INVESTIGATION OF DOG'S ABILITY TO DETECT KETOSIS IN DAIRY COWS

by

Amy Byers

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
April 2021

Dedication

This thesis is dedicated to my grandmother, the late Robina Harrington.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Cows can be in a state of sub-clinical ketosis before any of the clinical signs such as a decreased appetite, weight loss, and decreased milk production are present. In order to manage the incidence of ketosis it is important to focus on early detection. The objective of this study is to demonstrate the ability of dogs to discriminate between breath samples from dairy cows positive and negative for ketosis in a laboratory setting. Breath samples were collected from Holstein cows and presented to the dogs following a two-alternative forced choice (2AFC) procedure. Four dogs were assessed for their olfactory capabilities and performance. One dog was able to correctly discriminate between breath samples positive and negative for ketosis in 120 out of 130 sessions, or 92.3% of the time. These results highlight the potential of dogs, with further training and testing, as a tool for the early detection of sub-clinical ketosis.

LIST OF ABBREVIATIONS USED

2AFC: Two alternative forced choice

AcAc: Acetoacetate

Ac: Acetone

BHBA: Beta-hydroxy butyrate

CCAC: Canadian Council on Animal Care

EDT: Errorless discrimination training

FFA: Free fatty acids

GDP: Gross domestic product

NEFA: Non-esterified fatty acids

RAC: Ruminant Animal Center

SDT: Signal detection theory

VOC: Volatile organic compounds

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisors Dr. Simon Gadbois and Dr. Miriam Gordon for their vast amount of guidance, encouragement, and support throughout my degree. It was such an honour to work with two mentors filled with so much knowledge and a love for their profession. Miriam, thank you for coming to the barn with me in the early hours of the morning to hold up a cow's tail while I was learning to collect blood samples. Simon, thank you for trusting in me and allowing me into your lab to fulfill my passion. I have never known someone who was able to connect with an animal like Simon. My dog, Blue, would instantly light up when he would enter the room.

I would also like to thank my thesis committee members, Dr. Janine Gray and Dr. Nancy McLean for their guidance and contributions. A further thank you to the staff at the Ruminant Animal Center (Michael McConkey, Tracey Murdock and Stewart Yuill) for their assistance in blood and breath sampling. A special thank you to all of the cows at the RAC for their patience in allowing me to collect samples from them each week. My apologies for hoping you would become ketotic so that I could have samples for the dogs.

A most sincere thank you to all of the dogs and dog owners I had the pleasure of working with. Simon Gadbois (Ivy), Benjamin Rusak and Gail Eskes (Nellie), Allan and Lisa Byers (Rory), Beth Rutherford (Hazel), and last but not least my own dog Blue; without you, my thesis literally would not have been possible. Ivy, Blue, Nellie, Hazel, and Rory showed up to school each week full of excitement and ready to sniff. I was so lucky to be able to work along side such a wonderful group of dogs. I will truly cherish our many memories and especially the snuggles on the floor during break times.

My thesis also would not have been possible without the awesome group of students and volunteers I worked alongside each week. Josh Dort, Michael Klasz, Lindsay Colyn, Owen Ross, Kirsten Lebelle, Laura Kiiroja, Kadie MacDonald, Zeina Lawen and Shannon Neville. You all had a million other responsibilities to worry about but took the time to come into the lab each week to help me out or watch a dog when I couldn't be there. I cannot thank you all enough.

A special thank you to Meagan (and Beau) for allowing me to borrow her textbook while everything was shut down due to COVID-19.

I must express my deepest appreciation for my parents, my husband Sam, and my best friend Melissa for always supporting and encouraging me. I would not be where I am today without you all.

Finally, I must acknowledge my dogs Blue and Bindi for their unconditional love and endless hours spent sleeping beside me in my office. They are without a doubt the best co-authors I could ask for.

1.0 Introduction

The dairy industry is one of the largest agricultural sectors in Canada. Every year the industry contributes approximately $19.9 billion to Canada's gross domestic product (GDP) from nearly 11 000 farms and 945 000 cows across the country (Dairy Farmers of Canada 2017; Government of Canada 2018). Currently, the industry has placed its focus into two main areas of research; human nutrition and the health benefits of milk and milk products, in addition to increasing milk production and productivity on the farm (Dairy Farmers of Canada 2017).

A loss in milk production can be attributed to several factors. After calving, dairy cows experience a rapid increase in their milk production. Feed intake alone often cannot meet the energy demands required for this increase in milk production. Those cows unable to adapt to the increase in energy requirements descend into a state of negative energy balance known as ketosis (Herdt 2000). Ketosis affects an average of 40% of lactating dairy cows and is typically seen within the first two weeks post parturition (Duffield et al. 1998; Berge and Vertenten 2014). Cows can be in a state of sub-clinical ketosis before any of the clinical signs such as a decreased appetite, weight loss, and decreased milk production are present (Dobbelaar et al. 1996). In order to manage the incidence of ketosis and prevent the development of other metabolic diseases it is important to focus on early detection. Current cow-side tests for ketosis detection in milk and urine are highly variable in accuracy (Nielen et al. 1994). While on-farm blood testing is higher in accuracy, it can become too costly for routine monitoring of herd health. Alternative methods are needed for the detection of ketosis that are non-invasive and cost-effective for the producer.

Monitoring ketone levels in breath has been a newly proposed, non-invasive diagnostic tool in human medicine. This diagnostic method allows for more routine evaluation of ketone levels, without discomfort to the patient (Qiao et al. 2014). Preliminary research has also

evaluated the correlation between breath and blood ketone concentrations in dairy cows using gas chromatography. The high level of correlation between blood and breath ketone body concentrations shows potential for the development of a new non-invasive diagnostic method using the breath of dairy cows (Dobbelaar et al. 1996).

The use of scent detection dogs for biomedical applications is a growing field of research. Dogs have proven to be useful in the early detection of many human ailments, cancers, and agricultural diseases (Mayfield et al. 2008; Sonoda et al. 2011; Mendel et al. 2017). The ability of the dog to identify minute odour concentrations holds the possibility to detect ketosis on the breath of dairy cows in the subclinical stage, reducing the risk of subsequent diseases and further production losses. Evaluation of breath may also provide the industry with a detection method that is both convenient for repeated daily use and non-invasive for the animal. The overall goal of this study is to assess the ability of scent detection dogs to identify ketosis on the breath of dairy cows. Identification of ketosis on the breath would be less invasive than blood testing and may detect the disease at an earlier stage, effectively reducing production losses.

2.0 Literature Review

This review will highlight the need for alternative methods for the detection of ketosis and how scent detection dogs may aid in the development of alternative detection technologies. A general overview to ketosis will be provided, followed by the impacts of the disease, and the current detection methods. The physiology of canine olfaction will briefly be covered, along with examples of scent detection dogs currently used in the fields of human health and agriculture.

2.1 Ketosis in Dairy Cows

2.1.1 Overview

A common metabolic disease in dairy cows is known as ketosis. High producing dairy cows often go through a period of negative energy balance, where less energy is taken in than is expended in metabolism, when a large portion of their body fat is utilized for milk production. (Herdt 2000). During this time, the liver has a reduced ability to oxidize free fatty acids (FFA), resulting in an elevated concentration of ketone bodies in the blood (Ospina et al. 2010; Osorio et al. 2014). The three ketone bodies found in the blood are known as beta-hydroxybutyrate (BHBA), acetoacetate (AcAc), and acetone (Ac), with BHBA being the most prominent in ruminant animals (Kauppinen 1983).

Aside from an increase in ketone bodies, a decrease in blood glucose concentrations is another key metabolic change associated with ketosis (Bach and Hibbit 1959; Baird 1982). This decrease can be attributed to the increase in demand for glucose by the mammary gland for milk production and insufficient replenishment of glucose precursors through feed intake (Schultz 1968; Baird 1982). As glucose stores are depleted, insulin secretion declines and the body begins

to mobilize adipose tissue in the form of FFA to meet energy demands (Sjaastad et al. 2010). These FFA are carried in the plasma to the liver for further conversion to ketone bodies. The FFA are first oxidized in the mitochondria of the liver to form acetoacetyl-CoA, which can then be completely oxidized by acetyl-CoA to CO_2 in the citric acid cycle or be converted to AcAc (Littledike et al. 1981; Sjaastad et al. 2010). Acetoacetate is a fairly unstable compound and will either decarboxylate to Ac or can be converted enzymatically to BHBA (Bergman 1971; Littledike et al. 1981). In ruminants, BHBA is also formed from butyrate in the epithelium of the rumen (Sjaastad et al. 2010). Before metabolization in tissues, BHBA is converted back to AcAc and then to acetyl-CoA for oxidation in the citric acid cycle (Bergman 1971; Littledike et al. 1981). Ketone bodies provide the cow with an energy source when their glucose and glycogen stores cannot sufficiently meet their energy demands (Sjaastad et al. 2010). Both physiological and behavioural changes can be observed in the cow during this period of negative energy balance.

Ketosis can be broken down into two subtypes: clinical and subclinical. The clinical signs of ketosis include a reduction in feed intake, decreased milk yield, dry feces, dull appearance and weight loss (Dobbelaar et al. 1996). Clinical ketosis can be defined as a serum BHBA concentration greater than 2.99 mmol/L (Oetzel 2004). Subclinical ketosis is characterized by an increase in ketone bodies with the absence of any clinical symptoms. The disease affects an average of 40% of lactating dairy cows and is typically seen within the first two weeks post parturition (Duffield et al. 1998; Berge and Vertenten 2014). Various threshold values have been presented in the literature for subclinical ketosis; however, it is generally accepted as a blood serum BHBA concentration of 1.2-2.98 mmol/L with no observable symptoms (Oetzel 2004).

Despite the lack of observable symptoms, sub-clinical ketosis can have impacts comparable to that of clinical ketosis.

2.1.2 Impact of Ketosis

The clinical symptoms of ketosis have impacts on both the health status of the cow as well as their level of production. Ketotic cattle have reduced milk yield and show a decrease in reproductive performance (Duffield 2000). They are also subject to higher culling rates and an increased risk of developing other metabolic diseases. Cows with increased concentrations of BHBA have greater odds of developing metritis, lameness, and displaced abomasum (Suthar et al. 2013). Using a deterministic economic model, McArt et al. (2015) determined the average total cost per case of ketosis to be approximately $117 US. This estimate includes future reproductive losses, future milk production losses, diagnostics, and treatment. When also considering the possible development of metritis and displaced abomasum alongside ketosis, the average cost increased to $289 US (McArt et al. 2015). Aside from production costs, ketosis has also been shown to increase greenhouse gas emissions per kg of milk produced. The production efficiency and future reproductive performance of the cow is reduced, along with an increase in discarded milk. Greenhouse gas emissions have been shown to increase an average of 2.3% per case of subclinical ketosis, compared to the greenhouse gas emissions of a cow not in ketosis (Mostert et al. 2018). The early diagnosis of subclinical ketosis would help to reduce the risks of developing other metabolic diseases as well as subsequent production losses.

2.1.3 Detection of Ketosis

Ketosis can be diagnosed through the detection of ketone bodies in the blood, milk, and urine of the cow. Various cow-side tests are now available for use by the farmer, supplying immediate on farm results. Milk is one of the most convenient diagnostic methods, as it is easy

to collect and non-invasive. The KetoTest™ is used to determine the concentration of BHBA in milk (Larsen and Kristensen 2010). This is a dipstick test which turns purple based on its reaction with BHBA in the milk sample (Carrier et al. 2004). The concentration of BHBA is generally lower in milk than that of the blood and a threshold value of 0.2 mmol/L of milk is given by the manufacturer of the milk test kit for subclinical ketosis (Jezek et al. 2017). The KetoTest™ has a sensitivity, or the ability of a test to correctly identify those with the disease, of 73% and a specificity, the ability of the test to correctly identify those without the disease, of 96% as determined by Carrier et al. (2004). The ketone body acetoacetate (AcAc) may also be quantified on farm in urine using the Ketostix® urine strip. This test is based on a reaction between AcAc and sodium nitroprusside under alkaline conditions. The result is presented as a colour change from white to purple in the test well (Nielen et al. 1994). The degree of colour change corresponds to the severity of ketosis. The Ketostix® strip has a sensitivity of 78% and a specificity of 96% for detecting AcAc in urine samples (Carrier et al. 2004). Both milk and urine cow-side tests provide a semi-quantitative evaluation of ketosis. Their non-invasive sampling method allows for a large volume of cows to be tested more frequently and relatively quickly. Although they are quick and easy to use by the farmer, they are subject to visual interpretation of the colour change, and therefore lack sensitivity levels.

