Detecting Differences in Volatile Organic Compounds Produced by *Leishmania infantum* Infected and Uninfected Dogs

By

Monica Staniek
BSc

PhD Thesis
In Biomedical and Life Sciences

Lancaster University
Faculty of Health and Medicine
Biomedical Life Sciences

October 2019

Declaration

I declare that the content of this thesis is my own work and has not been submitted by myself in substantially the same form for the award of a higher degree elsewhere. Any sections of the thesis which have been published have been clearly identified.

Dedication

To the Brazilian dogs and their owners without whom this work would not have been possible.

Acknowledgements

First and foremost, my thanks go to my supervisors, Prof Gordon Hamilton and Dr Rod Dillon for their mentorship, supervision and endless supply of coffee over the years. I will always be grateful for your continued support, friendship and faith in me. Many thanks also to Dr Luigi Sedda for his support in the statistical analysis of the data in addition to his personal support and becoming a true friend.

I also render my thanks to Raquel Vionette and Fraser Mitchell for their technical support in the labs at Lancaster University and Tim Gibson of RoboScientific for his supply of the VOC Analyser and support in the sample analysis. Special thanks also go to the Central Control of Zoonosis (CCZ) in Governador Valadares for their practical support and permission to carry out the field work. I would also like to thank Ricardo Alves Miranda (CCZ phlebotomist) and Erika M. Costa (Veterinarian, FioCruz, Rio) for collecting the dog blood samples and initial clinical assessment of the dogs, as well as Melissa Bell (Lancaster University) and Cristian F. de Souza (FioCruz, Rio) for providing in-field technical support. Furthermore, I would also like to thank Prof P. Bates (Division of Biomedical and Life Sciences, Lancaster University) for supplying the cultured *Leishmania* promastigotes and Viv Dillon for helping with initial thesis corrections.

I would also like to extend my gratitude to the staff in the Division of Biomedical and Life Sciences at Lancaster University as well as the friends I have made during my PhD, special mention to Natalie Jones, James Tollitt, Alex Hardgrave, Marine Aublette and Jack Martin, for the endless supply of coffee breaks, entertainment and trips to the pub.

I am profoundly grateful to the dog owners of Altinópolos, Governador Valadares for allowing me to carry out this research on their cherished companions.

This work received financial support from the Lancaster University studentship.

Table of Contents

List of Figures

List of Tables

Abbreviations

^{0}C	Degrees Celsius
AD	Alzheimer's disease
amu	Atomic mass unit
ASSURED	Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment free and Deliverable to end users
BC	Border Collie (Rover)
BIC	Bayesian Information Criterion
CCV	Confounder Cross Validation
CCZ	Centre for the Control of Zoonosis
CD	Crohn's Disease
CKC	Cavalier King Charles (Bonnie)
CL	Cutaneous leishmaniasis
Ct	Cycle threshold
CV	Cross Validation
cVL	Canine visceral leishmaniasis
DA	Discriminant Analysis
DAT	Direct Agglutination Test
DDT	Dichlorodiphenyltrichloroethane
DFA	Discriminant Function Analysis
dH_2O	Distilled water
DHT	Dihydrotestosterone
dNTP	Deoxynucleotide
DPP	Dual-path platform
EDDA	Eigen decomposition discriminant analysis
EDTA	Ethylenediaminetetraacetic acid

EIC	Extracted ion chromatograms
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovin serum
GC	Gas chromatography
GC/MS	Gas chromatography coupled mass spectrometry
GV	Governador Valadares
H_2	Hydrogen
HIV	Human immunodeficiency virus
HOMEM	Hemoflagellate-modified minimum essential medium
IC	Immunochromatographic
IFAT	Indirect fluorescent-antibody test
IRS	Indoor residual spraying
ITC	Insecticide treated curtains
ITN	Insecticide treated bed nets
kDNA	Kinetoplast deoxyribonucleic acid
LAT	Latex agglutination test
Le.	*Leishmania*
LLITN	Long lasting insecticide treated nets
Lu.	*Lutzomyia*
M.	*Mycobacterium*
m/z	Mass to charge ratio
M199	Medium199
MCL	Mucocutaneous leishmaniasis
MHC	Major histocompatibility complex
MPP	Mass professional profiler
MRSA	*Methicillin-resistant Staphylococcus aureus*

MS	Mass spectrometry
MSSA	*Methicillin-sensitive Staphylococcus Aureus*
NIST14	National Institute of Standards and Technology library
P.	*Phlebotomus*
PC	Principle component
PCA	Principle component analysis
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction - restriction fragment length polymorphism
PD	Parkinson's Disease
PLS-DA	Partial least squares discriminant analysis
POC	Point of care
RDT	Rapid diagnostic test
Rel Freq	Relative frequency
RK26	Recombinant kinase 26
Rk39	Recombinant kinase 39
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
Rt-qPCR	Real time quantitative polymerase chain reaction
SCOT	Support-coated open tubular
SPME	Solid-phase microextraction
T.	*Toxoplasma*
TB	Tuberculosis
UTI	Urinary tract infections
V.	*Viannia*
VL	Visceral leishmaniasis
VOC	Volatile organic compound

WCOT	Wall-coated open tubular
WHO	World Health Organisation

Detecting Differences in Volatile Organic Compounds Produced by *Leishmania infantum* Infected and Uninfected Dogs

By

Monica Staniek
BSc

PhD Thesis
In Biomedical and Life Sciences

Lancaster University
Faculty of Health and Medicine
Biomedical Life Sciences

October 2019

Abstract

The leishmaniases are a complex of parasitic diseases which cause approximately 30,000 deaths annually. In Brazil, leishmaniasis is a significant burden (Bezerra *et al*., 2018). Dogs are the main reservoir host for *Leishmania (Le.) infantum* parasites and are the source of infection for the human population. *Le. infantum* is transmitted to people via the bite of an infected *Lutzomyia (Lu.) longipalpis* sandfly. To disrupt this transmission cycle, it is important to identify *Leishmania* infected dogs and remove them from the population. However, current diagnostic methods are complex, can be inaccurate and therefore limit effective reservoir control.

The aim of this study was to determine whether *Leishmania infantum* infected dogs could be discriminated from uninfected dogs using three different

methodologies; a VOC Analyser, behavioural bioassay and gas chromatography mass spectrometry (GC/MS). Previous studies have shown that animals (e.g. dogs, hamsters) infected with *Leishmania parasites* produce odours that are different to those produced by uninfected animals (De Oliveira *et al.*, 2008; Magalhães-Júnior *et al.*, 2014b). In this study the VOC analyser demonstrated the ability to reliably discriminate between *Leishmania* infected and uninfected dogs, with high sensitivity (97-100%) and specificity (95-100%). The accuracy demonstrated of this diagnostic is an improvement on the current in-field DPP test used for cVL diagnosis, with a sensitivity of 75-89% and specificity of 56-70% having been recently reported (Figueiredo *et al.*, 2018). This result suggests the potential for this approach for the rapid, non-invasive POC diagnosis of dogs infected with leishmaniasis. Studies on the behavioural response of male and female to the odour of infected and uninfected dogs showed that female *Lu. longipalpis* were significantly more attracted to infected odour whereas males were not. This result may suggest that *Leishmania* could be manipulating canine host odour in order to aid its own transmission. Increased attractiveness of infected dog odour suggests the potential of whole dog odour baited traps as a novel vector control methodology. Further GC/MS analysis confirmed the differences observed between the odour profiles of infected and uninfected dogs through both the VOC Analyser and behavioural bioassays. This analysis also allowed for the identification of the chemical structure and composition of compounds present in infected dogs which have the potential to not only enhance the VOC Analyser as a cVL specific diagnostic, but also could be investigated as artificial compounds for odour baited sandfly traps.

Contents

Chapter One - Introduction

1.1 Introduction to leishmaniasis

Leishmaniasis is a neglected vector-borne tropical disease caused by protist parasites from the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) (Ross, 1903). *Leishmania* affects both humans and animals, with over 20 different species of the parasite having been named (World Health Organisation, 2016). Leishmaniases are devastating diseases that have negative effects on both economic productivity as well as socioeconomic development; recurrent epidemics have caused high morbidity and mortality in affected communities (Camargo and Langoni, 2006). The World Health Organisation considers leishmaniasis as one of the most important zoonotic diseases, particularly in Brazil, where approximately 85,000 cases of visceral leishmaniasis were reported from 1990 to 2016, with the fatality rate reaching 20% (Luz *et al.*, 2018). Female *Phlebotomine* sandflies (Psychodidae: Phelebotominae) provide the primary route of *Leishmania* transmission (Bates and Rogers, 2004). Over 800 species of *Phlebotomine* sandflies have been recorded, with 98 having proven or suspected vectorial capacity for human leishmaniasis (Maroli *et al*., 2013). Females of two genera (*Phlebotomus* and *Lutzomyia*) have been proven to be successful *Leishmania* vectors for human pathogenic species and are therefore of medical importance (Killick-Kendrick, 1999). These include, 56 *Lutzomyia* species (Diptera: Psychodidae) found throughout the New World, and 42 *Phlebotomus* species (Diptera: Psychodidae) found throughout the Old World (Maroli *et al.,* 2013). Female sandflies are only able to transmit the species of the *Leishmania* that attach to the midgut (Lewis, 1971). Although the recognition of leishmaniases as a public health problem is growing, the

disease still remains one of the world's most neglected tropical diseases. A lack of easy diagnostics, safe and effective drugs and vector control tools are currently hampering the successful control of *Leishmania* (Hailu *et al.,* 2016).

1.1.1 Clinical Manifestations

The clinical outcome of *Leishmania* infection depends on a complex association between three main factors; the parasite, the host and the vector and is manifested in three different forms, all of which vary in symptoms and severity (MacMorris-Adix, 2009).

Cutaneous Leishmaniasis (CL) is the most common and widespread of the three diseases. It is characterised by painful skin ulcers that are located at the site of or close to the location of the sandfly bite (Hide *et al.,* 2007). The morphology of the ulcerative skin lesions can vary; they can become large and destructive ulcers which remain for many months before healing, or they can be relatively small with little effect on the patient (Oliveira *et al.,* 2004). Although these skin ulcers are largely self-healing with no need for any specific treatment, the scars that remain can often be socially debilitating (Hotez, 2008). Various etiological agents cause Old World and New World CL, including *Leishmania* major, *Le. tropica* and some zymodemes of *Le. infantum* in the Old World (Masmoudi *et al.,* 2013) and *Le. mexicana* or species of the *Le. Viannia* subgenus in the New World (Pace, 2014).

Mucocutaneous leishmaniasis (MCL) is a severe form of cutaneous leishmaniasis, mainly characterised by skin ulcers which develop around mucosal regions. These lesions often lead to disfiguring and extensive destruction of the mucous tissues of the mouth, nose and face. Arms and legs can also be affected which

can ultimately lead to serious disability (Hide *et al.,* 2007). Around 35,000 cases of MCL occur annually with 90% of all cases occurring within three countries; Brazil, Bolivia and Peru (Pace, 2014).

Visceral leishmaniasis (VL) is arguably the most severe form of Leishmaniasis with fatalities occurring approximately 95% of the time if left untreated (Ready, 2004). Visceral leishmaniasis is normally characterized by splenomegaly and hepatomegaly as well as severe weight loss, fever, pancytopenia and hypergammaglobulinemia (Sundar and Rai, 2002). Various etiological agents are known to cause VL with studies confirming *Le. donovani* as the main etiological agent in India, whereas *Le. infantum* is the primary etiological agent in Europe and South America. *Phlebotomus (P.) perniciosus* is the primary vector for *Le. infantum* in Europe, *P. argentipes* for *Le. donavani* in the Indian subcontinent and in South America, particularly Brazil, the primary vector for *Le. infantum* is *Lu. longipalpis* (Miró *et al.,* 2012). The risk factors involved in the transmission of VL including canine infection, socioeconomic factors and environmental features, are still very poorly understood (Miranda de Araújo *et al.,* 2013).

1.1.2 Transmission and Life Cycle

Leishmania has a digenetic life cycle, alternating between phlebotomine sandfly vectors and competent mammalian hosts (**Figure 1.1**). Mammalian hosts range from desert rats to canines and humans, who are often considered as accidental hosts. Outside of the mammalian host, the parasite life cycle is confined to the digestive tract of female sandflies. The precise location of the parasite differs between *Leishmania* subgenera. Species in the New World subgenus *Viannia* (e.g. *Le. braziliensis*) are

known as peripylarian parasites as they enter the hindgut before migrating to the midgut. Species in the *Leishmania* subgenus (e.g. *Le. mexicana*), however, are suprapylarian parasites as they develop in and are restricted to the midgut (Lainson *et al.,* 1977). *Leishmania* colonisation of the midgut is vital for parasite transmission. Promastigotes undergo specific development in the sandfly midgut which culminates in their differentiation into metacyclic promastigotes which are infective to and uniquely pre-adapted for survival in the vertebrate host (Monteiro *et al.,* 2017)

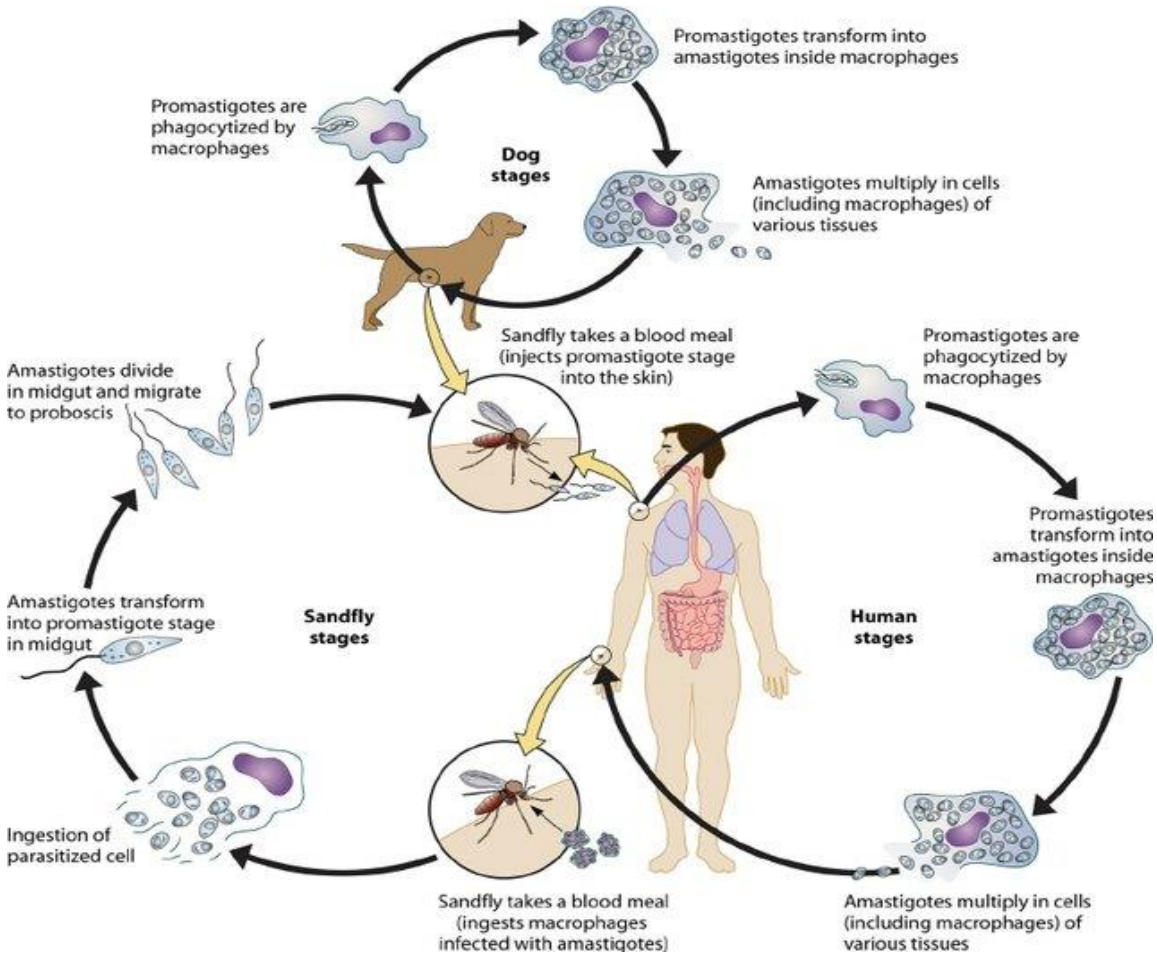


Figure 1.1 Life cycle of *Leishmania* species. When a sandfly takes a blood meal, infective promastigotes are injected into a susceptible host mammal. These promastigotes are then phagocytosed and transform into amastigotes, multiplying by simple division within these cells. The parasites continue to infect phagocytic cells either at the site of the bite (CL) or in secondary lymphoid organs (VL). When sandflies feed on an infected host, they become infected with amastigotes. These amastigotes transform into promastigotes in the midgut of the sandfly and migrate from the midgut to the stomodeal valve transforming into highly infectious metacyclic promastigotes (Esch and Petersen, 2013).

Parasite development in the vector begins following the bite of a female sandfly and ingestion of a blood meal containing macrophages that are infected with amastigotes; the immotile form of the parasite. Once ingested, a morphological change and development of the parasite occurs due to a notable difference in conditions in the sandfly, such as an increase in pH and a decrease in body temperature. The ingested amastigotes develop into procyclic promastigotes that are weakly motile and possess a short flagellum which beats at the anterior end of the cell. This form of the parasite is the first replicative form. The promastigotes proliferate in the ingested blood meal, being separated from the sandfly midgut by a type I peritrophic matrix. Approximately 48–72 hours after the blood meal, the parasites slow their replication and differentiate into motile, long nectomonad promastigotes. This parasite form is able to escape from the blood meal encased in the peritrophic matrix, migrate to the lumen of the midgut, and from there to the midgut anterior where they develop into short nectomonad promastigotes (or leptomonads) and, enter another proliferative cycle (Rogers *et al.,* 2002). Following detachment, migration and colonisation of the stomodeal valve, effective transmission of the parasite can occur. *Leishmania* ultimately transform into infective metacyclic promastigotes which, during a blood meal, are delivered to the mammalian host through regurgitation into the blood pool formed during feeding (Sacks and Perkins, 1985).

After the sandfly blood feeds on the mammalian host, the metacyclic promastigotes can infiltrate the host. In mammals, macrophages are the main cellular host for these parasites. These promastigotes can attach to the macrophages via receptor mediated mechanisms, being engulfed by phagocytosis forming a

phagolysosome within the macrophage. Promastigotes undergo various metabolic and biochemical changes within the macrophage which result in the formation of intracellular amastigotes (Rodriguez *et al.,* 2017). The infected macrophage bursts, releasing amastigotes which migrate to various areas of the host circulatory system i.e. dendritic cells, fibroblasts, new macrophages in the skin and the visceral system (Rittig and Bogdan, 2000). Due to macrophages being immune effector cells, the interaction of *Leishmania* parasites with these immune cells causes a distinctive response of the immune system. The observed parasite interaction with the mammalian host immune system cause distinct morphological changes, some of which have been linked to an increase in parasite transmission. Ulcerative lesions that are developed during cutaneous infection have been identified as areas that poses a high parasite load, which has been directly correlated with disease transmission due to the efficacy of sandflies to acquire parasites while feeding there (Aslan *et al.,* 2016). Furthermore, previous research has also demonstrated that the immune response to parasitic infections causes the production of disease biomarkers and volatile compounds which have been shown to increase vector attractiveness (De Moraes *et al.,* 2014). However, this phenomenon has currently not been demonstrated during *Leishmania* infection.

Various mammals have been implicated in the transmission of *Leishmania* with dogs and rats known to be among the major reservoirs in the New and Old World respectfully. Other mammals, including opossums (*Didelphis spp*.) and the crab-eating fox (*Cerdocyon thous*) have shown evidence of playing an epidemiological role in parasite transmission, however, these hosts have never been confirmed as reservoirs and the impact they have on the transmission cycle is unclear (Courtenay *et al.,* 2002;

Dantas-Torres, 2007). Bush dogs (*Speothos venaticus*) and maned wolves (*Chrysocyon brachyurus*) have also been proven to be infectious to sandfly vectors, however the relevance of these findings from an epidemiological standpoint is unknown (Luppi *et al.,* 2008; Mol *et al.,* 2015). Recent findings have demonstrated the potential of sheep as a host for *Leishmania infantum* in China, however, successful transmission of the parasite is unknown (Han *et al.,* 2018). Possibly more epidemiologically important results have identified rabbits that were infected with *Leishmania infantum* in Spain (Gracia *et al.,* 2014). The variety of different host animals that have been identified is an important factor to consider when understanding the mechanisms behind the successful transmission of *Leishmania*.

Although *Leishmania* transmission predominantly occurs via a sandfly vector, other insects could also be implicated as potential vectors. *Leishmania* DNA has been detected in ticks which were collected from naturally infected dogs in Brazil. The presence of parasite DNA suggests the participation of ticks in the zoonotic cycle of cVL (Campos and Costa, 2014). Additionally, research has also shown that *Leishmania enriettii* can develop a late stage infection the biting midge *C. sonorensis* (Seblova *et al.,* 2015). *Leishmania amazonensis* and *Le*. *braziliensis* have also been detected in biting midges in Brazil (Rebelo *et al.,* 2016). Additionally, research has also detected *Leishmania infantum* DNA in fleas that were collected from naturally infected dogs in Brazil (Colombo *et al.,* 2011). These studies support the theory that other biting and or blood feeding insects could be potential natural vectors for *Leishmania*. Other modes of transmission have also been reported that do not require an insect vector. The parasite has shown successful transmission venereally, vertically and through infected blood transfusions. Transmission of VL either directly or due to the production

of an artificial leishmaniasis cycle through blood transfusions, has been shown in both infected dogs and humans. In 2001, Owens *et al* documented the first transmission of *Le. infantum* by blood transfusion through foxhounds used as blood donors. Furthermore, *Leishmania* parasites have been detected in shared syringes collected in Spain, suggesting that needle sharing could promote the spread of *Leishmania* amongst intravenous drug users (Cruz *et al.,* 2002). There is further evidence to support this artificial means of transmission. In southern Europe approximately 70% of all adult VL cases are associated with HIV, with IV drug users accounting for 50-90% of all cases of HIV-leishmaniasis co-infection (Burton, 2001). Venereal transmission of cVL has also been reported, with *Le. infantum* having been shown to be transmitted between naturally infected male dogs who copulated with uninfected bitches, due to the shedding of *Leishmania* parasites into the semen (Silva *et al.,* 2009). Magno da Silva *et al.,* (2009), reported the first vertical transmission of *Le. infantum* from naturally infected female Brazilian dogs, with parasites found in the liver and spleen of two pups which were stillborn from a naturally infected mother. Interestingly, further direct dog to dog transmission has been recently reported, with the transmission of *Le. infantum* being demonstrated through the bite of a naturally infected dog that attacked another dog living in the same household causing multiple wounds (Naucke *et al.,* 2016).

