the chemical interreaction between the odorant molecules and receptor proteins in the nose. This means that detection accuracy is restricted by the concentration of the odorant present in that environment (17). In many real scenarios, target odor concentrations can even be below the dog's detection threshold. Therefore, other ways of enhancing odor-related response in the dogs are being actively investigated. Specifically, presence of nanoparticles of different metals such as copper, gold, silver, zinc, etc. are being researched. The results have mostly been unfruitful but for those with zinc. Studies have shown that the presence of zinc nanoparticles might enhance odorant responses of ORNs *in vitro* (18–20) as well as enhance functional Magnetic Resonance Imaging (fMRI)-based activation in the dog brain *in vivo* (21).

Basic olfaction as a process can be explained broadly in the sub events of sniffing, chemical binding of the odorant, signal transmission, recognition and interpretation. Each of these events involve different parts of the olfactory system (22–24). The olfaction process starts with sniffing which involves olfactory neuroepithelium of the nasal cavity. This enables the transfer of odorant molecules into the nose and to the mucus layer covering the olfactory epithelium (25). Next, the chemical binding of the odorant with a receptor protein (26, 27) initiates an intracellular cascade of signal transduction events of the G-protein-dependent adenilyl cyclase production of second messenger molecules (28) followed by opening of ion channels and passing of ion currents (29). This generates an action potential in the ORNs (30) that is projected to the olfactory bulb (OB) (31). The signal thus generated is transmitted to the regions of pyriform cortex, periamygdaloid cortex, and entorhinal cortex through olfactory stria. From pyriform cortex and periamygdaloid cortex, the signal is then transmitted to the thalamus and frontal cortex, where it is recognized and interpreted (32, 33). The regions of the Hippocampus receive the signal from entorhinal cortex for recognition purposes as well (34, 35). Apart from these, various regions of the brain such as the amygdala are involved in the emotional processing resulted from the odors recognized. A schematic of the olfactory pathway in dogs reconstructed based on previous literature is shown in **Figure 1**.

Based on previous *in vitro* (18, 20, 36) and *in vivo* (21) studies, we concluded that olfactory enhancement by the zinc nanoparticles is composed of two components. One component is based at the level of olfactory sensory receptors, and the second part of the olfactory enhancement is positioned at higher levels of olfactory perception. The first part was explained by a simple model: The endogenous zinc nanoparticles produce a certain number of functional receptor dimers that can be triggered by the odorant as well as take part in the generation of the olfactory signal. When the olfactory epithelium is subjected to a mixture of zinc nanoparticles and also the same odorant, extra receptor dimers are created by joining with each other pairs of previously unbound receptors (21). In this study, we investigate the second part of olfactory enhancement by zinc nanoparticles. We test the hypothesis that the connectivity of between brain regions that are situated above the olfactory sensory neurons have increased strengths in the presence of zinc nanoparticles.

We obtained the strength of paths between olfaction-related brain areas for the condition of dogs being exposed to odorant with zinc nanoparticles and compared them to those obtained for odorants without nanoparticles. We used two additional control conditions: a suspension of zinc nanoparticles in water vapor and just water vapor.

MATERIALS AND METHODS

Preparation of Dogs

A total of 8 dogs, raised in the Auburn University Canine Performance Sciences Program, with ages between 12 and 60 months were used for this experiment. Ethical approval for the study was obtained from the Auburn University Institutional Animal Care and Use Committee. We confirm that all methods were performed in accordance with the relevant guidelines and regulations. The concentrations of the zinc nanoparticles the dogs were exposed to are non-toxic to them (37) thus their use is not unethical in this study. The amount of zinc exposure from the sniffing is calculated as follows: the test concentration of metallic zinc in the test solutions was 0.02 nM, or 1.3 ng/L. The approximate volume of the solution applied per pulse (sniff) is 0.010 mL. For 5 sniffs per run, the volume of the solution is 0.05 mL. The daily dog exposure does not exceed 10 runs. Therefore the testing volume of zinc nanoparticle suspension does not exceed 0.5 mL. Thus, the amount of estimated zinc inhaled by a 30 kg dog per day is less than 0.5×10^{-3} L $\times$ 1.3 ng/L $= 6.5 \times 10^{-7}$ microgram/dog/day. The daily recommended amount of zinc per day for the 30 kg average body weight dog is 30 mg, or 3×10^4 microgram (37). This level of zinc intake is 50 billion times higher ($3 \times 10^4/6.5 \times 10^{-7}$) than daily exposure during fMRI experiments. Additionally, we have previously demonstrated that zinc nanoparticles are cleared from olfactory epithelium within 10 s (20). Also, zinc nanoparticle at the level we used in our work do not destroy olfactory epithelium in contrast to the zinc sulfide that is known to damage olfactory epithelium (38).

These dogs were trained to remain in the scanner bed with their heads inserted into the human knee coil (in prone position) for the duration of the scanning, carried out while the dogs were awake and unrestrained. Positive reinforcement behavior shaping procedures were used to keep them as still as possible and to desensitize them to the loud scanner noise.

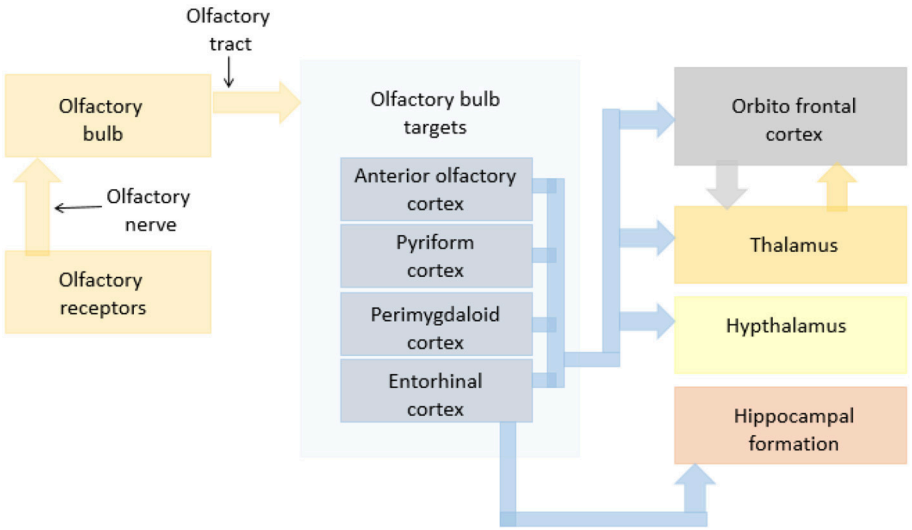

FIGURE 1 | A schematic flowchart indicating olfactory pathways in dogs.

Odorants

The odorant used in the experiment was a mixture of ethyl butyrate, eugenol, and (+) and (−) carvone in water at a concentration of 0.016 mM. This is well above the dog's LOD (level of detection) in air for odorants we used, which has been shown to be at the level of 5 pM (10^{-12} M) (39). This odorant mixture, as well as the training procedure, were the same as in Jia et al. (40). The odorant concentration was considered to be 0.016 mM as it was the low concentration in the previous work (40), for which the activation of olfaction related areas in the dog's brain could be detected. Nevertheless, we were able to detect a significant increase in activation when a higher concentration (0.16 mM) was utilized in that study. Saturation of the EOG signal takes place only at ∼10 mM of the same odorant mixture (20). These data reveal that using a low odorant concentration of 0.016 mM in the current work, there is sufficient dynamic range for zinc nanoparticles to enhance olfaction related activation in the brain without saturating the brain responses. It has been shown that the spatial clustering of principal responses to the individual odorants of this mixture show statistically distinct and different glomerular patterns (41). This fact may potentially enhance the odorant presentation in fMRI tests.

The concentration of odorant is given in the water solution. Because the water/air partition coefficient for all odorants we used in our experiments is very low (∼10^{-4}), the concentration of the odorants in the head space is in parts per billion range. For example, the concentration of Eugenol in head space can be estimated using Amoore-Buttery equation for the water/air partition coefficient, K_{aw}, from value of vapor pressure, solubility in water and molecular weight (42):

$$K_{aw} = \left(\left(\frac{55.5}{S - 0.0555}\right) \times M + 1\right) \times P \times 0.97 \times 10^{-6}$$

where P is vapor pressure in mm Hg, S is solubility in water in g/L of the pure odorant at 25°C and M is its molecular weight. For Eugenol, we have $P = 0.0226$ mm Hg; $S = 2.47$ g/L; $M = 164.2$ g/mol. According to the Amoore-Buttery equation, $K_{aw} = 8.08 \times 10^{-5}$. This value of K_{aw} for Eugenol agrees well with that obtain experimentally (43).

Thus, the concentration of Eugenol in head space (and consequently delivered to a dog) equals to

$C_h = K_{aw} \times C_b = 8.08 \times 10^{-5} \times 0.016 \times 10^{-3}$ M $= 1.3 \times 10^{-9}$ M, where C_h is a head space concentration and C_b is balk concentration in liquid. The head space concentration can be converted to nM and ppb as follows.

$C_h = 1.3 \times 10^{-9}$ M $= 1.3$ nM

C_h = (Mass in m^3)/molecular mass) × (volume of 1 mole) = (9.5 μg/m^3/164.2 g/mol) × 24.45 = 1.4 ppb.

Zinc Nanoparticles

The procedure of obtaining and mixing of the zinc nanoparticles was similar to that described in Jia et al. (21). The zinc nanoparticles were prepared by the underwater electrical discharge method as shown in Vodyanoy et al. (44). The produced particles were centrifuged at 20,000 × g for 1 h at 8°C. After centrifugation, the pellet is discarded and the supernatant is subjected to further centrifugations at 47,000 g for 1 h at 5°C to produce a fraction of nanoparticles enriched in particles of 1–2 nm. The particle physical properties were analyzed by electron microscopy, atomic force microscopy, and X-ray photoelectron spectroscopy (44). The total concentration of metal in suspension was measured by atomic absorption spectra (GTW Analytical Services, Memphis, TN, USA). Zinc nanoparticles had crystalline structure with an average diameter of 1.2 ± 0.3 nm. About 94% of metal atoms were not oxidized. The zinc nanoparticles were suspended in odorant solution at concentration of 0.02 nM.

Data Acquisition

The data acquisition procedure was described in detail in our previous publications (21, 40). Briefly, it consisted of: a 3T MAGNETOM Verio scanner (Siemens Healthcare, Erlangen, Germany), a 15 channel human knee coil adapted as a dog head coil, customized odorant applicator for computer-controlled delivery and evacuation of odorant stimulus, mask for receiving the odorant stimulus and covering the nose and mouth of the dogs, an external infra-red camera used to track head motion in dogs and retrospectively correct for motion artifacts in the data. Functional MRI data was obtained using an EPI (Echo-planar Imaging) sequence with the following parameters: repetition time (TR) = 1,000 ms, echo time (TE) = 29 ms, field of view (FOV) = 192 × 192 mm^2, flip angle (FA) = 90 degree, in-plane resolution 3 × 3 mm^2, in-plane matrix 64 × 64, and whole brain coverage. Anatomical data was obtained for registration purposes using an MPRAGE sequence with the following parameters: TR = 1,550 ms, TE = 2.64 ms, voxel size: 0.792 × 0.792 × 1 mm^3, FA = 9°, in-plane matrix = 192 × 192, FOV = 152 × 152 mm^2, number of slices: 104.

Data was obtained for each dog while being exposed to the following set of odorants: Odorants+ zinc nanoparticles (OZ), odorants alone (O), water vapor + zinc nanoparticles (WZ), water vapor alone (W). Each scanning session included 1 run of structural scan, 2 runs of functional scans involving odor stimulation with zinc nanoparticles, 2 runs with odorant alone, 2 runs of functional scans involving exposure to zinc nanoparticles alone in water vapor, and 2 runs of functional scans involving exposure to water vapor alone. These functional scans were run in random order for each dog.

Experimental Paradigm

As described in Jia et al. (21), each functional run with odorant stimulus had 5 blocks of odorant exposure each lasting for 10 s followed by 30 s of rest block to prevent the adaptation of the dog's olfactory response to the odorant (**Figure 2**). The stimulus block involved pumping of the odorant to the mask so as to expose the subject to it. The resting blocks consisted of an initial 10 s for vacuuming the odorant from the pipes and the mask followed by 20 s of no stimulation. Each run lasted for 200 s with the onset times of the stimulant in each run for the 5 blocks being 10, 50, 90, 130, and 170 s, respectively. The choice of 10-s odor-on condition and 30-s odor-off paradigm was guided by previous studies showing that it is effective for eliciting measurable neural response while preventing habituation (21).

A schematic of the experimental paradigm is shown in **Figure 2** and can be explained as follows. In the odorant sequence, green arrows indicate the onset time of the odorant stimulus in the 4 conditions (pure odorants, odorants + zinc nanoparticles, pure water vapor, and water vapor + zinc nanoparticles) and down arrows indicate the time when the stimulation ends. The four conditions above were presented randomly across runs within a session. In the vacuuming sequence, the green arrows indicate the beginning of the vacuuming or clearance of odorant, and red arrows indicate the ending. The block design represents the paradigm with "0" indicating absence of stimulus (OFF condition) and "1" denoting the presence of odorant (ON condition).

Data Processing

As described in Jia et al. (40), preprocessing of fMRI data was done using the software SPM8 (http://www.fil.ion.ucl.ac.uk/spm/software/spm8/, Functional Imaging Lab, The Welcome Trust Centre for NeuroImaging, in the Institute of Neurology at University College London). The basic steps of slice timing correction, realignment to the first functional image, spatial normalization to a template defined by us as in Jia et al. (21, 40), and spatial smoothing were done. Then the preprocessed fMRI data was input to a general linear model (GLM) and statistical tests were performed for obtaining voxels in the canine brain which were activated for the comparison of odorants + zinc nanoparticles with each of the following conditions: zinc nanoparticles alone, water vapor + zinc nanoparticles, water vapor alone, were obtained. Voxels significantly active in all of the following conditions, i.e., (odorants + zinc nanoparticles > zinc nanoparticles alone) n (odorants + zinc nanoparticles > water vapor + zinc nanoparticles) n (odorants + zinc nanoparticles > water vapor alone), were identified and used for definition of ROIs as discussed below. The GLM also modeled variance from confounding factors such as time and dispersion derivatives (in order to model the variability of the hemodynamic response function), motion parameters obtained from realignment, as well as motion parameters obtained from the external camera-based motion tracking device. We showed that adding zinc nanoparticles to a single low concentration of odorant, increases amplitude of the output signal, which is equivalent to the signal of 10 times stronger odorant (20, 21). The brain olfactory areas present a very complex connectivity system. Therefore, to analyze connectivity, we tried to keep the stimuli as simple as possible.

Considering the activations obtained from the contrast mentioned above (only the activated voxels) the following Regions of Interest (ROIs) were selected: Amygdala, Hippocampus, Olfactory bulb, Thalamus, Caudate, Pyriform lobe, Frontal cortex. While the voxels themselves were dictated by the contrast defined above, the nomenclature of the ROIs they belong to were identified using a dog atlas (45). For each of these ROIs, mean time series from activated regions were extracted for every run. These time series were then subjected to blind hemodynamic de-convolution using a cubature Kalman filter and smoother (46) to obtain the underlying latent neural variables. This was done in order to remove the confounding effect of HRF variability on connectivity results (47–53). Directional brain connectivity between the ROIs was then obtained for each condition using Dynamic Granger Causality (DGC) by using the analysis framework reported before (54–62). Connectivity for all possible paths between ROIs for the condition odorant +zinc nanoparticles were computed. Mean connectivity was also computed for each path for the conditions of odorant, water vapor + zinc nanoparticles and water vapor alone. Using two sample *t*-tests, paths whose connectivity strength was stronger for the condition of Odorant + zinc nanoparticles (OZ) as compared to other control conditions of odorant (O), water

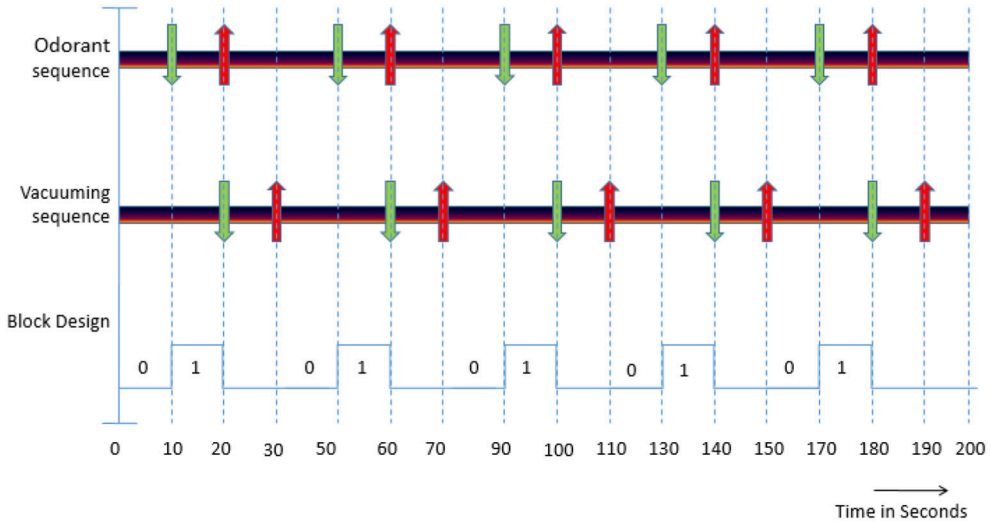

FIGURE 2 | (i) Odorant sequence: Green down arrow indicates the starting of the stimulant presentation and red up arrow indicates ending of the stimulation. (ii) Vacuuming sequence: Green down arrow indicates the starting of the vacuuming to remove odorant and the red up arrow indicates ending of vaccuuming. (iii) Block Design: "0" indicates the absence of an odorant (OFF condition) and "1" indicates the presence of an odorant (ON condition).

vapor + zinc nanoparticles (WZ), and only water vapor (W) were indicated.

RESULTS

All the paths with corrected $p < 0.05$ for the condition of Odorant + Zinc nanoparticles greater than the conditions of Odorant, water vapor + zinc nanoparticles, water vapor (OZ > O, WZ, W) were obtained and are listed in the **Table 1** along with their connection strengths. The paths are also shown pictorially depicted in **Figure 3**. It can be seen that many paths within the dog olfactory network show strengthening in the presence of zinc nanoparticles. When similar results were generated using different random splits of the data, the significant paths did not change. This provides some reassurance that the results are replicable.

Our previous fMRI analysis of the olfactory system in conscious dogs showed that an increase of odorant concentration of 10 times caused a considerable escalation of brain activity manifested by the growth of the total number of activated voxels from 379 to 759, at the ratio of 2.0 (40). When zinc nanoparticles were added to the odorant, we observed the doubling of the total number of activated voxels (21), which is equivalent to the activation obtained by a 10-folds higher concentration of odorant. In this work, we documented the robust increase in connectivity strength for odorant with zinc nanoparticles compared to the odorant alone, water vapor with zinc nanoparticles, and water vapor alone (**Table 1**). The mean value of connectivity increase was 3.14 ± 1.53 (SD) ($n = 16$), which was consistent with the ∼3-fold increase of electro-olfactogram (EOG) amplitude evoked by a 10-fold increase in odorant concentration in rodents (20, 36), and the brain activity increase observed in dogs (21, 40). Analysis of the cumulative frequency distributions (**Figure 4**) shows a ∼3-fold shift to larger values of connectivity in the presence of zinc nanoparticles.