Currently, the most accurate diagnostic method for ketosis is the laboratory evaluation of BHBA in the blood. Beta-hydroxybutyrate is more stable in the blood and can be found in higher concentrations than AcAc or Ac, yielding a higher accuracy of measurement (Herdt 2000; Oetzel 2004). This method of testing, however, comes with a delay in sample processing which may be problematic for early intervention and remediation. In order to replace the need for laboratory testing, a handheld meter has recently been introduced for cow-side monitoring of BHBA

concentrations. The Precision Xtra™ meter has traditionally been used in human medicine for those with diabetes (Iwersen et al. 2009). Once the blood has been drawn up the test strip to the sample well, BHBA dehydrogenase oxidizes the BHBA in the blood sample to AcAc. The electrical current generated by this conversion is proportional to the BHBA concentration in the blood sample (Zhang et al. 2012). The concentration of BHBA is then displayed by the meter in mmol/L. The Precision Xtra™ meter has been determined to have a sensitivity of 96% and a specificity of 97% at a ketone concentration of 1.4 mmol/L of blood (Iwersen et al. 2009). The high accuracy of on-farm blood testing is counter-acted by its high cost and invasive sample collection method. For these reasons, it is most often used for periodic assessment of ketosis within a herd.

While current milk and urine cow-side tests for the diagnosis of ketosis are easy to use and non-invasive, they are unreliable with their lower sensitivity levels. On-farm blood testing methods show high accuracy but are often too costly for routine monitoring of the prevalence of ketosis. Non-invasive technologies are needed to detect subclinical ketosis at an earlier stage, reducing the risk of developing other metabolic diseases and decreasing production losses.

2.1.4 Treatment of Ketosis

Detecting ketosis at the subclinical stage increases the chances of effectively treating the metabolic disease and minimizes further impacts on the cow's health. When treating ketosis, the main goal is to stimulate gluconeogenesis in order to increase plasma glucose concentrations. The stimulation of gluconeogenesis decreases the demand for glucose within the body, lowering the production of non-esterified fatty acids (NEFA) and decreasing plasma ketone body concentrations (Herdt and Emery 1992). Dairy cows may be supplemented with glucose precursors, such as propylene glycol or glycol, in a top-dress to their diet or as a drench in order

to stimulate gluconeogenesis. Propylene glycol is the most commonly used glucose precursor as it is more readily available to the cow, resulting in a quick absorption by the rumen and utilization by the liver for glucose production (Jeong et al. 2018). As ketotic cows typically show reduced feed intake, an oral propylene glycol drench is the preferred method of treatment over a top-dress for feed (Christensen et al. 1997). Piantoni and Allen (2015) determined the effective dosage of propylene glycol drench to increase plasma glucose concentrations to be 300 mL a day over the course of 3 to 4 days. Propylene glycol can have potentially toxic effects at too high of a dosage as sulfur-containing gases are produced during the fermentation of the glucose precursor in the rumen (Trabue et al. 2007). It is extremely important to monitor animals during their treatment to ensure they are recovering well and without any adverse effects. Treatment with glucose precursors may help mitigate the impacts of ketosis on the health and productivity of the cow and prevent the development of subsequent metabolic diseases.

2.2 Analysis of Breath

Monitoring ketone levels in breath has been a newly proposed diagnostic tool in human medicine. Breath collection is non-invasive for the patient, can be performed as often as desired, and can be analyzed in real time (de Lacy Costello et al. 2014). It has the potential to detect diseases in their early stages as exhaled breath contains many different volatile organic compounds (VOC) which can differ between healthy and diseased states (Phillips 1992; Lourenço and Turner 2014). Volatile organic compounds are secreted from cells as a result of various metabolic processes. They transverse the cellular wall and enter the bloodstream where they are carried to different locations within the body (Angle et al. 2016b). In the lungs, only a thin membrane separates the air in the alveoli from the blood in the capillaries. Volatile organic

compounds can cross the alveolar interface and appear in exhaled breath (Phillips 1992; Lourenço and Turner 2014). Volatile organic compounds have a low metabolic weight and can easily evaporate at normal temperatures and pressure (Angle et al. 2016b). Currently, 872 potential VOC have been identified in the breath of healthy individuals. One of the most prominent compounds found in breath is acetone, which has a characteristic sweet odour, like that of decaying apple (de Lacy Costello et al. 2014; Ruzsányi and Kalapos 2017). The detection of acetone in breath has proven to be a useful tool for the diagnosis and marker of treatment success of many disorders.

Diabetic ketosis is a condition which often occurs in patients with Type 1 diabetes when the pancreatic cells do not produce enough insulin, causing an imbalance in sugar, protein, and fats in the body (Bonadio 2013). Frequent blood sampling has traditionally been used to monitor blood ketone body concentrations. In both a rat model and trials using human adults, breath acetone concentrations were strongly correlated with blood acetoacetate and beta-hydroxybutyrate concentrations (Likhodii et al. 2002; Musa-Velosos et al. 2002). Daily monitoring of ketone concentrations is able to be employed using breath sampling. Measuring levels of ketones by breath offers a non-invasive and convenient method for the monitoring of ketosis with minimal discomfort for the patient.

A preliminary study by Dobbelaar et al. (1996) investigated the potential of detecting ketosis by the analysis of exhaled breath in dairy cattle. Acetone concentrations of the breath samples were measured by gas chromatograph and correlated to blood BHBA and milk AcAc concentrations. The high level of correlation between blood and breath ketone body concentrations shows potential for the development of a new non-invasive diagnostic method using the breath of dairy cows (Dobbelaar et al. 1996).

2.3 Scent Detection Dogs

2.3.1 Canine Olfaction

The unique structure of the canine nasal cavity, accompanied by a large volume of olfactory receptor neurons, yields a strong olfactory acuity. Dogs are capable of detecting odour concentrations as low as 1-2 parts per trillion (Walker et al. 2006). The olfactory abilities of the canine can largely be attributed to their anatomy, as those animals with a heightened olfactory acuity all share a similar nasal structure. The entrance to their nasal cavities can expand through muscular action to increase the volume of scent which is acquired (Jezierski et al. 2016). The dog has a unique nasal air flow pattern in that each nostril can acquire a separate odour sample during inhalation (Craven et al. 2010). Their unique air flow pattern optimizes odour transport to specific olfactory receptor neurons located in the back of the nasal cavity. The olfactory receptor neurons play a central role in the sense of smell, facilitating the detection of specific odour molecules. Compared to humans, whom have only five million olfactory receptor neurons, dogs have approximately 200 million (Lawson et al. 2012). The number of olfactory receptors allows the dogs to detect a greater variety of scents and differentiate the odours they are detecting. To date, there have been only a few investigations comparing the olfactory abilities of various dog breeds. Polgár et al. (2016) found breeds historically selected for scent work, such as the beagle and basset hound, performed significantly higher than non-scenting and short-nosed breeds in a detection task. While no differences have been found in the number of olfactory receptors between breeds, polymorphism of the olfactory receptor sequence has been found both within and between dog breeds (Tacher et al. 2005). This could potentially explain the vast differences in the olfactory capabilities of dogs. Sacharczuk et al. (2019) found individual differences in the olfactory receptor genes of dogs that completed similar training for drug and explosive detection.

Specific polymorphisms of the olfactory receptor genes corresponded with the differences in their detection performance. More research is needed to further investigate the differences in olfactory capabilities between and within specific breeds of dog.

2.3.2 Training Scent Detection Dogs

To date, there are no set standards or guidelines for the training and testing of dogs used in scent detection studies (Johnen et al. 2017). However, scent lineups, two-alternative forced choice (2AFC), Yes/No and Go/No-Go are the most commonly used training practices. Scent lineups are traditionally used for forensic investigations where dogs are used to identify criminals based on scent traces left at a crime scene (Schoon 1996; Gadbois and Reeve 2014). Their training involves a matching-to-sample task. The handler presents a sample from the perpetrator for the dog to sniff and the dog is required to identify the matching sample in a lineup of potential options (Schoon 1996; Gadbois and Reeve 2014). Scent lineups have been used all over the world as evidence for conviction, however, the reliability and validity of their results have often been called into question (Taslitz 2013). Vyplelová et al. (2014) investigated the possibility of human odour fallout onto an untouched object. Two different sample types were collected onto cotton squares. For the first, the person held the square in their left hand and for the second, their right hand hovered 5 cm over the square, each for three mins. The samples were presented to two police trained German Shepherd dogs in a scent lineup and asked to perform a matching-to-sample task. It was determined that a scent trace can be left on an object by humans, even if an object is untouched (Vyplelová et al. 2014). This, in effect, could lead to an incorrect identification of the perpetrator. For matching-to-sample tasks it is important to also consider the working memory of the animal (Gadbois and Reeve 2014). When the dog is asked to identify the matching sample, they must compare their memory of the sample with up to six potential

options, as the position of the matching sample is randomly determined. If the matching sample is located early in the lineup, it has been shown to result in a higher rate of correct selection. When the matching sample is further down the line, accuracy may decrease significantly (Gadbois and Reeve 2014). By the time the dog reaches the final sample, their ability to remember the characteristics of the original sample may have faded. One way to counteract this would be to use multiple dogs in each case identification (Taslitz 2013). Having multiple dogs agree on the correct sample would reduce the number of false positive identifications and improve accuracy. This issue could also be combated by presenting fewer choices to the dog, such as in the 2AFC, Yes/No or Go/No-Go tasks.

2AFC is a discrimination task which reduces the number of choices for the dog down to two, effectively increasing performance and accelerating learning (Gadbois and Reeve 2014). The dog must choose between the target odour (S+) and a foil (S-). In some cases, 3- or 4-AFC may also be used, however, performance in AFC tasks seem to be reduced significantly if the target scent is in position 4 and above (Gadbois and Reeve 2014). This type of training is useful for dogs that must discriminate between stimuli that often co-exist, such as different species of animals or strains of bacteria and viruses (Gadbois and Reeve 2016). Angle et al. (2016a) used this type of training for their investigation of dogs' ability to discriminate cell cultures infected with bovine viral diarrhea virus from uninfected cell cultures, as well as discriminate from cell cultures infected with bovine herpes virus 1 and bovine parainfluenza virus 3. For this task, there is only one odour for the dog to discriminate from a distractor scent and no "cue" or reminder of the scent is given prior to the task (Gadbois and Reeve 2016). In some cases, however, the dog does not always have the option to compare between samples. In these instances, Go/No-Go and Yes/No training becomes useful.

In real life scenarios, biomedical detection dogs are presented with one sample/patient at a time and asked to determine if the sample is positive or negative for the target odour. Go/No-Go and Yes/No procedures are used for a single sample presentation where the animal signals if the sample is an S+ or S-. In Go/No-Go, the dog indicates a S+ with a trained response (e.g., nose hold) and walks away/ ignores an S- (Gadbois and Reeve 2016). In a Yes/No procedure, the dog responds to a S+ as previously described and is also trained to respond to a S- with a different response (e.g., sitting) (Reeve et al. 2020). This training practice was used by Reeve et al. (2020) to train dogs to identify hypoglycemic breath samples from individuals with Type 1 diabetes. Biomedical alert dogs are required to "alert", give a yes response, only when the target odour is present (Gadbois and Reeve 2016). In the case of Reeve et al. (2020), when presented with a hypoglycemic sample the dog must alert by holding their nose over the sample for 5 secs ("yes" response) and must sit ("no" response) when presented with a normoglycemic or hyperglycemic sample. The Go/No-Go task allows us to more easily quantify the response bias of the dog to determine if they are more liberal or conservative in their decision making (Gadbois and Reeve 2016). The appropriate type of decision maker changes based on the risk assessment of task of the detection dog. A liberal dog is prone to commit more false alarms, whereas a conservative dog will produce more misses of positive samples. In the case of a biomedical detection dog, it may be useful to have a liberal decision maker as misses of positive samples are costlier than false alarms.

2.3.3 Scent Detection Dogs in the Field

The olfactory abilities of the dog have led to their use in a variety of different industries. Aside from law enforcement for drug detection and search and rescue, dogs have found themselves in the field of human health (Gadbois and Reeve 2014). For many cancers, early

detection is the key to successful treatment and survival. In the case of colorectal cancer, the fecal occult blood test is currently the most non-invasive screening method, however, it holds a positive predictive value of only 10% (Steele et al. 2009). This screening tool produces an abundance of false positive readings, sending a large volume of patients for additional screenings. The use of scent detection dogs was explored as an equally non-invasive tool for the early detection of colorectal cancer. Sonoda et al. (2011) found that dogs are capable of detecting cancer specific compounds on the breath of patients to a sensitivity and specificity of 91% and 99% respectively, and in stool samples to a sensitivity and specificity of 97% and 99%. Scent detection dogs have also been employed for the early detection of prostate cancer in men. Patients often seek out the most minimally invasive diagnostic method, and so canines were investigated as a possible alternative to current diagnostic tools. Urbanová et al. (2015) found that dogs trained for scent detection can accurately identify urine samples positive for prostate cancer with a sensitivity of 93.5% and specificity of 91.6%. Diagnosing prostate cancer in urine with the use of a scent detection dog is a non-invasive alternative which is comparably reliable to current diagnostic tools. Dogs hold the ability to detect extremely small quantities of a specific scent, providing an alternative diagnostic tool for cancers in which patients depend on early detection for survival (Urbanová et al. 2015).