The identification of other potential routes of transmission, other than via the bite of the sandfly vector, suggests the possible need for further research into *Leishmania* transmission as well as the current methods utilised for *Leishmania* disease control. This is particularly essential in areas without biological vectors, with vertical and sexual routes highlighted as being particularly important (Oliveira *et al.,*

2015). Despite this, health authorities still only consider the transmission of *Leishmania* by sandflies as being of epidemiological importance. Although other insects have demonstrated their potential as natural vectors for *leishmaniasis*, particularly ectoparasites in dogs, transmission capability and vector competence are yet to be confirmed. Although these insects may participate in the transmission cycle of *Leishmania* it is, however, unclear as to whether this is a terminal or incidental link, or whether these insects are epidemiologically important vectors (Rebelo *et al.,* 2016).

1.1.3 Epidemiology

Leishmaniases are distributed worldwide and are present in both tropical and subtropical regions, affecting both urban and rural communities (Akhoundi *et al.,* 2016). Leishmaniasis is endemic in 88 countries on five main continents; Africa, Asia, Europe and North and South America. Leishmaniases are predominantly found in poverty-stricken countries with an estimated 350 million people at risk of infection. In 2014, there were approximately 300,000 reported cases of leishmaniasis with an annual reported incidence of 900,000 to 1.6 million (**Table 1.1**) (Alvar *et al.,* 2012). The prevalence of leishmaniasis has, however, decreased over the past few years with new reports suggesting and estimated 700,000 to 1 million new cases each year with 25,000 to 65,000 deaths (World Health Organisation, 2019). Over 90% of global VL cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, Sudan and South Sudan. In contrast, CL is more widely distributed, with cases occurring in four main epidemiological regions; the Mediterranean, the Americas, Central Asia and the Middle East. Despite the presence of CL in Southern Europe and the Middle East, 95%

of CL incidences occur in seven countries; Afghanistan, Algeria, Brazil, Colombia, Iraq, Iran, and Syria (Alvar *et al.,* 2012).

	Reported CL cases per year	Estimated annual CL incidence			Average estimated annual CL incidence
Americas	66941	187,200	to	307,800	247,500
Sub-Saharan Africa	155	770	to	1500	1135
East Africa	50	35,300	to	90,500	62,900
Mediterranean	85555	239,500	to	393,600	316,550
Middle East to Central Asia	61013	226,200	to	416,400	321,300
South Asia	322	1900	to	3500	2700
Global Total	214036	690,900	to	1,213,300	952,145

	Reported VL cases/year	Estimated annual VL incidence			Average estimated annual VL incidence
Americas	3662	4500	to	6800	5650
Sub-Saharan Africa	1		-		-
East Africa	8569	29400	to	56700	43050
Mediterranean	875	1200	to	2000	1600
Middle East to Central Asia	2496	5000	to	10000	7500
South Asia	42623	162100	to	313600	237850
Global Total	58226	202200	to	389100	295650

Table 1.1: The reported cases per year and estimated annual incidence of both Visceral and Cutaneous Leishmaniasis. CL and VL has been recorded in 6 regions worldwide; Americas, Sub-Saharan Africa, East Africa, Mediterranean, Middle East to Central Asia and South Asia (Table adapted from Alvar *et al.,* 2012).

1.2 *Leishmania (Le.) infantum*

Leishmania infantum is an intracellular protozoan parasite that is part of the *Le. donovani* complex. This species of *Leishmania* is the only member of the complex that is present in South America, found over a wide area from northern Argentina to Southern USA (Ready 2014). Various wild and domestic vertebrates have been demonstrated as being reservoirs for *Le. infantum* in the New World, with infection recorded in carnivores, rodents and lagomorphs. Infection has recently been identified in red foxes in Argentina (Millán *et al.,* 2016) as well as domestic cats in Brazil (Medonca *et al.,* 2017). Despite these findings, canines are the main reservoir host of *Le. infantum* in endemic areas both in the Old and New Worlds. This has led to the

development of canine visceral leishmaniasis and an increase in the transmission of leishmaniasis to human hosts (Marcondes *et al.,* 2019). Although some VL infections by *Le. infantum* can be clinically characterised, many are asymptomatic with approximately 13 times more asymptomatic cases of leishmaniasis being reported in Brazil (Costa *et al.,* 2002). Although predominantly transmitted by the sandfly vector *Lutzomyia longipalpis* throughout the Americas (**Figures 1.2 and 1.3**), research has also demonstrated that *Le. infantum* can also transmitted by *Lutzomyia evansi* in Venezuela and Colombia (Feliciangeli *et al*., 1999; Bejarano *et al.,* 2001) and *Lutzomyia cruzi* in Brazil (Dos Santos *et al.,* 2003)

1.3 *Lutzomyia (Lu.) longipalpis*

The genus *Lutzomyia* consists of approximately 400 species, 33 of which have a medical importance as vectors of *Leishmania* (Beati *et al.,* 2004). The first description of *Lutzomyia longipalpis* was by Lutz and Neiva in 1912. *Lutzomyia longipalpis* is well adapted to live in a variety of different ecological environments and habitats and has the ability to adjust to various climate conditions (Costa *et al.,* 2013). In Brazil, the geographical range of *Lu. longipalpis* is expanding. Although previously found in forest/rural sites, *Lu. longipalpis* is now adapting to urban areas throughout the country. This adaptation has been demonstrated and research has suggested that the increase in the number of *Lu. Longipalpis* found in urban areas of Brazilian cities is potentially due to soil transfer from the natural environment (Brazil, 2013) as well as deforestation in order to build powerlines and other man-made constructions (Marzochi *et al.,* 2009; Almeida *et al.,* 2013). Additionally, the movement of *Leishmania* infected dogs to urban areas has also been shown to have a negative

impact on sanitary conditions in domestic environments allowing *Lu. longipalpis* to successfully establish and spread (Salomon *et al.,* 2015). The increased presence *Lu. longipalpis* in urban areas is a contributing factor to the observed increase in reported cases of human and canine VL throughout Brazil (Desjeux, 2004).

1.3.1 Taxonomy of *Lutzomyia longipalpis*

Sandflies are recognised by their slender legs, humped thorax and small hairy wings that are held in either an arched like manner or erect at roughly 40 degrees when at rest or blood feeding (**Figures 1.2 and 1.3**) (Sharma and Singh, 2008). They rarely exceed 5mm in length with females having a total body length that is generally larger than males (Azevedo *et al.,* 2000). Males are easily recognisable due to their prominent external structures known as coxites, which extend past the tip of the abdomen (Rutledge and Gupta 2009). Members of the subfamily Phlebotominae have relatively long mouth parts which possess cutting mandibles (Schmidt and Roberts, 1996). This results in sandflies being pool feeders with their mouth parts being well adapted to make an incision in the host skin and sucking up the blood that collects around the wound.

Lutzomyia longipalpis is recognized as being a complex of several sibling species that are found in different regions throughout the Americas (Araki *et al.,* 2009). Not only has morphological variation been identified, but biochemical, behavioural and physiological differences have also been reported (Souza *et al.,* 2017). The differences observed between the epidemiology as well as the variation in behavioural and physiological features have been shown to influence the vectorial capacity of *Lu. longipalpis*. It has been suggested that transmission dynamics differ among the *Lu.*

longipalpis sibling species with different species able to transmit specific *Leishmania* to different animal hosts (Maingon *et al.,* 2008). This observed variability in *Lu. longipalpis* could have a major effect on disease transmission. However, in order to gain a better understanding of the association between this species and VL incidence, further work needs to be undertaken on the geographical distribution of the *Lu. Longipalpis* sibling species as well as recent changes in species epidemiology.

Figure 1.2: Living female *Lu. longipalpis*, the most common vector of *Leishmania infantum* in Brazil. Sandflies are approximately 1.5–5 mm in length, with a hairy appearance, and wings which extended over the body at 40 degrees (Lerner *et al.,* 2007).

Figure 1.3: Dead *Lutzomyia* sandflies viewed under a microscope. A female (left) and male (right) sandfly of the genus *Lutzomyia* (Dantas-Torres, 2009). Not only are male sandflies smaller than females, but also the external genitalia of the male sandfly consists of paired structures which grip the female during copulation (Singh and Phillips-Singh, 2010).

1.4 Current *Leishmania* Prevention and Control Methods in Brazil

1.4.1 Reservoir Control

Methods that are employed to control the reservoir host of zoonotic leishmaniasis are influenced by which animals act as reservoirs. In Brazil, dogs are the primary reservoirs for leishmaniasis and it is therefore necessary to develop appropriate control measures (Ribeiro *et al.,* 2018) The use of reservoir control strategies as a possible intervention against visceral leishmaniasis is based on previous studies which have proven that the incidence rate of human infection is directly associated with the number of infected canines as well as the high observed efficiency of dogs able to transmit the parasite to sandflies (Dye, 1996; Courtenay *et al.,* 2017).

Canine Leishmaniasis (cVL) is notoriously difficult to control. This is due to the intricate transmission cycle between humans, sandflies and dogs as well as the complex ecology and biology of sandflies (Alexander and Maroli, 2003). Dog culling is officially recommended for the control of cVL throughout Brazil, where each year hundreds of thousands of canines with suspected infection are euthanized (Dantas-Torres *et al.,* 2012). This control method, which aims to break the parasite's transmission cycle, is in theory, an effective strategy (Costa *et al.*, 2013). The removal of the source of infection through euthanizing infected dogs not only removes the reservoir host from the environment, but also reduces the average duration of the infectious period (Werneck, 2014). This control programme has, however, been considered as ineffective with a high occurrence rate of cVL being observed in endemic areas throughout Brazil (Dantas-Torres *et al.,* 2012). Furthermore, studies have shown that not only is there is no significant reduction in human VL cases in Brazil but also a high occurrence of false positive results in the confirmatory test are still observed

(Silva *et al.,* 2019). Despite the identified problems with the current methodology, the culling protocol is still being maintained throughout Brazil.

The lack of specificity of the current diagnostic tests that are used for identifying infected dogs in the field is a major factor contributing to the ineffectiveness of the current control programme. If the canine culling strategy is to be successful, then a more effective and accurate method of in-field diagnosis is required.

1.4.2 Vector Control

Due to the transmission of *Leishmania* parasites occurring between animal reservoirs and humans via the bite of infected phlebotomine sandflies, current control methods have adopted protocols which aim to reduce human contact with infected sandflies. Current sandfly vector control measures that are employed throughout Brazil include the residual spraying of the internal walls of houses and animal shelters with insecticides, also known as indoor residual spraying (IRS), the use of insecticide-impregnated dog collars and the use of insecticide-treated bed nets (ITNs), curtains (ITCs) and clothing (Pinart *et al.,* 2015).

Despite the widespread use of these control methods, numerous problems and impracticalities have been identified through research. Although IRS is the most commonly-used sandfly control method, spraying needs to be repeated regularly, with research demonstrating a decrease in IRS efficacy after 1 month (Banjara 2019). This therefore decreases the long-term sustainability of this methodology. Furthermore, conventional ITCs and ITNs need to be retreated or replaced on a regular basis, approximately every 6 to 12 months (Pulkki-Brännström *et al.,* 2012). Although there

have been recent developments in long-lasting ITNs (LLITNs) which provide effective protection for 3 years, most sandfly activity tends to occur around sunset, thereby limiting the effects of LLITNs (Roberts 2006). Additionally, the use of insect repellents and protective clothing is not affordable or practical for poorer populations who live in highly endemic regions. Although it has been reported that sandflies are very sensitive to insecticides, some Dichlorodiphenyltrichloroethane (DDT) resistance has been reported (Dhiman and Yadav, 2016). This resistance is also a common phenomenon in malaria control programs with mosquito resistance representing a significant problem. Although IRS and ITNs have been demonstrated to generally reduce malaria incidence, it has been shown that they have little impact on the prevalence of malaria (Loha *et al.,* 2019). Due to the current limitations in vector control methodologies, new techniques and protocols need to be developed in order to reduce the number of female sandflies in endemic areas in Brazil. This will subsequently lead to a reduction in the transmission of *Leishmania*.

1.5 Canine visceral leishmaniasis (cVL)

CVL is a zoonotic vector-borne disease caused by the *Leishmania infantum* parasite. In Brazil, canines as the main reservoir hosts of *Le. infantum,* play a key role in the transmission of the parasite to humans. In terms of the incidence of cVL throughout Brazil, studies undertaken in endemic areas have shown a prevalence of up to 70% (Quinnell *et al.,* 2013). CVL has previously been regarded as a rural disease, however, studies have now demonstrated that it is firmly established in large urbanised areas therefore being a major risk to human health (Guimarães *et al.,* 2016). It has been suggested that the control of human VL is dependent on the effective

control of cVL. This has stimulated an increase in research into leishmaniasis infection in canines, and effective control methods (Pessoa-e-Silva *et al.,* 2019). Current methodologies employed for decreasing VL transmission in Brazil are based on vector and reservoir control programs (Werneck, 2014). However, trials from Brazil, which have evaluated the effect of infected dog culling on the incidence of leishmaniasis, report a relatively low effectiveness. Although a reduction in seroconversion was reported, the studies suggest an urgent need to revise the use of culling in the Brazilian VL control program (Costa *et al.,* 2007; Werneck *et al.,* 2014b).

1.5.1 Epidemiology and Clinical Manifestations

CVL is widespread throughout both rural and urban areas of South America having been implicated in human CL epidemiology (Dantas-Torres, 2009). Research has demonstrated that dogs from rural areas of Brazil are 3.4 times more likely to be infected than those in urban environments (Oliveira *et al.,* 2016). CVL also presents with a complex epidemiology in South America. This can be demonstrated through various factors. These include the multiple causative agents and culpable sandfly species that have been identified (Coelho *et al.,* 2009).

An extensive range of clinical manifestations and immune responses have been demonstrated in dogs infected with cVL. The infection in canines can range from being a subclinical or a self-limiting disease, to a very severe and often fatal illness (Solano-Gallego *et al.,* 2009). CVL can manifest itself asymptomatically or through nonspecific clinical signs with loss of body weight, splenomegaly, renal disease and fever being common, nonspecific clinical manifestations (Ciaramella *et al.,* 1997). The disease can range in severity from mild proteinuria to chronic renal failure; the main cause of

fatality due to cVL (Solano-Gallego *et al.,* 2011). Due to the asymptomatic nature and nonspecific clinical manifestations of cVL, diagnosis through the observation of clinical signs is unreliable and ineffective yet it is often used as an initial assessment of infection (Coura-Vital *et al.,* 2011). The current unreliability of cVL diagnosis is a major concern particularly as current control methods rely on an accurate diagnosis.

1.5.2 Canines as a Reservoir Host of *Leishmania infantum*

A natural reservoir host can be defined as an animal or species that is infected by a parasite, and subsequently serves as a source of the infection for humans and/or another species (Medical Dictionary, 2007). This basic description has been redefined numerous times with Ashford, (2003) describing a reservoir host as being an ecological system in which an infectious agent can survive indefinitely. It has been suggested that a good reservoir host is one that is in close contact with humans, is susceptible to *Leishmania* infection and available to the sandfly vector in high enough quantities to cause infection. A good reservoir should rest and breed within the same habitat as the sandfly, being the primary blood meal source, with the disease progressing at such an extent so that the animal can survive until, at least, the next transmission season. These suggested qualities therefore implicate the dog as a good reservoir for *Leishmania* particularly in the urban environment in Brazil (Bray, 1982).

In Brazil, canines have been found to be naturally infected by various species of *Leishmania* including *Le. amazonensis, Le. infantum* and *Le. braziliensis* among others (Monteiro de Andrade *et al.,* 2006). *Leishmania infantum* is the etiological agent of zoonotic visceral leishmaniasis, a vector borne disease that is potentially fatal to humans and canines, posing a significant risk to public health in Brazil (Buckingham-

Jeffery *et al.,* 2019). The domestic dog has long been identified as the main reservoir host of *Leishmania infantum* infection throughout Brazil (Moreno and Alvar, 2002). Research has shown that canines are highly susceptible to *Le. infantum* infection, being exposed to infected sandflies that readily feed on animals (Rogers and Bates, 2007). A consequence of the ability of canines to act as a reservoir host is a steady increase in the number of human VL cases reported throughout Brazil over the past 30 years (Conti *et al.,* 2016). It is very common for canines to become infected by *Le. infantum* and not display any apparent clinical manifestations whilst maintaining a high transmission rate for long periods of time (Moreno and Alvar, 2002). It is therefore important to develop further diagnostic techniques other than the observation of clinical signs as many infected dogs are asymptomatic. *Lutzomyia longipalpis* sandflies can adapt easily to the peridomestic and domestic environment (Salomón *et al.,* 2015) with studies including Macedo-Silva *et al.,* (2014) observing the presence of dog blood within fed *Lu. longipalpis* sandflies collected from domestic areas. There is sufficient experimental and epidemiological evidence to support the contention that canines are the main reservoir host of *Le. infantum* for human infection. It is therefore reasonable to conclude that domestic dogs play a significant role in the epidemiology of visceral leishmaniasis particularly in Brazil (Petersen, 2009).

1.5.3 Current Diagnosis of Canine Leishmaniasis

The anthroponotic transmission of *Leishmania*, where humans are the major reservoir for the parasite, occurs predominantly in India and East Africa. In the Americas however, zoonotic transmission of *Leishmania* occurs with dogs in Brazil

consisting of the main source of transmission (Werneck, 2014). CVL is notoriously difficult to control. This is due to the intricate transmission cycle between humans, sandflies and dogs as well as the complex ecology and biology of sandflies (Alexander and Maroli, 2003). Although it is suggested that a prompt diagnosis and treatment of human cases could be employed to reduce zoonotic VL, this methodology is not an effective solution as humans do not play an important role in *Leishmania* transmission in Brazil (Quinnell and Courtenay, 2009).

CVL diagnosis in Brazil is based on serological, parasitological and molecular methods. Parasitological diagnosis by microscopy of bone marrow and splenic aspirates is a common diagnostic used for cVL in endemic regions (Ribeiro *et al.,* 2018). Although these techniques offer reliable evidence of the presence of the parasite in samples (**Figure 1.4**), there are many associated drawbacks. These include a lack of sensitivity when the parasite number is small and the requirement of a trained individual to analyse samples (Ertabaklar *et al.,* 2015). Additionally, *Leishmania* growth capacity varies from one species to another, a further disadvantage (Rasti *et al.,* 2016). Due to the current limitations of direct examination, new approaches have been developed for diagnosis of cVL in the laboratory.

Polymerase chain reaction (PCR) based diagnostics provide the greatest diagnostic sensitivity in canines compared with other laboratory-based techniques. These molecular techniques involve a delayed sample processing time, generate a species-specific diagnosis, and have a high sensitivity all of which are essential for the accurate diagnosis of cVL (da Silva Solcà *et al.,* 2014). PCR and RT-qPCR are currently part of common veterinary diagnostic routines, which have proven useful for follow-ups as well as allowing for diagnosis to be performed on various biological samples

such as blood, skin fragments or bone marrow aspirates (Silva *et al.,* 2017). Serological based methodologies are also commonly-used in Brazil for cVL diagnosis. These techniques involve the detection of specific anti-*Leishmania* antibodies using quantitative serological techniques such as the enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent-antibody test (IFAT). Although the IFAT is considered the reference technique by the World Organization for Animal Health (OIE, 2016), various studies have reported ELISA as being more sensitive than IFAT for cVL diagnosis (Figueiredo *et al.,* 2009; da Silveira *et al.,* 2011;; Sobrinho *et al.,* 2012). However, the diagnosis of cVL through serological methods has a variety of limitations. These include cross-reactions with cutaneous *Leishmania* species, *Trypanosoma* parasites and other haemoparasites. In addition, false positive and negative results are common due to low antibody titres and the presence of antibodies in dogs that were previously treated (Lopes *et al.,* 2017). Despite these limitations, immunochromatographic assays are currently employed as routine serological laboratory diagnostic tests for the diagnosis of cVL due to the high sensitivity and specificity observed (Ribeiro *et al.,* 2018).

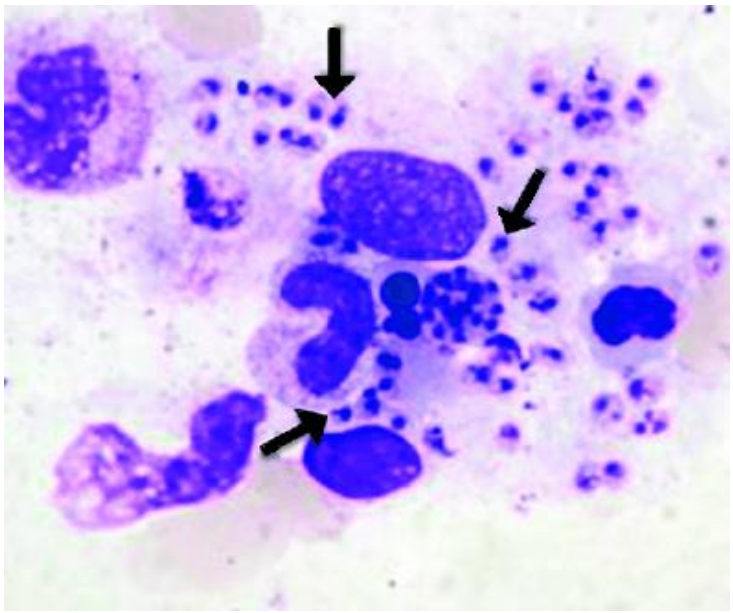


Figure 1.4: *Leishmania* amastigotes in skin lesion biopsy. Parasites are stained with either Giemsa or hematoxylin and eosin. Amastigotes appear as oval or round bodies that are 2-4ul in diameter with characteristic kinetoplasts and nuclei and are indicated by arrows (Hadjipetrou *et al.,* 2014).