TABLE 1 | Paths with significant increase in connectivity strength for the condition of odorant + zinc nanoparticles (OZ) compared to conditions of odorant (O), water vapor+ zinc nanoparticles (WZ) and water vapor alone (W).

Path origin	Path termination	*P*-value	Mean connectivity	
			OZ	O, W, WZ
Amgdala	Caudate	8.95×10^{-24}	0.252	0.052
Amgdala	Hippocampus	3.23×10^{-11}	0.194	0.067
Amgdala	Olfactory bulb	1.80×10^{-07}	0.159	0.056
Amgdala	Pyriformlobe	3.18×10^{-21}	0.254	0.06
Amgdala	Thalamus	5.79×10^{-18}	0.23	0.066
Amgdala	Frontal cortex	2.26×10^{-12}	0.217	0.072
Caudate	Amgdala	6.16×10^{-20}	0.204	0.053
Caudate	Hippocampus	1.74×10^{-23}	0.194	0.013
Caudate	Olfactory bulb	2.13×10^{-25}	0.194	0.038
Caudate	Pyriformlobe	5.13×10^{-18}	0.213	0.038
Caudate	Thalamus	7.45×10^{-11}	0.187	0.042
Caudate	Frontal cortex	2.13×10^{-21}	0.156	0.061
Hippocampus	Thalamus	4.56×10^{-2}	0.218	0.106
Olfactory bulb	Caudate	0.27×10^{-2}	0.136	0.114
Olfactory bulb	Pyriformlobe	1.53×10^{-2}	0.162	0.119
Olfactory bulb	Frontal cortex	0.04×10^{-2}	0.154	0.099

Resultant p-value of the t-test, mean connectivity values of the paths for conditions OZ and (WZ, W, O) are shown.

DISCUSSION

Canine olfaction has been very useful to mankind over decades for various tasks such as detecting explosives, people etc. However, they still do not seem to be accurate on occasions due to reasons such as the low concentrations of the odorant

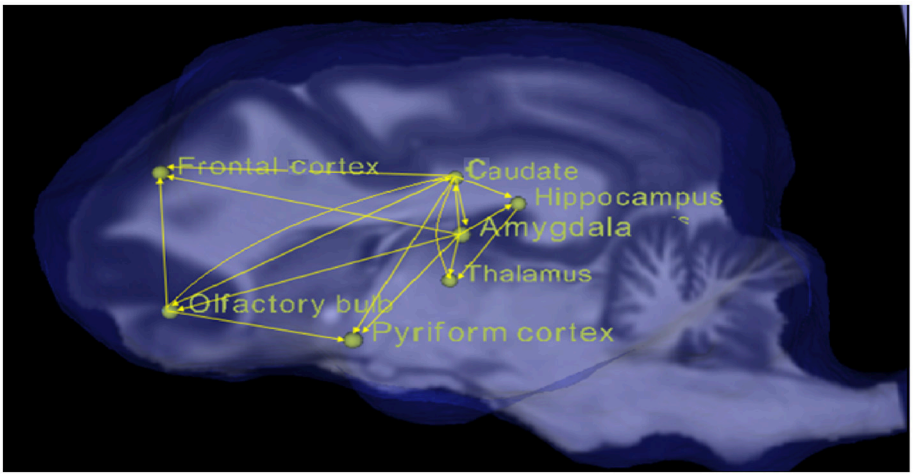

FIGURE 3 | Pictorial depiction of paths with significant increase in connectivity strength for the condition of odorant + zinc nanoparticles (OZ) compared to conditions of odorant (O), water vapor+ zinc nanoparticles (WZ), and water vapor alone (W).

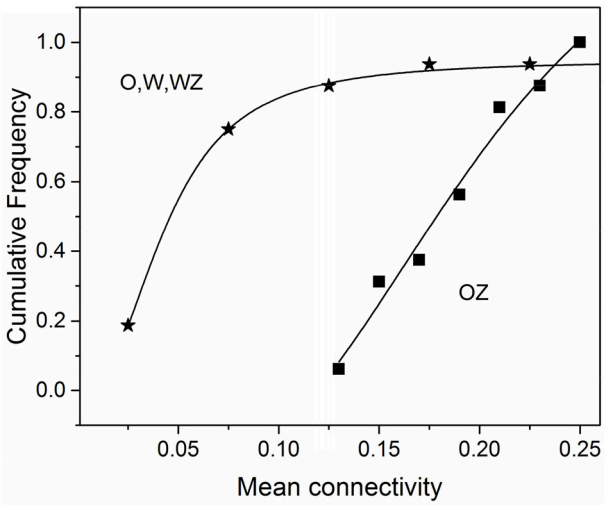

FIGURE 4 | Cumulative frequency distribution of the mean connectivity for the condition of odorant + zinc nanoparticles (OZ) compared to conditions of odorant (O), water vapor+ zinc nanoparticles (WZ), and water vapor alone (W). Data were taken from **Table 1**.

in the surrounding environment. Therefore, understanding the olfactory system in canines and methods of enhancing their olfactory capabilities are of high interest. Efforts have been made in this direction using *in vitro* cellular (63, 64) or behavioral approaches (65–68). *In vivo* imaging studies till now have mostly concentrated on activations in various regions of the brain (21, 40). Given the strides made in human imaging for gaining a perspective on brain function using connectivity modeling in the brain, our study is an attempt (likely the first, in awake dog imaging) to explore the canine olfactory system and its enhancement with zinc nanoparticles using connectivity modeling.

Perceived odor intensities by humans are observed to be highly correlated with the EOG amplitude (69). EOG studies indicate that neural activity at the human olfactory epithelium mirrors olfactory perception (70). Since its introduction, fMRI has become a very powerful instrument to noninvasively infer underlying mechanisms of brain function (71). Our prior work has demonstrated the use of fMRI for inferring the cognitive foundations of odor processing in fully conscious and unrestrained dogs (40).

Our hypothesis, that the connectivity of the various signal paths involved in the process of olfaction will increase in the presence of the zinc nanoparticles, is motivated by previous works which have shown *in vitro* enhancement of the olfactory response in olfactory sensory neurons in the presence of zinc nanoparticles (18, 20, 36, 72) as well as *in vivo* enhancement observed in terms of increased fMRI-based activation of olfaction-relevant regions of the dog brain (21). The sensory olfactory nervous system is a part of the peripheral somatic nervous system and transmits olfactory signals from olfactory sensory neurons to the brain. Using whole cell patch clamp, we demonstrated that zinc nanoparticles significantly increase electrical signals from individual neurons (20). Below, we discuss our results in the context of what we already know about the canine olfactory system.

Electrical potentials measured in the ORNs (73, 74) at the initiation of the olfaction are proportional to the logarithm of the concentrations of odorants (75, 76). The olfactory bulb, as described before, receives the signal from the receptor neurons (31) and transmits them to the amygdala, entorhinal cortex and pyriform cortex. The signal received by the olfactory bulb and further transmitted to the above regions is directly related to the odorant molecules reacting with the receptor neurons. We can observe from the results that the paths originating from the olfactory bulb and driving to the pyriform lobe and entorhinal cortex significantly increased their strength in the presence of zinc nanoparticles. The amygdala mainly contributes to the

processing of the emotionally salient content in the olfactory stimuli (77). We observed that all the paths originating from and towards the amygdala had enhanced connectivity in the presence of zinc nanoparticles. The caudate, in conjunction with the amygdala and hippocampus, participates in functions related to memory, goal oriented activities, and emotions i.e., they are involved in the higher order processing of the olfactory stimuli (34, 35). In addition, the frontal cortex is known to be involved in the interpretation and recognition of olfactory stimuli (32, 33) while the thalamus acts as a relay between cortical and sub-cortical structures in the olfactory network. Our results show a tight network of paths between the frontal cortex, thalamus, caudate, amygdala, and hippocampus whose connectivity was enhanced in the presence of zinc nanoparticles.

It is interesting to note that in our previous report (40), we had demonstrated that when the odorant concentration increased 10 times it caused the spatial extent of activation in conscious dogs to approximately double in area (40). This was corroborated in a followup study using zinc nanoparticles wherein the addition of nanoparticles to the odorant increases the spatial extent of activated region 2-fold (21). The finding indicates that zinc nanoparticles may be equivalent to a 10-fold increase in odor concentration. Analysis of connectivity data in the presence of zinc nanoparticles from **Table 1** shows a 3-fold shift to larger values of connectivity in paths belonging to the canine olfactory network (**Figure 4**). This is in agreement with a similar 3-fold increase in EOG amplitude evoked by a 10-fold increase in odorant concentration in rodents (20, 36). Our data are in agreement with results obtained by direct optical recording of the activity of rat glomeruli in rat olfactory bulb (78). They described the relative activity of glomeruli as a sigmoidal function of odorant concentration. However, the major increase of activity is proportional to a logarithm of stimuli, and a 10-fold increase in odorant concentration correspond to ∼3 times increase in the relative activity of glomeruli in the olfactory bulb (23). Our results agree well with those showing connectivity from olfactory sensory neurons expressing OR37 receptors into the higher brain centers visualized by genetic tracing (79).

The results of study suggest that zinc nanoparticles enhance the canine olfactory sensitivity by potentially upregulating both activity (21) and connectivity (current study) in the canine olfactory network. If corroborated by behavioral studies, this finding could provide a potential method of improving the detection capabilities of sniffer dogs in ultra-low concentration environments. The longer term implications of this work could provide an enhancement in the individual sense of smell in disorders such as Alzheimer's and Parkinson's, which show olfaction loss (80). In early Alzheimer's, olfactory deficits are a preclinical symptom that aggravates with disease progression (81, 82). Alzheimer's disease impacts ∼5.5 million Americans as of 2017 and is the 10th leading cause of death in the United States (80). We hope that upcoming therapies with zinc nanoparticles functioning on the olfactory receptor level at minimal concentrations could compensate the loss of smell and improve emotional well-being as well as quality of life.

AUTHOR CONTRIBUTIONS

GD, VV, TD, EM, and PW conceived the idea and designed the experiment. GD, NS, and RB optimized sequences and acquired the data. PW handled the dogs. OP prepared the odorants and operated the odor applicator device. CW trained GD and RB for using the external motion tracking device in addition to providing the device. GD, BR, and VV analyzed the data. GD, BR, VV, and JK interpreted the results. All authors contributed toward writing the manuscript.

ACKNOWLEDGMENTS

Funding from Auburn University's Intramural Grant and from the Defense Advanced Research Projects Agency (government contract/grant number W911QX-13-C-0123) is gratefully acknowledged. The views, opinions, and/or findings contained in this article are those of the authors and should not be interpreted as representing the official views or policies, either expressed or implied, of the Defense Advanced Research Projects Agency, US Department of Defense or the federal Government of the United States.

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Conflict of Interest Statement: Two of the authors are employed by commercial companies. CW is the founder and CEO of MRRA Inc., who supplied the optical motion tracking system and contributed to this work by training the first author on using this product. MRRA Inc., is the license holder for the patent covering the motion tracking product. NS is an employee of Siemens Healthcare, Malvern, PA who is stationed at the MRI Research Center in Auburn University. She contributed by optimizing the sequences used to acquire fMRI data in this study. However, note that these authors had no role in data analysis and its interpretation. These competing interests do not alter the authors' adherence to common policies on sharing data and materials.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

REVIEW
published: 14 August 2018
doi: 10.3389/fvets.2018.00188

Birds and Dogs: Toward a Comparative Perspective on Odor Use and Detection

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While canines are generally considered the gold standard for olfactory detection in many situations other animals provide alternatives and offer a unique opportunity to compare biological detection capabilities. Critical components in successfully studying biological detectors is not only understanding their anatomical evidence for olfaction, but also, understanding the life history of the species to better direct the potential of an olfactory task. Here, a brief overview is provided presenting a comparative viewpoint on the use of odors by birds and canines over a range of unique detection scenarios. Similar to canines, birds use olfactory information in various natural oriented contexts where odors are dispersed over a widespread spatial range. Comparing these two distinctive animal models, and current trends in physiological and behavioral assessments may open the door for novel uses of birds as biological sensors in forensic applications.

Keywords: biological detection, odor, canines, birds, olfaction

OPEN ACCESS

Edited by:
Cynthia M. Otto,
University of Pennsylvania,
United States

Reviewed by:
Alexandra Horowitz,
Columbia University, United States
Vera Baumans,
Retired, Zeist, Netherlands

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Specialty section:
This article was submitted to Veterinary Humanities and Social Sciences, a section of the journal Frontiers in Veterinary Science

Received: *10 April 2018*
Accepted: *24 July 2018*
Published: *14 August 2018*

Citation:
Prada PA and Furton KG (2018) Birds and Dogs: Toward a Comparative Perspective on Odor Use and Detection. Front. Vet. Sci. 5:188. doi: 10.3389/fvets.2018.00188

INTRODUCTION

The term "biological detector" is applied to organismal detectors including animals and plants that can be trained, conditioned or genetically modified to detect key molecules in the environment. The detection of target odor chemicals plays a key role for a variety of purposes within the forensic realm, thus the active research investigating a variety of animal models for the optimal and efficient detection of odors in practical field operations (1–3). With respect to mobile chemical detectors, canines have long been the biological detector of choice, and are currently widely used by law enforcement around the world for detecting a range of forensically important traces. Canines offer clear advantages over instrumental analytical detectors: dogs can easily operate in public; they can be trained to specific odor signatures of target materials, and can track a scent to its source over uneven terrain. These highly mobile biological detectors are also able to pick up and discriminate a specific "scent picture" even against a variety of different "noisy" odor backgrounds. Canine olfaction has been the subject of study from a range of different perspectives. From a physiological standpoint, researchers have been elucidating nasal airflow patterns and their role in odorant transport (4–7). Forensically, canines are one of the most important detection tools for homeland security and law enforcement purposes. Thus, a number of studies have focused on enhancing and understanding canine team performance (8, 9), training regimens (10–12) and clarification of relevant odor chemicals within forensic contexts (13). Clinically, the detection of various types of cancers by canines has been evaluated (14–16). Not surprisingly, the canine olfaction model is widely used when compared to other animal systems. However, it is important to keep in mind that other organisms also use odors in

various contexts as observed by their olfactory-related behaviors within their natural environment. Birds are one such animal model that has been largely ignored within an olfaction perspective and more so, in practical, detection capabilities. Birds may represent the next phase in understanding how olfactory cues used across different environmental contexts can prove useful as a biological detection model if directed toward more focused olfactory detection tasks. This paper outlines avian olfaction evidence presenting three bird species as models (i.e., homing pigeons, turkey vultures and domestic chickens) and highlights how birds' intrinsic life history olfactory traits, even though greatly overlooked for biological detection, can be potentially directed to similar detection tasks as that observed in canine forensic field-based operations.

WHAT ARE SOME USES OF ODORS IN AVIAN SPECIES?

Both canines and birds use olfactory evidence over a range of unique detection viewpoints. However, as opposed to canines, avian olfactory capabilities have been substantially overlooked by a historical belief that birds are anosmic (i.e., having little or no smell) (17). However, over the past 50 years, researchers have shown the use of olfaction by birds in a range of biological contexts ranging from navigation and foraging to species, sex, and individual odor recognition (18–23). Since the seminal work of Bang in 1960, the anatomical evidence for avian olfaction surfaced in the scholarly literature (17). As part of this morphological evidence in the olfactory functioning of birds, continuous research focused on comparing olfactory bulbs across species (24). This early survey suggested that kiwis, tube-nosed marine birds and some vulture species, had among the largest olfactory bulbs. However, the relative importance of this morphological value with the olfaction modality in avian species was not fully understood and subsequently has become an area of fruitful biological research (See **Table 1**) (23). Olfactory-driven behaviors in birds can be discussed in relation to specific natural contexts and for purposes of this paper, the bridge between these natural traits will be linked to their potential forensic approaches. A description of three avian model systems will be presented: homing pigeons, turkey vultures, and domestic chickens.

TABLE 1 | Comparison of olfactory bulb to brain ratios, adapted from Bang and Cobb 1968.

ORDER/Species	Olfactory Bulb Diameter (mm)	Cerebral Hemisphere (mm)	Bulb/ Hemisphere Ratio
APTERYGIFORMES			
Kiwi *Apteryx Australia*	12.0	35.0	34.0
PROCELLARIIFORMES			
Snow Petrel *Pagodroma nivea*	6.7	18.0	37.0
Wilson's petrel *Oceanites oceanicus*	3.6	10.8	33.0
Wedge-tailed Shearwater *Puffinus pacificus*	5.5	17.8	30.0
Greater Shearwater *Puffinus gravis*	6.0	20.0	30.0
Dove Prion *Pachyptila desolata*	4.1	14.0	29.5
Black-footed Albatross *Diomedea nigripes*	8.0	28.0	29.0
California Shearwater *Puffinus opisthomelas*	5.0	17.0	29.0
Cape Pigeon *Daption capensis*	5.5	20.0	27.5
Fulmar *Fulmarus glacialis*	5.7	21.0	27.0
Diving Petrel *Pelecanoides georgicus*	2.0	11.3	18.0
COLUMBIFORMES			
Wild Blue Rock Pigeon *Columba livia*	2.9	13.7	22.0
Domestic Rock Pigeon *Columba livia*	2.0	11.0	18.0
FALCONIFORMES			
Turkey Vulture *Cathartes aura*	6.0	24.0	28.7
GALLIFORMES			
Domestic Fowl *Gallus gallus*	2.0	13.0	15.0

Homing Pigeons

One of the most common avian models to study animal navigation has been the domesticated rock pigeon (*Columba livia*). Beyond their fascinating natural traits, the homing pigeon has been used in a variety of field operations including to transport messages and carry small light-weight packages, including smuggling contraband into prisons or carry messages in times of war/conflict. Undoubtedly their level of intelligence cannot be ignored, but more so, is understanding their ability to travel hundreds of kilometers to and from their home loft even after being released in completely unfamiliar territory. This is where the concept of olfactory navigation behavior is important for these types of potential applications. Back in the early 1970s, Papi et al. conducted pioneering studies with a group of pigeons with their olfactory nerves sectioned and an intact control group of pigeons (25). Only the intact group returned to the home loft. Furthermore, at around the same time, another experiment conducted by Wallraff introduced the hypothesis that indeed these birds used an environmental odor picture directly linked to their successful navigation back home (26). The idea behind an environmental odor picture is that pigeons are able to learn and associate these environmental "local" odors in conjunction with other factors such as wind. Hence, when left in unfamiliar territory, they are able to identify this odor "bouquet" and remember the direction and displacement of these environmental olfactory cues eventually leading them back home (**Figure 1**) [see review,(27, 28)]. This seminal olfactory navigation model basically branches into two distinctive steps, the first one where the pigeons learn the wind-borne odors in their home loft surroundings along with the wind direction (29) and secondly, an active operational step where the relocated bird can determine direction of displacement by identifying local odors and remembering where these local odors came from at the home loft (27, 30). The definition of this environmental odor blend was furthered validated by a model suggesting an explicit spatial "network" of odor gradients which is directly linked to location estimation relative to the loft. In this work, instrumental

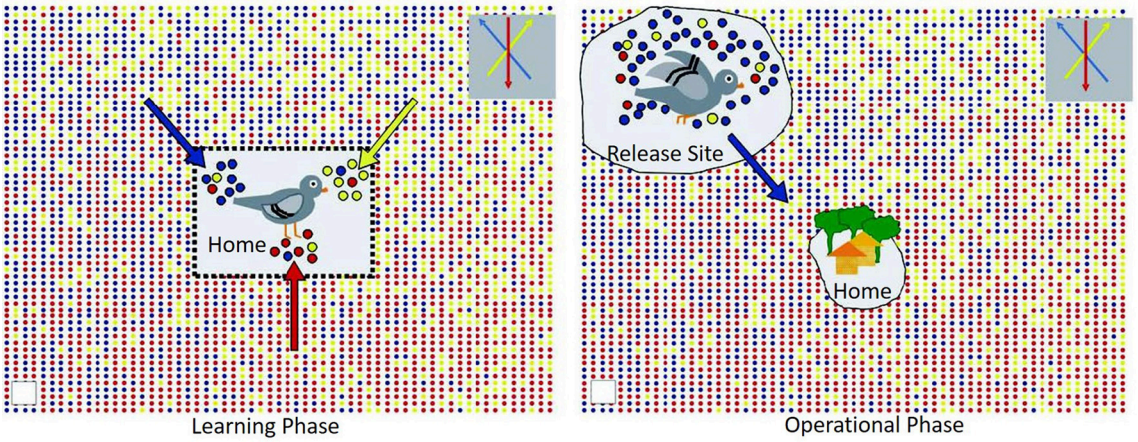

FIGURE 1 | Graphical representation of the environmental odor blend hypothesis in pigeon navigation; with permission Gagliardo (27).

analysis of air samples was studied at 96 sites over a radius of 200 km showing that indeed there is a rather stable gradient ratio of hydrocarbons that interact with wind patterns which birds could utilize for navigation (31). To this day, this "volatile atmospheric odor picture" is the subject of active research not only in pigeons (32) but extending the experimental approach to other wild birds (33). Thus, active experimental evidence seems to highlight the capability of the homing pigeon to sample their local odor gradients as a mechanism to establish navigation. Even though the actual compounds used for this purpose is still an area not yet fully understood, the potential for directing pigeons toward specific target odor chemicals by a constant exposure in their home loft environment could be an olfactory task developed for focused detection missions.