Aside from applications in human health, dogs are more recently being used in the field of agriculture. The efficient detection of estrus on dairy farms remains a challenge for many farmers and often relies on the visual observation of behavioural changes (Johnen et al. 2015). Estrus detection is key for successful reproductive performance and the use of artificial insemination. As visual observation is subject to individual interpretation and timing, alternative cost-effective methods are needed. Fischer-Tenhagen et al. (2011) determined that dogs are able

to successfully detect estrus indicating volatiles in the urine, milk, and vaginal fluid samples of dairy cows in a laboratory setting with an average accuracy of 80.3%. The dogs can generalize the odour of estrus not only across sample types, but also individual cows. Scent detection dogs trained for estrus detection have also been found to display a sensitivity of 71.9% and a specificity of 93.0% during an on-farm trial where the dogs identified positive cows from the feed alley (Johnen et al. 2015). The successful detection of estrus increases reproductive performance of high producing dairy farms, increasing overall production.

Dogs can also aid in decreasing production losses through the detection of agricultural diseases. Australian sheep are suffering losses in production due to infection by gastrointestinal nematodes. The nematodes result in anemia, anorexia, decreased fertility, and reduced wool growth for the sheep (Yu et al. 2000). The symptoms of nematode infection are not always visible until it is typically too late, and so dogs have been investigated as a method of diagnosis which can locate the nematodes at an early stage, reducing production losses. Dogs can correctly identify nematode infections in sheep 85% of the time, on average, 7 days post nematode infection (Mayfield et al. 2008). Plant-based diseases can also be detected by scent detection dogs. Laurel wilt disease affects the economically important avocado plant and is the result of an invasive species introduced into the United States through untreated wooden packaging material (Mendel et al. 2017). The plant succumbs to the disease soon after infection and so it is important to detect the Laurel wilt pathogen earlier on to stop the spread of the disease. Specially trained scent detection dogs are able to effectively indicate affected plants with an accuracy of 99.4% and a positive predictive value of 94.8% (Mendel et al. 2017). Scent detection dogs offer the possibility of becoming a successful management tool to control the spread of agricultural diseases.

At this time, scent detection dogs have not been used for the detection of ketosis on the breath of dairy cows. The ability of the dog to identify minute odour concentrations holds the possibility to detect the disease in the subclinical stage, reducing the risk of subsequent diseases and further production losses. Scent detection dogs may provide the industry with a detection method that is convenient for repeated daily use while being non-invasive for the animal.

3.0 Research Objectives

Current on-farm ketosis detection methods are highly variable in their accuracy or are too expensive and invasive for routine inspection of herd health. Monitoring ketone levels on the breath by scent detection dogs offers the possibility of a new, non-invasive detection method which can detect the disease early on.

The aims of this study were to:

1. Assess the ability of trained scent detection dogs to discriminate between breath samples from dairy cows positive and negative for subclinical ketosis in a laboratory setting.
2. Compare the reliability of the scent detection dogs trained to discriminate between breath samples from dairy cows positive and negative for subclinical ketosis to current ketosis diagnostic technologies.

4.0 Methodology

4.1 Participants

4.1.1 Dogs

Dogs were recruited by convenience sampling through word of mouth in the areas of Truro and Halifax. All dog participants of the Canid and Reptile Behaviour and Olfaction Laboratory are owned animals and brought into the lab once or twice a week by their owners for a 1 to 3-hour session. For this study, four dogs (n=4) were assessed and trained before final selection (see Table 1). Multiple dogs may have been present in the lab at any time, however, all training and testing sessions were completed on an individual basis without contact between the other dogs. During their time in the lab, volunteers would periodically take the dogs outside for short walks or play time. The appropriate animal care approval has been obtained from the Dalhousie University Committee on Laboratory Animals (2019-091) under the Canadian Council on Animal Care (CCAC).

Table 1: Description of dog participants

Name	Breed	Age in Years	Reproductive Status	Previous Obedience Training
Blue	Border Collie	6	Male, Neutered	Yes
Nellie	Standard Poodle	3	Female, Spayed	Yes
Rory	Duck Toller	4	Male, Intact	Yes
Ivy	Golden Retriever	4	Female, Spayed	Yes

It is important to note that this study used a small- n approach (Morgan and Morgan 2009). This is to say that four dogs were selected from a larger sample of dogs based on their ability to train and drive to work, as well as their previous history of performance. As a result of the careful selection, the small group of dogs in this study are considered to be different from the more general population of dogs. For this reason, the findings of this study are not to be generalized to all dogs, but specific to a few carefully selected and highly trained dogs. When training dogs for biomedical scent detection a larger sample size of dogs is not the focus. Instead, it is important to focus on a larger number of samples to present each individual dog. With each dog this study sought to validate a new "tool" for ketosis detection, and so the more samples that can be presented to each dog, the more confident one can be in the new tool for the detection of ketosis.

4.1.2 Cows

Holstein dairy cows located within the Ruminant Animal Center (RAC) at the Dalhousie Faculty of Agriculture were used for breath sample collection. Prior to breath collection, each cow was tested to determine their blood ketone concentration. The cows are routinely tested on days 7, 14, 21, and 28 post-parturition using a Precision Xtra™ meter to monitor their blood ketone levels (Figure 1). The blood sample was taken near the tailhead, where the coccygeal vein runs in the midline of the tail, using an insulin needle. The cow was restrained in a standing position using a tail jack and approximately 0.25 mL of blood was collected for analysis of ketones. Breath collection took place on regular blood testing days for each cow.

Figure 1: A Precision Xtra™ meter used to monitor the blood ketone concentrations of the cows at the Ruminant Animal Centre on days 7, 14, 21 and 28 post-parturition. A cow positive for ketosis will have a blood ketone body concentration > 1.2 mmol/L

4.2 Stimuli

4.2.1 Silicone-coated cotton balls

Breath samples were collected using cotton balls coated with silicone as per Reeve et al. (2018). The cotton balls were first weighed, and their combined weight recorded before placing into a 2.8 L glass dish. An equivalent weight (1 g silicone oil per 1 g of cotton balls) of 100% silicone oil was then dissolved in hexane in a 14 hexane: 1 silicone mass ratio. This solution was transferred to the glass dish with the cotton balls, ensuring each ball was completely and uniformly coated. The silicone-coated cotton balls were left inside a fume-hood for 24-48 hours, allowing the hexane to completely evaporate and leaving only silicone oil. The cotton balls were stored in an air-tight glass container until use for breath sampling. Coating the cotton ball in silicone oil improves the detectability of breath samples which are to be stored over longer periods of time (Reeve et al. 2018).

4.2.2 Breath samples

Cows were first habituated to the breath sample collection procedure. Breath samples were collected from cows both negative and positive for ketosis in a 20 mL glass scintillation vial (28 mm x 61 mm) containing a silicon oil coated cotton ball (Figure 2). The glass vial was held 2 cm in-front of the nose and mouth of the cow. After 3-5 strong breaths from the cow, the vials were quickly sealed, labeled, and cold-stored at 4 ℃ until ready for use. Two breath samples were taken from each cow at a time. Nitrile gloves and a face mask were worn and changed between each cow to avoid cross-contamination between samples.

Figure 2: A 20 mL glass scintillation vial containing a silicon oil coated cotton ball was used for collection of breath from dairy cows positive and negative for ketosis

4.3 Procedure

All training and testing procedures took place within the Canid and Reptile Behaviour and Olfaction Lab at Dalhousie University. The lab consists of three rooms: the largest room was for the preparation and cleaning of testing apparatus, along with two smaller rooms for testing.

4.3.1. Saliency training

The dogs were first trained to detect low salient stimuli using tea at saliencies ranging from 15 mins steeped down to 5 secs, and finally tea breath (Reeve et al. 2018). This training helped to select dogs with high trainability, performance, and drive to work. Water was boiled using an electric kettle and a tea bag of Red Rose Orange Pekoe tea was placed into a mug of boiling water, the timer began as soon as the tea bag touched the water. A cotton ball was then placed into a 20 mL glass scintillation vial where a 5 mL sample of tea was added to the cotton ball, using a syringe, for the target stimulus (S+) and plain boiled water placed onto a silicone coated cotton ball in an additional glass vial as the blank stimulus (S-). For tea breath samples, tea was steeped for 2 mins and cooled for 5 mins. An experimenter held the steeped tea in their mouth for 30 secs and then swallowed or expelled the tea. The experimenter then immediately exhaled two deep breaths onto a silicon coated cotton ball inside of a glass vial. The experimenter was unable to consume any food or drink 1 hour prior to providing a breath sample.

All training methods used positive reinforcement with a clicker as a secondary reinforcer and food treats as reward for the dog. Those dogs without previous clicker training experience spent their first sessions in the lab becoming exposed to clicker training. The nose hold behaviour, or the response to the target stimulus, was then shaped starting with a quick touch until a 5-sec long hold could be maintained. Dogs were asked to hold their nose for 5 secs over the top half of a stainless-steel funnel (Figure 3). The funnel ensures no contact is made with the sample to avoid contamination.

Initially, only one funnel containing the target stimulus was presented to the dog. When the dog simply approached the funnel or gave a quick sniff an experimenter would “click” and

give a treat reward. This was continued until the dog successfully sniffed and held their nose above the funnel with the target stimulus for 5 secs. Two funnels were then presented to the dog using a two alternative forced choice procedure (2AFC). One funnel contained a 20 mL glass scintillation vial with a silicon-coated cotton ball and sample of 15-min steeped tea inside, and the other with plain boiled water on the cotton ball. The dog was asked to smell both funnels and indicate which contained the target stimulus with a nose hold. Once the 5 sec nose hold was successfully shaped, tea trials began with 15-min steeped tea samples.

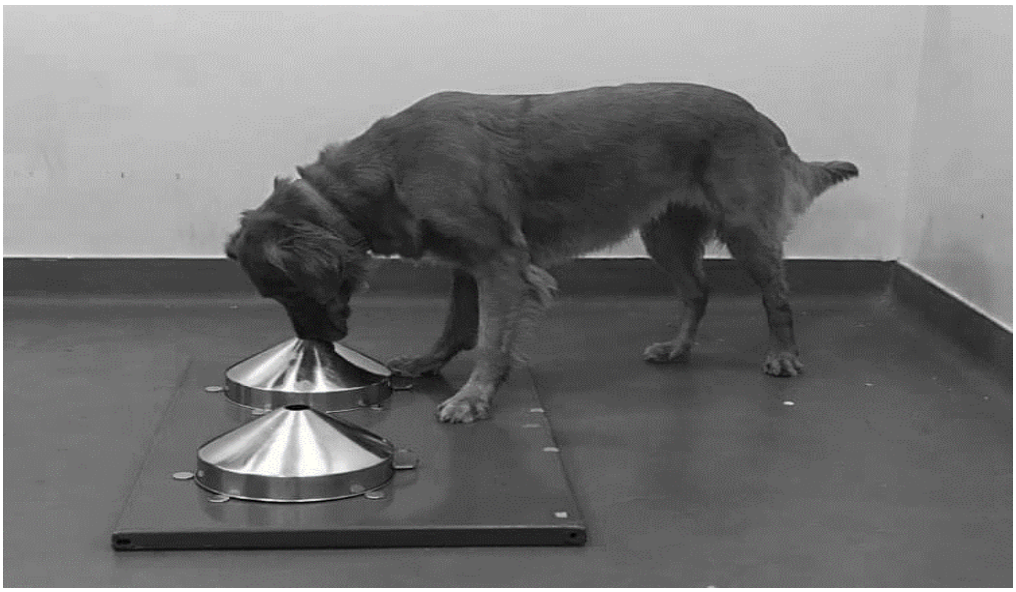

Figure 3: Glass scintillation vials containing a silicon-oil coated cotton ball and tea sample were placed beneath a metal funnel. The dog was asked to indicate a positive sample with a 5-sec nose hold

Sessions consisting of ten trials were run, asking the dog to correctly identify the location of the target sample with a 5-sec nose hold. The position of the target stimulus was determined by rolling a die. If the die rolled 1 , 3 , or 5 the target stimulus was in the left position and 2 , 4 , or 6 the target stimulus was in the right position. Some constraints were applied to the randomization, such that the target stimulus could not be in the same position for more than three

consecutive trials, and the position of the target stimulus could not follow a pattern for more than two repetitions (i.e., left, right, left, right) (Reeve et al. 2018). Dogs remained outside of the testing room with the handler while the positive and blank stimuli were randomly placed under the two funnels by an experimenter. The handler then allowed the dog into the testing room and asked the dog to identify the location of the positive stimulus. The trials were double-blind, with the handler orally communicating "left" or "right" to the experimenter behind a partitioned wall. The experimenter would then click to indicate a correct response and the handler gave the dog a treat reward. If an incorrect response was given the experimenter would say a gentle "nope" to announce to the dog it would be led back out of the testing room for another trial. A criterion was set for the dog to reach before moving on to the next saliency. The dogs had to reach a success rate of 80% or above correct responses three sessions in a row or two sessions in a row at 90% or above before moving on to the next saliency. Once they successfully completed each saliency of the preliminary tea training by reaching this criterion, they moved on to cow breath samples.