In addition to laboratory tests, the diagnosis of cVL also occurs in a field-based environment. Over the last 10 years, field diagnosis has involved the use of the recombinant K39 (rK39) antigen; a 39-aminoacid repeat recombinant leishmanial antigen. Rapid diagnostic tests (RDTs) such as the immunochromatographic (IC) rK39 test and DPP (Dual-Path Platform) diagnostic have been developed which detect rK39-reactive antibodies in sera. The rK39 antigen has been used widely to detect cVL as it was found that anti-rK39 antibodies are more prevalent in positive dogs than in humans (Scalone *et al.,* 2002). However, research has reported varying sensitivities for these RDTs with a range of sensitivities from 46% - 100% being reported in the diagnosis of cVL (de Lima *et al*., 2010; Quinnell *et al.,* 2013; Mendonça *et al*., 2017). Research has also demonstrated varying test specificities, ranging from 33% to 100%

among the literature. Therefore, additional laboratory based parasitological or serological tests are currently necessary in order to confirm in-field positive results.

In Brazil, the primary in-field diagnostic approach used is the dual-path platform (DPP) diagnostic test. This tool was recently developed by Chembio Diagnostic Systems, Inc (Medford, NY, USA) and is currently manufactured by Bio-Manguinhos/Fiocruz in Brazil. The DPP is a colloidal gold-based immunochromatography assay designed to detect antibodies that are present in dog blood against rK26/rK39. This rapid, disposable device has, in theory, great potential for the diagnosis of cVL especially when considering mass screening exercises. This is due to its proven technological advantages over other rapid diagnostic assays which, until recently, had been used as common practice in Brazil (Figueiredo *et al.,* 2018). The sensitivity and specificity of the DPP test is, however, under debate with few studies having been carried out to determine test accuracy. While some studies are reporting a sensitivity of 98% (Mendonça *et al.,* 2017), others have determined a sensitivity of 47% (Solcà *et al.,* 2014) with other results inbetween. Research from 2008 in Brazil also highlights the inaccuracy of this diagnostic test. It was reported that from August 2002 to July 2004, 61% of the canine population from an endemic area in Brazil were culled following a positive diagnosis using the current in-field DPP test, with only 27% of those confirmed as positive for the presence of anti-*Leishmania* antibodies following subsequent tests (Nunes *et al.,* 2008). Inconsistency in this diagnostic can be shown when analysing test specificity with results between 60% – 100% being reported across the literature. Further to the dispute about test sensitivity/specificity, additional disagreement on the capability of the DPP test to diagnose symptomatic and/or asymptomatic dogs can also been seen with some research suggesting the test

is able to detect both asymptomatic and symptomatic dogs equally (Laurenti *et al.,* 2014), while others suggesting the opposite, with a relatively low sensitivity being demonstrated when identifying asymptomatic dogs that are positive (Grimaldi *et al.,* 2012).

Despite the uncertainty surrounding the current diagnostics, in 2012 the Brazilian Ministry of Health established a standard protocol for the diagnosis of cVL. This currently involves a laboratory-based ELISA as a confirmatory test following a screening test using the Dual-Path Platform (DPP) rapid in-field diagnostic (Fraga *et al.,* 2016). In areas affected by cVL, a positive result from these tests is used as the main criteria indicating the need to euthanize suspected positive dogs. Even though the current diagnostic protocol for cVL has enhanced disease diagnosis, the ELISA technique used still requires a laboratory infrastructure and experienced staff. This restricts the ability to perform this diagnostic protocol to specialised laboratories only (Persichetti *et al.,* 2017). Furthermore, several challenges must be confronted by researchers when applying laboratory-based techniques directly in the field. These include the storing of reagents at room temperature, performing DNA isolation without a centrifuge, or performing a diagnostic analysis of the sample without sophisticated laboratory technology (Pessoa-e-Silva *et al.,* 2019). Additionally, the suggested inaccuracy of the DPP diagnostic also leads the ineffectiveness of this protocol. Although shown to be non-invasive, easy to use and rapid, the routine use of the current DPP tests in Brazil is somewhat to blame for the ineffectiveness of the current control programme (de Carvalho *et al.,* 2018). It therefore stands to reason that the currently used in-field diagnostics need to be reviewed in order to improve disease control. Research suggests that the DPP test is unlikely to be sensitive enough

for detecting canine carriers of *Leishmania infantum* (Fraga *et al.,* 2016). Therefore, in addition to a highly sensitive laboratory-based diagnosis, an accurate diagnostic test which could be implemented primarily in the field would prove advantageous for the ongoing control of cVL. Point of care (POC) diagnostics for cVL would achieve a rapid turnaround of results and therefore the efficient and prompt removal of infected dogs (Travi *et al.,* 2018).

1.5.4 Canine Leishmaniasis in Study Area; Governador Valadares (GV)

In the 1960s cases of human VL were first reported in Governador Valadares (GV), with the area being determined as highly endemic for leishmaniasis (Coelho and Falcão, 1966). Following this, VL control and surveillance activities were implemented throughout the region to control disease transmission (Barata *et al.,* 2013). However, an interruption in the control programme at the beginning of the 1990s has resulted in the re-emergence of human VL with cases of cVL also becoming more common and widespread throughout GV (Malaquias *et al.,* 2007). The high rate of cVL observed in the area is important as research suggests that an increase in cVL in endemic areas is correlated with an increase in cases of human VL and a high transmission rate (Lima *et al.,* 2018). Transmission of the parasite occurs intensely in GV, with research from 2008 to 2011 demonstrating a 30.2% infection prevalence in domestic dogs from 35 districts following in-field diagnosis (Barata *et al.,* 2013). The high transmission rate could also be attributed to the poor quality housing with only basic sanitation in the area, with many residents living with or near domestic dogs (Coura-Vital *et al.,* 2011).

Although epidemiological studies in the area have suggested that *Le. infantum* is the sole etiological agent of cVL, recent research has reported canine infection with

Le. amazonensis in GV (Valdivia *et al.,* 2017). Additionally, other studies have also demonstrated the possible emergence of *Le. braziliensis* in canines in the area (Malaquias *et al.,* 2007). In parallel with multiple *Leishmania* species being detected in dogs in GV, research in the area has also identified multiple *Leishmania* species in the insect vector *Lu. longipalpis*, the most abundant sandfly species in the region (Cardoso *et al.,* 2019). The high transmission rate of leishmaniasis in GV can therefore be attributed not only to the high incidence rate of cVL, but also the presence of multiple *Leishmania* species in infected dogs in the area and the ability of the local sandfly vector to transmit the parasite.

1.6 Parasite manipulation

The transmission of parasites has been shown to involve both passive transfer via ingestion of one host by another or active host-seeking by the parasite. Previous studies have shown that parasites are able to manipulate aspects of their host's behaviour. This manipulation has been shown to enhance the interactions between vectors and suitable hosts thereby increasing the rate of parasitic transmission (Holmes and Bethel, 1972). Parasites are thought to alter the behaviour of their hosts in a variety of different ways, all which aid in the survival and transmission of the parasite. Those parasites that are trophically transmitted (i.e. when one host is eaten by the next), can manipulate the behaviour of their intermediate host by increasing the predation by the definitive host on the intermediate host thereby aiding transmission (Lafferty, 1999). Parasites which thrive in a habitat different to the one in which the host lives can manipulate the host resulting in the movement of hosts to a different habitat. For example, hairworms (*Paragordius tricuspidatus*) induce crickets

to drown in water, which enables the parasite to exit into an aquatic environment which favours its reproduction (Biron *et al.*, 2006). Many studies have also reported that parasites are able to manipulate their vector in order to aid transmission (Moore, 1993). The most common report of this transmission aid is the behavioural manipulation of rats by the parasite *Toxoplasma (T.) gondii*. Studies have shown that *T. gondii* is able to cause permanent behavioural changes altering the rat's perception of cat predation risk (Webster *et al.*, 2007). Evidence suggests that these behavioural alterations are a product of selective pressures on the parasite to enhance its transmission from the rat intermediate host to the cat definitive host. The mechanism of action used by *T. gondii* to alter the intermediate host behaviour is however unknown (Berdoy *et al.*, 2000). Perhaps more relevant is research which has demonstrated *Leishmania* manipulation of sandfly feeding behaviour. *Leishmania* infection within sandflies has been shown to increase vector biting persistence on mice following feeding interruption as well as promoting feeding on multiple hosts. This suggests that *Leishmania* can enhancing their transmission efficiency through sandfly manipulation, increasing biting persistence (Rogers and Bates, 2007).

It would prove advantageous for the transmission of the parasite if its vector was more responsive to infected host odours, as this is the predominant cue that is used by vectors to find a blood meal. This parasite manipulation has been observed in research relating to both malaria and leishmaniasis. Recent research has demonstrated that malaria infection alters the odour of naturally infected human patients in Africa (de Boer *et al.*, 2017). Further it has also been demonstrated that *Plasmodium* associated changes in human odour increases the attractiveness to mosquitoes (Robinson *et al.*, 2018). Although this study did not confirm direct parasite

manipulation causing an increase in infection associated compounds, the increase in mosquito attraction which was demonstrated could lead to a higher number of vector–host interactions and increased transmission. Additional research by Rossignol *et al*., (1986) demonstrated that a higher number of *Aedes aegypti* mosquitoes were attracted to the odour of guinea pigs infected with *Plasmodium gallinaceum*. Both O'Shea *et al.*, (2002) and more recently Nevatte *et al.*, (2017) demonstrated that the odour of golden hamsters infected with *Leishmania infantum* was significantly more attractive to sand flies compared to before infection when the same hamsters were uninfected. This increased attraction could result in enhanced transmission of the *Leishmania* parasite with more sandflies becoming infected when feeding on a positive host. Furthermore, the identification of individual volatiles and whole odour profiles that are attractive to sandflies could be used in the practical application of attractiveness experiments in the field.

1.6.1 Host Odours for Vector Control

Effective sand fly vector control remains a key goal in order to reduce the world's burden of leishmaniasis. Although the development of new vaccines and drugs may eventually lead to a long-term solution for leishmaniasis control, the implementation of vector control strategies may lead to a reduction in the incidence of disease over a shorter timeframe (Bray *et al.*, 2014). However, the limitations observed for the current vector control methodologies used in Brazil, suggest that new protocols need to be established in order to reduce the female sandfly number and disease transmission. There are two primary limitations observed; insecticide resistance due to the negative impact of insecticide exposure, and the current control

measures only targeting those vectors that rest and feed indoors primarily during the night (Barreaux *et al.*, 2017). Targeting female sand flies specifically would be an effective strategy to decrease *Leishmania* transmission risk. Not only would specific targeting provide a short-term benefit by killing the vector-competent individuals but would also have benefits in the future by reducing the number of offspring and therefore enhance the control of sand fly population growth (Claborn, 2010).

The use of odours for vector control is not a new concept. An increase in the attraction of different insect vectors to human, animal and synthetic odour has long been established. Research has shown that the release of carbon dioxide in the vicinity of commonly-used mosquito traps enhances the capture of host-seeking mosquitoes (McPhatter and Gerry *et al.*, 2017). Research has also shown that mosquito traps baited with human odours, significantly attracts more mosquitoes than un-baited traps, with a 3.6-fold increase in attraction shown (Matowo *et al*., 2013). This research suggests that human odour could be utilised as an affordable source of attractants which would supplement odour baited mosquito traps. The use of odours for sandfly vector control has also been shown. Not only have sandflies demonstrated an increased attraction to the volatile components which were identified through the analysis of infected dog odour (Magalhães-Junior *et al.,* 2019), but an attraction to synthetic male sandfly pheromone has also been observed (Bray *et al.,* 2014). These observations suggest the use of natural odours and VOCs in the development of volatile baited traps. The use of such traps could increase the number of female sandflies caught, thereby reducing disease burden. In fact, the use of male pheromone for vector control has already been demonstrated in the field, with greater numbers of *Lutzomyia longipalpis* being caught and killed by pheromone baited light traps

placed in chicken sheds sprayed with insecticide (Bray *et al*., 2010). Furthermore, a decline in the use of Insecticide-treated bed nets and insecticide spraying of houses would also reduce the current risk of sandfly insecticide resistance, all of which would contribute to effective sandfly vector control.

1.6.2 Odours as Diagnostic Markers and Potential cVL Diagnostic

Detecting disease at an early stage increases the chance of successful treatment. There is therefore a need for non-invasive, inexpensive and early diagnostic techniques. The odour emitted by an individual infected with disease may be one of the first and most important clues that could lead to an early diagnosis. It has long been reported that all diseases, including those parasitic in both humans and animals, are associated with a change in the body odour profile of the infected individual with different volatile organic compounds (VOCs) being released (**Table 1.2**) (Liddell, 1976). Further gas chromatography experiments have identified many different VOCs in human clinical specimens could potentially serve as disease markers (Mitruka, 1975; Sethi *et al.*, 2013). Volatiles often present themselves as endogenous products of metabolic and physiological body processes in response to infection. During the pathogenesis of an infectious disease, the pathogen interacts actively with cells of the infected host, resulting in the production of a variety of different chemical metabolites. VOCs are also produced by the host's immune system in response to any microbial infections for example through the inflammatory response (Sethi *et al*., 2013).

The VOCs produced in response to disease are emitted through various means such as in exhaled breath and through sweat. The analysis of the breath volatiles of

infected individuals for disease diagnosis is not a new concept. During both healthy and pathological states, exhaled air contains many VOCs produced during metabolic processes due to metabolites moving to from the blood to the lungs (Cazzola *et al.,* 2015). The analysis of breath of individuals in various diseases states, has allowed for the study of any modifications observed in exhaled VOC profiles. Most notably has been the diagnosis of lung cancer through the analysis of exhaled breath with the breath of patients suffering from lung cancer being detected with a sensitivity of 79% and a specificity of 72 % using VOC analysis when distinguishing between patients with lung cancer and healthy individuals (Chang *et al.,* 2018). In addition to the analysis of breath, VOCs are also detectable in sweat, however there is very limited research in this area. The skin emits a variety of different volatile metabolites, many of them being odorous, with sweat containing various biomarkers of different diseases including diabetes and cystic fibrosis (Jadoon *et al.*, 2015). As it is known that infected individuals produce different VOCs compared to those that are healthy, it can be suggested that detecting a change in those VOCs through the analysis of skin and sweat could be achieved. In fact, many studies have noted a change in the odour of an infected individual in various disease states (**Table 1.2**). The identification of volatile biomarkers in clinical specimens offers a potential option for development of inexpensive, non-invasive and rapid diagnostic tools which can allow for the monitoring of several disease states.

The current ineffectiveness of VL control in Brazil has led to the expansion of leishmaniases cases creating a significant public health problem (Werneck *et al.*, 2014). Due to the role dogs play as a reservoir host for leishmaniasis in Brazil, research has been undertaken in order to identify new potential diagnostics for cVL. Research

has identified that the odour on the hair of dogs infected with *Le. infantum* consists of *Leishmania* specific volatiles, with distinct differences also being observed in the VOC profiles of symptomatic and asymptomatic dogs (De Oliveira *et al.,* 2008, Magalhães-Junior *et al.*, 2014b). This distinct difference in volatiles following *Leishmania* infection suggests the diagnostic potential of a technique which could differentiate between these volatiles and metabolites.

Disease/disorder	Body Source	Descriptive odour	References
Acromegaly	Skin	Strong, offensive	Daughaday, 1968
Anaerobic infection	Skin, Sweat	Rotten apples	Pavlou and Turner, 2000
Bacterial proteolysis	Skin	Over-ripe Camembert	Pavlou and Turner, 2000
Bromhidrosis	Skin	Unpleasant	Golding, 1965
Diphtheria	Skin	Sweet	Liddell, 1976
Gout	Skin	Gouty odour	Liddell, 1976
Hyperhydrosis	Skin	Unpleasant body odour	Liddell, 1976
Isovaleric acidemia	Skin, Sweat	Sweaty feet, cheesy	Pavlou and Turner, 2000
Phenylketonuria	Skin	Musty, horsey, mousy, sweet	Pavlou and Turner, 2000
Pseudomonas infection	Skin, Sweat	Grape	Pavlou and Turner, 2000
Rubella	Sweat	Freshly plucked feathers	Pavlou and Turner, 2000
Schizophrenia	Sweat	Mildly acetic	Smith *et al*, 1969
Scrofula	Skin, Body	Stale beer	Liddell, 1976
Scurvy	Sweat	Putrid	Liddell, 1976
Smallpox	Skin	Stench	Liddell, 1976
Squamous-cell carcinoma	Skin	Offensive odour	Liddell and White 1975
Trimethylaminuria	Skin	Fishy	Pavlou and Turner, 2000
Tuberculosis lymphadenitis	Skin	Stale beer	Pavlou and Turner, 2000
Typhoid	Skin	Freshly-baked brown bread	Pavlou and Turner, 2000
Vagabond's disease	Skin	Unpleasant	Liddell, 1976
Varicose ulcers (malignant)	Skin	Foul, unpleasant	Levine and Fong, 1957
Yellow Fever	Skin	Butchers shop	Liddell, 1976

Table 1.2: Studies describing the odours emitted from the skin in response to different diseases and metabolic disorders. Studies have associated these diseases with different odours that are released from affected human tissues, primarily the skin (Table adapted from Wilson and Baietto, 2011).

1.7 Volatile Organic Compound (VOC) Analyser

A volatile organic compound (VOC) Analyser, also known as an "electronic nose", is a device intended to detect and differentiate between odours (Stitzel *et al.,* 2011). The introduction of this device has allowed volatiles and odours to be used for many diagnostic situations. VOC Analysers can detect and differentiate between the volatile

organic compounds released as a result of various infections (Pavlou and Turner, 2000). The VOC Analyser was developed in order to emulate the mammalian olfactory system (**Figure 1.5**). Essentially, the procedure behind VOC Analyser analysis consists of headspace sampling using a sample delivery system, an array of sensors which respond to families of molecules, and a pattern recognition module which generates a signal pattern used to characterise odours. The odour molecules are drawn into the VOC Analyser using headspace sampling in which the air surrounding a sample which contains released volatiles is injected into the VOC Analyser (Pearce *et al.*, 2003). The VOCs within the odour sample are exposed to the array of sensors. The sensor array reacts to the individual volatile compounds upon contact, with the adsorption of each volatile on the sensor surface causing a specific physical change to the sensor. This physical change causes a subsequent change in electrical properties with each sensor responding to different VOCs at varying degrees (Harsányi, 2000). The specific response observed for each sensor is recorded by an electronic interface which

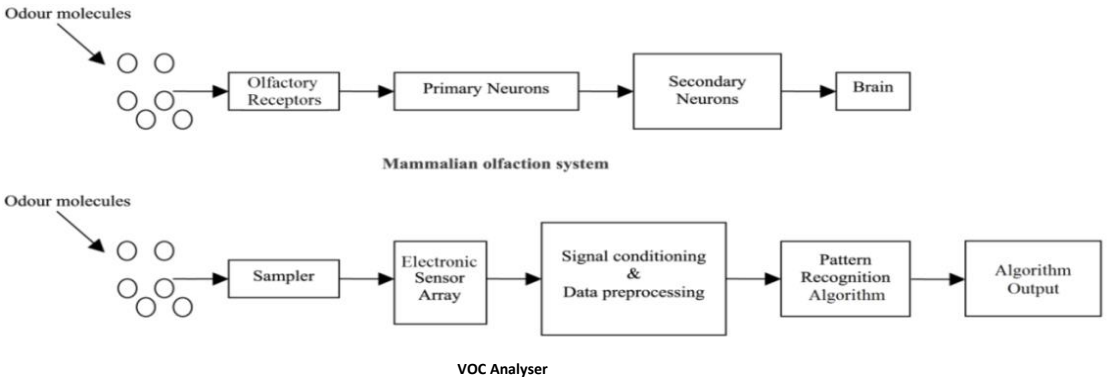


Figure 1.5: Flow diagram representation of human and artificial (VOC Analyser) olfactory systems. VOC Analysers are described as having three main components; the vapour delivery system, the electronic sensor array and the pattern recognition algorithm (Stitzel *et al* 2011). (Image adapted from Arshak *et al.,* 2004).

transforms the signal into a digital value. This value is recorded and subsequently analysed by pattern recognition algorithm systems (Arshak *et al.*, 2004). The VOC Analyser system has been designed so that the sensor array produces an overall response pattern for each individual odour, therefore allowing for identification.

1.7.1 Diagnostic Potential of VOC Analysers

The sense of smell has been used for centuries by medical doctors to determine the general health and physical state of their patients (Wilson, 2011). The basic principle behind VOC Analysers has been the development of a painless, simple and non-invasive screening method for the early medical diagnosis of various diseases. Studies have demonstrated that specific diseases and bacteria produce characteristic smells, all of which can be recognised and identified by VOC analysers (Shirasu and Touhara, 2011). Currently, the diagnosis of infection using traditional culture methods can take a minimum of 24 hours for the bacteria to be colonised and detected for diagnostic means (Bursle and Robson, 2016). Furthermore, the use of molecular and serological techniques for disease diagnosis using various biological samples (e.g. blood and urine) not only require sophisticated laboratory environments, but also have a slow turnaround time for results (Caliendo *et al.*, 2013). The slow turnaround time of these methodologies is a significant limitation of current diagnostic methods. The prevention and early treatment of disease which is essential in biomedicine, requires an early and accurate diagnosis. For both to be implemented, it is important that relevant rapid results are obtained using sensitive technologies. Currently, VOC Analyser technology has proven effective in numerous areas of medical diagnosis

including the detection of tuberculosis (TB) (Bruins *et al*, 2013), urinary tract infections (UTI's) (Pavlou *et al.*, 2002) and cancers (Thriumani *et al.*, 2014).

Various studies have demonstrated that VOC Analysers have a great ability to not only detect but also distinguish between a wide variety of fungi and bacteria from the human body, with specific biomarkers being identified (Chen *et al.*, 2013). *In vitro* studies have demonstrated that it is possible to distinguish between different species of aerobic bacteria based on differences in the quantity of ketones, terpenes and trimethylamines produced. For example, *Mycobacterium tuberculosis*; the causative agent of tuberculosis, has been identified in cultured sputum samples using enzymes which enhance *M. tuberculosis* growth and volatile production (Pavlou *et al.*, 2004). Subsequently, recent research demonstrated the ability of point of care VOC Analysers to distinguish between patients with confirmed tuberculosis cases and healthy individuals with 91% sensitivity and 93% specificity (Teixeira *et al.*, 2017). Further to this, six different bacterial species that are responsible for eye infections; *Haemophilus influenza*, *Escherichia coli, Moraxella catarrhalis, Streptococcus pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus* were successfully differentiated into six different classes using data which was obtained using a VOC Analyser (Dutta *et al.*, 2002). Additionally, recent research utilised VOC Analyser technology to different between methicillin-resistant *Staphylococcus aureus (MRSA)* and methicillin-sensitive *Staphylococcus aureus (MSSA*) as well as *Escherichia coli, Streptococcus pyogenes, Clostridium perfringens* and *Pseudomonas aeruginosa* with 78% sensitivity. This research supports the concept of using VOC Analyser technology to not only identify the relevant bacteria causing wound infections, but also promptly administer the appropriate antibiotics (Saviauk *et al.*, 2018).