Turkey Vultures

As observed from **Table 1**, the turkey vulture has one of the largest olfactory bulbs of any bird (23). These birds are normally associated with their rapid presence at scenes with decomposing tissue and dead animals. Hence, it is not unexpected that this avian specie has been the subject of study in numerous forensic taphonomic experiments. But before taking a forensic perspective, it is important to understand their olfactory tracking capabilities. Stager (34) conducted detailed field experiments where he noted that odors from both fresh and decomposing animal tissue, produced positive olfactory responses from turkey vultures. In his pioneering work, he also noted that this avian specie was able to detect the presence of hidden animal baits thus further strengthening the olfaction modality used in food location. Stager further describes how turkey vultures were attracted to a volatile organosulfur compound, ethyl mercaptan, used by oil company engineers as an odorant for gas leak detection (35). Hence, foundational observations led to the suggestion that olfaction indeed played a significant role in the life history of this bird. Other studies have even suggested that turkey vultures can discern the age of the carcass. Houston (36) performed experiments where turkey vultures were efficient at locating 1-day old carcasses while rejecting completely rotten meat. Thus, olfaction in this animal model can play a significant role for food location and also highlights a distinctive odor picture of the condition of their prey.

From a forensic viewpoint, vulture species have been the subject of study in terms of the effects of scavenging on human remains. For forensic investigators, animal scavenging can disrupt the crime scene by the dispersal of remains far from the location of interest and also represents a challenge in the estimation of the postmortem interval (PMI). However, regardless of the problems faced by crime scene investigation (CSI) teams by the effect of these scavenging activities, this avian model showcases a keen sense of olfaction for the decomposition odor plume. Reeves (37) used pig carcasses as scavenging targets in the central Texas region during summer months. Both black and turkey vultures waited 24 h before scavenging activities and skeletonized the carcasses in as fast as 3 h. Compared to this study, another experiment conducted in Southern Illinois highlighted that there was a delay in the time of first vulture arrival (up to 28 days), much slower feeding times on the pig remains. Hence, this study suggests an effect that vulture scavenging is directly linked to geographical region and climate (38). Furthermore, using spatial analytical methods, researchers have observed how skeletal remains are dispersed by vultures to lower elevations, and that such dismemberment and dispersal occurs during early phases of the scavenging activity (39). Even though olfactory studies in this avian species are limited, there is evidence of their olfactory detection toward a "decomposition odor blend" that has direct practical implications for future research in the area in terms of decomposition odor stages (as that seen with the condition of their prey) and in the potential identification of decomposing human remains.

Domestic Chicken

Like pigeons, the domestic chicken is a familiar avian model within various biological experimental contexts. In a review by Jones and Roper (40), the functional significance of an olfactory

modality is described in the domestic fowl. This animal system has been studied with respect to odorant exposure in terms of their rearing environment and chemosensory learning aspects. Studies with odorants such as isoamyl acetate, eugenol, and allyl sulfide demonstrated that 1-day old chicks showed differential sensitivity to different odorants at varying concentrations (41). Furthermore, evaluation of odorant exposure to chicks pre-hatching has also been investigated with stimulus such as strawberry demonstrating that a chick's chemosensory preferences are changed with a pre-hatching exposure to the desired stimulus thereby implying olfactory learning (42). Variations of these early exposure experiments have included a range of odorants (see review by Jones and Roper) (39), and also a variety of different methods of odor presentation (43–45). In other behavioral assessments, fecal predator odor was presented to domesticated chickens and showed that individuals can respond to predator olfactory cues, as observed by their decreased foraging and increased vigilance, without any prior odorant exposure or learning (46). Furthermore, it has been shown that even a blend of odorants representing a "motherly" scent reduces stress as determined by a range of physical and behavioral parameters (47). In a study conducted by Bertin et al. (48), a pre-hatch effect of the intensity of odor signals in the regulation of later feeding behavior was reported, thereby highlighting the capability of embryo chemosensory learning. Collectively, these findings provide growing support for the role of olfactory cues in this avian species in a series of chemical communication purposes.

HOW CAN AVIAN USE OF ODORS BE COMPARED TO CANINE BIOLOGICAL DETECTION?

The presented avian models emphasize the distinctive use of odors in their natural contexts. From navigation, food searching, to olfactory learning, these three presented avian species corroborate the use of olfaction. However, what has yet to be exploited is the potential uses of these natural olfactory-mediated behaviors in a more practical biological detection context, namely forensic detection and a direct comparison to the canine model. As stated previously, canines are the biological detector of choice, specifically in the realm of law enforcement and security purposes. In terms of biosensor applications, other animal models such as rats, bees, wasps (49), and elephants (50) have demonstrated such potential applications. To date, the avian species has not been the focus of any study for forensic odorant detection applications.

One reason for the neglect of avian biological detection could be that olfaction is not historically been considered a major sensory modality in birds. Perhaps, a general lack of recognition of the importance of olfaction in birds has misguided our efforts, despite the evidence, that avian species could be redirected for detection roles. When looking at the 3 avian models presented in this paper, olfaction in birds plays a key role for chemical signaling, communication, odor learning and exposure, early animal experience, and as a housing or environmental enrichment (as seen with navigation). The environmental enrichment can be observed by the ability of birds (as seen with the pigeon model) to learn environmental odors in association with wind direction, which highlights how the environment provides an odor source they are able to recognize to determine displacement direction (26). All of these olfactory-guided contexts are shared by the canine species. The only difference between these 2 species is the application to practical forensic detection roles.

The Environmental Odor Bouquet of Birds and Dogs

Using the "odor map" model of avian olfaction as that observed with pigeons for navigation, a comparative viewpoint can be made with the odor plume encountered by a canine during their search pattern behavior. This spatial odor gradient map suggested by Wallraff (25, 30, 51) in terms of sampling the air to obtain environmental odor cues for directionality, can be directly linked to canine's directional tracking. Whether it be for operational tasks such as finding a missing person or in search and rescue missions, the success of this olfactory role is in the canine's ability to sample the surrounding air for directionality as to the whereabouts of the target's location. In this case, the canine is not finding home (as the pigeon with the home loft) but his trained target odor. Studies in canine olfaction have embarked on evaluating the behavior of dogs during this olfactory tracking. Thesen et al. (52) evaluated 4 trained German shepherd tracking dogs using 20-min old tracks on grass and 3-min old tracks on concrete. They recognized three distinctive phases, an initial searching phase, a deciding phase (determination of directionality) and a tracking phase. Thus, the study demonstrated the need of the canine to obtain olfactory cues from the environment and points to the sensitivity in detecting specific substances for successful tracking. Other studies have even compared olfactory and visual cues for directionality of tracking (53) and also evaluated the amount of discrete information needed (5 sequential footsteps) to determine the direction of an odor trail (54). Thus, this tracking example within the canine model can be directly compared to the pigeon navigation model where the bird "samples the air" to determine directionality in relation to the home loft. The notion of an environmental odor blend capable of yielding these olfactory cues for navigation/tracking purposes exists for both species, thus the possibility of directing this navigation olfactory behavior (as in the case of birds) to specific chemical odor blends of forensic importance, within an avian species, is certainly not so implausible.

A Decomposition Outlook

A growing area of operational use and research needed within canine biological detection has been that observed in the detection of human remains, or the use of the so-called cadaver dog. Not only for particular crime scene processing issues (i.e., clandestine graves) but also in contexts such as those observed after natural disasters where these canine teams are deployed to help locate and identify deceased victims under difficult terrains and rubble piles. Many research groups have focused

on understanding the chemical odor blend of decompositional odor to gain a perspective on this volatile odor profile. Vass et al. (55–57) initiated the establishment of an odor database of human remains showing chemical trends for volatile organic compounds detected utilizing triple sorbent traps. The understanding of a human decomposition odor picture plays a key part in better directing optimal training procedures for biological detection. Hence, other work in the area has focused on the development and analysis of synthetic training aids (58–60), influence of age, soil textures, and surfaces on chemical odor profiles (61–63), to name a few. Recently, the introduction of 2-dimensional gas chromatography and time of flight mass spectrometry has been the subject of intensive decomposition odor profiling studies (64, 65). Having a context of the vast amount of research in the area of human decomposition odors, only calls for alternate pathways of detection. As observed in the above discussion on vulture scavenging, and their natural olfactory behavior in finding their decomposing prey, this avian specie could represent an experimental model to further investigate target compounds of interest within a decomposition odor picture. From the knowledge gained from decompositional odor databases using instrumental analysis, researchers can target compound validation using avian models such as turkey vultures to verify odor mixtures and concentration thresholds that yield positive avian response. Different odor blends can be prepared and demonstrate attraction or aversion to better understand human decomposition using an altogether novel biological system.

A Forensic Perspective on Odor Exposure

Under operational conditions, canine biological detection revolves around routine maintenance training of the target odor(s) for the corresponding mission. Hence, it is imperative that optimal performance be assessed not only on the behavioral aspect, but also in a thorough understanding of the target odor chemicals involved in that positive alert. Advances in the analytical forensic laboratory have resulted in increasingly lower detection thresholds allowing the elucidation of some of these volatile odor chemical signatures (13). Explored forensic areas have included narcotics (66–68), explosives (69, 70), and human scent (71). Regardless of the specific area of detection, a common factor of study is the underlying link between odor exposure and the behavior of the canine with respect to that odor mixture. Different studies have geared to understand effects of extraneous odors on canine detection sensitivity (72), while others have tested the number of substances trained with respect to detection performance (12). Just as that seen with the decomposition odorous blend, a key aspect in optimal detection is the ability to provide efficient training aids. Thus, comparing this need and area of research, a comparative perspective can be bridged with the domestic chicken as an avian model. Odor exposure pre-hatching and the link to odor learning can be extended to employing odor chemical of forensic importance in order to establish baseline odor thresholds within an avian species under controlled laboratory conditions. Comparing to practical canine models of experimentation, where different training aids, and/or target odor chemical are presented for behavioral responses, the domestic chicken can be trained to detect similar odor chemicals, developing the possibility of detection by indicating presence/absence of stimuli in natural environments. Initial work in this area has demonstrated that birds can be trained using the same forensic target odors used by dogs (73).

CONCLUSION

The role of biological detection in the area of forensic science and national security has witnessed an increase of active research investigating various animal models, with canines, being the most optimized and best-known working animal model to date. However, animal systems such as birds provide an olfactory foundational framework worth exploring further, and which to date, has largely been ignored within a practical operational perspective. It is important to inform how scientific support highlights an active and varied role of different olfactory-mediated behaviors within the avian species. Canine detection science has become a key tool in many forensic applications, but by working on a parallel level, birds can represent an avenue of olfactory detection that provides yet another pathway for implementation. It is critical to the success of biological detection to assess alternate models that represent solid evidence-based characteristics of fine-tuned olfactory capabilities. This review serves as a call for the research community to consider a different perspective on birds as a viable and working system for comparative olfactory tasks as that observed with working dogs simply taking into consideration already existing life history olfactory traits.

AUTHOR CONTRIBUTIONS

PP contributed with overall writing and preparation of the article, appropriate literature review and general outline of review. KF contributed with general outline of review, presentation of topic, editing and review.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

HYPOTHESIS AND THEORY
published: 06 September 2018
doi: 10.3389/fvets.2018.00206

Identifying and Resolving End of Session Cues in Substance Detection Canine Training

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When training and working a substance detection canine, a trained final response should be performed immediately upon recognition of odor (Generally, a 1–3 s window is preferred within our detection practices). Typical canine training places much emphasis on planning and setting up training scenarios to achieve specific objectives but not much consideration is given to how to end a training session. When the canine fails to maintain criteria, trainers are left trying to determine the cause of poor performance. One consideration often overlooked is a phenomenon called End of Session Cueing that may exist in detection training whereby a previously trained canine no longer responds to odor because it has taken on aversive association. This may be due to several factors associated with motivation. The sequence of events at the end of a session can be as equally important to maintain motivation for the task of scent detection in future sessions. This paper will identify and examine multiple factors associated with "End of Session Cues" in working dogs, how they may be responsible for poor final response performance and discuss potential strategies to address them.

Keywords: canine substance detection, canine behavior, substance detection canine training, working canine behavior, end of session cues, poisoned cues, premack principle, counterconditioning

OPEN ACCESS

Edited by:
Cynthia M. Otto, University of Pennsylvania, United States

Reviewed by:
Joseph Wakshlag, Cornell University, United States
Ken Ramirez, Karen Pryor Clicker Training, United States

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Specialty section:
This article was submitted to Veterinary Humanities and Social Sciences, a section of the journal Frontiers in Veterinary Science

Received: *20 April 2018*
Accepted: *08 August 2018*
Published: *06 September 2018*

Citation:
Topoleski J, Schultz CA and Warren WG (2018) Identifying and Resolving End of Session Cues in Substance Detection Canine Training. Front. Vet. Sci. 5:206. doi: 10.3389/fvets.2018.00206

INTRODUCTION

The trained final response behavior and how it relates to odor are a culmination of several factors which include but are not limited to individual and canine breed selection, behavioral genetics, trainer and handler skill levels and environmental experience (1, 2). The final response can be any behavior that is trained or conditioned during the initial odor imprinting process commonly associated with detector dog training. The Scientific Working Group on Dog and Orthogonal detector Guidelines (SWGDOG) and the National Institute of Standards and Technology's (NIST) Dogs and Sensors Subcommittee operationally defines imprinting as, "A phenomenon by which an animal, during a formative stage of life, forms a lasting attachment to, and preference for, some object or activity through exposure to the same independent of consequences. Operational usage: A method of initial odor/scent discrimination training" (3, 4).

The type of behavior selected as a final response is usually dependent on the target odor source and the ultimate utilization of the canine. For example, human remains detection canines may be trained to bark upon finding the odor of human remains so as to not disturb potential evidence and to alert handler of the presence of the target odor when out of view. Often times final response behaviors can be breed specific behaviors that naturally occur and are captured, or they are behaviors shaped to meet particular operational requirements.

In substance detection canines, a phenomenon may exist whereby newly trained or experienced canines, have progressed through odor imprinting/association and final response training phases with high rates of success and have demonstrated proficiency in various environments. However, over time as the training is moved to different locations or shifted to different contexts the canine seems to make a conscious decision to approach target odor, investigate the origin of the odor, then ignore it altogether and engage in other activities not associated with odor detection. This occurs even if the canine has had much exposure to a certain type of search environment/context with a high rate of reinforcement.

A seasoned handler or trainer would almost immediately identify the problem as a lack of focus, lack of motivation, or a reduced interest for the task of odor detection. All of these may be correct, however, how often do trainers/handlers observe the sequence of events at the ***end*** of a session and take that into consideration when evaluating these training challenges? This is why it is also important to understand and identify End of Session Cues and how these cues and events can negatively impact performance.

DISCUSSION

End of Session Cues

End of Session Cue(s) (EoSC) is simply a behavior or a series of behaviors or events that has been understood by the animal to mean that the training session is about to end (5). This could be purposely trained, such as a "Free" cue at the end of a training session indicating that the session is complete and the animal is released. Conversely, it can also be a cue that an animal has learned without formal training to associate experiences or certain stimuli with an aversive event. For example, marine mammals can tell by the sound of the trainer's near empty fish buckets that they are almost out of fish (reinforcer). When this happens, behavior performance may decrease as animals may start ignoring trainer cues because they may not have enough reinforcement left. Through association, the animal learns that the trainer is about to leave the area, and ultimately, the animal expects that the session is about to end, which equates to the animal perceiving that opportunities for reinforcement are decreasing or have ended. When this occurs, it may not be worth the animal's effort to continue to perform behaviors or interact with its trainer.

Under these circumstances the animal then finds something else of value in the environment to engage in. This is problematic as the animal then learns to reinforce itself for incorrect or undesirable responses, meanwhile, trained and desired behavior(s) may fade away and ultimately cease to occur if the EoSC is perceived by the animal as an aversive event. Whatever that stimulus in the environment is that the animal engages in may now become a competing stimulus to the task in which the animal was trained to do or asked to do by the trainer. This can then become even more problematic as the undesirable behavior continues to occur with no consequence if the trainer allows the animal to continue to rehearse undesirable behaviors (swimming away, foraging for fish at the bottom of the pool, engaging with other animals in the pool). In the working dog, an example would include not responding to a target odor, only to leave it and engage in a crittering behavior- such as smelling urine spots of other canines or animals, chasing animals, foraging for food, etc. Crittering is defined by SWGDOG and NIST as "A change in the canine's behavior where the canine becomes distracted by animal odor or some other animal distracter. Usually evident as there is a change in body language (head and tail position)" (3, 4).

As these undesirable behaviors continue to occur and are self-reinforced, one must contend with the possibility that the undesirable behavior (chasing animals) may soon replace the desired behavior (locating target odor) during detection tasks. This is due to a component of Hernstein's Matching Law whereby an animal's performance can be directly correlated to the rate of reinforcement the animal received for performing the task in previous trials when presented with choices. When faced with two choices an animal will select the choice that has been reinforced more frequently (6, 7). For the detection canine, if an undesirable behavior that is self-reinforcing (i.e., smelling urine spots) occurs more frequently than responding to target odor, Hernstein's Matching Law would predict that the K9 will most likely choose to engage in the behavior that has the highest rate of reinforcement; even if those behaviors are self-reinforced and undesirable (smelling urine).

In addition, each time undesirable behaviors are allowed to be self-reinforced, the strength of desired behaviors may become reduced and are subsequently either not performed or performed poorly (slower, less efficiently). When desired behavior (responding to target odors) starts to be offered less frequently or not to full criteria, trainers or handlers often fall into the trap of accepting/reinforcing a final response performed at a weaker criteria (slower, less intensity, etc.) simply to end the session. As they continue to reinforce the weakened criteria, it now becomes the new criteria.