4.3.2 Errorless Discrimination Training

To remediate any dogs with a lower success rate in saliency training, a method known as Errorless Discrimination Training (EDT) was employed as per Dort (2020). Initially, with this method, only the target sample is presented to the dog; effectively leaving the dog with only one choice to make. The concentration of the distractor sample is gradually and slowly increased until it is at the same concentration as the target sample. This process is described as "fading in" (Gadbois and Reeve 2014). This type of training teaches the dog to ignore the distractor sample and directs their attention towards the target sample, increasing the likelihood of correct responses by the dog.

Two metal funnels were placed on the floor with a small metal container to hold the sample underneath each funnel. The container with the target sample was left uncovered, while the container with the distractor sample was initially sealed with a square of aluminum foil over the open top so no volatiles could escape. As training progressed, holes were poked into the top of the aluminum foil in a systematic fashion which allowed more volatiles to escape from the jar (Dort 2020). The dog was asked to smell both funnels and choose the funnel containing the target sample with a 5-sec nose hold. The dogs had to reach a success rate of 80% or above correct responses three sessions in a row or two sessions in a row at 90% or above before moving on to the next saliency. The dogs began with a saliency of tea steeped for 5 secs and progressed to a saliency of tea steeped for 5 secs which was then diluted with water by 75% (Dort 2020).

4.3.3 Ketosis Trials

Those dogs which successfully completed saliency and EDT were selected to move on to ketosis breath trials. Training and testing for the discrimination of breath samples positive and negative for ketosis followed a 2AFC procedure. Two funnels were presented to the dog with a breath sample positive for ketosis (blood ketone concentration 1.2-1.6 mmol/L) placed under one funnel and a breath sample negative for ketosis (blood ketone concentration 0.6 mmol/L) under the other. Sessions consisting of ten trials were run, asking the dog to hold their nose for 5 secs over the funnel containing the positive ketosis sample. All sessions were randomized for sample location (left or right) and double-blind. Once a success rate of 80% or above correct responses in three sessions (of ten trials) each or two sessions in a row (of ten trials) at 90% or above had been reached data collection began.

4.4 Data Analysis

At the end of each session of ten trials, the percent of correct responses was determined. The results for each session were then graphed to display a learning curve for each dog over time.

$$\text{Percent of Correct Responses} = \frac{\text{Number of Correct Trials}}{\text{Total Number of Trials}} \text{ x } 100\%$$

5.0 Results and Discussion

As the findings of this study are not to be generalized to all dogs, the results of each dog will be discussed on an individual basis.

5.1 Saliency Training

Saliency training allowed for the initial assessment of the dogs for trainability and olfactory abilities. The dogs were introduced to the mechanics of the task while gradually training the detection of low saliency stimuli. Saliency training allowed for an assessment of the consistency of the dog in repetitive exposures to a target stimulus. A dog which is inconsistent, even if occasionally performing at an accuracy of 97%, may not be as valued as a dog that consistently achieves an accuracy of 90% over time.

5.1.1 Blue

During saliency training, Blue completed a total of 42 sessions over the course of 1 year. Figure 4 displays his performance to detect Red Rose Orange Pekoe tea at decreasing saliencies ranging from tea steeped for 15 mins, to tea steeped for only 5 secs, and finally tea breath. Blue was new to the lab with no previous scent training. It is also important to note that he is deaf, and so the hand signal of a “thumbs up” was used instead of a “click” to indicate a correct response.

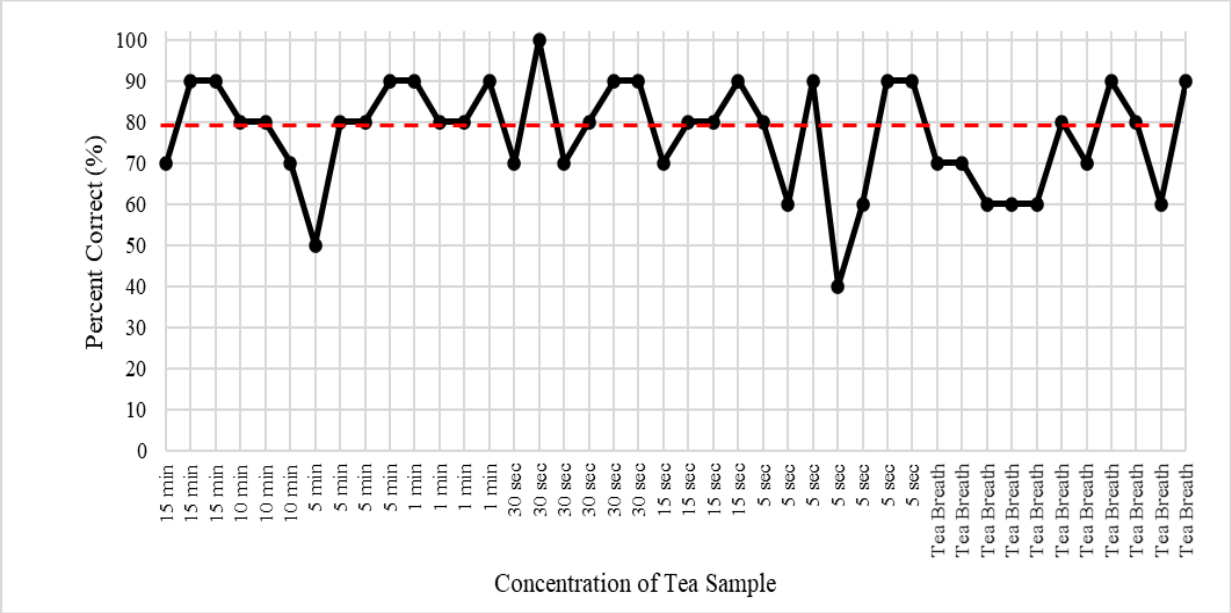

Figure 4: Blue's performance during saliency training where the dogs were trained to detect low salient stimuli using Red Rose Orange Pekoe tea at saliencies ranging from 15 mins steeped down to 5 secs, and finally tea breath. The dogs had to reach a success rate of 80% or above correct responses three sessions in a row or two sessions in a row at 90% or above before moving on to the next saliency, indicated by the dotted line

Blue quickly passed the 15-min steeped tea level, achieving 80% or above correct responses three sessions in a row. His performance dropped when introduced to 5-min steeped tea, but he was able to get back on track and move through the next levels in a timely manner. The majority of the saliency levels were considered passed within one day. His performance typically began below criterion when introduced to a new saliency, but by the end of the day he would reach, or go above, criterion. Blue spent a greater number of sessions on tea steeped for 5 secs; this saliency proved to be more difficult for him. If he was uncertain or he gave multiple wrong responses, Blue's motivation to work would decline. This was indicated by lying down, refusing to enter the testing room, or not smelling the samples. If this occurred, he was given a break for a walk and playtime so he would be in a positive mindset to try again. Occasionally, a quick re-fresher session would be required to get him back on track.

5.1.2 Nellie

During saliency training, Nellie completed a total of 42 sessions over the course of 1 year. Figure 5 displays her performance to detect Red Rose Orange Pekoe tea at decreasing saliencies ranging from tea steeped for 15 mins, to tea steeped for only 5 secs, and finally tea breath. Nellie was new to the lab with no previous scent training.

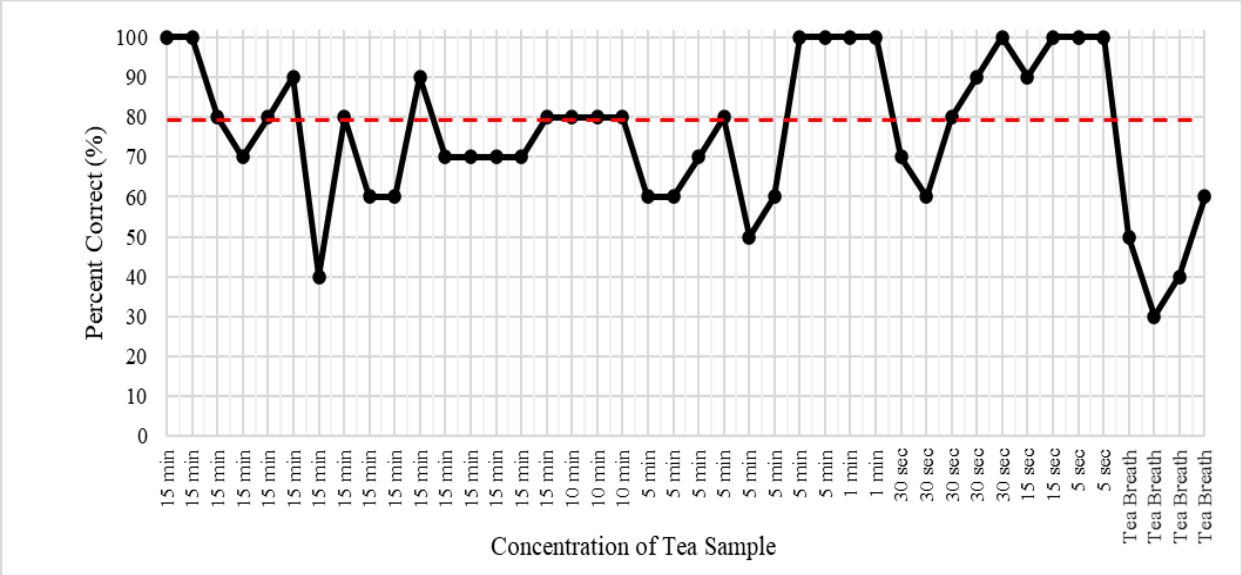

Figure 5: Nellie's performance during saliency training where the dogs were trained to detect low salient stimuli using Red Rose Orange Pekoe tea at saliencies ranging from 15 mins steeped down to 5 secs, and finally tea breath. The dogs had to reach a success rate of 80% or above correct responses three sessions in a row or two sessions in a row at 90% or above before moving on to the next saliency, indicated by the dotted line

Nellie struggled initially with her detection of tea samples. This is reflected by her inconsistent performance and the greater number of sessions to reach criterion at tea steeped for 15 mins. It was not until tea steeped for 1 min where she really started to grasp the concept and her percent correct per session increased to 100%. The transition from tea steeped for 5 secs onto tea breath was quite difficult for Nellie. A steep decline in her performance can be seen and a number of re-fresher session were required.

5.1.3 Ivy

Ivy completed a total of 19 sessions over the course of 3 months during saliency training. Ivy had previously worked in the lab with various different projects, as well as in the field, and has previously completed saliency training. For this reason, she began at 5 min steeped tea, instead of 15 mins as with Blue and Nellie. Her performance can be found in Figure 6.