In addition to the differentiation between bacterial pathogens, VOC Analysers have demonstrated the ability to detect various disease biomarkers produced by the human body during infection. A study by Di Natale *et al.*, (1999) demonstrated that patients suffering from kidney disorders produce distinguishable volatile organic compounds, which can be useful in the diagnosis as well as the control of renal dialysis. Further studies by the same group have also demonstrated that lung cancer can be identified through the analysis of patient's breath using a VOC Analyser (Di Natale *et al.,* 2003). This work showed promising results, with all lung cancer patients being successfully identified through the detection of aromatic compounds and alkanes. Additional recent research has also demonstrated that VOC Analyser technology can discriminate between the exhaled breath of patients with lung cancer and health individuals with 96% sensitivity and 91.5% specificity (Tirzïte *et al.*, 2018). Further research has also shown the potential use of VOC Analysers to identify Parkinson's disease (PD) and Alzheimer's disease (AD) sufferers. The technology was able to differentiate between patients with PD and AD from healthy controls with 94% accuracy. Patients with PD were also identified independently with 100% sensitivity and specificity (Bach *et al.*, 2015).

The use of VOC biomarkers in the diagnosis of parasitic diseases is expected to become increasingly important as more information on specific VOCs relating to various diseases becomes more available and as biomarker detection with VOC Analysers becomes a routine clinical. Numerous studies have already identified distinct odour profiles and associated biomarker VOCs for several different parasites and their associated diseases. In some cases, including cVL (de Oliveira *et al*., 2008; Magalhães-Junior *et al.,* 2014) and malaria (Kelly *et al.*, 2015; Berna *et al.,* 2015),

unique chemicals and/or chemical groups have been identified. A recent study by Gikunoo *et al.*, (2014) demonstrated that the parasitic protozoon *Plasmodium falciparum,* the predominant causative agent of malaria, could be detected using experimental carbon nanofibers grown on glass microballoons. This model system could detect *Plasmodium falciparum* histidine-rich protein-2 antigen concentrations as low as 0.025ng/ml. As previous research has demonstrated that VOCs bind readily to carbon nanofibers (Cuervo *et al*., 2008), it could be suggested that carbon nanofibers could be utilised in the development of VOC Analysers for parasitic disease diagnosis.

Despite the promising diagnostic potential and non-invasive ease of use, there are still limitations to the VOC Analyser and improvements that need to be made. For the diagnosis of disease, VOC analysers still have a relatively low sensitivity and specificity when compared to current molecular and microbiological techniques (Cellini *et al.,* 2017). Furthermore, their routine use in a clinical application is yet to be fully evaluated, with studies identifying a proof of concept rather than real world application (Wilson, 2018). Due to the current technology being independently developed, each individual VOC Analyser possesses a custom-built sensor array. Therefore, the technology will perform differently during routine clinical diagnosis of disease, even with a standardised protocol for sampling and analysis (Baietto *et al.,* 2010). Additionally, the device is reported to be affected by environmental factors such as humidity and temperature. These factors in addition to sensor degradation have been shown to cause sensor drift which produces poor batch-to-batch reproducibility (Sujatha *et al.*, 2012). However, despite these disadvantages, the use of VOC Analyser technology for disease analysis cannot be understated. The device

can assist with other laboratory based diagnostic techniques such as PCR, providing a rapid screening of samples which would optimise resource and time commitment during routine clinical practice. The use of VOC Analysers as a new possibility for real time assays as well as the fast and accurate diagnosis of disease, can ultimately allow for an earlier diagnosis as well as an evaluation of patient condition before clinical signs occur.

1.8 Aims of Project

The first aim of this work, was to determine whether *Leishmania* can manipulate canine host odour in order to aid its own transmission. The second aim of the research was to demonstrate whether a VOC analyser could identify and therefore diagnose dogs that are infected with leishmaniasis. If successful, this work could demonstrate the use of a VOC Analyser as a novel, rapid and non-invasive diagnostic test for cVL. Finally, GC/MS technology was utilised to confirm any discrimination observed between *Leishmania* infected and uninfected dogs, both previously (de Oliveira *et al*., 2008; Magalhães-Junior *et al.*, 2014) and following VOC Analyser analysis. GC/MS analysis also enabled the chemical structure and composition of potential *Leishmania* specific compounds to be preliminarily identified. These could, therefore, be used in the future development of both the VOC Analyser as a cVL diagnostic tool and odour baited vector traps.

Chapter Two - General Methods

2.1 Study Area

The municipality of Governador Valadares (18°51'S–41°56'W) is a city located in the eastern region of the state of Minas Gerais. It covers part of the Rio Doce basin, which is in the south east of Brazil (**Figure 2.1**). The city has an estimated population of 280,901 (Population Estimate 01.07.2017) distributed across 150 districts (citypopulation.de, 2017). The region experiences a hot and humid climate with temperatures that vary little throughout the year with an annual average of 32.6°C. Governador Valadares is an area of re-emerging focus for visceral leishmaniasis. From 2008 to 2017, 191 human cases of VL were reported mainly affecting children from 0 to 9 years of age and adult males, with a lethality rate of ~16% (DATASUS, 2019). *Leishmania* transmission occurs intensely in this region, with a high incidence rate of Canine Visceral leishmaniasis (cVL) (33.8%) being identified as an area of concern with many control programes focused on reducing cVL cases (Cardoso *et al.,* 2019). Collection of canine blood and hair samples was carried out in the Altinopolos neighborhood of Governador Valadares (**Figure 2.2**). This area was chosen because of the high prevalence of cVL observed there (Barata *et al.,* 2013) and the large number of dogs (Central Control Zoonosis survey).

Figure 2.1: Location of the municipality of Governador Valadares on a map of Brazil.

Coordinates: 18°51’12”S - 41°56’42”W. State of Minas Gerais. Mesoregion of Vale do Rio Doce. Image provided by Dr Luigi Sedda.

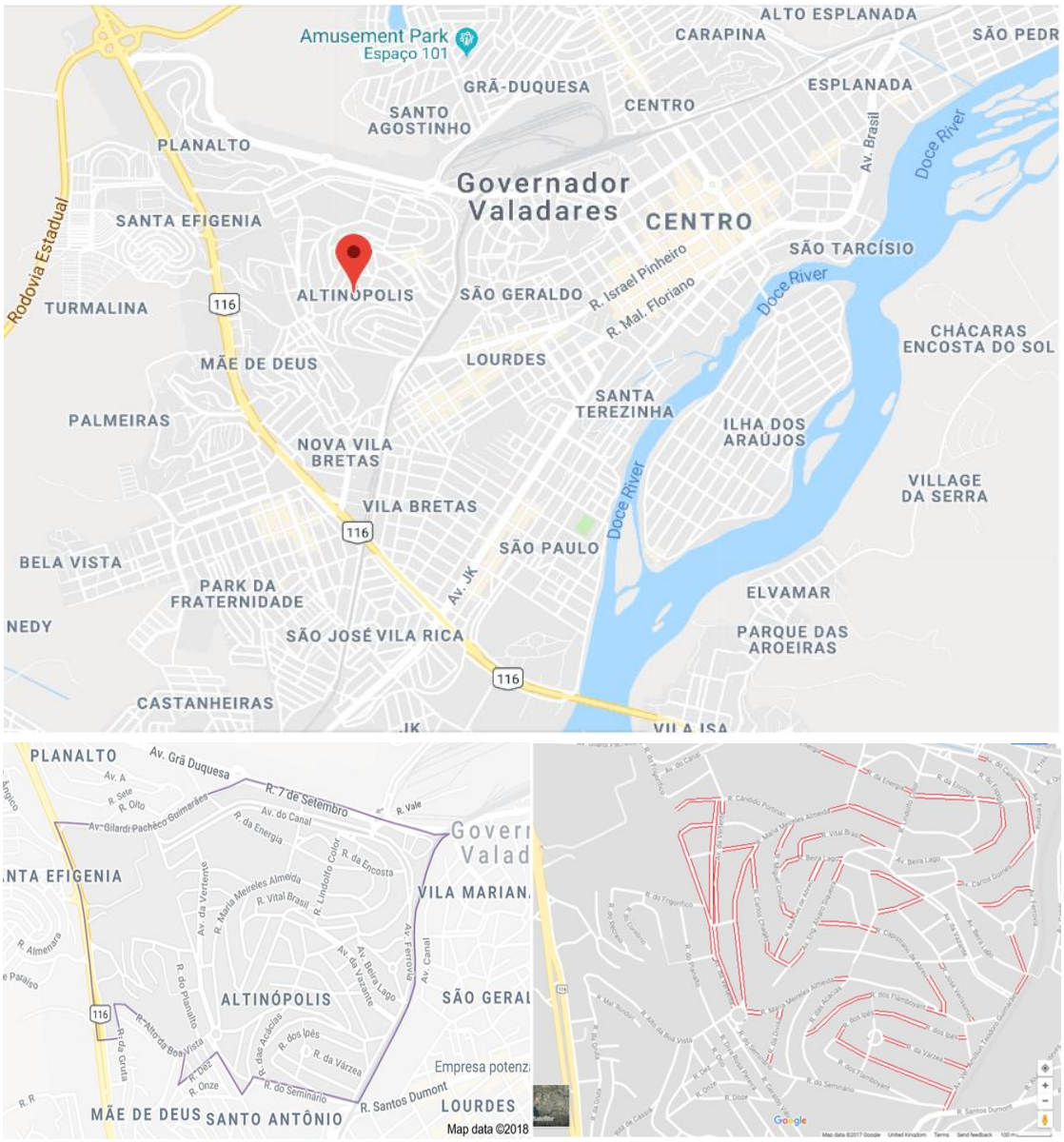


Figure 2.2: Altinopolis, Governador Valadares - State of Minas Gerais, Brazil. Map of Altinopolis displays the main streets and avenues that interconnect this neighbourhood with other parts of Governador Valadares. The location of all dogs sampled is highlighted in red (bottom right). A wide distribution of dogs sampled for this research can be observed (Images taken from Google maps).

2.2 Dog recruitment

Recruitment of domestic dogs in 2017 (n=185) and initial sampling was carried out during August in Altinopolis. The dogs were chosen randomly following owners consent obtained by signing a consent form and were microchipped to aid their identification in the 2018 follow up study. Inclusion criteria: dogs aged ≥ 3 months; dogs without previous clinical assessment or laboratory diagnosis for cVL. Exclusion criteria: pregnant/lactating bitches; aggressive dogs; stray dogs.

In April 2018 149 dogs were sampled, this number included 133 dogs that were resampled from the available 2017 cohort and an additional 16 “infected” dogs that were recruited from the CCZ facility, to compensate for loss of dogs between 2017 and 2018. The main reasons for dog loss included death, the owner having moved and the owners unwilling to give consent for sample collection. Inclusion and exclusion criteria were the same as previously stated.

2.3 Blood Samples

2.3.1 Collection of Blood Samples in 2017

Between 5ml and 10ml of peripheral dog blood was collected in 10ml K2 EDTA-coated tubes via venepuncture of the leg and neck. Blood samples taken in the field were stored in a cool box with a freezer pack before being transferred to a 4^{o}C fridge until processing.

2.3.2 Collection of Blood Samples in 2018

10ml of peripheral dog blood was collected in 10ml K2 EDTA-coated tubes via venepuncture of the neck. Blood samples taken in the field were stored in a cool box with a freezer pack before being transferred to a 4^{o}C fridge until processing.

2.3.3 Buffy Coat Extraction

200 ml of buffy-coat was obtained following centrifugation of collected blood samples at 2500 x g for 10 minutes at room temperature. Buffy coat samples were extracted using Pasteir pipettes and placed in 1.5ml Eppendorf tubes. The tubes were sealed and stored at -20^{o}C until DNA extraction.

2.3.4 DNA Extraction

The DNA was extracted from the buffy coat samples using the QIAamp DNA Blood Mini Kit (Qiagen) following the manufacturer's instructions using 200µl of buffy-coat. The lysis was based on protein kinase with a final elution volume of 50µl.

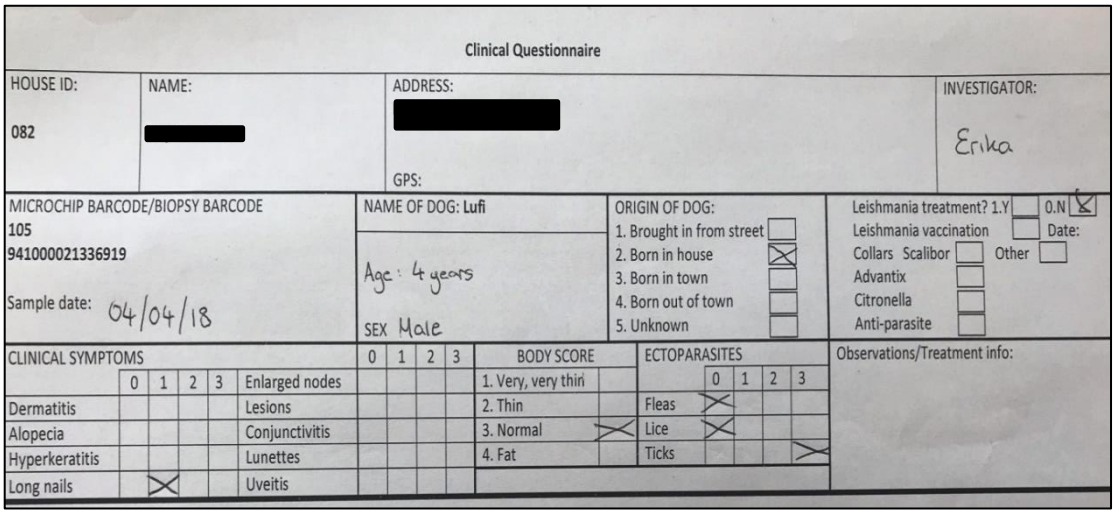
2.4 Collection of Hair Samples

Canine hair samples in both 2017 and 2018 were obtained by cutting the hair next to the skin of the dogs using surgical scissors that were sterilized using hexane prior to the collection of each sample. The sampling was performed in the dorsal area on all dogs, with a minimum of 2g of hair being collected. All samples were placed in individual sealed foil bags and stored at 4 °C prior to further analysis.

2.5 Clinical Questionnaire

A clinical questionnaire was completed by either CCZ cVL control specialists, or veterinarians for each dog sampled (**Figure 2.3**). This questionnaire allowed for a record of clinical signs, dog body score and presence and severity of ectoparasites to be kept for all dogs. This information was subsequently used following PCR, VOC Analyser and GC/MS analysis to determine any correlation between changes in odour, infection status and the symptoms observed. All dogs were assessed for clinical signs of *Leishmania* infection with information of all clinical signs observed recorded for each individual dog.

Animals were classified according to the presence of clinical signs suggestive of cVL. The main signs of cVL considered were onychogryphosis, ophthalmologic abnormalities, adenitis, cachexia, hepatosplenomegaly, alopecia, and crusted ulcers and lesions; dogs were classified as asymptomatic (the absence of clinical signs), oligosymptomatic (the presence of one to three clinical signs), or symptomatic (the presence of more than three clinical signs according to the criterion adapted from Mancianti *et al.,* 1988).



Clinical Questionnaire

HOUSE ID:	NAME:	ADDRESS:	INVESTIGATOR:
082	[redacted]	[redacted] GPS:	Erika

MICROCHIP BARCODE/BIOPSY BARCODE	NAME OF DOG: Lufi	ORIGIN OF DOG:	Leishmania treatment? 1.Y ☐ 0.N ☑
105 941000021336919	Age: 4 years	1. Brought in from street ☐	Leishmania vaccination ☐ Date:
Sample date: 04/04/18	SEX Male	2. Born in house ☒	Collars Scalibor ☐ Other ☐
		3. Born in town ☐	Advantix ☐
		4. Born out of town ☐	Citronella ☐
		5. Unknown ☐	Anti-parasite ☐

CLINICAL SYMPTOMS	0	1	2	3		BODY SCORE		ECTOPARASITES	0	1	2	3	Observations/Treatment info:
					Enlarged nodes	1. Very, very thin							
Dermatitis					Lesions	2. Thin		Fleas	☒				
Alopecia					Conjunctivitis	3. Normal	☒	Lice	☒				
Hyperkeratitis					Lunettes	4. Fat		Ticks				☒	
Long nails		☒			Uveitis								

Figure 2.3: A clinical questionnaire used during samples collection during sample collection. Owners address and personal information in addition to clinical signs, dog body score and presence and severity of ectoparasites were recorded for all dogs. Dogs were classified as either oligosymptomatic or symptomatic depending on the number of clinical signs observed, or asymptomatic.

2.6 Entrainment of Volatile Organic Compounds (VOC's)

Approximately 1g of collected hair from samples collected in both 2017 and 2018 were introduced into a portable entrainment system in order to collect volatiles present. The portable entrainment set up is made up of a pump and rotameter confined in a 16 inch x 12 inch x 6 inch case (**Figure 2.4**).

Charcoal-filtered air supplied by the portable entrainment system (flow rate, 15ml sec^{-1}) was introduced into the top of a 50ml round-bottom glass flask which contained individual hair samples. The supplied air was extracted from the top of the glass flask and the VOCs collected in a 3.5-inch glass vial containing Tenax as the absorbent. Air entrainments of each hair sample were run for 2.5hours. Collected

VOCs were recovered through elution of each Tenax tube with 2ml Hexane to provide 1ml - 1.5ml of extract. A fresh Tenax tube was used for each entrainment. All eluted samples were concentrated to a final volume of approximately 500µl using nitrogen and stored at -20°C until further analysis. A caryophyllene internal standard was added to each sample following elution with hexane. 10µl of 50ng/µl caryophyllene was added to the 500µl of each sample which therefore contained 500ng of caryophyllene.

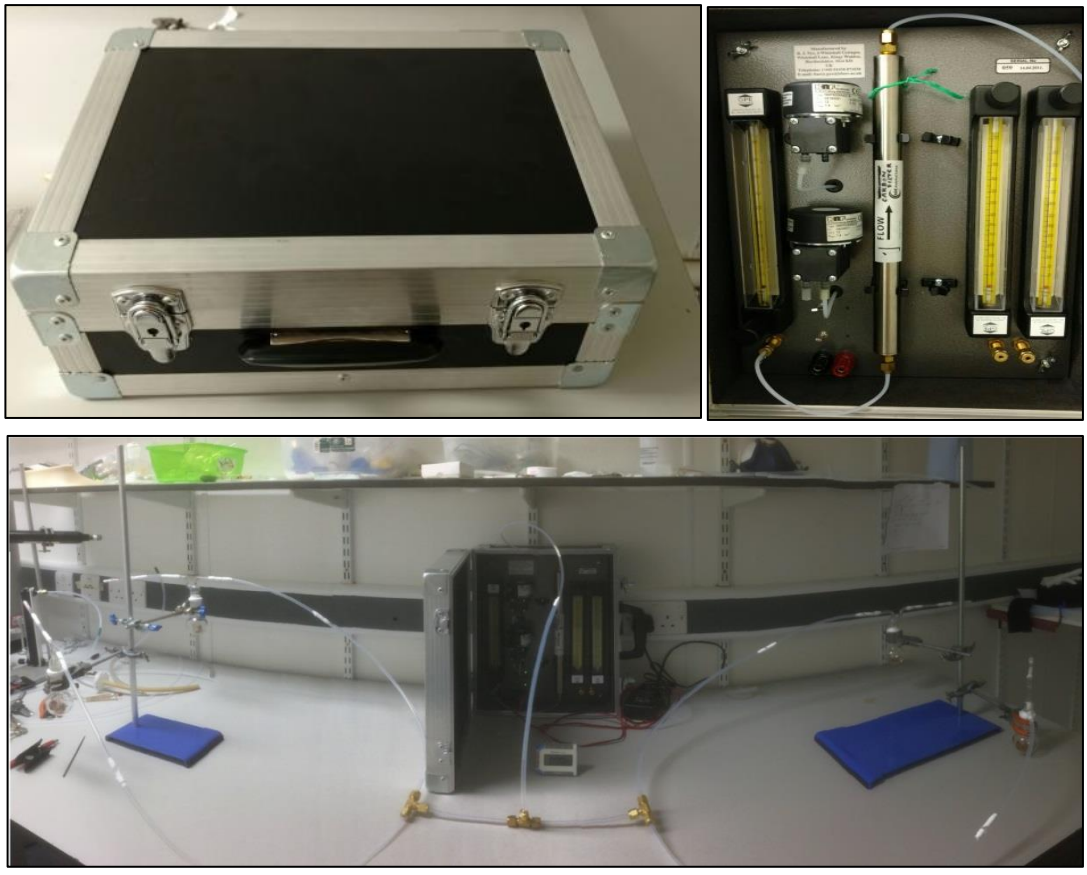


Figure 2.4: Portable entrainment system. The portable entrainment system is composed of a pump which supplies filtered air to a rotameter. Individual hair samples are placed in 50ml round bottom flasks and volatiles collected in Tenax vials. For the purpose of this experiment the flow rate was set at 15ml/sec.

Chapter 3- PCR and RT-RT-qPCR for the Diagnosis and Quantification of cVL

3.1 Introduction

Canine visceral leishmaniasis (cVL) is a zoonotic disease found in the Old and New Worlds. It is caused predominantly by *Leishmania infantum* and has been directly associated with human cases of visceral leishmaniasis (VL) (Nunes *et al.,* 2010). In urban areas, the domestic dog (*Canis familiaris*) is the main reservoir host for the disease which is transmitted through the bite of an infected phlebotomine sand fly. It is because these dogs live within close proximity to the human population that many cases of human VL have been reported (Alvar *et al.,* 2012). Accurate diagnosis in order to implement successful removal of these dogs from the environment is therefore essential in order to control the transmission of the parasite and reduce disease burden.