This relates to working canines in the following ways; all animals, including canines, learn through cause and effect consequences and associations. If choices are reinforced or punished, behaviors are learned or modified. At a more complex level, we see that not only does learning desired behavior occur through associations, learning of undesirable behaviors can arise through accidental reinforcement. Undesired behaviors can be created in the same way desired behaviors are learned if a trainer fails to realize that they are accidentally reinforcing a behavior (8). For the example, as discussed earlier in which the canine failed to respond to target odor and engaged in crittering behavior instead, the canine self-reinforcing itself for crittering because the trainer failed to acknowledge the undesirable crittering behavior, failed to stop the behavior and still reinforces the canine at the end or the search. Thus, crittering behavior increases.

Just as undesirable behaviors can become established through associations, certain stimuli can also be altered through associations. Stimuli that previously had a positive association can come to have a negative association if paired with something the animal finds aversive. An example for the working dog may include being corrected near target odor, over time, the canine may start to equate target odor with an aversive event and start

to avoid target odor all together. Another example may include immediately going back to the vehicle after a successful training session in which the canine found a target odor. In this example, the canine may perceive being returned to the vehicle kennel as a punisher.

A few notes worth discussing are that associations are not always created by singular responses, but can also be made between a series or chain of events. By linking a perceived aversive event (going back to the vehicle) with a previously learned task with positive reinforcement history (odor detection), the canine can develop an aversion that can be associated with a preceding event (interaction during search with handler, responding to directional control commands, etc.), a particular context (odor detection in open area search, wilderness, building, etc.), or even specific reinforcer(s) i.e., ending with a ball as a reinforcer on the last find (thus, deploying the ball may become the EoSC of going back to vehicle).

Additionally, fixed intervals of reinforcement can be inadvertently established through training that does not provide variability in time. This may lead to a decline in performance, however, it is unknown if the concept of "time" can be understood by the canine as an End of Session Cue. For the purposes of this paper, if the odor is perceived as the EoSC, it would be expected that the canine chooses *not* to give its trained final response in order to avoid going back to the kennel, regardless of time.

While not recognized as a technical term, referenced in scientific literature or acknowledged in the field of Behavior Analysis, the concept of "Poisoned Cues" is a term that is used within the animal training industry to illustrate the ability of stimuli that previously had a positive association to take on aversive associations. The term was originally coined by Karen Pryor in 2002 (9, 10). An End of Session Cue can become a form of a poisoned cue if some form of stimuli associated with the ending of a session takes on a negative association. This can also be related to the Premack Principle whereby an aversive activity or association occurs after the performance of a desired behavior thus, weakening it in future trials (11, 12). Use of the Premack principle will be discussed further in the Solutions section of this paper.

Consider the following choices by the canine: either leave odor and extend time in the environment, or, respond to the odor and go back to the vehicle. With that said, if a canine finds immense satisfaction, reward, or reinforcement in hunting or engaging with prey, could it stand to reason that "putting the canine up" at the end of a session for correctly identifying a target odor could be "aversive" or even a mild form of punishment? Granted, we *think we are rewarding* our dog for "the find" by praising, playing, and reinforcing it with what we think is rewarding- which to an extent we are. If one considers the canine that encounters an environment that is rich in stimulation combined with being kenneled all day (lack of activity/stimulation) and a reinforcer that fails to compete with the environment, the choice becomes easy. The canine is likely to value everything else other than the task of odor detection. Are we possibly, inadvertently, negatively punishing the canine by removing a reinforcing stimulus (environment)? Or at a minimum, offering a reinforcer that is of lesser value than the competing environment's value. Is there a way that you can incorporate the environmental experience into your reinforcement? You may also observe that when a session increases in duration or length, performance may drop in anticipation that the session will end and the canine starts to engage in other activities such as crittering. Crittering behavior then competes with target odor because it now becomes more reinforcing than making a find (a find equates to "the session is about to end and they are going back to the vehicle" where no stimulation occurs). Thus, the odor itself becomes "poisoned" and now becomes the EoSC.

In training, often the vehicle is used as a negative punisher in which a canine is placed back in a vehicle for failure to perform a task due to lack of focus or motivation. If the perceived punisher is accidentally applied after a correct response on a target odor, then it would stand to reason that the canine is being punished by going back to the vehicle. Furthermore, Classical Conditioning would show that repeating of this routine would suggest the "target odor" itself may *eventually* trigger a signal that a punisher would be coming soon, thus, giving way for the avoidance of odor and an explanation to why the canine would leave odor to engage in other extraneous behaviors that it finds to be of more value. Consider the following:

Negative Conditioned Stimulus or -CS (going back to vehicle = lack of reinforcement or reward)

Positive Conditioned Stimulus or +CS (odor = playtime, interaction, and stimulation is coming)
+CS (odor) ——> -CS1 (vehicle)

If reinforcement is weak or not "reinforcing", ultimately odor may take on the signaling of an aversive event, or, going back to vehicle:

-CS2 (odor) ——> -CS1 (vehicle)

Now that end of session cues or poisoned cues have been discussed and how they can develop, it is important to recognize behaviors associated with them so that we can mitigate them as quickly as possible. Some observable behaviors that handlers/trainers may observe include but are not limited to:

- Approaching, investigating and then leaving a trained odor source to engage in self- reinforcing- play behavior, scavenging or eating in environment, etc.
- "Crittering" behavior (smelling and investigating animal odors or non-targets for an extended amount of time).
- Running off or being unresponsive to the handler while trying to maintain search/directional control.
- Avoidance of the vehicle or hesitation while loading into the vehicle (This may occur during the initial load up or after a successful find).

These are just a few behavioral indicators that may be observed. These may also be observed with other issues as well and should not solely be considered to be end of session cues.

In addition to the behaviors outlined earlier in this article it is equally important to evaluate another part of the equation before determining if end of session cues are a possible culprit of poor training. Consider the canines' daily activity repertoire. If you work with a canine in a professional setting, and perhaps more typical in law enforcement applications, canines may spend a large amount of their day either in a yard, a kennel, a vehicle, or a combination of all. In general, they may only get trained or "worked" realistically for 1–2 h a day. This is actual work time; not putting the dog in the car or truck at the beginning of a shift and driving for 8 h. And if there is inclement weather or an extended lapse in training time, this number will most definitely decrease. This isn't a critique by any means, rather a harsh reality that must be recognized and addressed when training canines. Another important part in this equation is handler action and activity schedules. Consider some of the following questions:

Handler Action:

- How is the canine loaded into the vehicleon productive vs. unproductive sessions?
- How is the canine leashed up after each session or search?
- How long do you spend reinforcing the dog before kenneling?
- Is the kennel used as a punisher for poor performance?
- Does your canine get to take its toy with them before the search begins?

Canine Activity:

- How long does the canine spend in the kennel between sessions?
- Once the canine is reinforced does your session end immediately or do you continue the search scenario? How often?
- What is the ratio between time spent training or searching, to time spent in the kennel and being inactive in a 24 h period?

An important step in determining the underlying cause of final response regression is to test your assumptions as outlined earlier by setting up controlled field tests and testing the variables that are thought to be at the heart of the issue. The key is to test each assumption or variable separately. The assessment may be best done as part of a team; handler and at least one observer depending on what you are trying to assess. Preferably, the observer will be familiar with the scientific method or conducting blind assessments to help guide the process along and document the observations. To effectively assess the factors noted earlier in this article (behaviors associated with poor motivation, handler action and canine activity) we found it most beneficial to eliminate any extraneous variables that may contribute to the problem, or at least try to account for them when setting up the assessment(s).

Re-valuate the Reward System

It is generally accepted that, some canines, especially those with high prey drive or motivated to chase in the working dog industry, may consider the "act of the hunt" intrinsically reinforcing where no other behavior or object is as reinforcing. Each canine, regardless of breed or litter, may exhibit differences in preference then their closest siblings. This means that even their reward and value systems may be different. To strengthen your reinforcement, consider magnitude or "jackpot" reinforcers and make them an extended "event" not just a short occurrence. This can be accomplished by expanding the reinforcement in both intensity and duration at the end of a session. An example of this would be to reinforce the canine the entire way back to the vehicle, allow the canine to possess the reinforcement on the journey back to the vehicle and continue to praise the canine during the loading process. Emphasis should be placed on the level of arousal that occurs during reinforcement as increased arousal levels have been proven to enhance learning and memory consolidation (13–15). In fact, the reinforcement may not even need to be related to the task of substance detection for learning to occur as the dopamine release during unrelated pleasant experiences affect learning and memory (14, 15).

Something to consider at the end of a training session is occasionally letting the canine engage in extended reinforcement events long enough to reach satiation; letting the canine tell YOU when it is done being reinforced for a change. Engaging in play behavior after a training session has been shown to improve training performance in canines trained in discriminative tasks (16). While the application of play interaction has been historically considered as standard methodology in the substance detection canine training industry due to anecdotal observations; until now, there has been little work completed to quantify the role of playful activity as a reinforcement option in canines trained in discriminative tasks. Another consideration worth mentioning is varying up the type of higher valued reinforcement options. For example, One time the canine gets a lot of praise, receives a ball or tug and the next time it gets reinforced with free time or gets to engage in other behaviors that it values more. The key is getting the canine to associate the higher value reinforcement with the odor and trained final response behavior performance.

The above paragraph simply describes a concept called the Premack Principle whereby one can use an activity or engagement in behavior(s) in which a canine values as a reinforcement option; the more frequent activity will reinforce the other less frequent one (11, 12, 17–19). Premack theorized and proved that if an animal performed a behavior (behavior A) at a greater rate than another behavior (Behavior B), then behavior A can be used effectively as a reinforcer for performing behavior B (12). Lindsay further discusses this by stating the following:

> *"During an ordinary training session, the dog is going to prefer performing some exercises more than others. Determining at any moment what the dog would prefer to do and then providing access to that activity on a contingent basis is a sound and efficient incorporation of the Premack principle.*
>
> *It would be consistent with the Premack principle to follow the performance with an even more exciting and reinforcing opportunity." (11).*

In working dogs, this was discussed in Schutzhund and protection dogs where the activity that the canine preferred to complete was bite work. The activity of bite work was used to

reinforce obedience behaviors (20). If obedience behaviors were performed correctly then the canine was reinforced by being given the opportunity to engage in bite work. In the detection canine, an example of this would include choosing a behavior of higher value that the canine prefers to engage in and using it as a reinforcer for correctly responding to odor. For example, If the canine successfully responds to a target odor it will be given the opportunity to engage in a directional control session. The higher value behavior (directional control) can be independent and semantically unrelated to the lower value behavior (odor detection) so long as it is applied with immediacy, increases emotional arousal and is applied with duration (13, 14, 21, 22).

It is important to note the difference between how Premack principle activities are applied and how specific stimuli are perceived. The Premack principle relates to activities that are performed with frequency and their ability to be used to reinforce or punish behavior, whereas the majority of this paper discusses specific cues associated with performance regression of desirable behaviors. The Premack principle can strengthen or weaken responses based on behaviors performed. Before the activities are applied, specific cues can be associated before the activity is engaged in.

For example, going back to the vehicle (a perceived punisher) is an activity—where the odor becomes the cue that precedes the activity of going back to the vehicle, thus, weakening the trained final response to odor or creating avoidance of the odor altogether.

Evaluate the Canine's Routine

As discussed earlier in this paper, the canine's daily routine may not be stimulating enough. By taking a realistic inventory of your dog's daily activity a handler will know how many hours it spends in a kennel, or not training vs. learning and improving? This will be an excellent place to start the problem solving process. In addition, knowing the canine's strengths and weakness can make us aware of the length of sessions that we conduct and compare that to the time that they are alone without stimulation. If we think about the holistic activity of the canine; is the canine getting enough physical or mental stimulation?

Counterconditioning the Odor

While changing the perception of going back to the vehicle from an aversive event to a pleasurable event is necessary so to is counterconditioning the stimulus of the odor to "unpoison" the cue. Taking a few steps back in the training plan to review the odor association or imprinting training may prove to be beneficial. To change the perception of the odor stimulus, repetitiously reinforcing the canine for nose to contact or proximity to the target odor source. By increasing the rate of reinforcement for sniffing the target source without the consequence of immediately returning to the vehicle we are re-establishing the pleasurable experience associated with the detection of target odor. Once the canine demonstrates proficiency of detecting the source of the odor and not leaving it, start incorporating the trained final response criteria. Initially relax the trained final response criteria and increase criteria as the canine demonstrates fluency of the trained final response behavior. It is recommended that while the target odor/cue is being counterconditioned that the canine not immediately be returned to the vehicle upon completion of the exercise so as to not to continue to poison the target odor.

Evaluate the Health and Fitness of the Canine

Consider moderately exercising the canine prior to a training session or deployment (approximately 1–2 h prior to searching). Exercise benefits include an increase in serotonin and dopamine levels in the brain, which also assist in increased motor coordination, regulation of emotions, and pleasurable feelings (23). In fact, exercise with moderate length and intensity may improve learning and memory consolidation (24, 25). Robbins et al. (26) define fitness for the working dog as a "combination of cardiorespiratory function, balance, strength, flexibility, proprioception, stamina and muscle strength" it is worth noting that exercise should be limited to brief bouts of activity so as not to create fatigue, hyperthermia or impede detection capability. Environmental and physiological factors should be considered and exercise sessions should be structured to slowly increase a canine's fitness level to perform at the level desired. If a canine is not adequately acclimatized to heat and humidity, physical activity in those types of environments can increase heat stress or heat stroke in working canines as they are expected to perform in mentally and physically adverse environments (27).

In addition, be aware of how frequently the canine is working. Keep tabs on the duration of sessions and when your canine "checks out." It could be that searching has become aversive by reduced motivation over time or fatigue. Perhaps the canine has just been exposed to the same search scenarios and searching isn't as reinforcing, or conversely, that the training is too hard and the canine is perhaps mentally fatigued. An extended break from training and work may help learning in some instances. Rest is a vital component to learning and performance (28).

CONCLUSION

The end of session cue is a concept that is often overlooked, underused or even forgotten as a canine's detection proficiency improves. End of session cues can be correlated with the manner in which training sessions end, how and what type of reinforcers are delivered and the manner in which the canines are returned to holding kennels afterwards. Associations of events can be either positive or negative. In the case of poor performance, one should not only evaluate how sessions are conducted but also how they are ended. When evaluating poor performance, improper foundation should be discounted as an underlying cause. Once this is eliminated, the possibility of end of session cues should be explored.

By being aware of the behavior indicators associated with end of session cues and the canine's activity schedule canine handlers/trainers can be more equipped to mitigate their effects on working dog performance.

AUTHOR CONTRIBUTIONS

JT and CS developed the concept. CS researched the literature support. CS, JT, and WW participated in the manuscript preparation.

FUNDING

Funding for this article was provided by the Federal Bureau of Investigation.

ACKNOWLEDGMENTS

This is publication 18–19 of the Federal Bureau of Investigatio (FBI), Laboratory. Names of commercial manufacturers are provided for identification purposes only and inclusion does not imply endorsement by the FBI. The views expressed are those of the authors and do not necessarily reflect the official policy or position of the FBI or the U.S. Government.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

PERSPECTIVE
published: 16 November 2018
doi: 10.3389/fvets.2018.00284

A Solution for the Shortage of Detection Dogs: A Detector Dog Center of Excellence and a Cooperative Breeding Program

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OPEN ACCESS

Edited by:
Emily Patterson-Kane, American Veterinary Medical Association, United States

Reviewed by:
Janice Lauren Baker, Veterinary Tactical Group, United States
Joseph Wakshlag, Cornell University, United States
Robert Lee Gillette, Nutramax Laboratories Veterinary Sciences, Inc., United States

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Specialty section:
This article was submitted to Veterinary Humanities and Social Sciences, a section of the journal Frontiers in Veterinary Science

Received: 06 April 2018
Accepted: 25 October 2018
Published: 16 November 2018

Citation:
Leighton EA, Hare E, Thomas S, Waggoner LP and Otto CM (2018) A Solution for the Shortage of Detection Dogs: A Detector Dog Center of Excellence and a Cooperative Breeding Program. Front. Vet. Sci. 5:284. doi: 10.3389/fvets.2018.00284

Currently, demand for US-bred and born detector dogs exceeds available supply, while reliance on foreign-bred sources introduces many unnecessary and unwanted risks. With proper management of a domestic supply line, U.S. breeders can improve both health and behavior by applying scientific principles to breeding and raising of detector dogs. A cooperative national detector dog breeding and development program will mitigate the current shortage of domestic-bred dogs that meet the health and behavior standards required by government, military, and law enforcement agencies. To coordinate such a cooperative, we propose a Detector Dog Center of Excellence (DDCoE) led by representatives of academic canine science programs guided by an advisory board of stakeholders. As a non-governmental organization, the DDCoE will oversee selective breeding of dogs owned by breeders, purchase the resulting puppies, and its members will supervise puppy raising until dogs are of a suitable age to be purchased by government agencies or other working dog organizations. The DDCoE will serve as an approved vendor to facilitate the procurement process. Breeding decisions will be based on proven quantitative genetic methods implemented by a specialized database. A national working dog semen bank will ensure conservation of diverse genetic material and enhance selection response by providing numerous potential sires. As a data collection and genetic evaluation center, the DDCoE will lead research to define quantitative traits involved in odor detection, to understand how these traits develop, and methods to optimize training of dogs endowed with enhanced odor detection ability.

Keywords: detection dog, breeding, semen, cooperative breeding, center of excellence

INTRODUCTION

The increase in frequency of terrorist attacks and natural disasters in the U.S. over the last two decades and the increased understanding of canine olfaction and training have led to a greater demand for healthy detector dog candidates (1, 2). Despite the "Buy American Act" mandating that products for government use be purchased from domestic sources, the Federal government,

historically, and vendors currently supplying diverse United States agencies import most of their working dogs. These dogs frequently come with limited medical and training history data, often inaccurate ages, and their success rates are highly variable. Recent US legislation (3, 4) has encouraged domestic breeding of detection dogs, and some legislators have recommended restricting procurement of government working dogs to domestic sources.

To be prepared to meet the demands for domestic working dogs in the U.S., two major hurdles need to be surmounted. First is navigation of the government procurement process. Domestic suppliers of goods, including dogs, to the US Federal government must meet the Federal Acquisition Regulation (5). Potential vendors have a narrow window of opportunity to submit a cost proposal, which if accepted and the vendor meets all purchase regulation requirements, authorizes the vendor to bid on Federal contracts. These criteria differ among Federal agencies based on the type of work, working environment, training practices, and reward system (toy or food) used by each agency. Individual dogs could be matched with organizations based on behavioral phenotypes, since purpose-bred litters often contain pups with a range of behaviors. In the future, precise definition of work-related behaviors and standardization among agencies would facilitate breeding, selection, raising, and training. This hurdle associated with procurement, along with more lucrative sporting markets has reduced the incentive for domestic breeders to supply working dogs. The second hurdle is to increase the incentives and support for domestic breeders to breed working dogs.

Over the past 50 years, U.S. Federal government agencies such as Transportation Security Administration (TSA) (6), Customs and Border Protection (CBP; https://www.cbp.gov/border-security/along-us-borders/canine-program), U.S. Customs Service, and the U.S. Army (7) have initiated breeding programs. In every case, each of these breeding programs was either disbanded or dramatically reduced due to funding cuts. Clearly, decision-makers did not understand that steady, long-term genetic improvement requires at least three generations of selective breeding based on experience of guide (8), service, and military canine breeding programs (9).

To address current issues with quality and availability of detection dogs in the US, a plan for creating and administering a canine breeding cooperative is described. This cooperative will coordinate organizations and breeders to produce healthy, high-quality purpose-bred scent detection dogs for distribution across agencies. A basic tenet of the plan is to keep ownership of the breeding stock in the hands of private breeders and organizations. Doing so will help ensure the long-term survival of genetically advanced breeding stock, especially during times of scarce government funding.