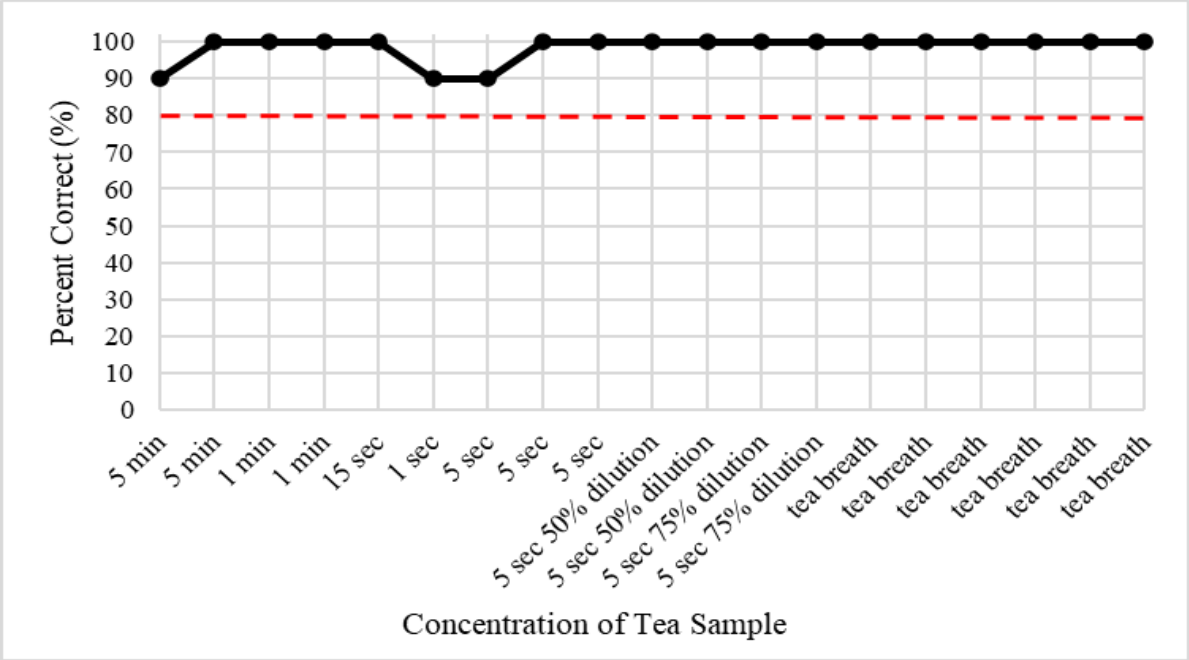

Figure 6: Ivy's performance during saliency training where the dogs were trained to detect low salient stimuli using Red Rose Orange Pekoe tea at saliencies ranging from 15 mins steeped down to 5 secs, and finally tea breath. The dogs had to reach a success rate of 80% or above correct responses three sessions in a row or two sessions in a row at 90% or above before moving on to the next saliency, indicated by the dotted line

Ivy showed the greatest consistency over time and percent correct responses of the three dogs. She correctly identified the target stimulus 90 to 100% of the time. This is as to be expected with her previous scent detection experience. Due to her success with low saliency stimuli, she also had further dilutions included in her training; tea steeped for 5 secs was diluted

by 50% and 75% with water. Ivy was selected to continue on to ketosis breath samples as a result of her success with low saliency stimuli, consistent performance, and high motivation to work.

5.2 Errorless Discrimination Training (EDT)

5.2.1 Blue

As a result of his inconsistent performance in saliency training with tea, Blue was chosen to move on to errorless discrimination training in an attempt to improve his ability to detect low saliency stimuli (Figure 7).

During saliency training, Blue quite often performed below criterion. His average percent correct across all sessions was 70%. Once Blue was switched to EDT his performance saw some improvement with his average percent correct rising to 84% (Dort 2020). When choosing a dog for biomedical detection, it is important that the dog has a strong drive to work and that they are consistent in their performance over time. Each day Blue typically completed a total of three sessions. His motivation to work would often decline after this point. This led to inconsistency over time with his percent correct per session. His performance would often be above criterion in the first two sessions and then fall to 60% or 70% correct in the last session. For these reasons, it was decided not to move forward with Blue to ketosis breath samples.

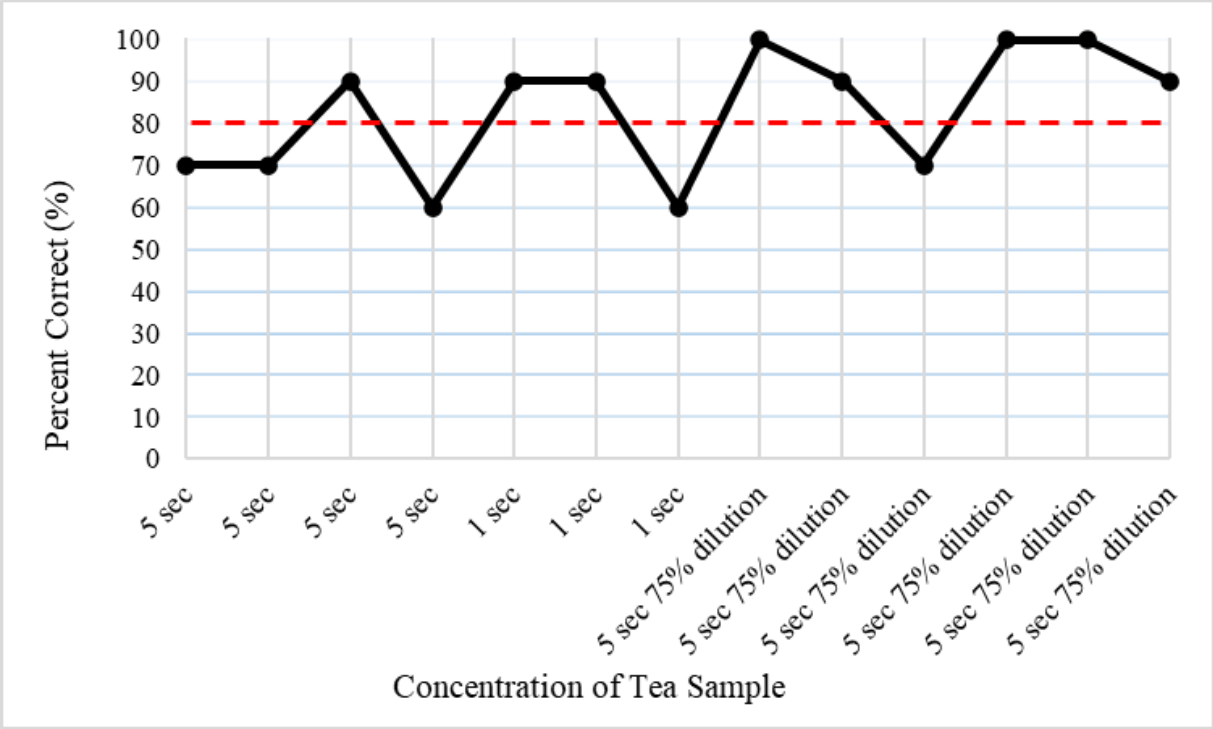

Figure 7: Blue's performance in Errorless Discrimination Training where dogs were trained to detect low salient stimuli using Orange Pekoe tea at saliences ranging from tea steeped for 5 secs to tea steeped for 5 secs and then diluted with water by 75%. The dogs had to reach a success rate of 80% or above correct responses three sessions in a row or two sessions in a row at 90% or above before moving on to the next saliency, indicated by the dotted line

5.2.2 Nellie

As a result of her inconsistent performance in saliency training with tea, Nellie was chosen to move on to errorless discrimination training in an attempt to improve her ability to detect low saliency stimuli (Figure 8).

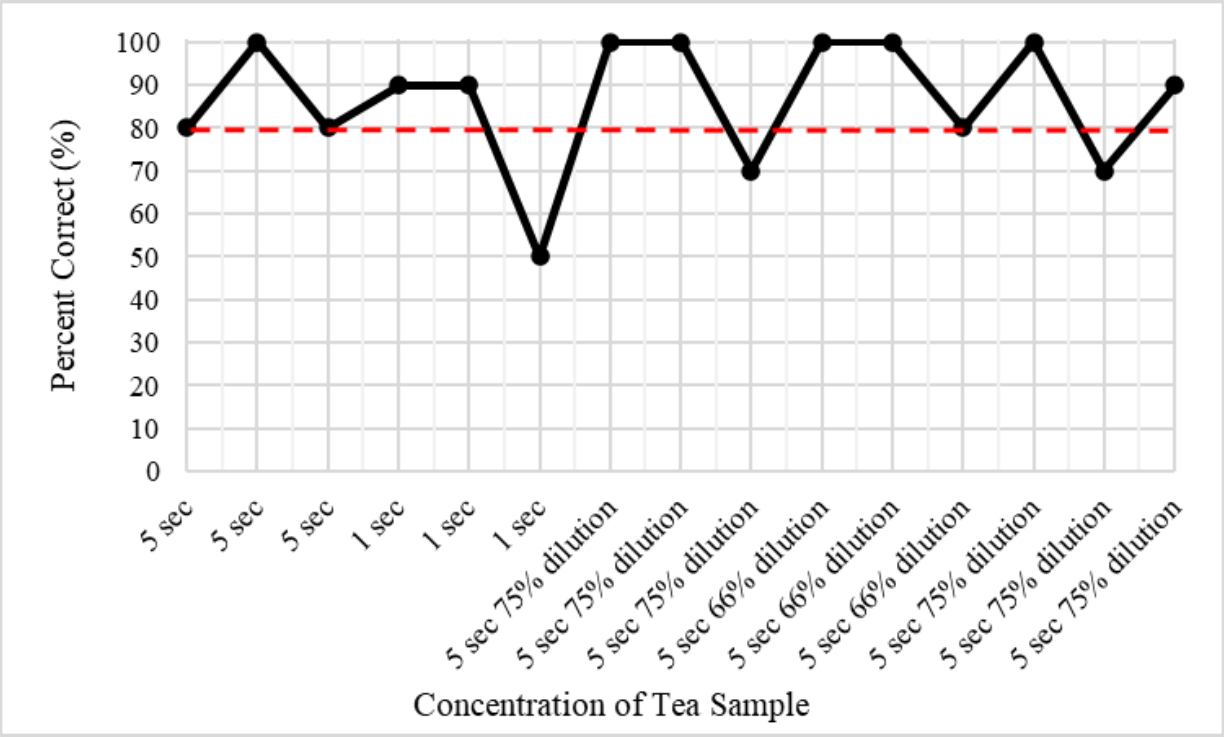

Figure 8: Nellie's performance in Errorless Discrimination Training where dogs were trained to detect low salient stimuli using Orange Pekoe tea at saliences ranging from tea steeped for 5 secs to tea steeped for 5 secs and then diluted with water by 75%. The dogs had to reach a success rate of 80% or above correct responses three sessions in a row or two sessions in a row at 90% or above before moving on to the next saliency, indicated by the dotted line

Nellie's performance during saliency training was inconsistent from session to session as well as week to week. It was difficult to determine a pattern to her performance. Her average percent correct per week was 77%. Switching Nellie to EDT allowed her performance to improve immediately, with her average percent correct rising to 85% (Dort 2020). She was above criterion for the majority of her sessions. Unfortunately, shortly after the completion of EDT, Nellie broke her leg and was unable to continue with training.

5.2.3 Rory

During Errorless Discrimination Training, Rory completed a total of 6 sessions over the course of two weeks. He was a participant of an honours research project exploring the use of EDT as a new training method and was introduced to ketosis breath samples using the methods outlined in section 4.3.2. Rory was new to the lab with previous training in scent detection.

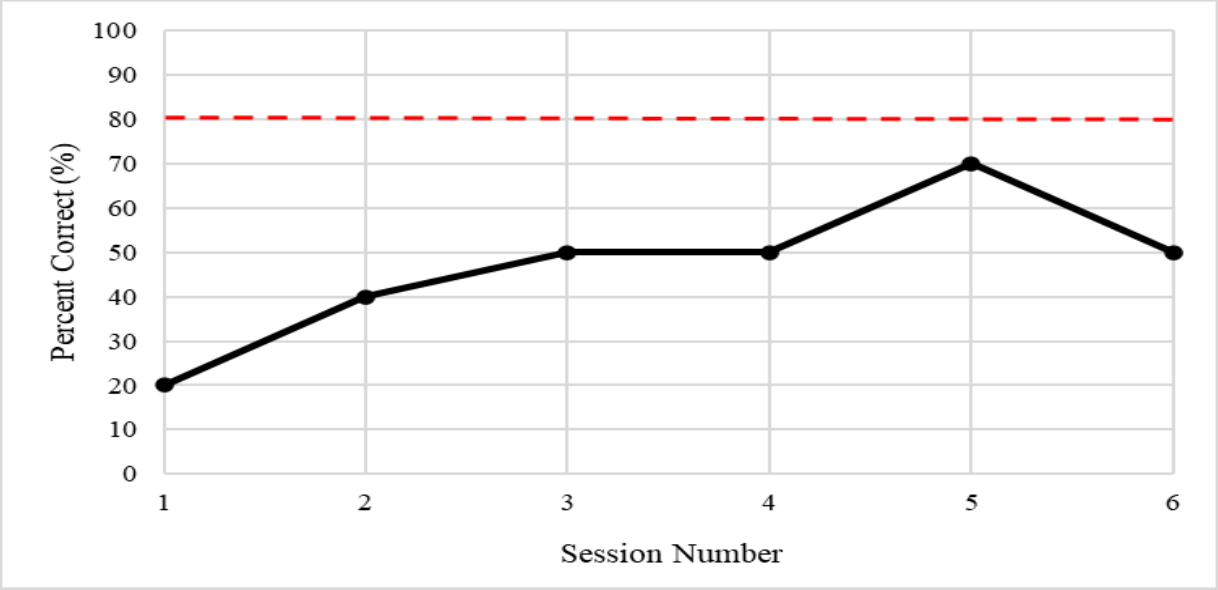

Figure 9: Rory's performance in Errorless Discrimination Training using breath samples from dairy cows positive and negative for ketosis

Unfortunately, due to the COVID-19 pandemic Rory was only able to train for 2 weeks. He was introduced to ketosis breath samples for the first time in session 1, obtaining 20% correct responses. His accuracy improved over the next four sessions, with a slight drop in the final session. Rory did not reach criterion of 80% correct responses in any of his sessions. He was brought back into the lab once restrictions from the pandemic were lifted, however, after multiple training sessions using breath samples his accuracy did not surpass 60% correct responses. For this reason, along with time constraints, his training was discontinued.