Traditional diagnosis was based on the direct identification of *Leishmania* in bone marrow aspirates by lab-based microscopy. However, the use of invasive procedures in order to obtain bone marrow samples, combined with a low sensitivity for the microscopic diagnosis of *Leishmania* (between 53% and 85%) demonstrate the limitations of this technique (Schallig *et al.,* 2019; Chappuis *et al.,* 2007). Serological testing was also a traditional methodology adopted for VL diagnosis (Varani *et al.,* 2017). Immunodiagnostic techniques, particularly IFAT and ELISA which detect antigens/antibodies in biological samples are still used widely for the diagnosis of both human VL and cVL. However, limitations have been identified (**Table 3.1**). Recent research has demonstrated a low sensitivity and specificity for these methods when

diagnosing VL. The IFAT demonstrated an inadequacy with sensitivities ranging from 18% to 93% for the diagnosis of human VL being reported (Mendonça *et al.,* 2017). Furthermore, studies have reported a low sensitivity of these methods for the diagnosis of *Leishmania* in immunosuppressed patients. The IFAT sensitivities ranged from 11% to 82% (Cota *et al.,* 2012). Molecular tests for the diagnosis of VL have also been developed over the past 10 years. Studies have demonstrated that PCR which targets the *Leishmania* kinetoplast DNA within clinical samples is a highly sensitive technique for VL diagnosis. Sensitivities as high as 98% have recently been reported (Khatun *et al.,* 2017). The analytical sensitivity of PCR has also shown the ability to detect between 0.1 and 0.001 parasites per ml of sample, with this methodology able to correctly diagnose vaccinated dogs; a limitation of serological methods (Travi *et al.,* 2018). However, there is still a lack of standardisation for PCR tests, with each individual laboratory differing in the setup of assays, DNA preparation, the PCR target and the reaction optimisation (Cota *et al.,* 2012).

Due to the limitations of the currently used diagnostic techniques, there are still challenges associated with the control of VL. The accurate identification of infected dogs is of importance, as a reduction in the number of positive dogs would in turn reduce the incidence of human VL. However, many countries currently rely on serological diagnostic methods rather than molecular ones. The resulted misdiagnosis of negative and positive dogs has led to controversy which surrounds the culling of seropositive, but clinically healthy dogs as well as allowing false negative dogs to

remain in the domestic environment. Therefore, the use of molecular methods should be considered as an alternative to alleviate the misdiagnosis which currently occurs.

Method and test/tissue used	Sensitivity (%)	Specificity (%)	Advantages	Disadvantages
Immunodiagnosis by:				
Antibody detection:				
IFA test	55-70	70-89		Do not differentiate between disease and symptomatic infection (Ghatei *et al.*, 2009).
Direct Antiglobulin Test	91-100	72-95	Low cost, high sensitivities observed for all methods Can be non-invasive with a diagnostic technique having been developed that can detect leishmania antibodies in urine using ELISA (Islam *et al* 2002).	Not always suitable for diagnosis of VL in immunocompromised patients like HIV/VL co-infected cases (Cruz *et al.*, 2006). Do not correlate the levels of antibodies that are circulating with the stage of the disease.
ELISA	72-100	87-97		Cross-reactions with other diseases reported (Sundar and Rai., 2002)
rK39 Rapid Diagnostic Test	46-78	90-99.9	Point of care, non invasive and cheap. Increased ease and rapid; a diagnostic result generated in minutes without the need for any specialist equipment (Quinnell *et al.*, 2013). Ease of use and interpretation (Paiva-Cavalcanti *et al* 2015)	Considerably varied sensitivity and specificity observed between different studies with a low sensitivity generally reported (Chapuis *et al* 2006) Sensitivity to diagnose canine infection much lower than detection of clinical disease (Quinnell *et al.*, 2013). Cross-reaction with other trypanosomatids possible (Paiva-Cavalcanti *et al* 2015)
Antigen detection:				
Latex Agglutination Test (LAT)	27-96	82-100	Rapid, inexpensive, point of care, reliable, easy to perform, relatively high sensitivity and specificity. Distinguish between the past and the current infections (Ghatei *et al.*, 2009) Antigen detection is more specific than antibody based diagnostics (Srivastava *et al.*, 2011).	Different sensitivities and specificities in different regions. Urine used for diagnosis must be boiled (Ghatei *et al.*, 2009).
Molecular Based Methods:				
PCR:			High specificities and sensitivities observed for all methods. Qualitative and semi-qualitative diagnosis. Practical, safe and reliable with promising results for leishmania diagnosis (Paiva-Cavalcanti *et al* 2015). Many different variatons of PCR possible such as nested-PCR or qPCR. Allows for characterization of sepecies of Leishmania (Mohammadiha *et al.*, 2013). Variety of different samples can be used e.g urine, conjunctival swabs, skin, blood etc. (Paiva-Cavalcanti *et al* 2015).	Not a point of care diagnostic. Relatively expensive to use. A high variability in the sensitivities of conventional PCR methods has been obsereved in the diagnosis of leishmania in both humans and dogs (Mohammadiha *et al.*, 2013). Taq Polymerase inhibitors are found within collected clinical samples as well as being commonly used for DNA extraction and sample collection (Yang and Rothman, 2004)
Blood	96	Depends on primers used.		
Bone marrow	100	Up to 100% reported		
Skin	93.8			
Microscopy	77	86	High specificity and cheap to use Ability to grade the parasites load on a logarithmic scale particularly in splenic smears (Chulay and Bryceson, 1983)	Variability in the sensitivity of detection. Requires a specially trained, expert microscopist. Time consuming technique.

Table 3.1: Current diagnostic methods used for cVL diagnosis. The sensitivities, specificities, advantages and disadvantages of the different methods used for the diagnosis of leishmaniasis are shown in the table above. Table adapted from Sundar and Rai, (2002).

3.1.1 Current Laboratory Based cVL Diagnosis

CVL is a widespread disease, found throughout both the Old and New Worlds. The incidence rate and distribution of cVL depends not only on human behaviour and location of the transmission sites, but also on the local ecological features and the characteristics of the *Leishmania* species present (Franco *et al.,* 2011). The diagnosis of infected dogs should be established as soon as possible in order to improve disease prognosis as well as avoid both dog and human transmission from false negative cases and preventable euthanasia from false positive ones (Gharbi *et al.,* 2015).

The current diagnosis of infected dogs considers not only the clinical signs observed, but also the epidemiological origin of the dog (**Figure 3.1**) (Solano-Gallego *et al.,* 2011). However, due to the nonspecific nature of some clinical signs, with other diseases found in areas of endemicity, such as mange (demodicosis), an inflammatory disease caused by the Demodex mite in dogs, presenting with very similar clinical signs (Mozos *et al.,* 1999), cVL diagnosis relies heavily on laboratory support. The origin of the infected dogs is an important factor considered when determining which laboratory test will be utilised; different serological, molecular and parasitological techniques are performed in different regions (Ribeiro *et al.,* 2018). Furthermore, although cVL is predominantly caused by *Le. infantum*, other *Leishmania* species have also been identified in infected dogs. These include *Le. tropica* in Europe (Ntais *et al.,* 2013) and *Le. amazonensis* (Valdivia *et al.,* 2017) and *Le. braziliensis* (de Lima Cunha *et al.,* 2006) in the Americas. Therefore, a diagnostic technique that is considered successful allows for the correct identification of *Leishmania* species. This in turn leads

to a greater accuracy in diagnostic tests resulting in a smaller percentage of false negatives and positives, that are a major hurdle facing cVL control.

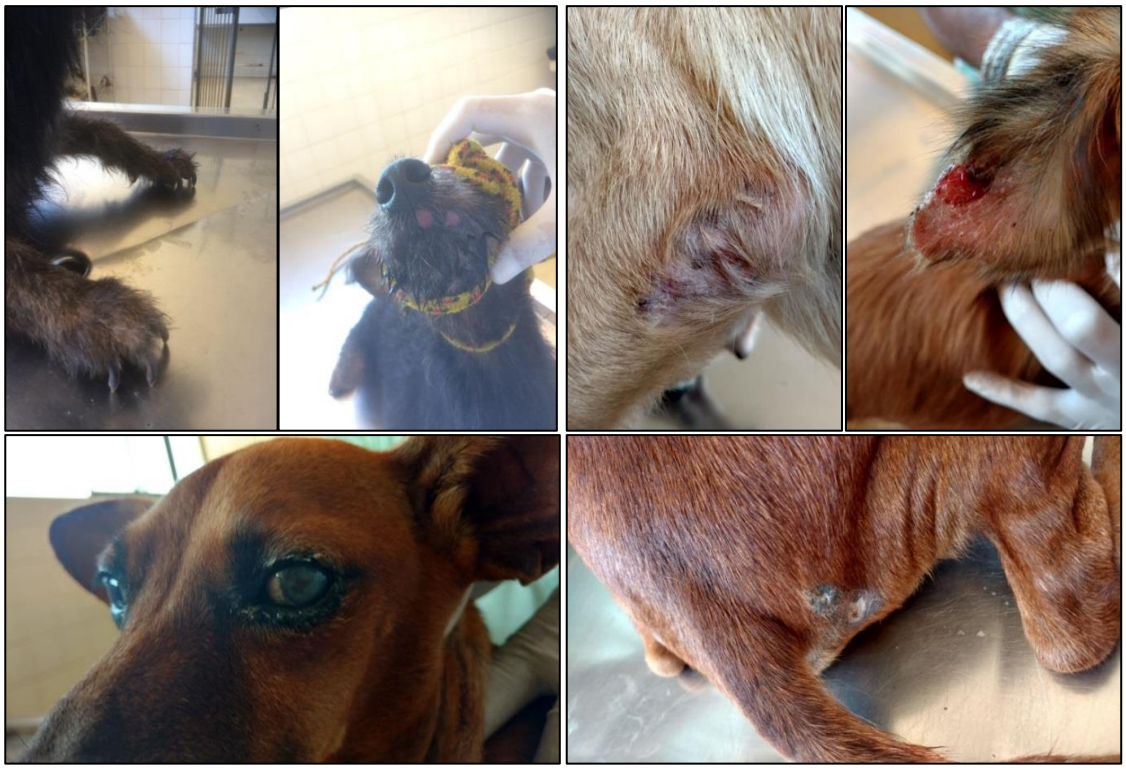


Figure 3.1: Clinical signs observed during sample collection: The main signs of cVL which were observed were crusted ulcers and lesions, dermatitis, onychogryphosis, cachexia, ophthalmologic abnormalities, and hepatosplenomegaly.

3.1.1.1 Europe (Old world)

Old world cVL was originally thought to be a disease found around the Mediterranean basin (**Figure 3.2**), where it was believed to have originated, as well as parts of Asia and Africa (Ready, 2014). In Europe, the main proven vectors for cVL are species from the *Phlebotomus* genus including predominantly *P. ariasi* and *perniciosus* (Ready, 2014). Cases of cVL have also been reported in countries in the western parts of Europe as well as the Balkan region (Franco *et al.,* 2011). Research has provided

evidence to show that cVL is expanding throughout Europe where dogs have been diagnosed as positive in areas such as north western Italy (Muccio *et al.,* 2015), Portugal (Maia *et al.,* 2016) and a recent case of leishmaniasis being reported in a UK dog with no travel history (McKenna *et al.,* 2019). The expansion of cVL northwards is in part due to territorial contiguity. This is often associated with global warming that is favouring vectorial transmission, but also by the importation of infected dogs to these areas (Maia and Cardoso, 2015). It has been estimated that CVL affects about 2.5 million dogs in the Mediterranean area each year (Moreno and Alvar, 2002). In 2011, it was reported that the seroprevalence rate of visceral leishmaniasis in domestic dogs in countries throughout Europe was 25%, with an estimated 700 new cases of visceral leishmaniasis in humans being reported (Franco *et al.,* 2011).

Currently, laboratory diagnosis of cVL in Europe is based on a combination of different criteria which include the presence of clinical signs. These include adenopathy, squamosis, onychogryphosis, localised alopecia and loss of weight (Solano-Gallego *et al.,* 2011). Microscopic identification of *Leishmania* parasites in lymph node aspirates and bone marrow is used extensively in Europe (Travi *et al.,* 2018). However, the low sensitivity of this technique makes diagnosis unreliable. Therefore, positive cases of cVL are confirmed by serological techniques, particularly ELISAs (Travi *et al.,* 2018). ELISAs have previously demonstrated 100% sensitivity for the detection of *Leishmania infantum* antigens in dogs in different states of infection in Italy (Solano-Gallego *et al.,* 2014) and 100% when diagnosing canine leishmaniasis using a dot-ELISA in Spain when a 1/800 cut off titre was established (Fisa *et al.,* 1997). Diagnostic PCR is also undertaken to identify infected dogs in Europe due to its high sensitivity and specificity as well as European countries being able to afford the

equipment required. A recent study demonstrated the accuracy of PCR protocols for the diagnosis of canine leishmaniasis in Portugal, with the higher overall percentage (37.6%) of infected dogs being detected using this technique (Albuquerque *et al.,* 2017). Prior studies also demonstrated a sensitivity of 100% when using PCR to diagnose cVL using lymph node aspirates in Italy (Reale *et al.,* 1999). Furthermore, PCR can also be utilised as a species-specific technique due to the availability of *Leishmania* species specific primers. Therefore, this methodology is currently being utilised more frequently due to other *Leishmania* species which have been identified as causing cVL in some regions of southern Europe (Ntais *et al.,* 2013).

Despite the apparent accuracy of the methodologies used throughout Europe, the diagnostic procedures used are very different from the cVL diagnostics that are undertaken in countries throughout South America. There are many reasons for this; the cost of veterinary diagnostic services which many cannot afford, the lack of guideline for CVL management and the lack of availability of molecular diagnostic tools (Dantas-Torres *et al.,* 2012).

3.1.1.2 The Americas (New World)

New World cVL is found primarily in Southern and Central America (**Figure 3.2**). However, several cases of cVL have also been reported in 21 U.S. states and 2 Canadian provinces predominantly in foxhound kennels (Petersen and Barr, 2009). Although primarily thought of as an imported disease in North America, the sporadic appearance of leishmaniasis not only in foxhounds but also in other species of dogs with no travel history outside North America indicates the potential of the disease to spread to new regions (Duprey *et al.,* 2006). The *Leishmania infantum* parasite is

believed to have been introduced to the Americas through domestic dogs brought over from Spain and Portugal during the last 500 years (Lukes *et al.,* 2007). Throughout the Americas, *Leishmania infantum* is primarily transmitted by *Lutzomyia longipalpis* sand-flies, however, other *Lutzomyia* species including *Lutzomyia migonei* have also been shown to be potential vector for this parasite (Guimarães *et al.,* 2016).

In South America, Brazil is of importance with a reported 90% of the total annual cVL cases. Until 2012, the IFAT was the primary laboratory diagnostic test adopted by the Brazilian Ministry of Health for dog screening and culling campaigns. However, this technique has shown limitations. Not only is the IFAT restricted to a laboratory setting (Solano-Gallego *et al.,* 2011) but varying specificities and sensitivities have also been reported. The sensitivity of the IFAT has been shown to range from 68% to 100% with the specificity ranging from 52% to 100% when diagnosing cVL in Brazil (da Silva *et al.,* 2006; Ferreira *et al.,* 2007). Further notable limitations include potential cross-reactivity with other pathogens including trypanosomes (Paltrinieri *et al.,* 2016) and a significantly lower sensitivity for the diagnosis of asymptomatic dogs when compared with ELISA (Coura-Vital *et al.,* 2014). The relatively low sensitivity of this test for asymptomatic diagnosis, the complicated execution of the technique and the long turnaround time between sample collection and culling of the infected dogs are possible reasons for the poor effectiveness of culling campaigns (Romero and Boelaert *et al.,* 2010). ELISA is currently the most utilised methodology throughout Brazil, generally demonstrating a higher sensitivity for the diagnosis of cVL. ELISA sensitivity has been shown to range from 91% to 97% with the specificity ranging from 83% to 98% when diagnosing cVL in Brazil (da Silva *et al.,* 2006; Ferreira *et al.,* 2007). It is due to the higher sensitivity of the technique that in 2012 the Brazilian Ministry of Health

adopted the ELISA as the recommended laboratory-based protocol for cVL diagnosis. Research has shown that the modification of this protocol should have a significant impact of cVL diagnosis. When analysing the plasma samples of 1,226 dogs using the old protocol a prevalence rate of 6% was reported. However, when analysing the same samples using the new adopted methodology, a prevalent prevalence of 8% was demonstrated (Coura-Vital *et al.,* 2014).

3.1.2 Molecular diagnosis of cVL

3.1.2.1 PCR as a Diagnostic Technique for cVL

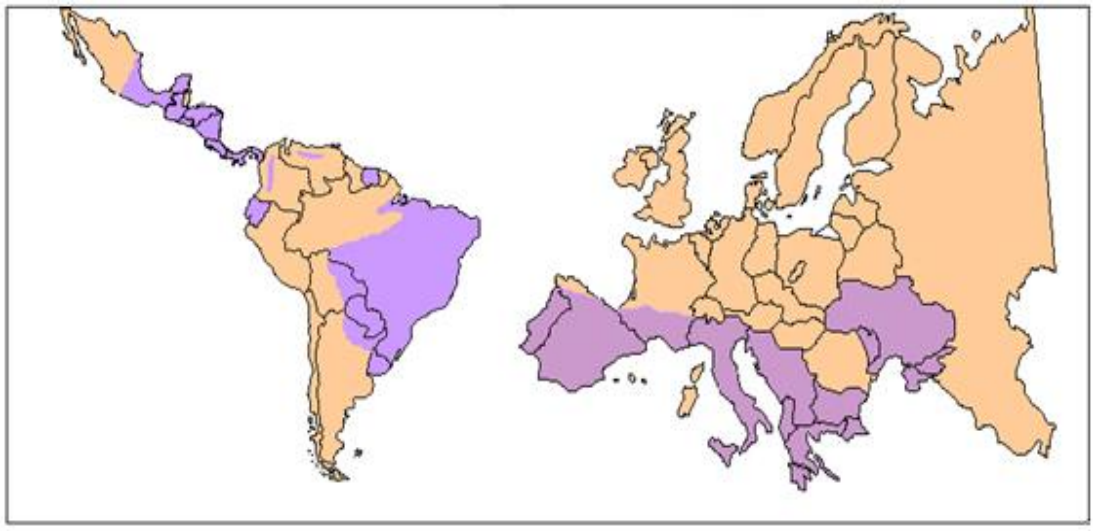


Figure 3.2: Distribution of Canine Visceral Leishmaniasis. Distribution of Canine Visceral Leishmaniasis in the Americas (left) and Europe (right) Pictures adapted from University of East London.

An accurate diagnosis of cVL is essential for the control of visceral leishmaniasis. A variety of molecular techniques have proven to be highly effective in the diagnosis of canine leishmaniasis, none more so than several PCR methods. Over the past decade, many PCR based methodologies have been developed for the detection, quantification and identification of *Leishmania* species using specific

primers to target DNA sequences. These include *Leishmania* rRNA genes and kinetoplast minicircle constant regions. Many studies have reported the use of PCR for the diagnosis of cVL using a variety of biological samples including blood, tissue fragments and aspirates (Lachaud *et al*., 2002; Almeida *et al.,* 2013). PCR has proven more effective and accurate in cVL cases which had not been resolved by classical diagnostic methods demonstrating a gain in sensitivity and specificity (Moreira *et al.,* 2007). Gomes *et al* (2007) demonstrated the effectiveness of PCR compared to traditional microscopy and culture parasitological methods. PCR showed a positive result for 100% of the parasitological confirmed positive cases, also demonstrating a high specificity when identifying negative dogs, detecting all 61 dogs (100%) which were negative for cVL. The parasitological test however, only identified 56 negative dogs (92%), with 5 of the dogs generating a false positive result. Furthermore, when compared to serological methods, PCR not only showed a similar sensitivity to an ELISA, but also demonstrated a much greater homogeneity for the diagnosis of asymptomatic and symptomatic dogs when compared to the ELISA and DPP protocol currently used in Brazil (de Carvalho *et al.,* 2018).

Due to different *Leishmania* species having been identified as causative agents for cVL, the identification of the *Leishmania* species involved in infection is extremely important. This is also essential in endemic areas which have both the cutaneous and visceral forms of the disease (Quaresma *et al.,* 2009). With respect to cVL, serological methods have shown an inadequacy when differentiating between different species of *Leishmania*. This is a major limiting factor given the presence of *Le. braziliensis*, *Le. infantum* and *Le. amazonensis* in canines particularly in the area studied (Dantas-Torres, 2007, Valdivia *et al.,* 2017). The use of species-specific primers in PCR diagnosis,

which amplify conserved sequences found in the Kinetoplast Deoxyribonucleic acid (kDNA) minicircles of different strains of *Leishmania*, has allowed for species specific diagnosis. This has proven highly advantageous in the development of new treatments, with many endemic regions having multiple *Leishmania* species all of which require different therapy (Schriefer *et al.,* 2009). Studies have reported that PCR based techniques including conventional PCR, PCR-RFLP (restriction fragment length polymorphism) and real-time quantitative PCR (RT-qPCR), have the ability to differentiate between various *Leishmania* species (Andrade *et al*., 2006; Marcussi *et al.,* 2008, de Morais *et al.,* 2016). Primers are being continuously designed for the identification of different *Leishmania* species using PCR. Primers are currently developed in order to identify genus, subgenus and species of *Leishmania,* therefore making them highly specific (Marcussi *et al.,* 2013). The adoption of specific primers for the diagnosis of infected dogs allows for PCR protocols to become standardised, a current limitation of molecular methods for cVL diagnosis.

The substantial advantages of PCR including *Leishmania* species specificity and ability to diagnose symptomatic, asymptomatic and vaccinated dogs suggest this methodology should be adopted throughout Brazil as a means to diagnose cVL. The possibility of less invasive sampling using peripheral blood and a better negative predictive value, are also influencing factors to be considered when adopting new diagnostic protocols. Therefore, the utilisation of PCR for the diagnosis of cVL in this study was suggested, with specific primers chosen in order to identify those dogs infected with *Le. infantum*. However, despite the advantages of PCR based *Leishmania* diagnostics, there is still a lack of standardisation for the PCR tests and it has not been

clarified as to whether molecular techniques should be considered as the “gold standard” for VL diagnosis (Varani *et al.,* 2017).

3.1.2.2 RT-qPCR Quantification of *Leishmania infantum* Parasitic DNA

Although highly accurate in the detection of symptomatic and asymptomatic dogs, conventional PCR is only, at best, semi-quantitative. An accurate and rapid method to detect and quantify *Leishmania* parasites is vital. This not only allows for an early diagnosis of leishmaniasis but also monitoring the effect of anti-*Leishmania* therapy (Verma *et al.,* 2010) and determining transmission capabilities of an infected dog. This is due to research showing that animals with a higher parasite load transmit a high percentage of the parasite to sand flies (Kimblin *et al.,* 2008).