To execute this plan, a federally supported Detector Dog Center of Excellence (DDCoE) is needed to coordinate the breeding plan, provide oversight of puppy development, collect data for continued genetic and training improvement, and negotiate the many complex issues of US Government purchase orders. The DDCoE will be led by representatives of academic canine science programs focused on working dogs in U.S. veterinary schools. An advisory board will be comprised of stakeholders from Federal, state, and local government agencies, academic institutions, working dog training organizations, researchers, and breeders. One of this board's functions will be to balance the DDCoE's requirements to facilitate diverse participation while maintaining standards that will result in genetic improvement over generations.

Breeders and working dog breeding organizations will be invited to apply for membership in the breeding cooperative. DDCoE managers will screen both the people and the breeding bitches they are nominating for enrollment based on clearly defined standards. Because the DDCoE will serve as the product supplier, during times of reduced US government demand, the DDCoE will be able to sell dogs to state or local governments, working dog organizations, and individuals. This freedom will preserve the ability to remain fiscally solvent. Dogs in the DDCoE program that are unsuited for government scent detection work may be sold to agencies who will place them into other forms of detection or other service work. Ownership of puppies born to bitches enrolled in the program will be pre-determined by contractual agreement made before a bitch is bred. The DDCoE will assume responsibility for puppy raising and for phenotype measurements to identify the best dogs for replacement breeders and for the different working disciplines. When a dog is between 8 and 14 months old, it will enter the inventory of young adult dogs available for sale. The overall intent is to enable government and law enforcement agencies to buy American-bred working dogs selected for an innate scent detection ability, thus ensuring the nation a secure supply of healthy, well-socialized dogs working to maintain public safety, while providing a coordinated approach for the sale of these dogs.

The DDCoE will establish and adhere to ethical standards for the treatment of dogs in breeding and puppy raising activities. Selection of dogs and breeders will be made with the overall goal of producing dogs that are willing and enjoy their work, and will have long, healthy careers. Unethical treatment by breeders, raisers, or DDCoE personnel and volunteers will not be tolerated. Affiliated veterinary colleges and specialty trained veterinarians will provide high quality medical care. An adoption program for dogs not meeting selection criteria and retired dogs will be established. In the event that an alternative career cannot be found (e.g., as a service dog), the dog will be offered to puppy raisers or for placement as a sport or pet dog.

GUIDELINES FOR THE DETECTION DOG CENTER OF EXCELLENCE

Organization

a) The DDCoE shall be a non-governmental organization so that it can be sustained for the decades required to establish and maintain a successful working dog breeding program. The network of cooperating private organizations will meet government vendor requirements and can sell dogs to the federal government, but at times of low government demand, dogs can also be sold to state and local governments, service dog organizations, dog sport enthusiasts, and pet homes.

b) The DDCoE shall be a cooperative consisting of non-governmental working dog organizations sharing the goal of producing sufficient healthy, high-quality, purpose-bred dogs for scent detection work.
c) Breeding stock shall be owned by these non-governmental organizations and private breeders to ensure that genetic improvement is not lost because a single breeding program is disbanded. A wide geographical distribution will prevent large losses from local disease outbreaks or disasters.
d) Academic working dog centers located at veterinary schools, and other similarly interested institutions shall comprise the DDCoE governing board. This board will set goals for the breeding program and coordinate selective breeding decisions for individual dogs. Other stakeholders will serve on an advisory board.
e) The DDCoE will be a 501(c)(3) non-profit or part of a non-profit so it can provide receipts for donations by private individuals, including donations of dogs.
f) The DDCoE will be an aggregator of young dogs from working dog organizations, puppy raising programs, and breeders that meet the contract specifications of agencies such as the TSA.
g) If the DDCoE model can work with academic working dog centers as the initial proof of concept, then the DDCoE Board of Governors will entertain the option of expanding institutional membership to include other veterinary schools and academic institutions with canine science programs and appropriate veterinary support. This would increase geographic diversity among owners of the breeding bitches. Furthermore, it would distribute some of the workload for supplying high-quality working dogs among a larger group of similar, but geographically separated schools and organizations.

Breeding

a) Semen Bank

i) To enable the use of genetically superior males as sires of puppies born into the DDCoE, a frozen semen bank must be established to augment natural service or fresh-chilled semen breeding. Proper use of frozen semen requires a network of veterinarians (theriogenologists) skilled in trans-cervical insemination (TCI), vaginal insemination, surgical insemination. and timely semen placement (10, 11).
ii) Frozen semen on each stud collected shall be permanently stored in multiple geographic locations to prevent loss due to physical or natural disaster.
iii) For optimal semen quality, collected semen must meet minimum standards for post-thaw motility, morphology and sperm count (12, 13).

b) Puppies

i) Identification of Breeding Bitches

(1) Private breeders may nominate their bitches to participate for one or more litters.
(2) Bitches with superior characteristics that enter the DDCoE program may also be identified for breeding. In this case, "superior" characteristics will be determined by application of estimated breeding values combined into an overall selection index that emphasizes the traits that need the most improvement.

ii) Each nominated bitch that meets defined health and behavior standards will receive extensive health screening and phenotypic evaluation from the DDCoE.
iii) Acceptance of a bitch into the program means that the owner will have an opportunity to negotiate a litter ownership agreement with the DDCoE. The DDCoE has the right to refuse litters to ensure that demand is met, but not exceeded.
iv) Coordination by the DDCoE will ensure that insemination, prenatal, and whelping care are provided for every bitch by qualified veterinarians, veterinary technicians, or by the dog's private owner, if that is their choice. Veterinarians or veterinary technicians may be affiliated with one of the participating veterinary schools, or with a private veterinary practice.
v) Before a mating is initiated, the bitch's owner will choose from several litter ownership options including

(1) Donation of the entire litter to the DDCoE,
(2) Sale of the entire litter to the DDCoE
(3) Owners could retain ownership of up to two puppies if seven or more puppies are weaned, or one puppy if between three and five puppies are weaned.
(4) The breeder could retain ownership of the litter and risks associated with selling the litter. DDCoE would have the first right of refusal to purchase as many weaned puppies as it requires.

vi) Young breeding quality bitches identified among puppies born into the DDCoE program may be bred for up to two litters beginning on their first heat cycle after they pass 18 months of age, while continuing training. After whelping their second litter, each bitch will be ovariectomized and will enter the work force in an appropriate career path. Bitches will produce no more than two litters to help maintain genetic diversity and to keep the generation interval short. Genetic improvement occurs by a combination of generation turnover in concert with the application of selection pressure. By keeping the generation interval short, genetic change per year is maximized (14).

c) Population Scaling

The number of matings required to produce 100 Labrador Retriever (LR) detector dogs can be scaled. The following guidelines can be used in the calculations:

i) Assuming the best case scenario, conception rate is ∼85%.
ii) Average litter size is 7.5 puppies. In a study on Norwegian Kennel Club registrations, mean litter size was 6.9 ± 0.2 for LR (15). In The Seeing Eye breeding program, mean litter size at birth for LR was 6.8 ± 2.3 (16). In one study of litter size using frozen semen, average litter size was 5.4 pups (11). These statistics describe the specific populations in which they were measured and are likely to vary for other populations such as LRs bred for detection work in the US. Mean litter size varies

between breeds, so results will vary by breed. In the Norwegian study, German Shepherd Dogs and Belgian Shepherds (also known as Groenendael) had smaller litter sizes (6.1 ± 0.1 and 6.4 ± 0.4, respectively, and Golden Retrievers had larger litter sizes (7.5 ± 0.2) (15). In The Seeing Eye study, German Shepherd Dogs had a mean litter size of 6.4 ± 2.5 (16).

iii) Ninety four percent of puppies born will survive until weaning. In The Seeing Eye breeding program, mean litter size at weaning for LR was 6.4 ± 2.4 (16).

iv) Although early puppy screening at 8 weeks still remains a goal, many studies to date have failed to identify accurate predictors at this age (17–19). For the purpose of this calculation and until more accurate screening methods are developed, retention of 100% of the puppies is assumed.

v) Ninety five percent of dogs will successfully complete the puppy raising phase.

vi) Eighty percent of dogs will pass medical screening at the end of the puppy raising phase.

vii) In the beginning, it is anticipated that between 30 and 50% of dogs will meet government contracting standards, but with the production of genetically improved puppies born into future generations, the success rate will improve. This estimated success rate is based on findings in working and guide dog programs and the experience of the authors. A study on the Swedish Dog Training Center (20), which trained dogs for patrol, detection, and guide work, reported that about 50% of the dogs selected for training were disqualified. A 30% success rate was reported for dogs entering advanced training at the South African Police Dog Center (21) and for dogs at the Tokyo Customs Canine Training Center (22). In work on predicting success in multiple guide and assistance dog programs, Duffy and Serpell (23) report the programs have training success rates between 30 and 50%. For Australian guide dog programs, success was reported as 50–56% (24). A survey study involving an international group of guide dog schools found success rates between 23 and 100% for dogs completing training and 13 to 100% for dogs still working 1 year after completing training (25).

Under these assumptions, 53 litters will need to be born in a year to produce 100 puppies that meet contracting requirements. This example may over or underestimate some of the factors influencing successful number of puppies. Data collection from the program is necessary to provide more accurate estimates. Some of the success rates will improve as the DDCoE refines processes for insemination, whelping, puppy raising and training, and as genetic improvement is made across the population over generations. The approach of early, flexible training that depends on each puppy's aptitudes is likely to significantly improve success rates as dogs can be prepared for careers in explosives (object or passenger screening) and narcotics detection, search and rescue (SAR), human remains detection (HRD), and other specialties (www.vet.upenn.edu/wdc). Although these fields of work all involve odor detection, different sets of behaviors are optimal for different settings. This flexibility is likely to allow for a higher success rate than working dog breeding programs that focus solely on a single criterion for a successful outcome. This approach requires clear definition and consistent scoring of phenotypes associated with olfaction ability and aspects of behavior to ensure that each dog is placed into its optimal working discipline (26).

Puppy Raising

a) The DDCoE will have complete ownership of the puppies it purchases, enabling it to sell puppies to any government or private working dog organization. The DDCoE will be responsible for raising and socializing each puppy to maximize the probability that a young dog will meet government procurement requirements.

b) The procurement process for each agency sets the price of a dog but does not require the government to purchase any minimum number of dogs. The DDCoE will be able to sell dogs not needed by agencies within the Federal government to state and local agencies, private working dog organizations (e.g., search and rescue), and private individuals.

c) Assuming that puppies are born with a strong genetic foundation, then their socialization experience during their first year of life will largely determine each puppy's ultimate success or failure. To ensure proper socialization, DDCoE must utilize puppy raising protocols designed to meet the special needs of scent detection dogs, perhaps by adapting already existing protocols in use by academic working dog centers. Opportunities may exist to engage local college students to be puppy raisers, as well as other local residents within a reasonable driving time of one of the DDCoE member organizations. In some settings, it may be possible to use the 4-H youth program and other social agricultural infrastructures to recruit puppy raisers who could either be volunteers or paid a stipend. Alternatively, correctional institution-based detector dog programs may be recruited, expanded and replicated to provide a scalable solution to needed dog raising resources. This variety of puppy raising models may be necessary due to the difficulty of recruiting individuals willing to live with and provide basic training for dogs that will not stay with them. Data from all of the alternative raising strategies, including puppy raising professionals, will be collected to determine the most cost-effective methods to produce the highest success rates.

Genetics

a) The choice of a mate for a participating bitch will be made by the DDCoE using a data-driven quantitative genetics protocol set up by its governing board to select for priority traits.

b) Organization of pedigree and phenotype data on a large number of dogs is needed to apply quantitative genetic selection methods to the complex traits important for working dogs. The International Working Dog Registry (IWDR; https://www.iwdr.org), newly developed by the International Working Dog Breeding Association (https://www.iwdba.org), provides a database with specialized features for storing canine pedigree, phenotype, and in future, genotype data.

c) The IWDR calculates inbreeding coefficients for dogs and their potential offspring, providing a tool for minimizing the

accumulation of inbreeding in the working dog population. Including inbreeding information in mating decisions will preserve genetic variability in the population and avoid deleterious health and reproductive effects associated with increases in inbreeding (27–30).

d) The IWDR will be able to fit quantitative genetics models to phenotype data. The potential for improving quantitative traits can be assessed in part by calculating the heritability of the trait (31). These models can also provide estimated breeding values (EBV) for each dog for each trait. An EBV depends on the phenotype of the dog and of its relatives, with close relatives contributing more information to the value than more distant relatives. EBVs can be used to select the individuals most likely to pass on favorable alleles for complex traits to their offspring (32). This method has been used to improve mean hip joint conformation score in The Seeing Eye dog population (8), and for distraction index and search-related behaviors in the TSA breeding program (unpublished data). Although the heritability of behavior traits is often considered to be low, moderate heritabilities have been found for some working dog behaviors. A study on German Shepherd Dogs (GSD) and Labrador Retrievers (LR) at the Swedish Dog Training Center (SDTC) found heritabilities above 0.25 for courage (GSD: 0.26 ± 0.06; LR 0.28 ± 09), prey drive (GSD: 0.31 ± 0.07), nerve stability (GSD: 0.25 ± 0.06), affability (GSD: 0.37 ±0.08), and ability to cooperate (GSD: 0.28 ± 0.07; LR (0.35 ± 0.09) (33). The personality trait for shyness-boldness had heritabilities of 0.25 in the GSD and 0.27 in the Rottweiler on the Dog Mentality Assessment given at the SDTC (34). In a population of U.S. guide dogs, heritabilities for dog-directed aggression was 0.27 ± 0.12 and for non-social fear was 0.27 ± 0.09 in Golden Retrievers (GR) as assessed by the C-BARQ questionnaire [described in Duffy and Serpell (23)]. The C-BARQ trainability score had heritabilities of 0.46 ± 0.07 in LR, 0.47 ± 0.07 in GSD, and 0.20 ± 0.08 in Golden Retrievers (35). A study of a guide dog breeding program in the UK found heritabilities of 0.25 ± 0.09 for "Following when called" in LRs, GRs, and their crosses (36). As generations of genetic improvement accumulate, it will, almost certainly, be necessary to develop new selection criteria for traits and characteristics that are not part of the selection criteria chosen as most important at the onset.

e) The application of these genetic principles to a large population of dogs such as the potential DDCoE program is more feasible and effective than their application to small working dog organizations or single private breeders (14, 37). In the past, these methods have been applied widely for the improvement of livestock species [e.g., milk yield in cattle (38), growth rate in beef cattle (39), and body weight in chickens (40)] and hip dysplasia in dogs (8).

f) The production of crossbred dogs for scent detection may be considered as an option, especially if "market forces" indicate a need and a demand for utilizing crossbred dogs for odor detection. This option will be most viable by choosing purebreds as parents of the crosses that will yield crossbred females suitable for breeding. Crossbred males and females not chosen for breeding would be available for training as odor detection dogs, or for alternate career paths as determined by each dog's abilities.

Research and Development

a) The DDCoE will develop and validate a quantitative scent detection aptitude test to determine whether a dog meets government contracting specifications. As a general measure of a dog's scent detecting ability, it will also be used as a phenotype for selective breeding decisions by the DDCoE.

b) This aptitude test result could provide a basis for the release of dogs unlikely to be successful from the program, saving financial resources. Academic working dog centers are currently collecting phenotype data on pups as early as 8–12 weeks, so these tests can be validated.

c) The decreasing costs of high-density whole-genome marker panels and even genome sequencing, along with the DDCoE's access to data on large numbers of dogs, can lead to progress in understanding the complex health and behavior traits in detector dogs (41). Genetic factors affecting complex traits such as behavior and hip joint conformation have been elusive because these traits are the result of genes at many loci along with environmental effects. Large data sets are required to find genes of small effect (42, 43).

d) The use of genomic selection methods also depends on the availability of large data sets. In this method, breeding dogs are selected based on possession of sets of genetic markers contributing to variation in desired traits. These methods have been used with livestock species with large data sets available, but not with dogs.

e) A refinement of our understanding of how puppy raising and training strategies influence success rates at older ages will be undertaken. Comparisons between different methods and environments, both within and between DDCoE member organizations, could facilitate this understanding. Using knowledge gained from these studies, it may be possible to create puppy rearing strategies that maximize the number of Federal agency acceptable puppies produced by a single strategy. This could enable the targeting of a specific Federal agency's needs with puppies reared by a particular strategy.

f) At present, criteria defining what makes a dog acceptable for Federal government purchase differs among agencies (5). The DDCoE will work with the Federal government to standardize the definition of acceptance criteria for Federal government purchase across agencies, especially those that focus on purchasing dogs destined for odor detection.

Education

a) The DDCoE will be an educational center for the dissemination of knowledge obtained through breeding, selection, and raising of detection dogs.

b) Educational programs will be directed at veterinarians, researchers, agencies, trainers, and handlers.

Benchmarks of Success

a) Dog performance: an annual increase in the percentage of puppies enrolled in the program that are able to enter training based on a reduction of health issues and an improvement in puppy development.
b) An annual increase in the percentage of dogs that enter the workforce.
c) An annual increase in the average duration of working life.
d) An annual reduction in the cost of producing the dogs.
e) The research benchmark will be the collection of data from the stages of development and the analysis and application of the data that allows a target 10% annual improvement in genetics, training, and performance measures.

In summary, a comprehensive approach to increasing the availability and improving the overall quality of detection dogs is proposed. This approach incorporates experiences from working dog programs over the past several decades. Key components that set this program apart from early programs that no longer exist include (1) the cooperative non-governmental structure, (2) application of the most current genetic, reproductive, medical and behavioral knowledge to the breeding and raising of dogs, and (3) the ability to distribute dogs to a wide variety of end-users.

AUTHOR CONTRIBUTIONS

CO, EL, EH, ST, and LW all provided content, edits, and review. All authors approved the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 22 January 2019
doi: 10.3389/fvets.2018.00345

Canine Olfactory Thresholds to Amyl Acetate in a Biomedical Detection Scenario

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Dogs' abilities to respond to concentrations of odorant molecules are generally deemed superior to electronic sensors. This sensitivity has been used traditionally in many areas; but is a more recent innovation within the medical field. As a bio-detection sensor for human diseases such as cancer and infections, dogs often need to detect volatile organic compounds in bodily fluids such as urine and blood. Although the limits of olfactory sensitivity in dogs have been studied since the 1960s, there is a gap in our knowledge concerning these limits in relation to the concentration of odorants presented in a fluid phase. Therefore, the aim of this study was to estimate olfactory detection thresholds to an inert substance, amyl acetate presented in a liquid phase. Ten dogs were trained in a "Go/No go" single scent-detection task using an eight-choice carousel apparatus. They were trained to respond to the presence of solutions of amyl acetate diluted to varying degrees in mineral oil by sitting in front of the positive sample, and not responding to the 7 other control samples. Training and testing took place in an indoor room with the same handler throughout using a food reward. After 30 weeks of training, using a forward chaining technique, dogs were tested for their sensitivity. The handler did not assist the dog during the search and was blind to the concentration of amyl acetate tested and the position of the target in the carousel. The global olfactory threshold trend for each dog was estimated by fitting a least-squares logistic curve to the association between the proportion of true positives and amyl acetate concentration. Results show an olfactory detection threshold for fluid mixtures ranging from 40 parts per billion to 1.5 parts per trillion. There was considerable inter-dog difference in sensitivity, even though all dogs were trained in the same way and worked without the assistance of the handler. This variation highlights factors to be considered in future work assessing olfactory detection performance by dogs.