5.3 Ketosis Trials

Ivy was the only dog chosen to move from saliency training to breath samples collected from dairy cows. Prior to data collection Ivy completed 3 training days, over the course of three weeks, where she was introduced to the cow breath samples for the first time. After demonstrating an understanding of the task, data collection trials began. Breath samples were presented from five individual cows positive for ketosis (blood ketone concentration 1.2-1.6 mmol/L) and five individual cows negative for ketosis (blood ketone concentration 0.6 mmol/L) (Table 2). Each testing day a new set of samples were presented from two different cows, one positive for ketosis and one negative for ketosis. On testing day 3, the sample set was changed out between sessions 9 and 10 with two new individual cows.

Table 2: Breath samples presented to Ivy during data collection trials

Testing Day	Session Number	Positive Sample Blood Ketone Concentration (mmol/L)	Negative Sample Blood Ketone Concentration (mmol/L)
1	1	1.2 (Cow A)	0.6 (Cow B)
2	2	1.2 (Cow C)	0.6 (Cow D)
2	3	1.2 (Cow C)	0.6 (Cow D)
2	4	1.2 (Cow C)	0.6 (Cow D)
2	5	1.2 (Cow C)	0.6 (Cow D)
3	6	1.2 (Cow E)	0.6 (Cow F)
3	7	1.2 (Cow E)	0.6 (Cow F)
3	8	1.2 (Cow E)	0.6 (Cow F)
3	9	1.2 (Cow E)	0.6 (Cow F)
3	10	1.2 (Cow G)	0.6 (Cow H)
3	11	1.2 (Cow G)	0.6 (Cow H)
3	12	1.2 (Cow G)	0.6 (Cow H)
4	13	1.6 (Cow I)	0.6 (Cow J)

During data collection, Ivy completed a total of 13 sessions, of 10 trials each, over the course of 4 separate days (Figure 10). These days were not consecutive but spread out over the course of one month.

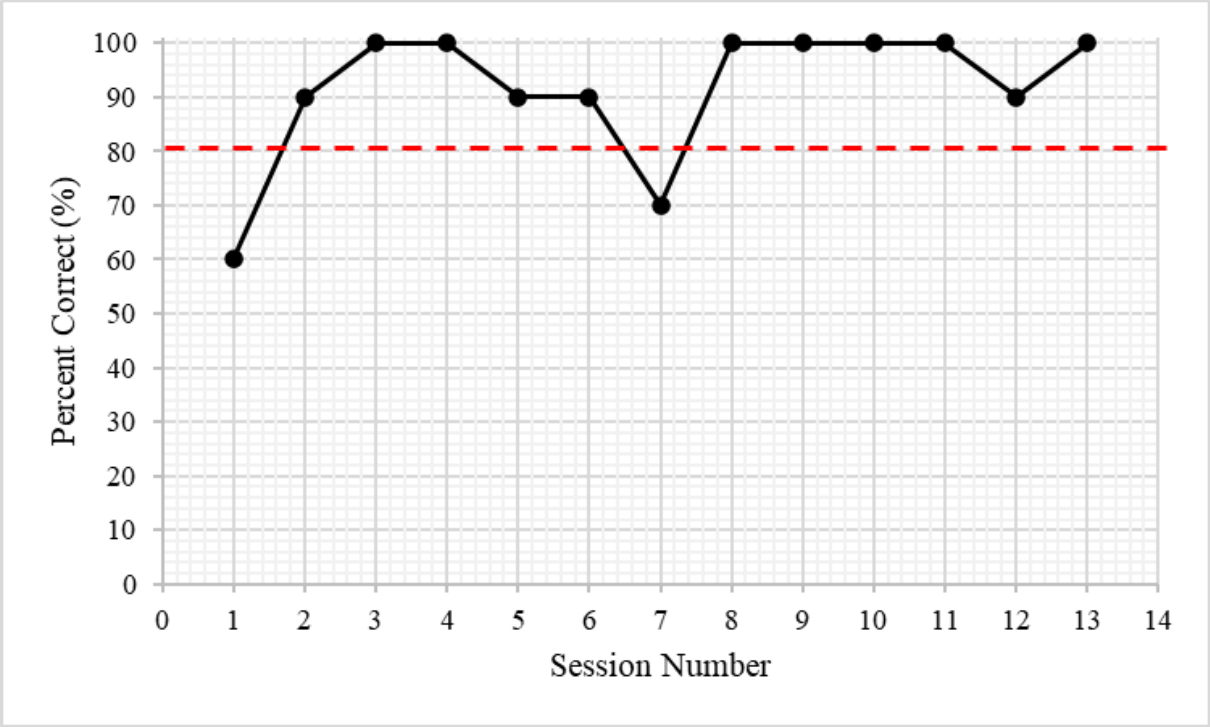

Figure 10: Ivy's performance in the discrimination between breath samples from dairy cows positive for ketosis (blood ketone concentration > 1.2 mmol/L) and cows negative for ketosis (blood ketone concentration 0.6 mmol/L)

Ivy's performance was initially low for her first session achieving 60% correct responses, however, she quickly increased to 90 and 100% correct. She showed a decrease in her performance at the beginning of her third day of data collection. After a quick walk outside, she was ready to try again and her performance was back up to 100%. Ivy will quickly inform you when she is unmotivated to work. During these times, she is taken out for walks or given a break to allow her to re-focus. Sessions numbered 6 to 12 were all run on the same day. Sessions 6 to 9

were run using a different set of samples than sessions 10 to 12. Ivy's performance was maintained at 100% moving from session 9 to 10 with the switch in sample sets, meaning a change in individual cows. Overall, she was successfully able to identify a breath sample positive for ketosis in 120 out of a total of 130 trials, with an accuracy of 92.3%.

This study also sought to compare the reliability of the dog trained to discriminate between breath samples from dairy cows positive and negative for subclinical ketosis to current ketosis diagnostic technologies (see Figure 11). The accuracy of the current ketosis diagnostic technologies are represented on the graph as compared to Ivy's average percent correct per testing day; the KetoTest™ has an accuracy of 84.5% for milk samples, the Ketostix® has an accuracy of 87% for urine samples, and the Precision Xtra™ meter has an accuracy of 96.5% for blood samples.

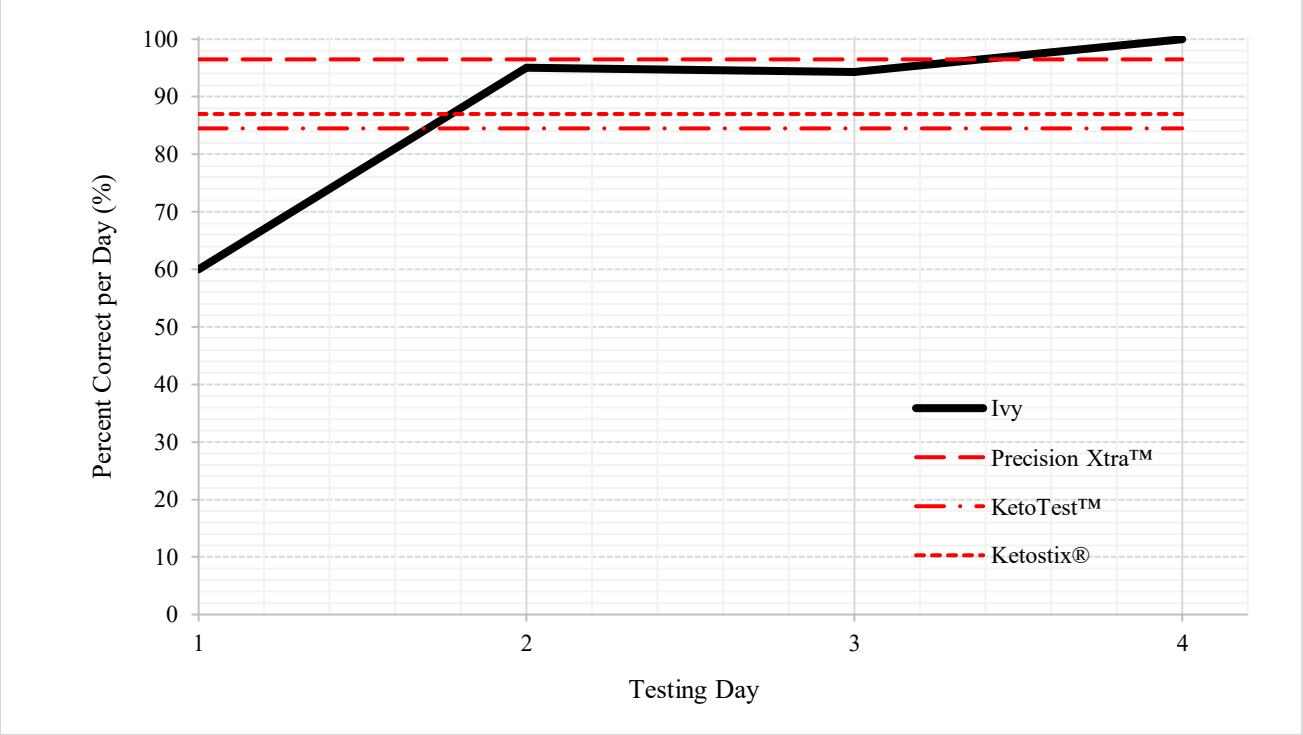

Figure 11: Ivy's percent correct responses over the four testing days compared to the accuracy of the current ketosis detection technologies

By the end of the second day of testing, Ivy's performance was above that of the KetoTest™ and the Ketostix® at an average of 95% correct responses. She quickly surpassed the accuracy of the Precision Xtra™ meter by the end of the fourth and final day of testing, reaching 100% correct responses.

Unfortunately, time limitations related to the COVID-19 pandemic prevented the completion of further testing which had initially been planned. Further considerations on future analyses will be discussed in the General Discussion (section 6.0).

6.0 General Discussion

The overall goal of this study was to assess the ability of trained scent detection dogs to discriminate between breath samples from dairy cows positive and negative for ketosis in a laboratory setting. Four dogs underwent preliminary saliency training and errorless discrimination training before selection to move onto testing using ketosis breath samples. One dog, a golden retriever "Ivy", was able to correctly discriminate between breath samples positive and negative for ketosis 92.3% of the time.

There is currently no standard for the length of time or number of sessions to successfully train a biomedical scent detection dog. Ivy was successfully able to discriminate between samples positive and negative for ketosis after 13 sessions, with four testing days over the period of one month. Over a period of 6 months Richards et al. (2008) were able to train 2 dogs to differentiate between sheep feces infected and uninfected with three species of nematodes. The dogs were able to detect fecal samples infected with a mixture of three nematode species 92% of the time (Richards et al. 2008). In contrast to Johnen et al. (2015), who trained a dog to detect cows in estrus, first from vaginal mucus samples and eventually under practical conditions cow side, over the course of 15 months. Within that time, the dog was successfully able to detect cows in estrus with a sensitivity of 71.9% and a specificity of 93.0% (Johnen et al. 2015). In comparison to the literature, the time to train Ivy to discriminate between ketosis breath samples seems very promising. It is important to consider the preliminary training Ivy was subject to, along with her history of scent detection fieldwork and participation in additional research projects. Prior to her participation in ketosis breath trials, she was previously involved in scent detection work and familiar with the mechanics of saliency training. This is in contrast to Blue and Nellie, who had no previous training in scent detection. Her history of training may have

been a crucial factor in the speed at which she acquired the ability to accurately discriminate between breath samples positive and negative for ketosis.