Real Time quantitative PCR (RT-qPCR) has become progressively more popular over the last few years not only for the detection and quantification of *Leishmania*, but also for species specific identification (Galluzzi *et al.,* 2018). Previous research demonstrated the ability of RT-qPCR to diagnose and distinguish between malaria species with 97.4% sensitivity (Mangold *et al.,* 2005) as well as demonstrating a detection limit of 100 parasites/mL of blood (0.1 genome equivalents) when used to diagnose trypanosomiasis (Becker *et al.,* 2004). Recently, research has demonstrated a high sensitivity of 100% when using RT-qPCR to detect *Le. infantum* in the spleen and skin of infected dogs (Nunes *et al.,* 2018). Furthermore, studies have also demonstrated that RT-qPCR is as equally effective as conventional PCR for the detection of *Leishmania* parasites in blood (Pereira *et al.,* 2014). Although RT-qPCR based methodologies have proven effective in the diagnosis of cVL, a standardised

method still does not exist, a limiting factor when considering the use of RT-qPCR as a routine cVL diagnostic (Galluzzi *et al.,* 2018).

In addition to the high sensitivity observed, the ability to quantify parasite load in infected dogs through RT-qPCR allows for the determination of the status of positive dogs. The determination of the parasite load in clinical cases of cVL has become an indispensable tool for the diagnosis of leishmaniases. The continuous data generated through RT-qPCR could provide a better understanding for clinicians managing cases of cVL by differentiating between dogs infected with *Leishmania* or dogs with the active leishmaniasis (Otranto and Dantas-Torres, 2013). Larger studies are, however, necessary for the clarification of the dividing line between disease and infection.

3.1.3 Chapter Aims

PCR has previously been used as an analytical method for the diagnosis of cVL in numerous studies, directly revealing the presence of small parasite numbers in samples in addition to demonstrating a high sensitivity and specificity (Travi *et al*., 2018). In this chapter I aim to use PCR as a diagnostic tool for canine leishmaniasis, identifying positive dogs for further VOC Analyser analysis. Furthermore, using RT-qPCR I aim to determine the parasite load in those dogs which are infected, allowing for the diagnostic threshold of the VOC Analyser to be determined and to provide evidence on the origin of the VOCs identified in positive dogs.

3.2 Methods

3.2.1 Optimization of Parasite Detection

3.2.1.2 Parasite culture

Leishmania infantum promastigotes (strain M4192) supplied by Paul Bates at Lancaster University, were grown in 20% Foetal Bovine Serum (FBS) hemoflagellate-modified minimum essential medium (HOMEM) in 25ml plastic culture flasks and stored in a 26^{o}C incubator. Multiplication was observed over three days (72 hours) when the medium contained flagellate forms of the parasite (promastigotes) that were appropriate to perform a passage. Successful 1:100 passages in medium were made to a total volume of 10ml. Passages were performed every three days to continue the optimal growth of the culture.

3.2.1.2 DNA Extraction of seeded samples

200 ml of buffy-coat was obtained following centrifugation of 5 ml of beagle whole blood (K2 EDTA) supplied by Seralab at 2500 x g for 10 minutes at room temperature. Seeded samples were made by adding [10^{5}] live pelleted *Le. infantum* promastigotes (M4192) to the canine buffy coat. Serial dilutions (1:10) were performed in order to obtain parasite concentrations ranging from [10^{5}] to [10^{-3}] parasites mL^{-1} of blood, corresponding to DNA equivalents of 1000, 100, 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 parasites per PCR tube. The DNA was extracted from these samples using the QIAamp DNA Blood Mini Kit (Qiagen) following the manufacturer's instructions with 200µl of buffy-coat. The lysis was based on protein kinase with a final elution volume of 50µl.

3.2.1.3 Primer Optimisation

Two sets of primers were compared for sensitivity when used in conventional PCR for detecting low parasite concentrations; MaryF (5' – CTT TTC TGG TCC TCC GGG TAG G – 3'), and MaryR (5'- CCA CCC GGC CCT ATT TTA CAC CAA – 3') which amplify a 140bp conserved region of the *Leishmania* kDNA minicircle (Mary *et al.,* 2004) (**Figure 3.3**) and LeF (5' –TCC GCC CGA AAG TTC ACC GAT A - 3') and LeR (5' – CCA AGT CAT CCA TCG CGA CAC G - 3') which amplified a 400bp region of *Leishmania* rRNA ITS-1 (Spanakos *et al.,* 2008).

The reactions were performed in a final volume of 25µl with 0.5µl DNA template (100ng μl^{-1}), 12.5µl Mastermix (dH_2O, Buffer 5x, MyTaq redmix polymerase, dNTP's) and 10µM of each primer (MaryF/R or LeF/R). The PCR amplification was performed in a TECHNE® Prime Thermal Cycler (Bibby Scientific) using the following conditions: 95°C for 5mins and 30 cycles of 95°C for 30sec, 57°C for 30sec and 72°C for 60sec, followed by 72°C for 10min.

1 aatggtcaaaaatagcccaaaattccaaa**ctttttctggtcctccgggtag**gggcgttctg
61 cgaaaaccgaaaaatgggtgcagaaatcccgttcaaaaaatggctgaaaatgccgaaaat
121 cggctccggggcgggaaactgggggg**ttggtgtaaaatagggccgggtgg**tggctggaaat

Figure 3.3: *Leishmania infantum* kDNA minicircle DNA partial sequence and primer location. The bold underlined sequences represent the MaryF and MaryR primer pair (Mary *et al.,* 2004).

3.2.1.4 Primer Specificity

Due to recent research identifying dogs infected with *Le. (L.) amazonensis* and *Le. (V.) braziliensis* in Brazil (Souza *et al.,* 2019), MaryF/R primer specificity was determined. Canine buffy coat was seeded using live pelleted *Le. (L.) infantum* (strain M4192), *Le. (L.) amazonensis* (strain M2269) and *Le. (V.) braziliensis* (strain LTB300) promastigotes stabilites. DNA was extracted according to the protocol in Chapter 2 and conventional PCR performed under the same conditions as mentioned above.

3.2.2 Molecular Diagnosis of Dogs

3.2.1.1 Real time qualitative PCR detecting *Leishmania* DNA

Extracted DNA from canine blood from both sampling occasions (August 2017 or April 2018) was tested using conventional PCR to diagnose those dogs which were positive for *Leishmania* infantum. Primer pair MaryF and MaryR (Mary *et al.,* 2004) was used due to their specificity for *Le. infantum* and their ability to detect low parasite concentrations. Reaction conditions as described above.

3.2.1.2 Agarose gel electrophoresis

Gel electrophoresis was performed using 2% agarose gels prepared with 1x TAE buffer. The gels were run at 90 V for 1hr 30 minutes and visualized under UV light following the addition of 1µl of 40x gel red to each sample when loading the gel.

3.2.1.3 RT-qPCR

Real-time quantitative PCRs (RT-qPCR) for the detection and quantification of *Le. infantum* DNA in positive dog samples from both sampling occasions (August 2017

or April 2018) were performed using MaryF/R primers. The RT-qPCR amplifications were performed on a Bio-Rad C1000™ Thermal Cycler with each reaction consisting of a final volume of 13.0µl; 12.0µL of PCR mix plus 1µL of DNA (approximately 75-100 ng μl^{-1} per reaction). The RT-qPCR mix was composed of 6.25 µL 2x QuantiNova SYBR Green PCR Master Mix, 0.5 µL of each primer (MaryF/R, corresponding to 10 mmol) and 4.75 µL of water as described by Costa Lima Junior *et al,* (2013).The amplification was performed under the following conditions as described by Ceccarelli *et al,* (2014): 94°C for 10 min, followed by 40 cycles at 94°C for 30 sec, 60°C for 20 sec and 72°C for 20 sec. At the end of each run, a melt curve analysis was performed from 55°C to 95°C in order to identify the formation of non-specific products as well as primer dimers. Each reaction was performed in triplicate. A standard curve was established using extracted *Le. infantum* DNA; 1:10 serial dilutions, ranging from 10,000 to 0.01 parasites per ml.

3.2.1.4 Internal control

The genomic DNA extracted from the canine buffy coat underwent agarose gel electrophoresis in order to detect its integrity as well as its molecular size and to roughly determine the DNA content. Furthermore, in order to determine the effectiveness of the DNA extraction and the transport of samples from Brazil to the UK, and to verify negative results for both PCR and qPCR, amplification of the constitutive canine globin gene was performed using the primers: '5 - CAA CTT CAT CCA CGT TCA CC – 3' and '5 - ACA CAA CTG TGT TCA CTA GC – 3' (Greer *et al.,* 1991) under the same conditions as the samples. A positive result with the canine housekeeping gene was considered a successful validation of sample integrity.

3.3 Results

3.3.1 Optimisation

3.3.1.1 PCR

Buffy coat samples extracted from canine blood supplied by Seralab were seeded with $[10]^5$ live *L. infantum* (M4192) promastigotes. In order to determine sensitivity of both sets of primers (Mary F/R and LeF/R), 1:10 serial dilutions were performed with a minimum concentration of $[10]^{-3}$ parasites/ml. The sensitivity of each PCR reaction was assessed by determining the intensity of each banding pattern in the gel red stained agarose gel (Figure 6). At higher concentrations ($\geq[10]^3$ parasites/ml), the bands produced by MaryF/R primers showed a greater intensity. However, there was no difference in PCR sensitivity of both sets of primers and both were still able to detect *Leishmania* DNA at high concentrations (**Figure 3.4**). The differences between the primer pairs became significant at low concentrations ($\leq[10]^2$ parasites/ml). LeF/R were able to detect a minimum parasite concentration of $[10]^0$ whereas MaryF/R were able to detect a minimum concentration of $[10]^{-2}$, therefore, showing a higher sensitivity for *Le. infantum* detection in canine buffy coat.

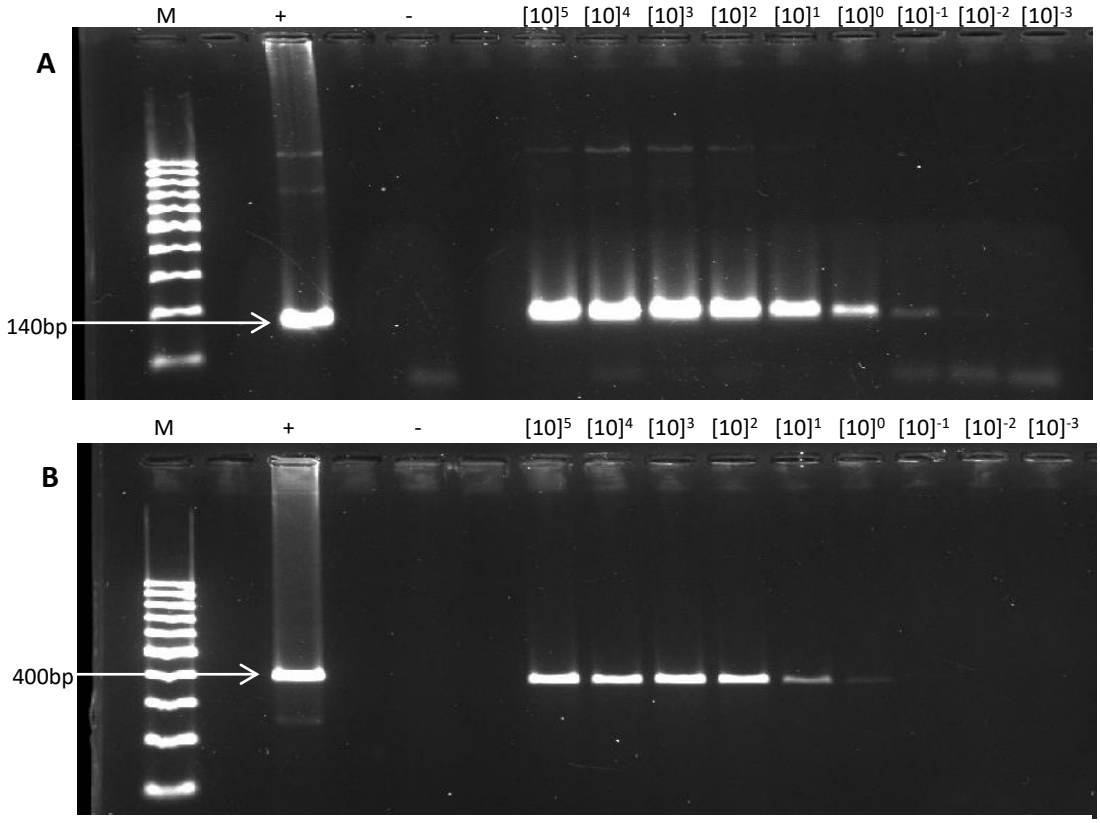


Figure 3.4: Gels showing sensitivity of two sets of primer pairs for the detection of *Le. infantum*. Two PCR reactions using A; MaryF/R primers which amplify a 140bp conserved region of the *Leishmania* kDNA minicircle and B; LeF/R primers which amplify a 400bp region of *Leishmania* rRNA, was carried out using template DNA extracted from cultured *Leishmania infantum* promastigotes (M4192). M, molecular weight marker (100bp DNA ladder); +, positive control; -, negative control (water).

3.3.1.2 Primer specificity

Due to the high sensitivity of MaryF and R, combined with recent research identifying dogs infected with *Leishmania amazonensis* and *braziliensis* in Brazil (Souza *et al.,* 2019; Valdivia *et al.,* 2017), the specificity of MaryF and MaryR primers for New World species was determined. PCR assays were performed using *Leishmania* species: *Le. (L.) infantum, Le. (L.) amazonensis* and *Le. (V.) braziliensis* seeded in dog buffy coat.

As represented in **Figure 3.5**, primers MaryF and MaryR were able to amplify all these New World species with varying sensitivity. An additional BLAST search however highlighted a lack of sequence homology for MaryF and MaryR primers in *Le. (L.) amazonensis* and *Le. (V.) braziliensis* kDNA minicircle sequences.

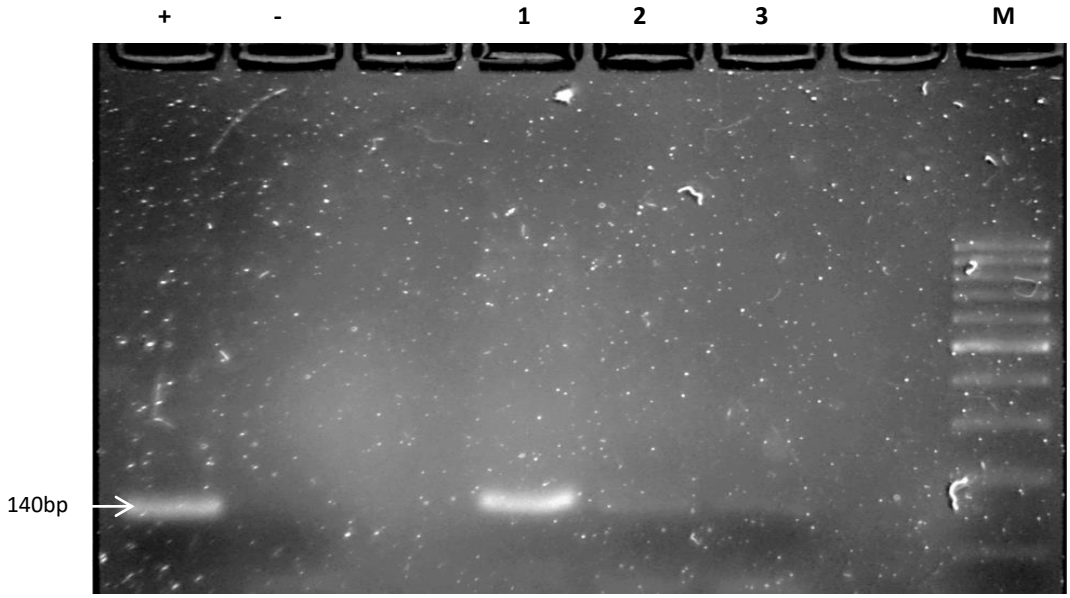


Figure 3.5: MaryF/MaryR specificity PCR. PCR was conducted using MaryF/R primers in order to determine primer specificity for *Leishmania infantum*. 1: *Le. infantum* (140.2ng/μl); 2: *Le. amazonensis* (99.5ng/μl); 3: *Le. (V.) braziliensis* (125.9ng/μl). A 100bp ladder (M) was used as a reference panel. +, positive control; -, negative control.

3.3.1.3 SYBR green qPCR

The sensitivity of the SYBR green RT-qPCR reaction using MaryF/R primers was tested by using 1:10 serial dilutions of *Leishmania infantum* DNA extracted from a known quantity of cultured parasites. **Figure 3.6** presents the standard curve, efficacy and slope generated with concentrations ranging from $[10]^4$ to $[10]^{-2}$ *Le. infantum* parasites per ml of canine blood. The generated standard curve was subsequently used

to detect unknown parasite concentrations in positive canine blood samples collected from Brazil during both sampling occasions (August 2017 or April 2018).

The results show the *Le. infantum* amplicon having melting temperature of approximately 81^{o}C, without the formation of primer dimers or non-specific products (**Figure 3.7**). The RT-qPCR assay showed a sensitivity ranging from 10,000 parasites per ml of blood to 0.01 parasites/mL using calibration curves constructed with serial dilutions of *Le. infantum* DNA. The MaryF/R pair of primers was able to amplify the DNA of *Le. infantum* effectively.

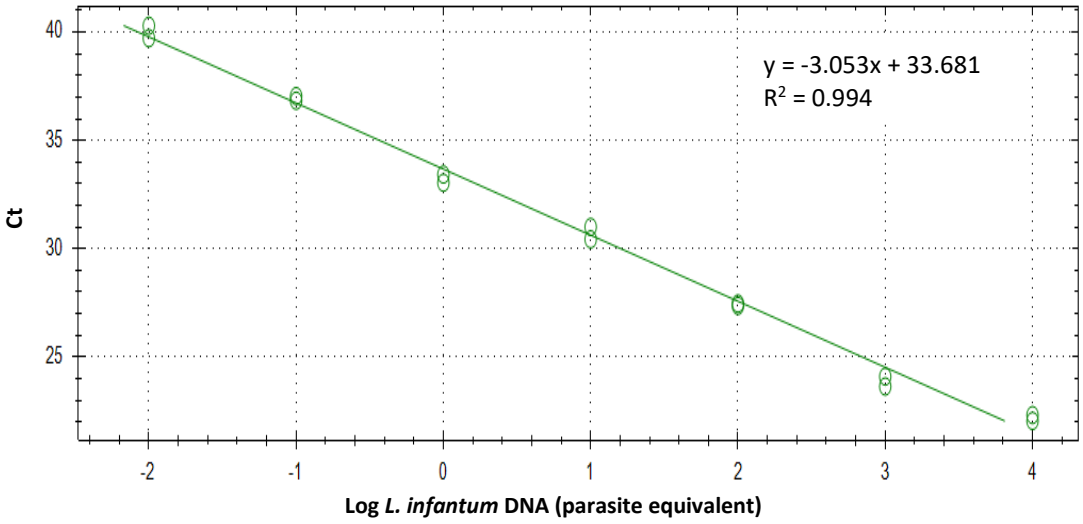


Figure 3.6: Standard curve constructed using serial dilutions of *Le. infantum* DNA. A standard curve was obtained using serial dilutions of *Le. infantum* M4192 DNA with primers MaryF and MaryR. The standard curve was obtained with serial dilutions ranging from 10,000 to 0.01 parasites equivalent/mL of whole canine blood. Slope, −3.053; PCR efficacy, 112.6%; R squared, 0.994.

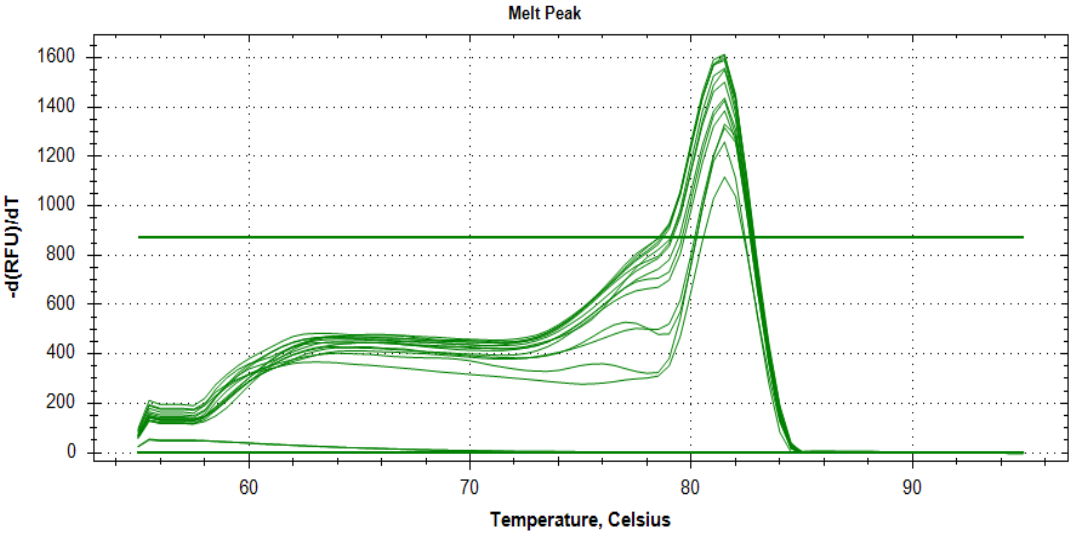


Figure 3.7: Melting curve analyses of PCR products. Dissociation curve of the amplification products of *Le. infantum* obtained using primers MaryF/MaryR showing peaks at 81.50 °C and from three negative samples.

3.3.2 DNA degradation and purity

3.3.2.1 DNA Degradation

The purity and integrity of the DNA extracted during both sampling occasions (August 2017 or April 2018) were analysed using gel electrophoresis in order to determine DNA integrity and identify any degradation.

As shown in **Figure 3.8**, the molecular weight of the extracted genomic DNA was greater than 1 kilo base, indicating that the genomic DNA was not only extracted intact, but also that the DNA integrity and purity were high. The brightness of the extracted DNA was uniform across all samples, confirming that the extraction efficiency was consistent.