Keywords: olfactory thresholds, amyl acetate, detection, accuracy, sensitivity

OPEN ACCESS

Edited by:
Emily Patterson-Kane, American Veterinary Medical Association, United States

Reviewed by:
Lowell Paul Waggoner, Auburn University, United States
Mitsuaki Ohta, Tokyo University of Agriculture, Japan

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Specialty section:
This article was submitted to Veterinary Humanities and Social Sciences, a section of the journal Frontiers in Veterinary Science

Received: *23 August 2018*
Accepted: *27 December 2018*
Published: *22 January 2019*

Citation:
Concha AR, Guest CM, Harris R, Pike TW, Feugier A, Zulch H and Mills DS (2019) Canine Olfactory Thresholds to Amyl Acetate in a Biomedical Detection Scenario. Front. Vet. Sci. 5:345. doi: 10.3389/fvets.2018.00345

INTRODUCTION

The olfactory abilities of dogs are widely documented in the literature and are generally thought to be superior to currently available man-made sensors (1–6). Accordingly, dogs are used worldwide in a variety of chemical detection tasks for civilian, military, wildlife, and medical detection purposes [e.g., (7–10)]. Despite their importance as biological sensors protecting life and property,

relatively little research has focused on the measurement of the limits of the dog's olfactory sensitivity. The olfactory detection threshold, [ODT, (11)] is the minimum concentration of an odorant stimulus an individual is able to reliably detect and differentiate from a blank sample (12–15), and may be defined, alternatively, in terms of a performance criterion relating to a detection task (e.g., percent of correct responses/true positives) (16, 17). The dog's olfactory threshold has been estimated as being within the parts-per-billion (ppb) to parts-per-trillion (ppt) range for a variety of chemical odors. For example, Moulton et al. (18) reported a detection threshold for aliphatic acids such as propionic acid at 10,000 ppm and acetic acid at 100,000 ppm; by contrast, Marshall et al. (17) determined a threshold for n-pentanoic acid of between 1 and 100 ppb using the performance criterion of a 50% correct response. The detection threshold for more complex chemical odors such as methyl benzoate, cyclohexanone, and nitroglycerin has been determined to be between 0.1 and 10 ppb (12, 19). Although data derived from laboratory studies are expected to provide substantial information about olfactory sensitivity, determinations may be unreliable or lack reproducibility. A major issue for assessing the threshold levels reported by different studies is that varied methodologies have been used, which gives rise to very different threshold estimations for the same odors (19–21), even when performed by the same investigators (18). For instance, (22) using a conditioned suppression paradigm to determine the dog's olfactory sensitivity to amyl acetate in six Beagles, reported it to be between 52 and 32,600 ppt, while (23) observed a positive spontaneous electroencephalographic olfactometry response only at a threshold concentration of 1 ppm in six Beagles. Finally, (24) trained two dogs (Standard Schnauzer and Rottweiler) in field conditions to recognize n-amyl acetate in retriever tubes and then, tested them using a chamber box. This resulted in detection values of 1.9 and 1.14 ppt. According to the authors, training methods based on positive reinforcement, non–restrained conditions and a more natural search scenario, were the main reasons for the much higher sensitivity, roughly 30–20,000 times lower than the thresholds reported in previous studies produced by more conventional laboratory procedures (e.g., using water deprivation and punishment) (17, 18, 22).

Over the past decade, dogs have been widely trained to work under controlled laboratory settings to check different samples and discriminate between target (i.e., the conditioned odor) and non-target samples using a reward-based approach (i.e., food or toy rewards) and non–restrictive searching systems, such as multi-choice apparatus and line-ups [e.g., (2, 25–27)]. In these non-restrictive searching systems, the samples with different odors are placed next to each other in a straight line-up or a circular one (carousel) and the dog has to identify the target sample by showing a trained alert response, and ignore the non-target samples. Scent detection tasks performed by dogs in a laboratory environment have involved forensic human scent match-to-sample tasks (28, 29) and diagnostic procedures for biomedical applications (8, 30). In a biomedical detection scenario, dogs detect disease biomarkers in human samples, which may relate to a particular cancer, bacterial or viral infections [e.g., (3, 30–33)]. As a biomedical detection sensor for human diseases, dogs can be trained to detect volatile organic compounds (VOCs) in low concentrations that might range from parts per million or even parts per trillion. The metabolism of infected cells slightly changes the odor of these VOCs compared to those of someone who is healthy (34–36) and so unique, chemical compositions are naturally emitted into the blood and bodily fluids when someone has a disease. Potential volatile organic compounds biomarker concentrations are reported to be in the range of parts per billion in blood and urine (34), which may be detected by dogs with a high degree of olfactory acuity. Although VOC biomarkers appear to be within the potential detection range of a dog's olfactory sensitivity, these values are derived from studies using odorant diluted in a gas phase; and there appears to be a lack of reports based on the odorant presented in a fluid phase, which is the norm in a biomedical detection scenario. In the last decade, there are also no reported attempts to estimate dog olfactory detection thresholds using the more prevalent reward-based detection training methods and a standardized laboratory setting. Therefore, the aim of the present study was to estimate the olfactory detection thresholds of several dogs to amyl acetate presented in a liquid phase in such a setting.

MATERIALS AND METHODS

Subjects

This study involved 10 detection dogs from the charity Medical Detection Dogs (UK charity registration number 1124533): 4 females and 6 males, ranging in age from 30 to 138 months (mean ± SD: 64.3 ± 38.52 months), with body weight from 10.5 kg to 24.0 kg (mean ± SD: 19.24 ± 3.97 kg), of the following breeds: Labrador Retriever ($n = 3$), Working Cocker Spaniel ($n = 3$), English Springer Spaniel ($n = 2$), and Border Collie ($n = 2$) (**Table 1**). These dogs were not specifically selected for their breed or type, but rather simply selected as potential working dogs by the charity.

This study was approved by the delegated authority of the School of Life Sciences Ethics Committee at the University of Lincoln, United Kingdom. All dogs were trained according to the ethical guidelines established by the charity Medical Detection Dogs.

Odor Sample Preparation

The dogs were trained to detect solutions of amyl acetate (CAS 628-63-7; ≥99% Sigma Aldrich, W504009) diluted in mineral oil (Sigma Aldrich, M8410) at different concentrations. Amyl acetate was chosen on the basis of previous studies testing olfactory detection thresholds in humans (37), rodents (38, 39), and dogs (22–24). Mineral oil was used as solvent because it produces higher concentrations of volatile gases within the headspace than other potential solvents such as water (40).

A stock solution at 1:1,000 amyl acetate:mineral oil (0.5 mL amyl acetate plus 499.5 mL mineral oil) was made up to ensure consistency in the preparation of the target odor (amyl acetate concentration). A simple stepwise dilution from this stock solution was used to prepare samples with concentrations >1:1,000,000. This simple stepwise dilution consisted of 2 μL of

TABLE 1 | Demographic data relating to the dogs included in the study.

Dog	Breed	Age (years)	Sex
Dog 1 (Casper)	Springer Spaniel	6.10	Male, castrated
Dog 2 (Molly)	Labrador Retriever	3.4	Female, not spayed
Dog 3 (Hamish)	Working Cocker Spaniel	11.6	Male, castrated
Dog 4 (Tangle)	Working Cocker Spaniel	11	Male, castrated
Dog 5 (Sye)	Springer Spaniel	2.1	Male, castrated
Dog 6 (Amberly)	Labrador Retriever	3.5	Female, not spayed
Dog 7 (Kizzy)	Working Cocker Spaniel	3	Female, not spayed
Dog 8 (Ozzy)	Border Collie	2.6	Male, not castrated
Dog 9 (Lacey)	Border Collie	5.7	Female, spayed
Dog 10 (Chester)	Labrador Retriever	3.5	Male, castrated

the stock solution being mixed with an appropriate volume of mineral oil to achieve 1 mL of the desired concentration. One to three steps of 1.25-, 1.5-, and 2-fold serial dilutions of the stock solution were used to prepare target odor concentrations below 1:1,000,000. In these serial dilutions, the concentration of amyl acetate required for each step came from the diluted solution of the previous dilution step.

One milliliter of the target odor concentration was deposited in a sterile 60 mL screw-top polypropylene container (4 cm diameter, item number 360103PP; Wheaton, Rochdale, UK). Likewise, seven controls, each made up of 1 mL of mineral oil, were placed in identical sterile containers. The target odor and controls were opened and situated in an octagonal carousel (**Figure 1A**) similar to the circular stainless-steel odor presentation apparatus that has been used in other studies (32, 41). Each of the 8 carousel arms was removable which allowed changing of the position of the target odor on the carousel. The containers with the odor stimuli were placed underneath the plate of the arm and fixed to the arm with a metal spring clip (**Figure 1C**). The dogs searched for the target odor by sniffing the hole located in the center of the plate on the arm (**Figure 1B**).

To avoid the risk of cross contamination between controls and target odors, controls were made up first followed by the target. The target and controls were made up 10 min before the session started and set up by the researcher within the carousel. Each set of containers were used for a single session and subsequently discarded.

Similarly, a new clean set of arms was placed on the carousel for each session. The carousel was cleaned with distilled water and the set of arms washed in a dishwasher (Clasic-XX Bosch) for 45 min after each session.

For optimal estimation of the concentration of the odor stimulus, calibration curves were performed using solid phase microextraction (SPME) combined with gas chromatography-mass spectrometry (GC-MS) [Perkin Elmer Clarus 600 operated with Perkin Elmer TurboMass (2008) software] to identify the compounds and obtain direct measurement of liquid concentrations within the headspace from the stock dilution 1:1,000 amyl acetate: mineral oil (0.01 ppm) and for each 10-Fold dilution step (1:1,000; 1:10,000; 1:100,000; 1:1,000,000; 1:10,000,000; 1:100,000,000; 1:1,000,000,000). Three concentrations of amyl acetate were presented daily for each dog in a training session. Additionally, blank runs (i.e., sessions with the eight positions arms containing controls) were randomly included throughout the sessions.

Training Procedure

The dogs worked in an indoor training room at the charity Medical Detection Dogs (see 24). During training and testing, the room was maintained at a constant temperature (∼20°C) and humidity (51%).

The dogs worked with the same handler (R.H.) throughout the study to perform a "Go/No go" task. This requires the dog to issue a trained alert response in the presence of the conditioned odor (i.e., "Go" to target odor) and to withhold a response when the odor is not present ("No" go) (42).

The training involved six steps (**Table 2**):

Step 1. The dogs were classically conditioned to a clicker with food (Educ Royal Canin®) (43).

Step 2. A piece of tennis ball (2 cm, Head team®, yellow) was used as the initial target scent to make it easy for the dog to learn the trained alert response and use of the carousel without variations in the target odor (44, 45). Training to search the carousel was achieved by the handler presenting the dog to two carousel arms, one with a piece of tennis ball in a polypropylene sterile container and the other with an empty identical sterile container. When the dog showed interest, sniffing longer at the carousel arm with the piece of tennis ball, the dog was clicked and rewarded with Educ Royal Canin®. This was repeated until the dog reached the criterion of more than 80% correct alerting to the arm with the piece of tennis ball. Afterwards, dogs were trained to search for the piece of tennis ball in different positions on the carousel, while the remaining seven arms held empty sterile containers.

Step 3. After a few sessions, a conditioned alert response was introduced, so that when the dog correctly identified the position with the piece of tennis ball on the carousel, a verbal "sit" command was given to the dog by the handler, once sat, the dog was clicked and rewarded with food.

Step 4. The piece of tennis ball was replaced with the target odor, starting with a dilution of 1:1,000 (amyl acetate: mineral oil). The dog was clicked and rewarded with food as soon

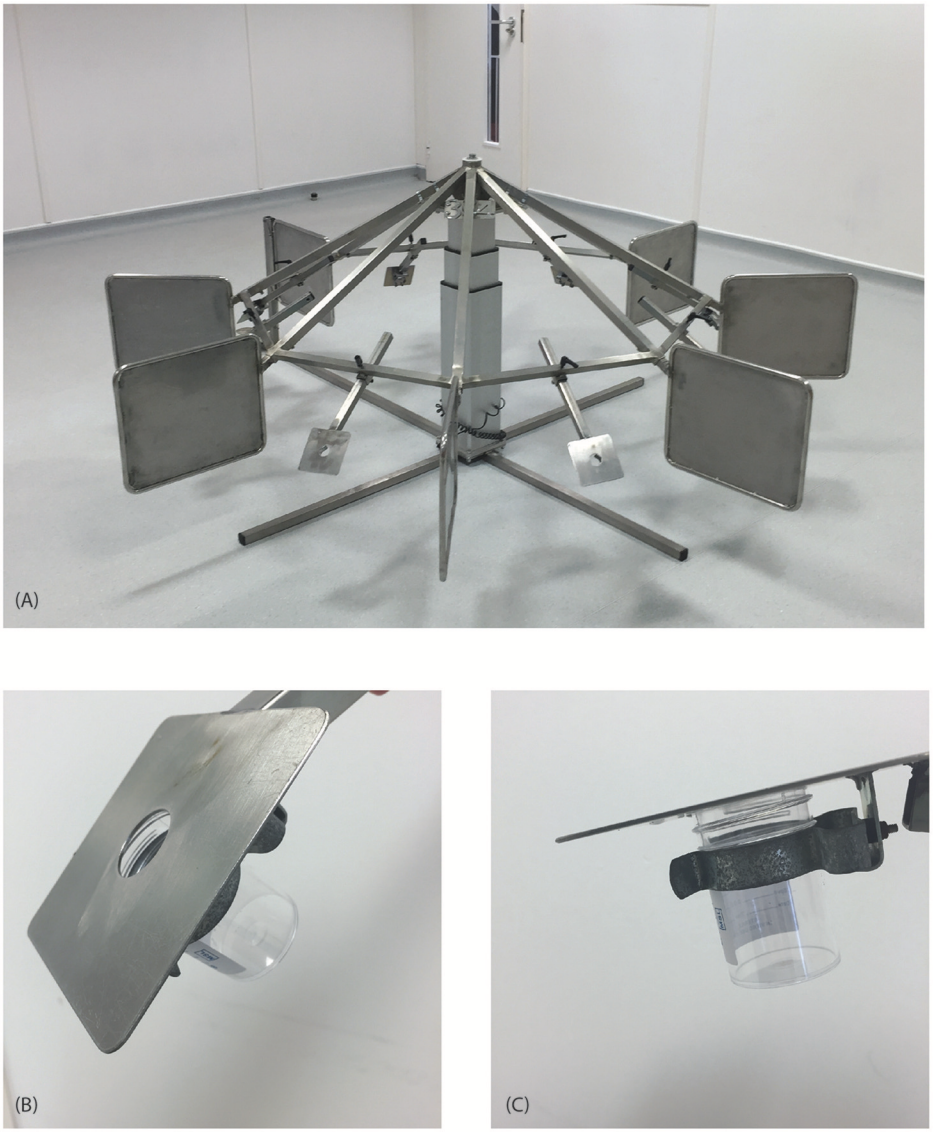

FIGURE 1 | (A) The odor stimuli were presented using the multi-choice "carousel," an octagonal stainless-steel stand with 8 removable arms. Each arm had a letter identified from A to H (in alphabetical order) to identify in which arm the target odor had been placed. Each position in the carousel had a number located on the base (1–8), which allowed recording the position of target odor in the carousel. **(B)** The dog sniffed the odor stimulus though a hole located in the center of the plate of the arm. **(C)** The odor stimuli were placed in a polypropylene container underneath the plate of the arm.

as it sniffed the target odor placed in a sterile container on the carousel. The rest of the arms contained empty sterile containers.

Step 5. Once the dog was able to identify and alert to the presence of the target odor with the trained alert response, controls (tubes containing mineral oil) were introduced and placed on the carousel arms to start the discrimination between the target odor and controls. The dogs had to identify either one target sample among eight samples, or ignore all the samples in a run of only control samples (a blank run). When the latter condition was introduced, the dog was recalled from the carousel once it had investigated the eight samples. In this way, the dog learned there may not always be a positive sample present and to come away from the carousel when a target was not present, positioning itself next to the handler to indicate a blank run.

Step 6. Detection threshold training involved the dogs working in pairs, based on their prior performance in detecting similar concentrations; each pair worked the same set of samples (target odor and controls) within a session. The order in which dogs worked (first or second) was counterbalanced during each session over different target concentrations.

TABLE 2 | Training steps to teach the dog to respond to the presence of the target (amyl acetate diluted in mineral oil) and not respond to the control samples (mineral oil).

Training phase	
Step 1. Clicker training	- The clicker was classically conditioned to food (Educ Royal Canin®). The clicker was employed as a marker when the dog detected the target odor in the carousel.
Step 2. Training to search on the multiple-choice apparatus ("carousel")	1. The dogs were trained to detect a piece of tennis ball (Head team®, yellow) in a sterile container. 2. The handler presented to the dog a piece of the tennis ball in a sterile container placed in the carousel arm. 3. Odor discrimination was trained between an empty sterile container and sterile container with tennis ball in different positions on the carousel.
Step 3. Introduction of trained alert response	- When the dog displayed an alert response or showed interest in the tennis ball, the handler gave a "sit" command to the dog and rewarded it.
Step 4. Training target odor (amyl acetate diluted in mineral oil)	- The piece of tennis ball was replaced with the target odor starting with a concentration of 1:1,000 (amyl acetate:mineral oil). The dog was clicked and rewarded with food as soon as the dog sniffed the target odor placed in a sterile container on the carousel. The rest of the arms remained empty.
Step 5. Detection threshold	**Stage 1—weeks 1–16** - The target dilution was presented to the dog with a systematic lowering of concentration through the stage - The handler stood next to a screen but was visible to the dogs. - The position of the target odor in the carousel was selected randomly (Excel® random number generation) and was not blind to the handler. –Screening for control samples only (searching for blanks) was also performed, where the eight positions contained only controls (i.e., the target odor was not present in the carousel). **Stage 2—weeks 17–30** - A mixture of dilutions was presented in a random fashion. - Handler was not visible to the dogs. - The position of the target in the carousel was determined by a custom-made computer target selector program and it was blind to the handler.
Step 6. Discrimination	- Once the dog was able to identify and alert to the presence of the target with the trained alert response, the controls (mineral oil) were introduced and placed on the carousel arms to discriminate between the target odor and controls. The dogs had to identify one target sample out of eight samples.
Determination of threshold criterion	Blind testing continued with serial dilutions until the proportion of true positive indications declined to consistently below 40% (4 true positives over 10 exposures to the target odor).

This detection threshold training consisted of two stages. In the first stage (weeks 1–16), target dilutions were presented to the dog with a systematic lowering of concentration. The decrease in concentration was 50% below the previous level detected by the dog, once the proportion of true positives detected by the individual at the previous concentration above 80%. During this stage, the handler stood next to a screen but was visible to the dogs. The position of the target odor in the carousel was randomly selected (Excel® random number generation) and was not blind to the handler. Blank runs were included, in which only controls were present in the apparatus.

In the second stage (weeks 17–30) a mixture of dilutions was presented in a random fashion to minimize any sample order bias. The handler stood behind the screen where he could watch the dog through a one-way mirror without being seen by the dog. The position of any target in the carousel was determined randomly using custom-made computer software, and the handler was blind with respect to the target concentration tested and the position of the target in the carousel. To reveal if the dog had alerted to the correct position, the handler pressed a keypad with the number of the carousel arm that was indicated by the dog. If the dog had indicated correctly it was clicked and rewarded.