The variations in time to train a dog may also be in part due to the saliency of the sample. Certain VOC may be more easily detected by dogs than others, depending on their volatility. Tanaka et al. (2020) discovered two compounds through gas chromatograph analysis of extracts from the culture media of breast cancer cell lines to be of moderate volatility. Urbanová et al. (2015) were able to train a dog to correctly identify urine samples positive for prostate cancer with a sensitivity of 93.5% and a specificity of 91.6%. Training of the dog took place over a period of 11 months with 1-2 training sessions a day, 4-5 days per week (Urbanová et al. 2015). In comparison, acetone, with a characteristic sweet odour, is defined as a very volatile, or highly volatile, organic compound by the world health organization (Salthammer 2014). The higher volatility of acetone may have contributed to the shorter length of time to train Ivy to discriminate between breath samples positive and negative for ketosis as compared to the time to train a dog for cancer detection.

The dog itself is another important factor to consider, as the trainability and motivation of the dog can impact the length of time to teach a specific task. This was seen during saliency training with Blue and Nellie. It took a greater length of time and more sessions to reach tea breath when compared to the amount of time for Ivy to reach the same level. Although classified as a pet dog, Ivy was bred from a working line of Golden Retrievers. Dog breeds with a higher baseline level of the neurotransmitter dopamine, typically seen in working breeds such as the Border Collie and Belgian Malinois, have been shown to have higher levels of motivation and consistency, and the ability to work for longer hours (Gadbois and Reeve 2014; Arons and Shoemaker 1992). Dopamine plays a role in the reward system of the brain, motivational and

anticipatory processes, as well as exploratory behaviours, such as sniffing (Gadbois and Reeve 2014). A higher baseline level of dopamine may have contributed to Ivy's consistency in performance and high motivation to work. To date, there have been only a few investigations comparing the olfactory abilities of various dog breeds. Polgár et al. (2016) found that breeds historically selected for their olfactory capabilities, such as the beagle and basset hound, performed significantly higher than non-scenting and short-nosed breeds in a detection task. In contrast, a study comparing the ability of German Shepherds, Greyhounds, and Pugs to perform an olfactory task found the Pug to outperform both other breeds (Hall et al. 2015). More research is needed to further investigate the differences in olfactory capabilities between and within specific breeds of dog.

The training environment could also impact the length of time to train a biomedical detection dog. Johnen et al. (2015) took 15 months to train a dog to detect cows in estrus. However, in that time they were able to transition the dog from within a laboratory setting to out in the field working directly beside the cow. In a laboratory setting, where Ivy completed her training, there are fewer auditory, visual, and olfactory distractions than out in the field. Notably, there are fewer background scents to interfere with the detection of the target scent. Many factors play a role in determining the length of time to train a dog. It is therefore difficult to compare the length of time and accuracy of the detection dog between various experiments.

Future investigations should include a greater number of sample sets, minimizing the repetition of individual samples. Due in part to time constraints, Ivy was introduced to five individual cows positive for ketosis and five individual cows negative for ketosis as determined by the Precision Xtra™ meter. The number of positive samples was limited by the number of cows which became ketotic during the sampling period. With a greater number of samples,

testing could be run with positive and negative samples from the same cow (within subject) and be compared to the dog's ability to discriminate between samples from different cows (between subjects). Minimizing the repetition of samples would also ensure the dog is generalizing the odour of ketosis across samples and not simply memorizing the individual samples. Elliker et al. (2014) set out to determine if dogs could discriminate between urine samples from men with prostate cancer from control samples. After introducing the dogs to an entirely new sample set, however, their ability to identify urine samples positive for prostate cancer dropped below 50% (Elliker et al. 2014). A greater number of samples across all levels of subclinical ketosis (blood ketone concentration of 1.2-2.98 mmol/L) would also further ensure that the dog is able to generalize the odour of the sample. Thorough testing of the dogs with a greater volume of samples and variation of sample sets is important to ensure dogs are generalizing the scent of the target odour, and not memorizing large numbers of individual samples.

Future investigations should also explore the effect of sample collection on the ability of the dog to discriminate between breath samples positive and negative for ketosis. There is currently no standardization for breath sample collection (Jezierski et al. 2015). Factors including the effect on shallow versus deep breathing, along with the number of actual breaths, during sample collection should be explored.

Due to time restraints, further testing was unable to be completed. Once training and testing with a 2 AFC procedure had been completed, training and testing would have moved on to a Yes/No procedure. During the Yes/No sessions the dog is presented with one sample at a time, either positive or negative for ketosis, and asked to give a response. The dog is asked to give a 5-sec nose hold when presented with a positive sample and sit or lie down when presented with a negative sample. This type of testing is more practical for a biomedical detection task. If

the dog was inside a barn, they will not be given a lineup of cows and asked to locate the one cow positive for ketosis. The dog, instead, must determine if each individual cow is either positive or negative for subclinical ketosis with a "yes" or "no" response. If the sample presented is positive for ketosis, the dog may correctly identify the sample ("hit") or incorrectly identify the sample ("miss"). If the sample is negative for ketosis, the dog may correctly identify the sample ("correct rejection") or incorrectly identify the sample ("false alarm") (Kingdom and Prins 2016). The total number of each possible outcome presented by the dog, along with the total number of trials, would have been used alongside Signal Detection Theory (SDT) to measure the dog's ability to distinguish between a breath sample positive for ketosis and a breath sample from a healthy cow. The response bias of the dog (Criterion C) would also have been determined. Quantifying the internal bias of the dog would determine if the dog was a more liberal or conservative decision maker (Kingdom and Prins 2016). A liberal decision maker would be prone to more false alarms than misses. A conservative decision maker would be prone to more misses than false alarms (Gadbois and Reeve 2016). The appropriate type of decision maker changes based on the task of the detection dog and so it is important to determine which type of decision maker the specific dog is. Knowing the bias of the dog during training also helps in re-directing the training of the dog to change the bias if desired. For instance, a conservative dog could be reinforced more generously when hesitating over a target stimulus, subsequently making the dog more liberal, or more likely to indicate "yes" when uncertain. In the field of biomedical detection, and in the case of ketosis detection in cows, it may be more useful to have a liberal decision maker as misses are costlier than false alarms. It is easier to double check the blood ketone concentration of the cow than it is to miss a cow positive for ketosis and manage the cost of treatment and production losses.

This study also sought to compare the reliability of the dogs trained to discriminate between breath samples from dairy cows positive and negative for subclinical ketosis to current ketosis diagnostic technologies. Cows can be in a state of sub-clinical ketosis before any of the clinical signs such as a decreased appetite, weight loss, and decreased milk production are present. In order to manage the incidence of ketosis, it is important to focus on early detection. For this reason, this study focused on presenting Ivy with breath samples collected when blood ketone concentrations were at 1.2 mmol/L when at all possible. Ivy was able to correctly discriminate between breath samples positive and negative for ketosis 92.3% of the time. The KetoTest™ for milk samples has an accuracy of 84.5% as determined by Carrier et al. (2004). The Ketostix® strip for urine samples has an accuracy of 87% for detecting AcAc in urine samples and the Precision Xtra™ meter for blood samples has been determined to have an accuracy of 96.5% at a ketone concentration of 1.4 mmol/L of blood (Carrier et al. 2004; Iwersen et al. 2009). Ivy's overall performance was higher than that of the KetoTest™ and Ketostix®. By the end of the final testing day she was able to surpass the accuracy of the Precision Xtra™ meter, obtaining 100% correct responses that day. These results indicate the potential of Ivy and her ability to discriminate between breath samples from dairy cows positive and negative for subclinical ketosis given more time to complete further testing.

7.0 Conclusions

The onset of ketosis in dairy cows has many negative impacts on farm productivity. A cow in ketosis shows a reduction in milk yield and an increased risk of developing subsequent diseases, including displaced abomasum and metritis. Current cow-side tests for ketosis in milk and urine are highly variable in accuracy. While on-farm blood testing is higher in accuracy, it can become too costly for routine monitoring of herd health. Early detection by scent detection dogs will potentially minimize these productivity losses and reduce the time to test large groups of animals, allowing for more routine testing. This study demonstrated the ability of a dog to accurately discriminate between breath samples positive and negative for ketosis in a laboratory setting 92.3% of the time. By the end of the final day of testing, the dog was able to surpass the accuracy of all three of the current on-farm ketosis diagnostic technologies. These results highlight the potential of dogs, with further training and testing, as a tool for ketosis detection in the future.

References

Angle, T.C., Passler, T., Waggoner, P.L., Fischer, T.D., Rogers, B., Galik, P.K. and Maxwell, H.S. 2016a. Real-time detection of a virus using detection dogs. *Front. Vet. Sci.* **2(79)**: doi: 10.3389/fvets.2015.00079.

Angle, C., Waggoner, L.P., Ferrando, A., Haney, P. and Passler, T. 2016b. Canine detection of the volatilome: a review of implications for pathogen and disease detection. *Front. Vet. Sci.* **3(47)**: doi: 10.3389/fvets.2016.00047.

Arons, C. D. and Shoemaker, W. J. (1992). The distribution of catecholamines and beta-endorphin in the brains of three behaviorally distinct breeds of dogs and their F1 hybrids. *Brain Res.* **594**: 31–39.

Bach, S.J. and Hibbitt, K.G. 1959. Biochemical aspects of bovine ketosis. *Biochem. J.* **72**: 87-92.

Baird, G.D. 1982. Primary ketosis in the high-producing dairy cow: clinical and subclinical disorders, treatment, prevention, and outlook. *J. Dairy Sci.* **65(1)**: 1-10.

Berge, A.C. and Vertenten, G. 2014. A field study to determine the prevalence, dairy herd management systems, and fresh cow clinical conditions associated with ketosis in western European dairy herds. *J. Dairy Sci.* **97**: 2145-2154.

Bergman, E.N. 1971. Hyperketonemia-ketogenesis and ketone body metabolism. *J. Dairy Sci.* **54(6)**: 936-948.

Bonadio, W. 2013. Pediatric diabetic ketoacidosis: an outpatient perspective on evaluation and management. *Pediatr. Emerg. Med. Pract.* **10**: 1-14.

Carrier, J. Stewart, S., Godden, S., Fetrow, J. and Rapnicki, P. 2004. Evaluation and use of three cowside tests for detection of subclinical ketosis in early postpartum cows. *J. Dairy Sci.* **87**: 3725-3735.

Christensen, J. O., Grummer, R. R., Rasmussen, F. E. and Bertics, S. J. 1997. Effect of method of delivery of propylene glycol on plasma metabolites of feed-restricted cattle. *J. Dairy Sci.* **80**: 563-568.

Craven, B. A., Paterson, E.G. and Settles, G. S. 2010. The fluid dynamics of canine olfaction: unique nasal airflow patterns as an explanation of macrosmia. *J. R. Soc. Interface* **7**: 933-943.

Dairy Farmers of Canada. 2017. Canadian dairy sector overview. (Online) Available: https://www.dairyfarmers.ca/content/download/4898/46392/version/2/file/Dairy_Sector_Overview_2017.pdf

De Lacy Costello, B., Amann, A., Al-Kateb, H., Flynn, C., Filipiak, W., Khalid, T., Osborne, D. and Ratcliffe, N.M. 2014. A review of the volatiles from the healthy human body. *J. Breath Res.* **8** (014001).

Dobbelaar, P., Mottram, T., Nyabadza, C., Hobbs, P., Elliot-Martin, R.J. and Schukken, Y.H. 1996. Detection of ketosis in dairy cows by analysis of exhaled breath. *Vet. Quart.* **18(4)**: 151-152.

Dort, J.S. 2020. Determining the effectiveness of errorless discrimination training for dogs (*Canis lupus familiaris*). Unpublished undergraduate thesis. Dalhousie University, Canada.

Duffield, T.F. 2000. Subclinical ketosis in lactating dairy cattle: metabolic disorders of ruminants. *Vet. Clin. North Am. Food Anim. Pract.* **16**: 231-253.

Duffield, T. F., Sandals, D., Leslie, K.E., Lissemore, K., McBride, B.W., Lumsden, J.H., Dick, P. and Bagg, R. 1998. Efficacy of monensin for the prevention of subclinical ketosis in lactating dairy cows. *J. Dairy Sci.* **81**: 2866–2873.

Elliker, K.R., Sommerville, B.A., Broom, D.M., Neal, D.E., Armstrong, S. and Williams, H.C. 2014. Key considerations for the experimental training and evaluation of cancer odour detection dogs: lessons learnt from a double-blind, controlled trial of prostate cancer detection. *BMC Urol.* **14**(22).

Fischer-Tenhagen, C., Wetterholm, L., Tenhagen, B. and Heuwieser, W. 2011. Training dogs on a scent platform for oestrus detection in cows. *Appl. Anim. Behav. Sci.* **131**: 63-70.