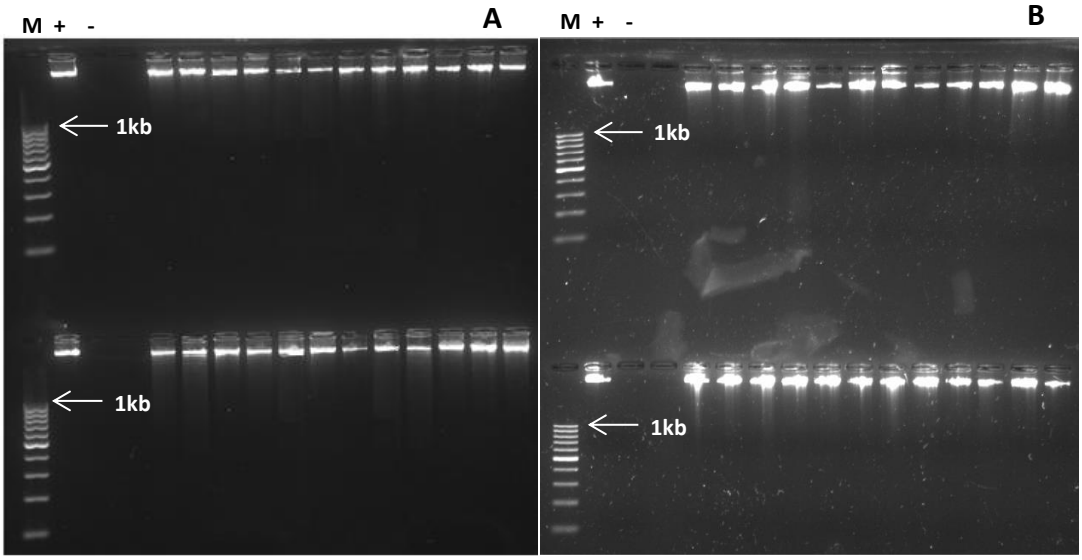


Figure 3.8: Agarose gels of genomic DNA of 24 random samples extracted from canine buffy coat in A; August 2017 and B; April 2018. Blood samples were collected, and genomic DNA extracted from 200µl of buffy coat. The whole genomic DNA obtained was analysed using agarose gel electrophoresis. M, molecular weight marker (100bp DNA ladder); +, positive control; -, negative control.

3.3.2.2 Canine beta-globin

In order to determine the effectiveness and integrity of each PCR reaction, and to check for DNA degradation within the samples due to transport of the DNA from Brazil to the UK, the amplification of the constitutive canine beta-globin gene (internal control) (Quaresma *et al.,* 2009) within 24 random samples for both August 2017 or April 2018 were performed (**Figure 3.9**). Canine beta-globin, has constant expression levels and a relatively conserved gene sequence under various conditions. If there is a relatively complete canine genome DNA template, canine beta-globin can be amplified in all collected samples. Also, if the purity of the extracted DNA is high, canine beta-globin can also be easily amplified. Gene amplification confirmed that the extracted canine genomic DNA was complete and its purity was high, indicating that it can be amplified effectively in vitro.

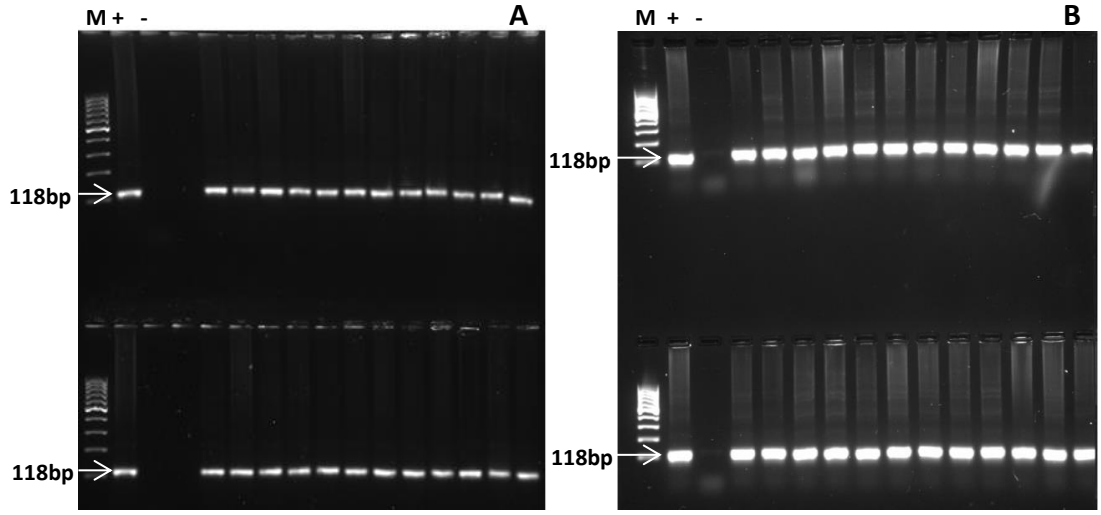


Figure 3.9: Agarose gels showing the amplification of the constitutive canine - β globin gene for 24 random samples from A; August 2017 and B; April 2018. DNA integrity gels show that all DNA samples used were intact indicating no DNA degradation during sample transport from Brazil to UK. M, molecular weight marker (100bp DNA ladder); +, positive control; -, negative. control.

3.3.3 Molecular diagnosis

3.3.3.1 Evaluation of PCR Diagnosis

Results from the molecular diagnosis of each sample collected in the field in both August 2017 and April 2018, identified 11/185 (6%) and 34/133 (26%) dogs as being positive for *Le. infantum* respectively (**Table 3.2 and 3.3**). As the same dog population was revisited in 2018, a 20 percent increase in infection rate can therefore be confirmed over this period. This increase in infection rate over the 9-month period with 33 dogs becoming infected in this period implicates this area (Altinopolis, Governador Valadares) as an endemic area for canine leishmaniasis.

In 2017, three out of the 11 positive dogs presented with one or more clinical signs of cVL, the others being asymptomatic. While in 2018, ten out of 34 positive dogs presented with clinical signs. According to the clinical evaluation performed on all the dogs, the most frequently observed clinical signs were skin lesions including dermatitis (18%; 2017, 27%; 2018) and ulcerative lesions (25%; 2018), long nails (9%; 2017, 25%; 2018) and some dogs also showed signs of conjunctivitis (18%; 2017, 14%; 2018).

Following sample collection from 16 dogs which were diagnosed as being positive by the Brazilian Central Control of Zoonosis in April 2018, only ten (63%) classified as being infected following the DPP and ELISA analysis by the CCZ were confirmed as positive following the PCR analysis undertaken in this research. The rest (6 cases) were deemed to be false positives diagnosed incorrectly by the CCZ.

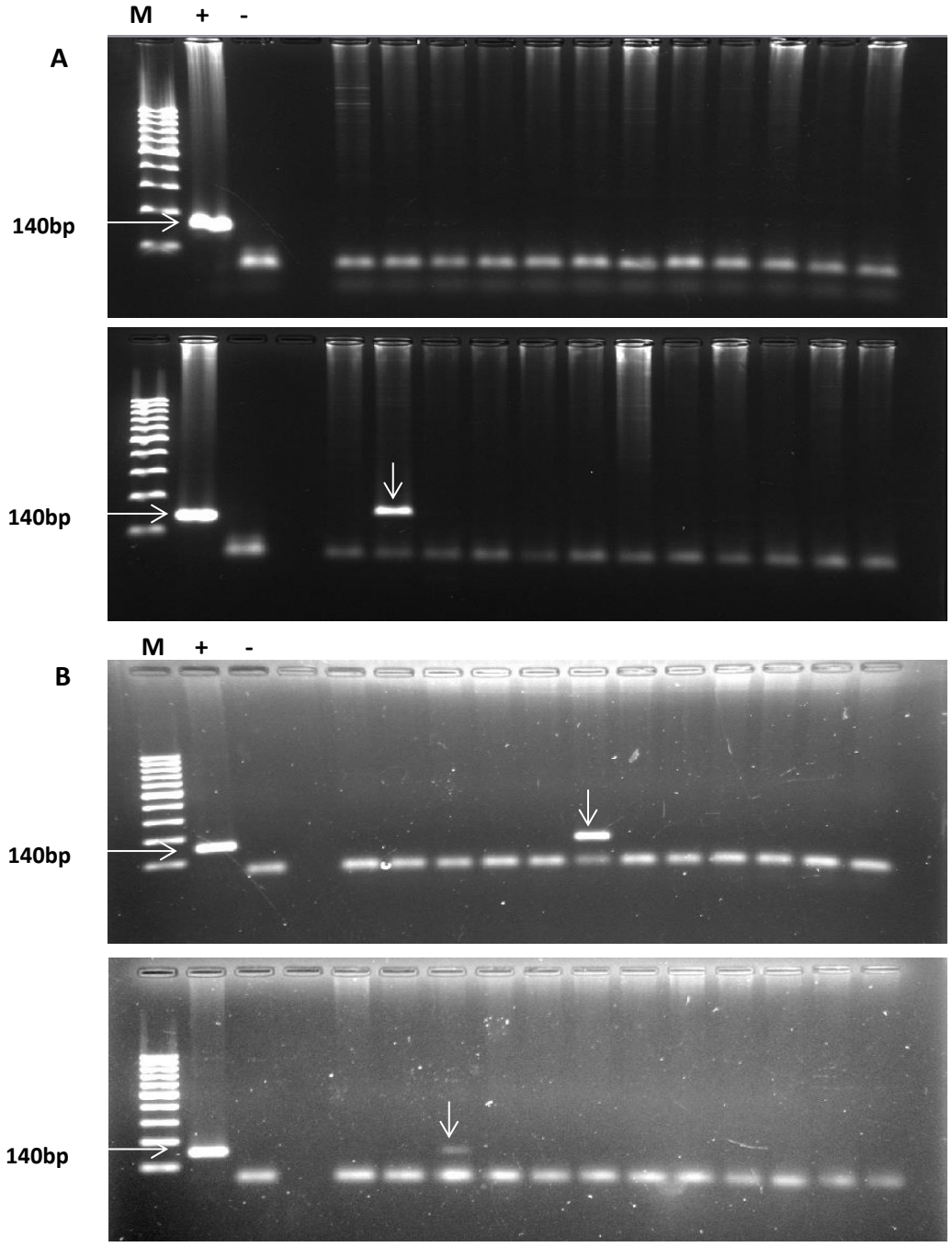


Figure 3.10: SYBR safe-stained 2% agarose gels showing the results of electrophoresis of 24 random PCR products from A; April 2017 dogs and B; August 2018 dogs. Positive dogs are represented by a 140bp band visible in line with the PCR product for the *Leishmania* positive control (indicated by white arrows). M, molecular weight marker (100bp DNA ladder); +, positive control; -, negative control.

	Aug-17			Apr-18					
	In field			In field			Central Control Zoonosis		
Samples Collected	185			133			16		
	Replicate 1	Replicate 2	Replicate 3 (confirmatory)	Replicate 1	Replicate 2	Replicate 3 (confirmatory)	Replicate 1	Replicate 2	Replicate 3 (confirmatory)
Positive dogs	8	10	11	26	30	34	9	9	10
Negative dogs	177	175	174	107	103	99	7	7	6
% infection rate	**5%**	**6%**	**6%**	**20%**	**23%**	**26%**	**56%**	**56%**	**63%**

Table 3.2 Summary of PCR results for *Leishmania infantum* detection in dogs from Governador Valadares, Minas Gerais, Brazil. 185 and 133 dogs were collected in the field in both August 2017 and April 2018 respectfully along with 16 dogs diagnosed as 'positive' by the Central Control of Zoonosis over a period of 2 weeks in April 2018. PCR experiments were performed in triplicate with positive and negative dogs identified.

Date of collection	Location of dogs	Number of dogs examined	Positive Dogs			Prevalence (%)
			S	O	A	
Aug-17	In field (Altinópolis)	185	0	3	8	**6.0**
Apr-18	In field (Altinópolis)	133	3	7	24	**25.6**
	CCZ (Governador Valadares)	16	7	1	2	**62.5**

Table 3.3 Percentage infection rates and clinical symptoms of positive dogs. Dogs were classified as asymptomatic (A) (the absence of clinical signs), oligosymptomatic (O) (the presence of one to three clinical signs), or symptomatic (S) (the presence of more than three clinical signs) according to the criterion adapted from Mancianti *et al.,* 1988)

3.3.3.2 RT-qPCR Evaluation of Parasitic Burden in Dogs with Suspected cVL

The kDNA RT-qPCR assay allowed for the quantification of the parasite load in all positive dogs from August 2017/April 2018 and the CCZ. Parasite loads ranged from 103 to 0.4 parasites mL^{-1} in 2017 compared to 2018 (both in the field and from the CCZ) where the range was from 850 to 1 parasite/mL (**Figure 3.11**).

This large variation in parasitic load over the two years can be observed through analysis of the median values which ranged from 5.06 parasites/mL (dog #116) in 2017 to 28.32 parasites/mL (dog #178) in 2018. Comparisons of parasitic load among the samples revealed that dog #126 in 2018 (853.44 parasites/ml) exhibited the highest degree of parasitism with dog #146 in 2017 (0.41 parasites/ml) exhibiting the lowest.

The average value of Ct (ΔCt) obtained for dog #126 in 2018 (highest degree of parasitism) and for dog #146 in 2017 (lowest degree of parasitism) were the following: dog #126; 20.43 and dog #146; 30.45. A lower Ct value correlates with a higher parasitic load per ml of blood.

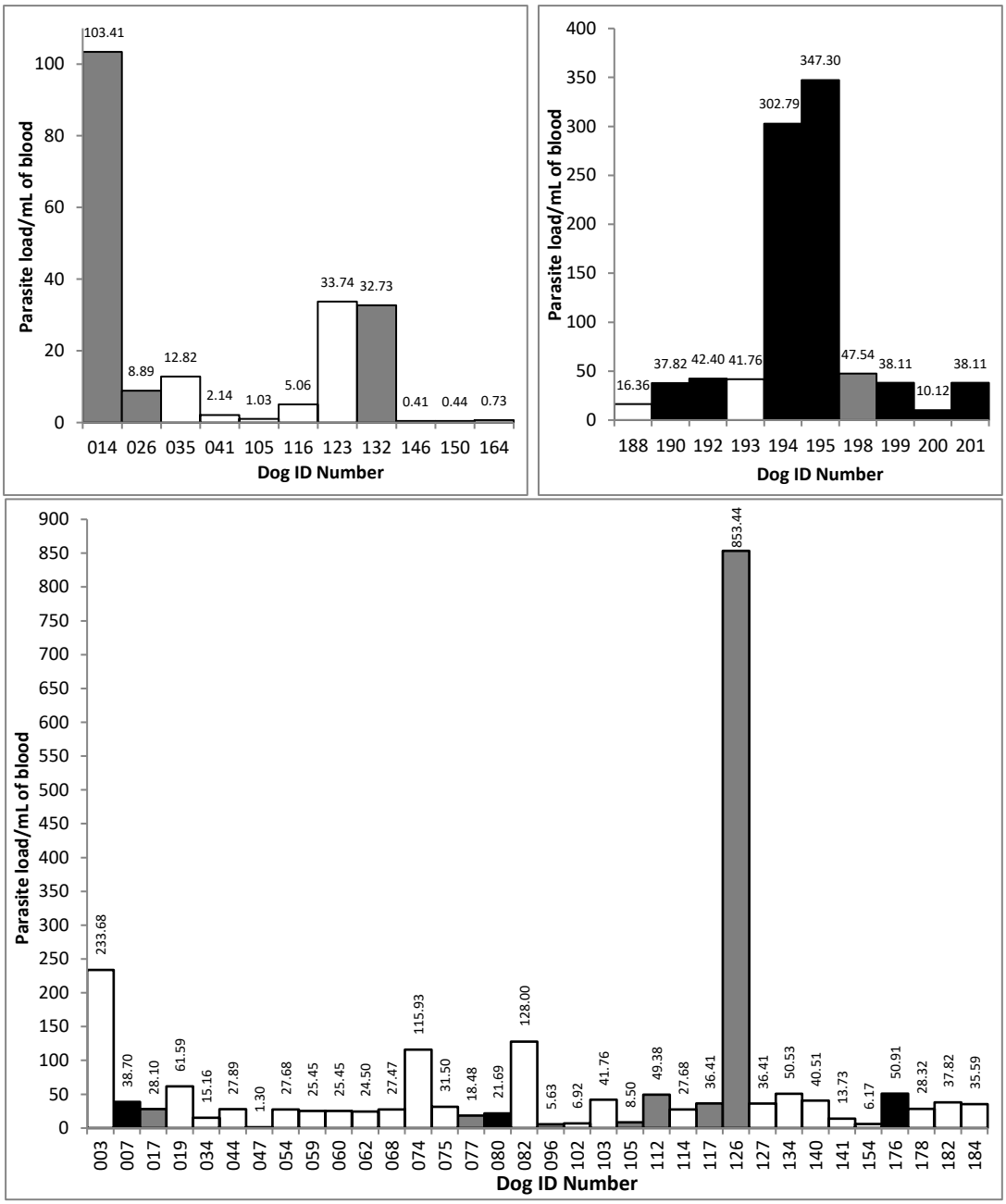


Figure 3.11: kDNA RT-qPCR assay of all positive samples from A; in the field (August 2017) n=185, B; CCZ (April 2018) n=16 and C; in the field (April 2018) n=133. Positive samples previously determined by PCR were qualitatively analysed by RT-qPCR to determine parasite loads of each positive dog. Range of parasite load; 0.41 parasites mL^{-1} to 853.44 parasites ml^{-1}. White bars; asymptomatic dogs, grey bars; oligosymptomatic dogs and black bars; symptomatic dogs.

3.4 Discussion

An ideal diagnostic test for canine visceral leishmaniasis (cVL) would show 100% sensitivity, identifying all dogs infected as well as those uninfected: a 'gold standard' for infection. The inaccuracy of the current tests used in Brazil will lead to high proportion of false positives and negatives therefore hampering cVL control. In fact, recent research has demonstrated that the official recommended diagnostic protocol for cVL in Brazilian endemic areas fails to successfully diagnose infected animals (Lopes *et al.,* 2017). Although serological tests are currently employed as the recommended diagnosis for cVL in Brazil, there are known limitations to the sensitivity of these techniques for the diagnosis of asymptomatic dogs. Furthermore, in comparison to molecular methods, serological tests demonstrate a low capacity to detect *Leishmania* infection in vaccinated dogs (Solano-Gallego *et al.,* 2017). Therefore, the rate of infection in endemic areas may be currently underestimated (Nunes de Carvalho *et al.,* 2018).

Several studies have also shown the use of PCR for the detection of cVL is a more sensitive method for *Leishmania* detection in blood samples than traditional diagnostic methods such as microscopy (Moreira *et al.,* 2007). Another advantage of PCR as a diagnostic tool is the ability to perform this technique using various biological samples. These include blood and buffy coat, lymph node aspirates, lesion scrapings, urine and canine conjunctival scrapings. Therefore, not only is this technique highly sensitive, but also has the potential to be relatively non-invasive. The impact of the high sensitivity of PCR could have a positive impact on the success of the euthanasia-based measures currently in place for VL control. However, although a high accuracy has been demonstrated, the technique is still not used throughout Brazil. This is

primarily due to high cost, requirement of sophisticated laboratory infrastructure and lack of standardisation of the technique. Despite these limitations, the development of safer and faster molecular diagnoses is more advantageous than the continued use of conventional techniques (Gualda *et al.,* 2015).

3.4.1 Optimisation of PCR

Due to the limitations observed for current conventional methods for cVL diagnosis, it is important that new methodologies are standardised in order to maximise repeatability and quality of the diagnosis. Research has demonstrated PCR as a rapid and sensitive tool for the diagnosis of leishmaniasis identifying parasites in different clinical samples from reservoirs, vectors and humans. Despite the high sensitivity observed for PCR diagnosis of cVL, the performance and quality of the technique is also related to the extraction protocol used, as well as the primers chosen. The identification of various *Leishmania* species in infected dogs in Brazil allows the opportunity for this technique to become highly specific for specific parasites if the correct primers are chosen (Souza *et al.,* 2019). In endemic areas, such as the study area chosen here, it is highly possible that different *Leishmania* species are found. Research has demonstrated the presence of *Le. amazonensis* and *Le. braziliensis* in addition to *Le. infantum* in infected dogs in Governador Valadares (Valdivia *et al.,* 2017). Therefore, the utilisation of specific primers that are not only highly sensitive, but also detect the correct *Leishmania* species in clinical samples is highly important. This is not only to allow for correct treatments to be used, but also for later work which aims to identify *Leishmania* species specific volatiles in infected dogs.

The results discussed here determine the effectiveness of two sets of primers; MaryF/R and LeF/R, for the amplification of varying concentrations of *Le. infantum* ranging from $[10]^5$ to $[10]^{-3}$ parasites/ml. The MaryF/R primers (Mary *et al.,* 2004) have been widely used for the detection of *Le. infantum* using qPCR. Good efficiency, sensitivity and specificity have been shown throughout research (Ceccarelli *et al.,* 2014). The LeF/R primers (Spanakos *et al.,* 2002) have been used in various conventional PCR experiments for detection of generic *Leishmania* DNA without the possibility of species identification but have shown a high sensitivity (Chargui *et al.,* 2013; Siriyasatien *et al.,* 2016). Although both sets of primers showed excellent performance with both detecting a minimum concentration of $[10]^0$ parasites/mL of blood, MaryF/R shows both greater specificity and sensitivity, amplifying at concentrations of $[10]^{-2}$ and generating more intense bands at higher concentrations. This will in turn lead to a more accurate diagnosis of *Leishmania* infection in dogs.

Despite the high sensitivity of these primers for *Le. infantum* diagnosis, the results here demonstrate that these primers can also amplify *Le. amazonensis* and *Le. braziliensis* (**Figure 3.5**). The ability of MaryF and R to amplify New World species has already been confirmed. Together with the results presented here, it could be suggested that these primers amplify a subclass of minicircles that are conserved among different *Leishmania* subgenera or species (Ceccarelli *et al.,* 2014). However, a BLAST search which revealed a lack of homology for these species combined with the faint bands observed confirm these primers as being more specific for *Le. infantum*. This in turn will allow for a more species-specific diagnosis. Despite some amplification of other New world species, the PCR method described using the primers MaryF/R is very effective at diagnosing seeded blood samples at very low concentrations of

parasites. This protocol was therefore used for diagnosis of canine blood samples collected from Brazil in both 2017 and 2018.