Structure of a Training Session

The structure of a training session has been described in detail previously by the authors [see (26)]. Each training session involved a new concentration of amyl acetate, and consisted of "runs" and "search passes": a "run" related to the searching allowed when the target odor was in a given position on the carousel (e.g., when the odor was on arm 2); a "search pass" was a single search of arms 1–8 of the carousel. Up to three "search passes" were allowed within a "run," with a third search pass allowed either when the dog appeared, in the handler's opinion, to show at least some hesitation on a particular carousel arm during the previous search pass or when the dog did not appear to have searched all the arms of the carousel in the previous two search passes (i.e., missed a position). A training session consisted of two changes of position of the target on the carousel per concentration (i.e., 2 "runs").

The target and control odors were set up in the carousel by the same researcher (AC), while the dog and handler (RH) were in a separate room. The researcher left the room after setting the odor samples and entered the room between runs to change the position of the target on the carousel according to the computer program. Once the researcher left the room, the handler and the dog entered the room together and left the room between runs, but remained inside between search passes.

The session started with the handler standing next to or behind the screen (depending on training step) with the dog positioned next to him. The handler gave a verbal command to the dog to start the search. The dog sniffed the individual carousel arms without the assistance of the handler. When the dog showed the trained alert response (i.e., sit) at a position on

TABLE 3 | Pairs of dogs and concentrations of amyl acetate tested for each dog, the concentrations used with each subject were determined according to the individual dog's ability as revealed in the training phase.

Dog	Concentration of amyl acetate:mineral oil
Dog 1	1: 1,000,000
Dog 2	1: 15,000,000
	1: 30,000,000
	1: 45,000,000
Dog 3	1: 10,000,000
Dog 4	1: 30,000,000
	1: 50,000,000
	1: 70,000,000
Dog 5	1: 10,000,000
Dog 6	1: 40,000,000
	1: 70,000,000
	1: 100,000,000
Dog 7	1: 10,000,000
Dog 8	1: 100,000,000
	1: 750,000,000
	1: 1,500,000,000
Dog 9	1: 10,000,000
	1: 100,000,000
	1: 500,000,000
	1: 1,000,000,000
Dog 10	1: 1,000,000
	1: 15,000,000
	1: 30,000,000
	1: 45,000,000

The dogs were paired on the basis of apparently similar threshold levels during training.

the carousel, the handler confirmed the position through the use of key pad linked to the custom-made computer program; if the indication of the dog was correct (true positive) it was clicked, the dog left the carousel position and returned to the handler to be rewarded with food (Educ Royal Canin®). By contrast, if it was a false positive, the behavior of the dog was not reinforced (negative punishment). Blank runs (once introduced) were correctly indicated by the dog positioning itself next to the handler at the end of the run, it was clicked and rewarded as long as a false alert was not performed during the blank run.

The dogs were trained until their performance fell to below 40%, i.e., 4 true positive indications over 10 exposures to a target odor of a given concentration.

Testing

After 30 weeks of training followed by a 7 day break, dogs were tested for their detection sensitivity. Olfactory detection threshold testing consisted of up to 3 sessions per day for 4 consecutive days for each dog. As described above, each session involved one concentration of amyl acetate. Four concentrations were chosen for each dog based on the statistical estimation of their global olfactory detection threshold trend given the individual's previous olfactory performance. Each dog was exposed 3 times to each concentration. Dogs were paired for testing within a session on the basis of similar detection threshold levels according to their previous olfactory performance, 2 dogs could not be paired (**Table 3**).

Data Analysis

The olfactory detection performance of the dog was assessed for conformity with signal-detection theory (42, 46, 47) as follows: (1) True positive: The dog indicates the target odor in the manner in which it was trained ("sit" response), (2) True negative: The dog does not alert in the absence of the target odor, (3) False positive: The dog alerts to a non-target position (control), (4) False negative: The dog fails to exhibit the trained alert in the presence of the target odor.

To estimate the olfactory detection threshold of amyl acetate for each dog in both training and the test, a constrained logistic function was fitted to the curves describing the relationship between the proportion of true positives and amyl acetate concentration exposure to the dog. Specifically, this function was fitted using non-linear least squares, as implemented in the "minpack.lm" package for R (48) and detection thresholds estimated as the concentration at which true positives would have resulted by chance (i.e., 12.5%, 1 out of 8 possible locations). The dog's accuracy was calculated based on the number of correct assessments (true positive + true negative) over the number of all assessments (true positive + true negative + false positive + false negative) of the test data (47, 49). The accuracy of the threshold assessment was determined by how close the threshold estimation was to its true value (50). In other words, how reliable the estimation was to the actual olfactory capability revealed by the dog's ability to detect a given threshold level of amyl acetate concentration. It was predicted that low accuracy at the lowest concentration detected by the dog was a result of an increase in false negatives and false positive responses (51).

RESULTS

Olfactory detection threshold levels of amyl acetate were estimated to be between 1:40,000,000 (30 ppb) and 1:1,500,000,000 (1.5 ppt) on the basis of the fitted curve to the testing data (**Figure 2**, Dogs 1–10).

Accuracy measurements for the lowest concentrations detected by each dog were determined as being between 81.71 and 96.49% (**Table 4**). This indicates a low rate of false indications over control samples.

DISCUSSION

Any attempt to quantify odor detection and discrimination needs to consider the simplest measure of the individual's olfactory performance limits: odor threshold concentration (11, 16, 37). Below this limit, the physical stimulus is subliminal or not detectable (52). Olfactory detection thresholds vary for different chemicals and compounds and different individuals may have different thresholds for the same odorant (37). Variation in threshold performance may be influenced by genetic polymorphism of olfactory receptors (53, 54), the proportion of functional against non-functional genes (55, 56), the individual's ability to focus on searching (57), a temperament suitable for the high demands of detection training (58, 59), individual learning

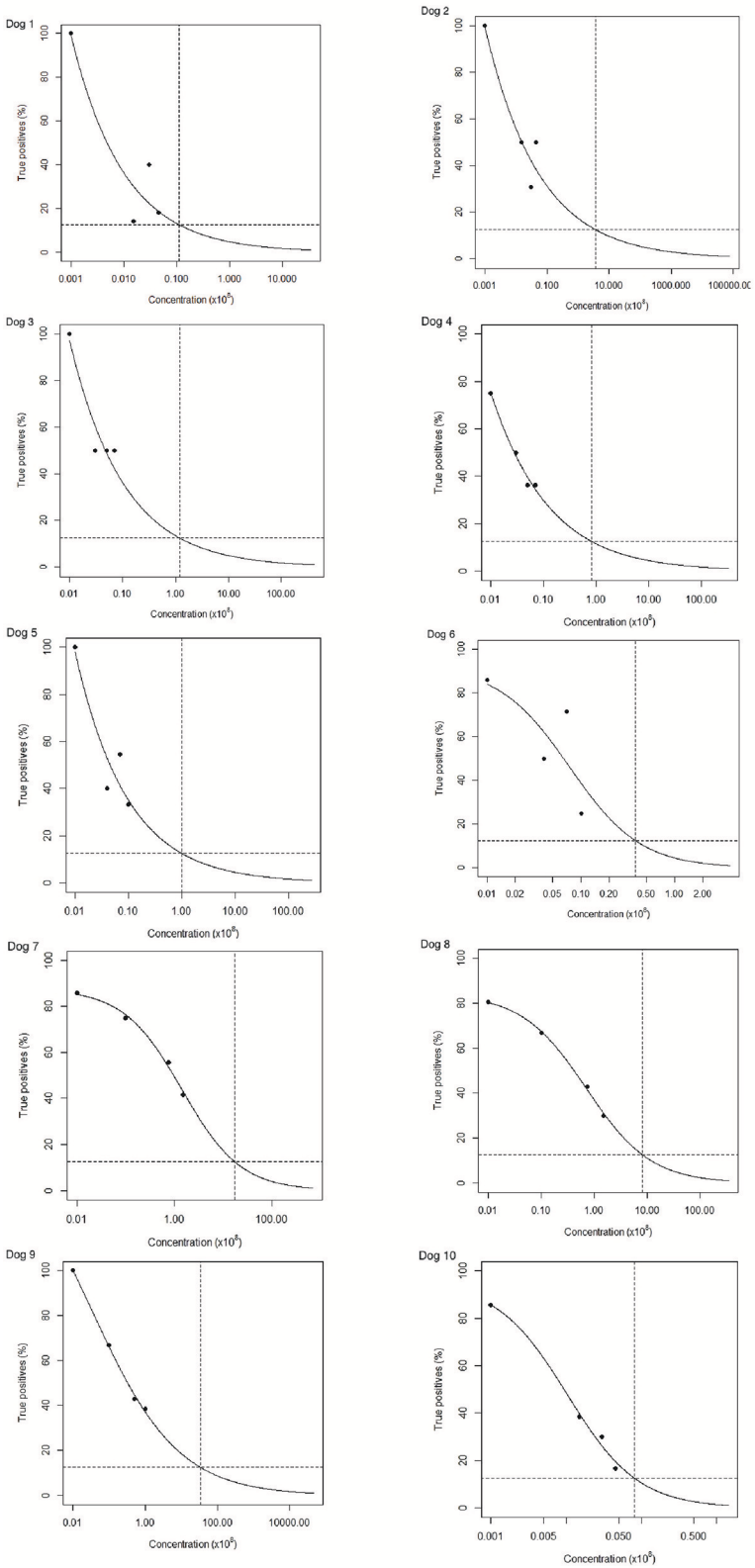

FIGURE 2 | Summary of performance and estimation of thresholds of dogs 1–10 over 12 sessions of olfactory detection thresholds. Graphs show the proportion of true positives at the different concentrations of amyl acetate tested (dots) and an estimation of the global threshold trend (slope). The detection thresholds were estimated as the concentration at which the true positive rate was the equivalent of chance at 12.5% (i.e., the concentration at which the horizontal and vertical dashed lines intersect).

TABLE 4 | Detection performance as a function of accuracy at the lowest concentration detected by each dog.

Dog	Lowest concentration of amyl acetate: mineral oil detected	Accuracy (%)
Dog 1	1: 45,000,000	81.71
Dog 2	1: 45,000,000	87.50
Dog 3	1: 70,000,000	96.49
Dog 4	1: 70,000,000	84.73
Dog 5	1: 100,000,000	90.90
Dog 6	1: 100,000,000	83.11
Dog 7	1: 1,500,000,000	87.05
Dog 8	1: 1,500,000,000	92.86
Dog 9	1: 1,000,000,000	83.33
Dog 10	1: 45,000,000	86.59

The accuracy was calculated as the proportion of correct assessments (true positive + true negative) over total number of assessment (true positive + true negative + false positive + false negative) of the test data (47).

abilities (46, 60) and motivation (61, 62). These factors may have different degrees of impact on olfactory detection performance, which is reflected in inter-dog variability in detection thresholds to amyl acetate estimated in this study, irrespective of the fact that the dogs were trained under the same conditions. Although it was not part of this study to investigate how these factors influence the olfactory detection performance, we believe that it is possible that perceptual learning may have played an important role in the lower levels of detection thresholds observed at the end of the training and testing period (37, 63). This is in line with many studies on olfactory perceptual learning that demonstrate that the more an animal is trained to detect an odorant, the easier it is to separate that odorant from background odors (12, 64). Thus, repeated exposure is an important factor in developing olfactory sensitivity (24, 39, 65, 66) contributing to an improvement in odor acuity (63) so that the individual is able to detect at much lower thresholds than during the initial training (20, 63, 65–70).

However, as reported by Walker et al. (24) training dogs for threshold testing tasks consumes a great deal of time. For instance, one can spend approximately 6 months training two dogs for an olfactory threshold task. Likewise, this study involved 30 weeks of training for 10 detection dogs.

Previous studies assessing olfactory sensitivity in dogs have been performed using custom-fabricated devices to present odor stimuli in a standardized controlled manner (i.e., automated air stream olfactometer and test chamber) for the integration of an optimum odorant stimulus (71). However, an olfactometer controls the amount of odorant delivered to the dogs but does not necessarily facilitate effective transport of the odorant molecules into the nose. Dogs actively sniff to acquire an odor sample even when a flowing stream has been used, thus dogs dynamically control the access of odorants to the nose through sniffing (72–74) regardless of the method chosen for odor stimulus presentation (i.e., air flow or into a jar). Moreover, these laboratory measures to improve precision are not easy to reproduce. In our study, some of the dogs reached detection levels to amyl acetate at parts per trillions (ppt), yielding thresholds approximately 30-fold lower than that reported in previous work (22). This suggests that the presentation of the odor stimulus in a liquid phase using serial dilution steps provides a convenient and replicable alternative for quantifying concentrations to assess olfactory thresholds.

This study also showed that dogs achieved a high level of accuracy at the lowest threshold concentrations detected. Accuracy is used to determine how well a measure, such as olfactory detection threshold, matches the event that the test is intended to obtain, such as the actual ability of the dog to detect the target odor. Lowering the detection stimulus may produce less accurate responses due to an increase in the number of false negative and false positive responses. For instance, in the current study, the solvent (mineral oil) used in the binary mixture (i.e., amyl acetate diluted in mineral oil) was also the control (negative sample) and therefore, the dogs could be falsely responding to a similar component in the mixture at the lowest concentrations of the target odor.

Thus, the apparent difference in olfactory detection thresholds could simply reflect different tradeoffs between false and true responses and not necessarily indicate real differences in the olfactory capabilities of the dogs (51). It might be argued that, ideally, olfactory detection accuracy in dogs should be close to 100% if it is to truly reflect the dog's capabilities (60). In the present study, the accuracy was determined to be over 81.71%. Similar rates over 80% have been found for different target odors involving accelerant detection, cadaver search, and explosive detection (12, 60, 75, 76).

Several studies on scent detection dogs in the diagnosis of human disease have been reported, providing evidence for using dogs as a viable non-invasive biomedical screening method. In this biomedical detection scenario, dogs are able to detect volatile organic compounds released into body fluids such as blood and urine as a consequence of human diseases (31, 34–36). Although these VOCs are in the detection range of the dogs' olfactory sensitivity demonstrated in previous studies using odorant diluted in a gas phase, to the best of our knowledge, our study is the first investigating detection thresholds in odorants presented in a fluid phase as occurs in a biomedical detection scenario. Nevertheless, the binary mixture of amyl acetate diluted in mineral oil tested in our study only contain one hundred volatile compounds identified through the analysis using with the solid phase microextraction (SPME) combined with gas chromatography-mass spectrometry (GC-MS). By contrast, human fluids samples, such as urine, contain over seven hundred VOCs, which are present in very low concentrations and with a range of volatilities in the headspace gas (77).

Further investigation is needed to examine dogs' olfactory sensitivity to a wider range of odor stimuli, such as simple and complex odor mixtures, that would help us to better understand

how dogs use their olfactory skills and strategies to optimize detection of volatile compounds within human biofluids.

CONCLUSION

The first major practical contribution of the present study is that it provides much needed data on olfactory detection thresholds to amyl acetate, which is widely used in olfactory studies in dogs. This information is important given that the only other comparable study reported data for only two dogs and dates back more than 10 years. Additionally, detection thresholds reached, and accuracy level determined in our study using the olfactory stimulus presented in liquid phase evidence a reproducible alternative method to assess olfactory function in dogs.

The inter-dog variability in detection thresholds performance estimated in this study brings attention to how factors inherent to the individual (e.g., olfactory capabilities, performance and personality traits, perceptual learning abilities) can influence olfactory detection performance and the need for further investigation of these so that dogs can achieve their potential. Future studies should assess the range of factors, which may influence olfactory sensitivity in dogs and investigate dog's olfactory sensitivity in a range of odor stimuli, such as simple and complex odor mixtures.

AUTHOR CONTRIBUTIONS

All the authors listed have made a substantial, direct and intellectual contribution to the work, and approved for publication. The idea for the paper was conceived by AC, CG, DM, AF, HZ, RH, and TP. The study was part of Dr. Concha's Ph.D. dissertation. This work was designed by AC with guidance from DM, HZ, TP, AF, and CG. Data collection was performed by AC, RH, and CG, and data were analyzed by AC with guidance from TP, DM, and HZ. This article was primarily written by AC and the co-authors were involved with various phases of editing this article. All authors have approved the final article for submission.

FUNDING

This work was supported by Royal Canin SAS.

ACKNOWLEDGMENTS

The authors would like to thank Mr. Gordon, Pam Jones, Maria Jeskins-Powell, Betty Horwood, Becky Harris and the volunteers of the charity Medical Detection Dogs for their support. Dr. William Hayes of University of Lincoln for help in chemical techniques. This material is based on work completed as part of a Ph.D. dissertation authored by AC at the University of Lincoln.

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Conflict of Interest Statement: Royal Canin SAS supported the study financially; they approved the experimental design, financially supported AC, and provided the food and Edu®; food treats for the dogs used in this research. AF is employed by Royal Canin SAS. This author and co-authors have neither patent nor stock ownership, which would affect this research or publication nor do they have any membership of a company board of directors, membership of an advisory board or committee for the company.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 09 April 2019
doi: 10.3389/fvets.2019.00098

The Ability of Narcotic Detection Canines to Detect Illegal Synthetic Cathinones (Bath Salts)

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Twelve certified narcotic detection canines were tested for their ability to detect confiscated illegal synthetic cathinones (bath salts). These canine teams were randomly assigned to two different groups and each group imprinted on one of two types of bath salts, ethylone and alpha-pyrrolidinovalerophenone (α-PVP), over the period of 1 month; while simultaneously documenting the imprinting procedure. The newly imprinted canines were validated by field testing and found to not only detect the imprinted bath salt to which they were trained, but they were able to detect other bath salts. The imprinting procedure and results are the first scientifically validated studies on the ability of canines to detect these harmful and illegal substances. Analytical headspace analysis using Solid Phase Microextraction (SPME) on several ethylone and α-PVP samples revealed compounds common in both. These compounds can be used to create a safe and reliable synthetic cathinone mimic training aid for canine teams.

Keywords: canines, bath salt, training, volatiles organic compounds, cathinones

OPEN ACCESS

Edited by:
Cynthia M. Otto,
University of Pennsylvania,
United States

Reviewed by:
George E. Moore,
Purdue University, United States
Lisa A. Murphy,
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Specialty section:
This article was submitted to Veterinary Humanities and Social Sciences, a section of the journal Frontiers in Veterinary Science

Received: *04 December 2017*
Accepted: *11 March 2019*
Published: *09 April 2019*

Citation:
Shellman Francis V, Holness HK and Furton KG (2019) The Ability of Narcotic Detection Canines to Detect Illegal Synthetic Cathinones (Bath Salts). Front. Vet. Sci. 6:98. doi: 10.3389/fvets.2019.00098

INTRODUCTION

Currently, there are thousands of canine teams within the United States deployed for the detection of narcotics, explosives, cadavers, live humans, ignitable liquids, biological threats, currency, and various forms of agricultural contraband (1–4). Deemed the "Gold Standard" for detection, canines are efficient, cost effective, fast, easy to train and are more sensitive than most instrumental detection devices (2, 5, 6). A major advantage for canine detection is the dog's ability to locate a target odor while simultaneously ignoring all interfering non-targeted odors (6–8).

Through the use of active sniffing (inhaling short voluminous breathes), as is the case during a field search, and the possession of more than 200 million olfactory cells; a canine's short breathes enhance the amount of odorous compounds that flow through the nostrils into the olfactory organs (6–8). The canine is then aware that there is an odor and begins to determine whether they recognize that odor or not. Although canines possess a smaller brain in comparison to their human partners, a canine's olfactory bulb is three times the size of a humans (8). This explains their increased olfactory sensitivity and why a canine can detect odor from a given substance while a human deems it odorless.