Gadbois, S. and Reeve, C. 2016. The semiotic canine: scent processing dogs as research assistants in biomedical and environmental research. *Dog Behav.* **3**: 26-32.

Gadbois, S. and Reeve, C. 2014. Canine olfaction: Scent, sign, and situation. Domestic dog cognition and behavior. Springer, New York, NY. 3-29.

Government of Canada. 2018. Overview of the Canadian dairy industry at the farm. (Online) Available: http://www.dairyinfo.gc.ca/index_e.php?s1=dff-fcil&s2=farm-ferme&s3=nb

Hall, N.J., Glenn, K., Smith, D.W. and Wynne, C.D.L. 2015. Performance of pugs, German shepherds, and greyhounds (*Canis lupus familiaris*) on an odor-discrimination task. *J. Comp. Psychol.* **129 (3):** 237-246.

Herdt, T.H. 2000. Ruminant adaptation to negative energy balance: influences on the etiology of ketosis and fatty liver. *Vet. Clin. Food Anim.* **16**: 215-230.

Herdt, T.H. and Emery, R. S. 1992. Therapy of diseases of ruminant intermediary metabolism. *Vet. Clin. North Am. Food Anim. Pract.* **8**: 91-106.

Iwersen, M., Falkenberg, R., Voigtsberger, D., Forderung, D. and Heuweiser W. 2009. Evaluation of an electronic cowside test to detect subclinical ketosis in dairy cows. *J. Dairy Sci.* **92**: 2618-2624.

Jeong, J., Choi, I., Moon, S., Lee, S., Kang, H., Jung, Y., Par, S. and Kim, I. 2018. Effect of two treatment protocols for ketosis on the resolution, postpartum health, milk yield, and reproductive outcomes of dairy cows. *Theriogenology* **106**: 53-59.

Jezek, J., Cincovie, M.R., Nemec, M., Belic, B., Djokovie, R., Mlinkon, M. and Staric, J. 2017. Beta-hydroxybutyrate in milk as screening test for subclinical ketosis in dairy cows. *Polish J. Vet. Sci.* **20(3)**: 507-512.

Jezierski, T., Ensminger, J. and Papet, L. E. 2016. Canine olfaction, science, and law: advances in forensic science, medicine, conservation, and environmental remediation. CRC Press, Boca Raton, USA.

Jezierski, T., Walczak, M., Ligor, T., Rudnicka, J. and Buszewski, B. 2015. Study of the art: canine olfaction used for cancer detection on the basis of breath odour. Perspectives and limitations. *J. Breath Res.* **9**: 1-12.

Johnen, D., Heuwieser, W. and Fischer-Tenhagen, C. 2017. An approach to identify bias in scent detection dog testing. *Appl. Anim. Behav. Sci.* **189**: 1-12.

Johnen, D., Heuwieser, W. and Fischer-Tenhagen, C. 2015. How to train a dog to detect cows in heat- training and success. *Appl. Anim. Behav. Sci.* **171**: 39-46.

Kauppinen K., 1983. Correlation of whole blood concentrations of acetoacetate, beta-hydroxybutyrate, glucose and milk yield in dairy cows as studied under field conditions. *Acta Vet. Scand.* **24**: 337-348.

Kingdom, F.A.A. and Prins, N. 2016. Psychophysics. A practical introduction. Elsevier, London, England.

Larsen, M. and Kristensen, N.B. 2010. Effect of a lucerne feeding strategy in the first week postpartum on feed intake and ketone body profiles in blood plasma, urine, and milk in Holstein cows. *Acta Agr. Scand. A-An.* **60**: 239-249.

Lawson, M. J., Craven, B. A., Paterson, E. G., and Settles, G. S. 2012. A computional study of odorant transport and deposition in the canine nasal cavity: implications for olfaction. *Chem. Senses* **37(6)**: 553-566.

Likhodii, S.S., Musa, K. and Cunnane, C. 2002. Breath acetone as a measure of systematic ketosis assessed in rat model of the ketogenic diet. *Clin. Chem.* **48(1)**: 115-120.

Littledike, E.T., Young, J.W. and Beitz, D.C. 1981. Common metabolic diseases of cattle: ketosis, milk fever, grass tetany, and downer cow complex. *J. Dairy Sci.* **64**: 1465-1482.

Lourenço, C. and Turner, C. 2014. Breath analysis in disease diagnosis: methodological considerations and applications. *Metabolites* **4**: 465-498.

Mayfield, A.E., Smith, J.A., Hughes, M. and Dresden, T.J. 2008. First report of laurel wilt disease caused by Raffaelea sp. on Avocado in Florida. *Plant Dis.* **92(6)**: 976.

McArt, J.A.A., Nydam, D.V. and Overton, M.W. 2015. Hyperketonemia in early lactation dairy cattle: a deterministic estimate of component and total cost per case. *J. Dairy Sci.* **98**: 2043-2054.

Mendel, J., Furton, K.G. and Mills, D. 2017. An evaluation of scent-discriminating canines for rapid response to agricultural diseases. *Proc. Fla. State Hort. Soc.* **130**: 17–23.

Morgan, D.L. and Morgan, R.K. 2009. Single-case research methods for the behavioural and health sciences. Sage.

Mostert, P.F., van Middelaar, C.E., Bokkers, E.A.M. and de Boer, I.J.M. 2018. The impact of subclinical ketosis in dairy cows on greenhouse gas emissions of milk production. *J. Clean Prod.* **171**: 773-782.

Musa-Veloso, K., Likhodii, S.S. and Cunnane, C. 2002. Breath acetone is a reliable indicator of ketosis in adults consuming ketogenic meals. *Am. J. Clin. Nutr.* **76**: 65-70.

Nielen, M., Aarts, M.G., Jonkers, A.G., Wensing, T. and Schukken, Y.H. 1994. Evaluation of 2 cowside tests for the detection of subclinical ketosis in dairy-cows. *Can. Vet. J.* **35**: 229-232.

Oetzel, G.R .2004. Monitoring and testing dairy herds for metabolic disease. *Vet. Clin. Food Anim.* **20**: 651-674.

Osorio, J.S., Trevisi, E., Ji, P., Drackley, J.K., Luchini, D., Bertoni, G. and Loor, J.J. 2014. Biomarkers of inflammation, metabolism, and oxidative stress in blood, liver, and milk reveal a better immunometabolic status in peripartal cows supplemented with Smartamine M or MetaSmart. *J. Dairy Sci.* **97(12)**: 7437-7450.

Ospina, P.A., Nydam, D.V., Stokol, T. and Overton, T.R. 2010. Evaluation of nonesterified fatty acids and β-hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. *J. Dairy Sci.* **93**:546–554.

Phillips, M. 1992. Breath tests in medicine. *Sci. Am.* **267(1)**: 74-79.

Piantoni, P. and Allen, M.S. 2015. Evaluation of propylene glycol and glycerol infusions as treatments for ketosis in dairy cows. *J. Dairy Sci.* **98**: 5429-5439.

Polgár, Z., Kinnunen, M., Újváry, D., Miklósi, Á. and Gácsi, M. 2016. A test of canine olfactory capacity: comparing various dog breeds and wolves in a natural detection task. *PLoS ONE* **11(5)**: e0154087.

Qiao, Y., Gao, Z., Liu, Y., Cheng, Y., Yu, M., Zhao, L., Duan, Y. and Liu, Y. 2014. Breath ketone testing: a new biomarker for diagnosis and therapeutic monitoring of diabetic ketosis. *Biomed Res. Int.* **2014**: 1-5.

Reeve, C., Cummings, E., McLaughlin, E., Smith, S. and Gadbois, S. 2020. An idiographic investigation of diabetic alert dogs' ability to learn from a small sample of breath samples from people with type 1 diabetes. *Can. J. Diabetes.* **44**: 37-43.

Reeve, C., Wentzell, P., Wielens, B., Jones, C., Stehouwer, K. and Gadbois, S. 2018. Assessing individual performance and maintaining breath sample integrity in biomedical detection dogs. *Behav. Processes* **155**: 8-18

Richards, K.M., Cotton, S.T. and Sandeman, R.M. 2008. The use of detector dogs in the diagnosis of nematode infections in sheep feces. *J. Vet. Behav.* **3**: 25-31.

Ruzsányi, V. and Kalapos, P. 2017. Breath acetone as a potential marker. *Breath Res.* **11** (024002).

Sacharczuk, M., Walczak, M., Adamkiewicz, E., Walasek, A., Ensminger, J., Presch, M. and Jezierski, T. 2019. Polymorphism of olfactory and neurotransmitters receptor genes in drug and explosives detection dogs can be associated with differences in detection performance. *Appl. Anim. Behav. Sci.* **215**: 52-60.

Salthammer, T. 2014. Very volatile organic compounds: an understudied class of indoor air pollutants. *Indoor Air* **26**: 25-38.

Schoon, G.A.A. 1996. Scent identification lineups by dogs (*Canis familiaris*): experimental design and forensic application. *Appl. Anim. Behav. Sci.* **46**: 257-267.

Schultz, L.H. 1968. Ketosis in dairy cattle. *J. Dairy Sci.* **51(7)**: 1133-1140.

Sjaastad, O. V., Sand, O. and Hove, K. 2010. *The physiology of domestic animals*. 2nd Edition. Scandinavian Veterinary Press, Norway.

Sonoda, H., Kohnoe, S., Yamazato, T., Satoh, Y., Morizono, G., Shikata, K., Morita, M., Watanabe, A., Morita, M., Yoshihiro, K., Inoue, F. and Maehara, Y. 2011. Colorectal cancer screening with odour material by canine scent detection. *Gut* **60**: 814-819.

Steele, R.J., McClements, P.L., Libby, G., Black, R., Morton, C., Birrell, J., Mowat, N.A., Wilson, J.A., Kenicer, M., Carey, F.A. and Fraser, C.G. 2009. Results from the first three rounds of the Scottish demonstration pilot of FOBT screening for colorectal cancer. *Gut* **58**: 530-535.

Suthar, V.S., Canelas-Raposo, J., Deniz, A. and Heuwieser, W. 2013. Prevalence of subclinical ketosis and relationships with postpartum diseases in European dairy cows. *J. Dairy Sci.* **96**: 2925-2938.

Tacher, S., Quignon, P., Rimbault, M., Dreano, S., Andre, C. and Galibert, F. 2005. Olfactory receptor sequence polymorphism within and between breeds of dogs. *J. Hered.* **96(7)**: 812-816.

Tanaka, M., Hsuan, C., Oeki, M., Shen, W., Goda, A., Tahara, Y., Onodera, T., Sanematsu, K., Rikitake, T., Oki, E., Ninomiya, Y., Kurebayashi, R., Sonoda, H., Maehara, Y., Toko, K. and Matsui, T. 2020. Identification of characteristic compounds of moderate volatility in breast cancer cell lines. *PLoS ONE* **15(6)**: e0235442.

Taslitz, A. E. 2013. The cold nose might actually know: science & scent lineups. *Crim. Just.* **28(2)**: 4-8, 55-56.

Trabue, S., Scoggin, K., Tjandrakusuma, S., Rasmussen, M. A. and Reilly, P. J. 2007. Ruminal fermentation of propylene glycol and glycerol. J. Agric. *Food Chem.* **55**: 7043‑7051.

Urbanová, L., Vyhnánková, V., Krisová, S., Pacík, D. and Nečas, A. 2015. Intensive training technique utilizing the dog's olfactory abilities to diagnose prostate cancer in men. *Acta Vet. Brno* **84**: 77-82.

Vyplelová, P., Vokálek, V., Pinc, L., Pacáková, Z., Bartoš, L., Santariová, M. and Čapková, Z. 2014. Individual human odor fallout as detected by trained canines. *Forensic Sci. Int.* **243**: 13-15.

Walker, D. B., Walker, J. C., Cavnar, P. J., Taylor, J. L., Pickel, D. H., Hall, S. B. and Suarez, J. C. 2006. Naturalistic quantification of canine olfactory sensitivity. *App. Anim. Behav. Sci.* **97**: 241-254.

Yu, F., Bruce, L., Calder, A., Milne, E., Coop, R., Jackson, F., Horgan, G. and MacRae, J. 2000. Subclinical infection with the nematode *Trichostrongylus colubriformis* increases gastrointestinal tract leucine metabolism and reduces availability of leucine for other tissues. *J. Anim. Sci.* **78**: 380-390.

Zhang, Z., Liu, G., Wang, H., Li, X. and Wang, Z. 2012. Detection of subclinical ketosis in dairy cows. *Pak. Vet. J.* **32(2)**: 156-160.