With the overwhelming amount of discrimination that can be achieved by using canines for detection of substances; the first step is training the dog to make an association with the particular target substance. A canine's olfactory neurons live for approximately 30–60 days before they die and are naturally replaced with new ones (7, 9). When a dog becomes routinely exposed to a

certain odor for detection and is rewarded, there is a shift in the neurons produced. This means the newer neurons will contain more of the receptor sites of the odors that the canines are routinely encountering; thereby increasing precision and accuracy for detection of that substance (7, 9). The detection of the substance is called an "alert" defined as a characteristic change in ongoing behavior in response to a trained odor/scent, as interpreted by the canine handler. The components of the alert may include: change of behavior (COB), interest, and final response or indication.

In an effort to standardize training practices used by different canine organizations, working individuals gathered nationally to develop best practice guidelines for properly caring, training, and testing any canine for detection work. The Scientific Working Group on Dog and Orthogonal detector Guidelines (SWGDOG, www.swgdog.org) developed these best practice guidelines. SWGDOG was a federally funded partnership between local, state, federal, and international agencies dedicated to improving the reliability, accuracy, consistency of detector dog teams (10). The guidelines set forth by this group have been used and cited by numerous agencies; including the work conducted here. This work is now being continued through the Dogs and Sensors Subcommittee of the Organization of Scientific Area Committees (OSAC).

Traditional target odors for narcotic detection canines typically include: marijuana, heroin, cocaine, methamphetamine, and any other substance required to meet the training objectives (11, 12). The concern for canines and the method in which the drugs are introduced for training purposes has brought about the need for safe alternatives that still yield positive results. However, synthetic cathinones (bath salts) are not included as one of these substances for narcotic detection canines.

Instances of synthetic cathinone or bath salt intoxication by substances abusers has been increasing due to over usage. Oftentimes users of these substances will increase their intake for an increased feeling or longer duration of euphoria. Others will overlay doses in an effort to stop the adverse effects of coming off the drugs, during the down phase. When an individual consumes quantities outside the typical range for bath salts they experience increased psycho-stimulant effects such as paranoia, hallucinations, excessive agitation, anxiety, talkativeness, time lost, sweating, vomiting, muscle twitch, suicidal thoughts, tachycardia, vertigo, and many more (13–16). The length of time these adverse effects last can range from hours to months, with some cases resulting in death. On September 8th 2011, in an effort to combat the drastic increase of cases pertaining to bath salt overdosing; the Drug Enforcement Administration (DEA) issued a notice of intent to temporarily schedule three synthetic cathinones [mephedrone, methylone, and Methylenedioxypyrovalerone (MDPV)] under the Controlled Substance Act (CSA)(17, 18). The notice was issued as a response to the "imminent hazard to the public's safety" in regards to the listed drugs. Though the DEA's emergency schedule banned the possession and consumption of the previously listed drugs, amateur chemists continue to modify these compounds to avoid such regulations. By slightly altering the chemical structure of these drugs, new generations of these substances are created with slightly different chemical structures, thereby avoiding DEA regulation, while producing the similar euphoric effects when abused. The ability to quickly and inexpensively modify these drugs has made control of these substances particularly challenging for law enforcement, requiring them to use new tools to detect and confiscate these rapidly evolving bath salts.

Studies have shown that by performing simple google searches of names such as "bath salts" or "ivory wave," consumers are brought to secure websites for retail or wholesale of various types (19). Websites routinely advertise bath salts as "legal highs," where encryption is implemented for consumer safety, "buy one get one" advertised specials, expedited shipping, and many more aggressive marketing tactics are openly used to encourage sales of these narcotics (20, 21). Although regulations have been placed to halt incoming traffic of these drugs, a large portion still remains readily available throughout many local neighborhoods at gas stations and corner stores (The Schedule of Controlled Substances at 21 CFR 1308.11).

To the authors' knowledge, no certified narcotics detection canines are able to detect these bath salts, which leaves a significant gap for law enforcement to find and seize these substances. Field detection using canines offers a solution to the overwhelming problem with the increasing influx of these drugs into the United States that go undetected by standard procedures currently employed.

This study established whether canine teams currently certified for narcotic detection can alert to various types of synthetic cathinones (bath salts) and demonstrated the feasibility to imprint and train these canines to detect bath salts.

MATERIALS AND METHODS

All canines teams used for the study were previously certified following guidelines of the International Forensic Research Institute (IFRI) for narcotics detection (https://ifri.fiu.edu/research/detector-dog-research/index.html). The teams tested were trained and certified to detect a wide range of routinely encountered narcotics including cocaine, marijuana, heroin, methamphetamine, and MDMA.

All synthetic cathinones used were provided after special permission and under onsite supervision at the Miami Dade Police Department (MDPD, Doral, FL) and the Palm Beach Sheriff's Office (PBSO, West Palm Beach, FL). Confiscated samples used included α-PVP, methylone and ethylone; these were verified using headspace gas chromatography mass spectroscopy. Headspace analysis was conducted using polydimethylsiloxane/divinylbenzene (PDMS/DVB) solid phase microextraction (SPME) fibers. SPME fibers were exposed for 6 h to samples and then analyzed via gas chromatography mass spectrometric (GCMS) analysis. The GCMS used was a Varian 3800 GC and Saturn 2000 Ion Trap MS, equipped with a Solgel-wax capillary column, 30 m length, 0.25 μm phase thickness, and 0.25 mm internal diameter using helium a carrier gas at 1 ml per minute. Heroin, MDMA, methamphetamine, and marijuana samples were provided by the canine teams deployed by the Miami Dade Police Department and Palm Beach Sheriff's Office. Canine trials were

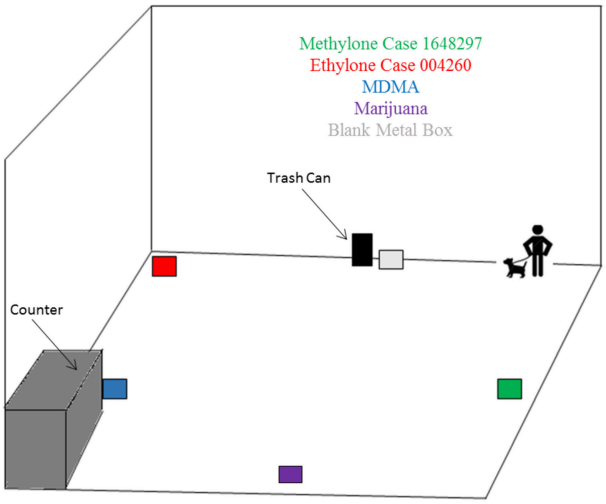

FIGURE 1 | The schematic of the Miami-Dade Canine Trial (not to scale).

performed on location at the Miami Dade Police Department crime lab (Doral, FL). Additional testing was conducted at Palm Beach Central High School (West Palm Beach, FL) using hallway lockers. The imprinting process was performed at the Palm Beach Sheriff's Office Canine Training Facility (Palm Beach, FL). The containers used during the imprinting phase were K-9 BSD-2 HDPE Kit purchased from EliteK9 (Boaz, KY).

Prior to imprinting, preliminary canine trials were conducted at both the Miami-Dade and Palm Beach facilities, as demonstrated in **Figures 1**, **2**. These canine trials were used to assess whether the canines could detect synthetic cathinones while only being imprinted on the substances previously listed. For the Miami-Dade trial, the allotted space used was the lab's cafeteria, which provided a connecting outside ramp for easy access. The section used for testing was closed off and controlled. As depicted in the diagram the hides consisted of the methylone and ethylone for testing, the positive control, and a blank. The blank did not contain any odor which would cause an alert by the canine. The positive controls (confiscated marijuana and 3,4-methylenedioxymethamphetamine) and the cathinones were located at the least 10 feet apart. Each case and control was placed in pre-washed metal boxes provided by the canine teams; also used during routine training. Each hide was allotted a minimum of 30 min for the volatile organic compounds (VOCs) to be released and made available for the canines to detect.

Canine trials were conducted in a single blind test scenario as the evaluators knew the outcome but the canine-handler team did not. Each canine team was allowed to search the room in the same manner in which they would typically conduct a normal search. The handlers were instructed to inform the observer whether their dog alerted, showed interest, or failed to alert to the hides. The canine handlers were also informed that they could only reward their canines if they alerted to the positive control, as confirmed by the researcher, and were instructed not to reward their canines for alerting to a hide that contained any bath salt. As each canine team completed their first run, they were ushered out of the testing area, the order of the teams were again randomized and the teams performed a second and third run after being randomized again. The canine trial in Palm Beach County was set up in the manner depicted in **Figure 2** (2 runs per team).

Each synthetic cathinone or hide was placed in a separate locker corridor. Only the bottom half of the lockers were used to optimize canine odor interaction as the trainer had previously conducted canine work in this manner. The cases were supplied by PBSO through the required standard for drug retrieval for canine detection work. Each case arrived in heat seal non-permeable bags and was opened to retrieve the inner bags. Each case contained approximately 10 grams of the bath salt. All designated lockers were opened and with gloved hands, the inner bags were taken out and placed in the seam of the locker. The lockers were then closed and the locks were fixed in a manner similar to the unopened surrounding lockers.

Similar instructions were given to the canine handlers at Palm Beach as were given in the Miami-Dade canine trials. The alert, interest, and failure to alert indicated by the handlers were noted.

After the preliminary trials, this study's imprinting phase was completed utilizing PBSO's canine detection team. These canines were divided into two sets, those imprinted on α-PVP only and those imprinted on ethylone. Cleaned mason jars containing the selected material were screwed into the open slot within a Behavior Shaping Device (K-9 BSD-2 kit; popper box). Clean hand towels (rewards) were rolled, taped in place and positioned in the shooter hole (open slots attached to the device's back) of the BSD. For imprinting purposes 5 boxes were used; one box contained the target odor and the remaining four boxes were blank. The odor was allowed to accumulate for 30 min before allowing canines to search. Canines were led to walk along the boxes and brought to the odorant box where it was allowed to sniff. At this point, the BSD would eject the towel (reward) from the box and the canine was allowed to grab the reward. This was repeated at least three times per session (3 sessions per week).

The second phase objective was to develop the searching pattern. The canine was brought in and given the command to search, if the canine passed the odorant box, they were corrected by the handler pulling on the leash and brought back to be reintroduced to the box. When the canine remained stationary at the box, the shooter ejected the towel and the canine collected its reward. This process was repeated for several weeks until the dog was able to sit or alert. The third phase incorporated distractors to test the canine's focus, alertness, sensitivity, and reproducibility of positive indications. The boxes remained idle for the first 30–40 min prior to the trial. The canines were given the active command to search. Corrections were given where needed, until satisfactory results were achieved for the trainer.

After completion of the imprinting process a validation test was conducted. The seized bath salt cases were placed in unmarked boxes approximately 10 feet apart. The search included distractors and MDMA as the positive control.

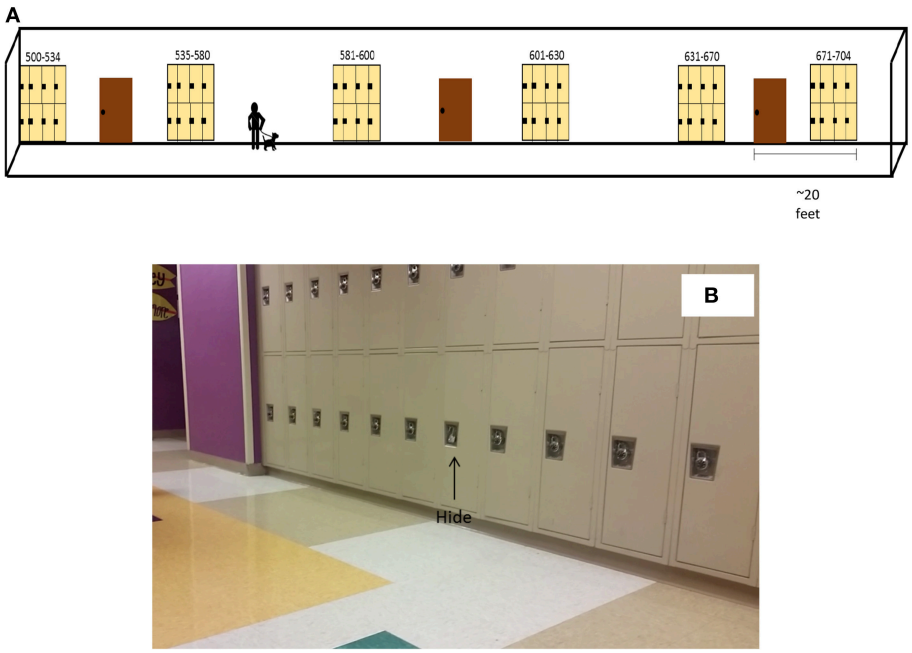

FIGURE 2 | (A) Setup of Palm Beach Canine Trial **(B)** Picture of lockers.

Based on the guidelines following SWGDOG, the canine teams conducted the search and the alerts were recorded as indicated by the handler. The canines had to receive a minimum score of 90% correct responses to be confirmed as being successfully imprinted on the new drug. Canine teams that scored lower than 90% were asked to reinforce the imprinting process and perform the validation test after another week.

RESULTS

Initial Response of Narcotic Detection Canines to Bath Salts

As synthetic cathinones are structurally similar to both MDMA and methamphetamine, it was initially theorized by law enforcement officials that their odor would be similar to bath salts. This led to the expectation that those similarities would allow canines that can detect MDMA and methamphetamine, to also successfully detect synthetic cathinones. However, as shown in **Tables 1**, **2**, all of these certified narcotic detection canines ($n = 12$), though able to alert to the presence of their positive controls (PC1) and (PC2), failed to alert to bath salts.

A more detailed evaluation of the Palm Beach trial (**Table 2**) revealed that canines could not reliably alert to synthetic cathinones such as α-PVP and ethylone. The interest percentage was approximately 7% (negative predictive value was 95%). Although there was interest shown for the three baths salts in **Table 2**, no canine produced a final alert. The confiscated currency used as a blank during this trial also produced some interest by one canine during the first run but no response upon the second exposure. The Ethylone had an overall alert rate of approximately 28% with a low PPV (positive predictive value) of 27%.

TABLE 1 | Detection capabilities of narcotic detection teams deployed in Miami-Dade County.

Canine No.	PC 1 (Marijuana)	PC 2 (MDMA)	Methylone	Ethylone	Blank
1	3/3	3/3	0/3	0/3	0/3
2	3/3	2/3	0/3	0/3	0/3
3	3/3	3/3	0/3	0/3	0/3
4	3/3	3/3	0/3	0/3	0/3
5	3/3	3/3	0/3	0/3	0/3
Alert Response	100%	94%	0%	0%	0%

Imprinting of Canines on Bath Salts

In order to correct the inability of canine teams to detect bath salts, they were divided and imprinted on two types of synthetic cathinones. This division was used to test the ability of each canine to accurately detect the presence of other cathinone derivatives, even though they may not have been previously imprinted on them. Introducing the canine to the odor was the first stage of imprinting. The popper boxes employed by the trainers were devices equipped with a launcher that housed a reward and ejected it for positive reinforcement. With this device, it was noticeable that the canines appeared to develop odor

TABLE 2 | Detection capabilities of narcotic detection teams deployed in Palm Beach County.

Canine No.	PC 1 (Marijuana)	PC 2 (MDMA)	αPVP Case # 17–426	αPVP Case# 14–1856	Ethylone Case # 15–02913	Ethylone Case # 14–65213	Blank (Currency)
1	2/2	2/2	0/2	0/2	0/2	0/2	1/2
2	2/2	2/2	0/2	0/2	0/2	0/2	0/2
3	2/2	2/2	0/2	0/2	2/2	0/2	0/2
4	2/2	2/2	0/2	1/2	0/2	0/2	0/2
5	2/2	2/2	1/2	0/2	0/2	1/2	0/2
6	2/2	2/2	0/2	0/2	2/2	0/2	0/2
7	2/2	2/2	0/2	0/2	0/2	0/2	0/2
Alert Response	100%	100%	7.1%*	7.1%*	28.5%	7.1%*	7.1%*

**Interest Rate Calculated when canine showed interest but no alert was confirmed by the handler.*

recognition rapidly in comparison to other methods of rewarding for imprinting (i.e., towel tugging and PVC pipes). During the odor introduction stage, after the first session all canines began to familiarize themselves with the odor associated with each specific bath salt. However, the odor introduction continued for 1 week.

During the search pattern stage of imprinting, improvement was observed for the canines. One canine team in particular developed a strong alert to α-PVP after the first week, indicated by the handler's attempt to solidify the canine's confidence by employing the "walk-away" method. This method is where the handler will actively walk away to test whether the canine will break from their alert or hold fast. Nonetheless, all canine teams were able to actively search and identify the presence of each bath salt after approximately two and a half weeks of routine imprinting sessions.

The last stage of the imprinting process incorporated distractors such as dog food, tennis balls, and play toys. During this phase four of the canine teams struggled initially. The canines were continuously given various commands from the trainer and handler and removed from the active search line as part of a corrective measure. Each corrective action was performed to reinforce the canine's drive to actively search and detect (work vs. play). This part of the training required the most work for these canine teams with successful completion after approximately 1 month of onsite and at home reinforcement. After completion of the imprinting process, 12 canines were tested for validation; two separate test days. Using the same samples from **Table 2**, the validation trial concluded that all canines had been successfully imprinted on the odor of these drugs. The canines imprinted on α-PVP (group A) were able to detect the Ethylone cases and the same was witnessed with group B (imprinted on the Ethylone); combined alert rate of 100% (based on 2 canine trials).

DISCUSSION

Assessment into the detection capabilities of currently certified narcotic detection canines reveals that they failed to reliably alert to synthetic cathinones (bath salts). Headspace analysis of confiscated bath salts, methylone, ethylone, α-PVP and 3,4-methylenedioxypropiophenone using Solid Phase Microextraction has revealed that these bath salts do in fact have different headspace profiles, these results have been previously reported (22). However, substantial overlap does exist with compounds such as methylone being detected in the headspace of all the confiscated samples allowing for canines to use one or more of these compounds as the active odorant that is responsible for them producing an alert. Studies are ongoing to further isolate these active odorants to ultimately create a mimic canine training aid for the detection of synthetic cathinones. More than 85% of the canines tested in this study from both counties (26 of the 29 runs) were not able to detect any of the bath salt cases presented; while only 20% (Palm beach canine teams; 3 of the 14 runs) showed interest in the bath salts without producing a final alert. Twelve canines were successfully imprinted on confiscated bath salts within a 1 month period. The canine trials conducted have shown that certified narcotic detection canines can in fact be quickly imprinted and trained to detect these new threats within a matter of weeks with sufficient reliability to pass a certification with 90% accuracy. Testing also revealed that canines that were imprinted on one type of bath salt α-PVP (group A) were able to detect the Ethylone and the same was witnessed with those imprinted only on Ethylone (group B) who were able to alert to α-PVP. Analytical headspace analysis using solid phase microextraction on several ethylone and α-PVP samples revealed compounds common in both samples, helping to explain how canines are able to detect either bath salt. Further studies are being conducted to identify and isolate the active odorant in these bath salts responsible for a canine alert, which can be used to create a safe and reliable mimic training aid for canine teams.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of The Institutional Animal Care and Use Committee (IACUC) at FIU. The protocol was approved by the FIU Animal Welfare Assurance.

AUTHOR CONTRIBUTIONS

VS conducted all experiments described and data analysis within this manuscript. She was also the lead author responsible for recording and assembling the text within this manuscript. HH assisted in the experimental design, data analysis, and writing of the manuscript described. KF designed, funded and extended collaborative partnerships used in the execution of this project. He was also responsible for reviewing and crafted conclusions of the manuscript.

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