3.4.2 PCR Diagnosis of cVL

Currently in Brazil, the use of an ELISA for the confirmation of infected dogs following the screening using DPP diagnostics using blood samples is a cause for concern (Lopes de Mendonça *et al.,* 2017). Not only has a low sensitivity and specificity of the DPP test been demonstrated, particularly among sub-clinically infected dogs (Grimaldi *et al.,* 2012), but serological techniques present with a variety of limitations as previously mentioned. The low effectiveness of the current diagnostic protocol can be demonstrated in the results presented here. In 2018, samples were collected from 16 dogs confirmed as being cVL positive by the CCZ following the use of the current diagnostic protocol. After sample collection, these dogs were subsequently culled. However, further diagnosis using PCR in this study identified only 10 dogs (63%) as actually being positive for *Leishmania*. Although the unnecessary culling of negative dogs is of concern, more worryingly is the fact that positive dogs are being misdiagnosed as negative, leaving positive dogs in the environment to continue disease transmission. The lack of accuracy observed with the current tests in Brazil, highlights the need for a more accurate diagnostic test to be adopted in order to have a positive effect on disease control.

Further to an improvement in the diagnostic sensitivity of the current protocol, a diagnostic technique which remains relatively non-invasive would also be highly advantageous. The use of PCR for the detection of cVL in peripheral blood has many advantages. First, circulating parasites have been identified in dogs (Reithinger *et al.,*

2000). Second, taking blood samples is far less invasive and simple compared to other sampling methods such as lymph node biopsies or bone marrow aspiration. And third, research has demonstrated that the sensitivity of cVL diagnosis by PCR when using blood is in concordance with bone marrow aspirates (Pedrosa *et al.,* 2013). Using peripheral blood, however, has limitations which need to be considered when performing diagnosis. Research has shown that parasite concentration in peripheral blood is lower than that found in the lymph nodes, spleen and bone marrow aspirates. Furthermore, inhibitors which affect the sensitivity of PCR have been found in peripheral blood (Reithinger *et al.,* 2003). Despite these limitations the less invasive nature of blood sampling and similar sensitivity to other biological samples has resulted in an increased popularity of performing diagnosis using peripheral blood samples (Paiva-Cavalcanti *et al.,* 2015). Research has demonstrated that conventional PCR has sensitivities ranging from 89% to 100% when using blood samples from potentially infected dogs (Carson *et al.,* 2010). Therefore, the use of PCR as a diagnostic for cVL using peripheral blood is a technique that should be considered as a replacement for the current Brazilian protocol.

In order to confirm the sensitivity of PCR for the diagnosis of cVL, a conventional PCR protocol using Mary F and Mary R primers was used for the diagnosis of leishmaniasis in canine samples collected from Governador Valadares (GV). Analysis of the PCR results obtained in this study, not only demonstrated the accuracy of PCR based techniques for cVL diagnosis, but also confirmed GV as an endemic area for cVL in agreement with Cardoso *et al.,* (2019). An infection rate ranging of 6% in 2017 and 26% in 2018 was demonstrated. These results are in concordance with previous studies showing the prevalence of cVL in GV to be 30.2% (Barata *et al.,* 2013). These

results also coincide with recent research which has demonstrated similarities between the accuracy of ELISA and PCR for the diagnosis of cVL (Nunes de Carvalho *et al.,* 2018). This is due to previous studies in this area using serological based methods for the diagnosis of infected dogs.

Not only was the high infection rate previously observed in this area confirmed, but the use of PCR as an effective diagnosis of cVL was also demonstrated with both symptomatic and asymptomatic dogs being diagnosed. Overall, although the PCR diagnosis of cVL using peripheral blood presents with some difficulties, numerous advantages can be observed. These include the high level of sensitivity observed when compared to other conventional methods in addition to the ability to detect asymptomatic as well as seronegative dogs. With the current methods used in Brazil showing a need for improvement, the development of a more accurate diagnostic protocol using PCR can be suggested. Increasing the effectiveness of the current protocol would prove advantageous for the ongoing control of leishmaniasis.

3.4.3 PCR Diagnosis of Asymptomatic Dogs

Recent research has proposed the use of a reliable clinical score based on clinical signs as a potential tool for the diagnosis of cVL in endemic areas which have limited diagnostic resources (da Silva *et al.,* 2017). In fact, a severity score that is based on cVL clinical signs has already been proposed in Italy (Manna *et al.,* 2009). Although diagnosis using clinical signs could be used as an initial tentative diagnosis, clinical signs are highly variable. Clinical manifestations can depend on the immune response in each dog, disease history and other factors such as other diseases presenting with similar clinical signs (Ribeiro *et al.,* 2018). Efficient diagnosis of cVL also requires the

correct diagnosis of infected dogs that are asymptomatic as these dogs not only have a great epidemiological importance, but also represent a large proportion of all dogs with infection. Research has also shown that asymptomatic dogs are not only highly infective, but also highly competent of establishing *Leishmania* infection in sandflies. In fact, a higher proportion of infected sandflies have been shown to feed more on asymptomatic dogs (Laurenti *et al.,* 2013). This research not only indicates the role of asymptomatic dogs in maintaining the Le *infantum* cycle, but also demonstrated the involvement of these dogs in the spread of VL in endemic areas. Therefore, a diagnostic technique that is able to distinguish between both symptomatic and asymptomatic dogs is important (Michalsky *et al.,* 2007).

Recent research has demonstrated that the sensitivity of some serological tests is significantly decreased when there are no clinical signs (Mendonça *et al.,* 2017). A sensitivity of 29% has been demonstrated for the diagnosis of asymptomatic dogs using the IFAT diagnostic, with a slightly higher sensitivity of 53% being observed when diagnosing asymptomatic cases using the ELISA (Mettler *et al.,* 2005). Previous research has demonstrated that PCR could be used as a highly sensitive diagnostic tool for cVL diagnosis in asymptomatic dogs (Ferreira *et al.,* 2009) In order to demonstrate the ability of this PCR protocol to diagnose asymptomatic dogs, clinical manifestation was recorded for each dog sampled in the field. Clinical signs were observed in 3 (27%) of the 11 positive dogs sampled in 2017 and 10 (29%) of the 34 positive dogs sampled in 2018. The clinical signs observed most included skin lesions, long nails and conjunctivitis. However, more dogs were asymptomatic with 73% of positive dogs presenting with no clinical signs in 2017 and 71% in 2018. The high proportion of asymptomatic dogs identified by this PCR protocol demonstrates the use of PCR as an

effective diagnostic tool permitting good sensitivity for cVL diagnosis of asymptomatic dogs.

3.4.4 Parasite load in positive samples

The ability to accurately monitor parasite replication and survival using real-time quantitative PCR (RT-qPCR) is an important concept. Determining parasite quantity is vital in order to understand the biology of parasitic infections by distinguishing between parasite and host-mediated pathology as well as to monitor the effect of therapeutic interventions (Antonia *et al.,* 2018). In fact, recent research has demonstrated that malaria treatment that is based on RT-qPCR diagnosis can not only reduce the prepatent period, but also the number of adverse events which occur before treatment (Walk *et al.,* 2016). Previous research has also demonstrated the accuracy of RT-qPCR not only in the diagnosis but also quantification of various parasitic infections including malaria and Chagas disease. A developed RT-qPCR was able to simultaneously detect three *Plasmodium* targets with high sensitivity as well as provide absolute quantification of the malaria parasite (Kamau *et al.,* 2013). Results have also suggested the use of RT-qPCR for the diagnosis and monitoring of *T. cruzi* infection due to a high sensitivity observed for the detection and quantification of the parasite (D'Avila *et al.,* 2018). Regarding leishmaniasis, RT-qPCR is considered one of the most reliable diagnostic methods due to its high sensitivity (Travi *et al.,* 2018). Previous research has investigated the accuracy of different molecular tests when diagnosing cVL in bone marrow samples from dogs. It was shown that RT-qPCR was the most sensitive technique having a sensitivity of 91% when compared to other PCR based methods which had sensitivities ranging from 54% to 72% (Carson *et al.,* 2010).

Furthermore, it has also been shown that RT-qPCR can detect parasite levels as low as 0.2 parasites/mL, when compared to conventional PCR which only recognised a positive result when bone marrow samples contained >30 parasites/mL (Francino *et al.,* 2006). Additionally, a rapid and low-cost RT-qPCR assay for leishmaniasis has been developed through the reduction of reaction volumes as well as DNA amplification time. This diagnostic test was able to amplify *Le. infantum* and *Le. braziliensis* in infected dogs with good sensitivity (Dantas-Torres *et al.,* 2017). This is highly important given that cVL infection can be caused by both species of *Leishmania*.

The ability to determine parasite load in *Leishmania* positive dogs allows for a correlation, if any, to be seen between symptomatic/asymptomatic dogs and parasite load as well as allowing for a threshold level of detection to be determined for innovative diagnostic techniques. This quantification also allows for further information on the status of positive dogs diagnosed by conventional PCR. In order to quantify *Leishmania* parasite loads in our positive canine blood samples, a real-time RT-qPCR was developed using MaryF and MaryR primers (Mary *et al.,* 2004). To achieve the best sensitivity, kinetoplast DNA was the molecular target for these primers with Lachaud *et al.,* (2002) finding that kinetoplast DNA based methods show a minimum sensitivity of 3 parasites per PCR tube. A wide range of parasite loads were observed in this study ranging from 0.41 to 853.44 parasites/mL over the two separate sampling occasions. In August 2017, not only were there fewer positive dogs as demonstrated by conventional PCR diagnosis, but the parasite burden was also relatively low with a maximum parasite load of 103 parasites/mL identified. However, in April 2018, not only was there an increase in the number of infected dogs, but the parasite burden was also significantly increased with a maximum parasite load of 853

parasites/mL determined. Due to the longitudinal nature of this study, and the significant increase in number of infected dogs and parasite burden over a 9-month period, this particular study area could be considered as an endemic area for *Leishmania infantum* infection in dogs. The high parasite loads observed could have a direct impact on the identification of dogs which have a high transmitting ability. This is due to recent research which has shown that the parasite load in dogs that are naturally infected with *Le infantum* is directly correlated with their ability to infect sand fly vectors (Borja *et al.,* 2016). Through these results it can also be suggested that RT-qPCR may be a useful tool in the prediction of the outcome of cVL infection, as high parasite loads have previously been correlated with disease severity (Verma *et al.,* 2010).

Further results from this study also determined that parasite load could be directly correlated to clinical status, with dogs with the largest parasite burdens; 126, 195 and 194, showing clinical signs upon sample collection. This is an observation that can be correlated to the results obtained in recent *Leishmania* research which has demonstrated a positive correlation between parasite load and common clinical signs observed in cVL (Torrecilha *et al.,* 2016). This is also reflected in other parasitic diseases, with research demonstrating that clinical severity in malaria is correlated with a higher parasite load (Dormond *et al.,* 2015). It has also been shown that a high parasite load is directly related to a greater inflammatory response that is associated with Chagas disease (Wesley *et al.,* 2019). The correlation observed between *Leishmania* parasite load and clinical signs may allow veterinarians to make more informative decisions regarding infection prognosis and therapy that is based on routine initial examinations of clinical signs in the field (Torrecilha *et al.,* 2016).

The results obtained here demonstrate that Governador Valadares, which is an endemic region for cVL, has a high prevalence of infected dogs which present with a wide range of parasite loads (Penaforte *et al.,* 2013). Despite only a few dogs presenting with a high parasite load (>100 parasites/ml) it has previously been suggested that a small number of highly infected dogs would have a more significant impact on parasite transmission than a larger number of dogs with a low parasite load (Courtenay *et al.,* 2017). This reinforces the need for strict *Leishmania* control actions, particularly through the culling of seropositive dogs. The accuracy of RT-qPCR for cVL diagnosis has previously been shown. Additionally, the added advantage of being able to quantify parasite burden not only allows for the status of positive dogs diagnosed by PCR to be confirmed, but also allows for an estimation of the parasite load kinetics and provides information on the response to treatment (Francino *et al.,* 2006). However, despite RT-qPCR-based methodologies proving to be highly effective in leishmaniasis diagnosis, as with conventional PCR, a standardised method does not exist.

Chapter 4 - VOC Analyser Analysis of *Leishmania infantum* Infected and Uninfected dogs

4.1 Introduction

4.1.1 Current in Field Leishmaniasis Diagnostics in Brazil

Currently, in Brazil, the main method adopted for the control of visceral leishmaniasis in endemic areas is through the euthanasia of infected dogs to reduce the transmission of the parasite. Accurate as well as early detection of infected dogs is therefore critical but challenging. In 2012, the Brazilian Ministry of Health replaced the exisiting protocol which used an ELISA test as an initial screen followed by Indirect Immunofluorescence Test for confirmation, with a new protocol which uses the DPP (®) canine visceral leishmaniasis (cVL) rapid test as the initial screening test followed by an ELISA cVL as the confirmatory test.

The Dual-Path Platform (DPP) rapid diagnostic test (**Figure 4.1**) is a novel, immunochromatographic assay which was launched by BioManguinhos/Fiocruz, Rio de Janeiro, Brazil. Following confirmation of DPP positive dogs with an ELISA these dogs are culled, while DPP negative dogs are considered free of infection. A rapid in-field diagnostic test such as this is generally preferred due to easy use in the field, and thus useful for mass screening of cVL (Figueiredo *et al.,* 2018). These infection control programmes however tend to fail, not just due to the long delay between diagnosis and culling in highly endemic areas, but also due to a lack of accuracy observed with this current rapid diagnostic test in use throughout Brazil.

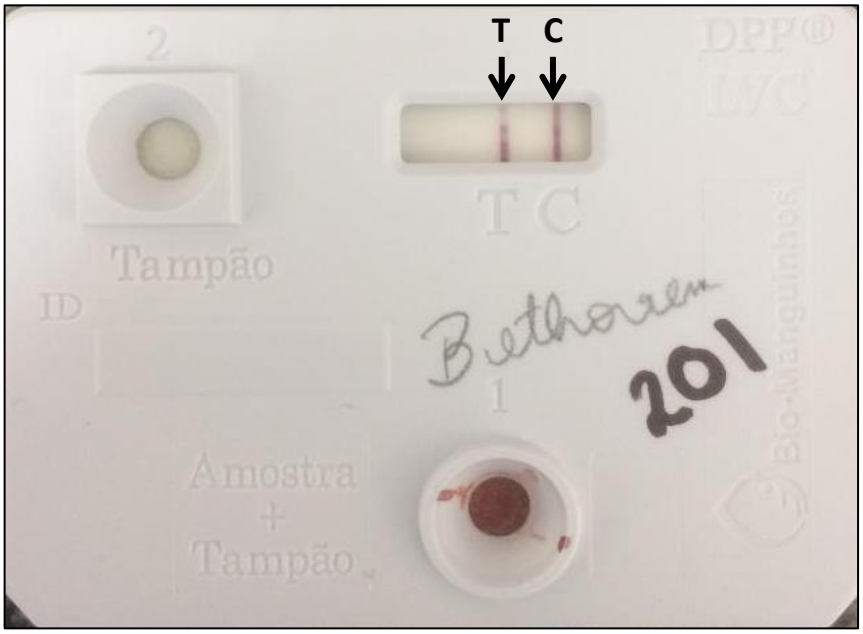


Figure 4.1: Positive result obtained by K26/K39-specific antibody Dual-Path Platform rapid diagnostic test (DPP®) for Dog #201: In the test window, the right line represents the control band (C). The left line indicated by an arrow represents the test band (T). If a sample is negative, the test will only develop a single control line (C) which demonstrated that the test has worked. A positive sample, however, develops both the control line (C) and test line (T).

Despite the sensitivity of an ELISA being as high as 94%, suggesting that the current Brazilian protocol should be successful, there is still debate as to the accuracy of the DPP test. The DPP has been assessed several times since it was introduced with the most recent studies carried out in Brazil demonstrating an overall sensitiy of 86%-89% a specifcity of 70%-94% (Fraga *et al.,* 2016; Figueiredo *et al.,* 2018). Additional research has also reported a sensitivity of 47.1% for the DPP diagnosis of splenic aspirates from cVL infected dogs compared to a sensitivity of 66.7% when using an ELISA and 100% when using RT-qPCR (Solcà *et al.,* 2014). This low sensitivity coincides with results

presented earlier in this study showing the DPP to have an accuracy of 63% when diagnosing dogs in Governador Valadares (**Table 3.2 and 3.3**, Chapter 3). Further research has also demonstrated a low sensitivity and specificity of this rapid diagnostic test when diagnosing asymptomatic dogs. Sensitivities as low as 75% and specificities as low as 47% have been observed which is of particular concern, especially as many infected dogs never display any clinical signs (Grimaldi *et al*., 2012; Figueiredo *et al.,* 2018;). Furthermore, research has demonstrated a low sensitivity of the DPP cVL diagnosis when compared to current parasitological methods. The lack of accuracy demonstrated for this rapid diagnostic test, highlights the need for better in-field diagnostic tests that will allow for more effective canine removal regimes than those currently used by public health services in Brazil.

4.1.2 Odour and Infection Status

Research has revealed a variety of ways in which odour could signal both non-infectious and infectious disease. First, it has been suggested that infections can change the composition of microbes both internally and externally which play important roles in the development of an individual's distinctive odour. The bacterial population on the skin in particular has been shown to play an important role during infection, particularly in the canine population, changing its composition as a response to disease (Weese, 2013) with an increase in dysbiotic skin microbiota being observed during leishmaniasis infection (Gimblet *et al.,* 2017).

Second, it has been demonstrated that infection triggers an immunological response that can change an individual's odour. For example, genes of the major histocompatibility complex (MHC) are not only important in the control of

immunological non-self/self-recognition but also have a major influence on body odour and mating (Janes *et al.,* 2010). Research has found that changes in the levels of MHC have an influence on the number of volatile compounds produced by mammals including sulphur-containing compounds, male pheromones and carbonyl metabolites (Novotny *et al.,* 2007). As a decrease in the expression of MHC class I and II on *Leishmania* infected cells has been shown (Nyambura *et al.,* 2018) it has been suggested that this decrease could be influencing the odour of a *Leishmania*-infected individual however, it is still unclear how the MHC genes alter odour.

Third, it has been suggested that activation of the immune system during infection could alter the metabolic by products that are excreted from the endocrine system. For example, individuals who have an infection tend to have a lower concentration of androgens and corticosterone; hormones that are suspected to regulate the production of sex pheromones and `alarm odours' respectively. Interestingly, dihydrotestosterone (DHT), the main androgen produced by males, has been found to have a significant role in *Leishmania* infection. In the presence of DHT, *Leishmania* promastigotes were found to have a significantly enhanced replication rate, a higher rate of survival in macrophages and an increased infection rate, as well as producing significantly larger lesions in mouse earlobes (Sánchez-García *et al.,* 2018). These results suggest that DHT plays an important role during *Leishmania* infections. Furthermore, research has also shown that during visceral leishmaniasis infection there are abnormalities in the hypothalamus-pituitary-adrenal axis, as well as irregular ADH and aldosterone secretions, magnesium depletion and thyroid insufficiencies (Verde *et al.,* 2017).

4.1.3 Vector-borne Parasite Manipulation of Host Odour

The survival of vector-borne parasites is dependent their ability to pass from one host to another via an insect vector. Parasites that are predominantly found in the circulatory system or superficially in the skin, have been shown to influence the odour of infected individuals which subsequently increases vector attraction and aids in parasite transmission (Hughes and Libersat, 2019). This phenomenon has been demonstrated for both malarial infections (De Moraes *et al.,* 2014) and leishmaniasis (Tavares *et al.,* 2018). Although it has been demonstrated that parasitic infection induces a change in host odour there is still a significant lack of research examining the potential of *Leishmania* parasites to alter the odour of their host animal in a natural environment. Through further understanding of such a manipulation, the potential for novel diagnostics based on disease odour detection could be suggested.

4.1.4 Diagnosis of cVL based on VOCs

Observed changes in the volatile organic compounds (VOCs) emitted by the parasitized host provides further evidence linking parasitic infection and host odour. In canine visceral leishmaniasis (cVL), studies by De Oliveira *et al* (2008) and Magalhães-Junior *et al* (2014b) provided evidence which suggested a difference between the profiles of the VOCs emitted by dogs infected with *Le. infantum* and those which were uninfected. Furthermore, Magalhães-Junior *et al* (2014b) reported a distinct difference in the odour profiles of infected canines which were presenting clinical manifestations compared to those infected canines which were showing no clinical manifestations. Although these studies suggest that an early diagnosis of infected dogs with and without clinical signs could be achieved, there are a few

limitations surrounding the research. Most notably is the small sample size which was used during the investigations; 12 healthy and 24 infected dogs (Magalhães-Junior *et al.*, 2014b) and 8 healthy and 16 infected dogs (De Oliveira *et al.*, 2008). The use of plastic bags during the study by Magalhães-Junior *et al* (2014b) may also interfere with the analysis. Plastic polymers have been shown to generate interference and chemical noise when undertaking metabolomics analyses due to the highly sensitive equipment used (Yin *et al.*, 2015). Additionally, solid-phase microextraction (SPME) is an equilibrium method and all of the compounds found on the dog hair may not be completely extracted or in the same proportions from the sample, another limitation of the previous analysis (Lappas and Lappas, 2016).

The work by Magalhães-Junior *et al* (2014b) identified six specific VOCs that could be determined as potential biomarkers of *Leishmania* infection; all six showed significant variations between infected dogs and healthy dogs. In contrast however, de Oliveira *et al*, 2008 identified 24 VOCs which they reported as being significant enough to discriminate between infected and non-infected canines, three of which showing the most significant difference; 2-hexanone, benzaldehyde and 2,4-nonadienal. These studies suggest that the identification of the biomarkers in canines that are infected with *Leishmania* could potentially be used as a painless, non-invasive diagnostic tool for the identification of cVL. Despite these results, there is still no evidence that demonstrates a threshold level or minimum parasite concentration that could be detected by observing the change in the odour profiles of canines. Furthermore, the origin of the different odour profiles produced during infection has not been determined; the change might be related to an alteration of the animal's behaviour once infected or even the microbial composition of the skin (Salgado *et al.*,

2016). In order to further understand the observed differences and origin of the infected odour profiles, the link between parasite load and infected odour could be identified. By identifying the parasite threshold level at which the observed change in the VOC profiles occur, the use of odour as an early stage diagnostic could also be determined.

4.1.4.1 *Leishmania* biomarkers in Hair

The use of hair as an analytical matrix could potentially be used for the diagnosis of cVL. Hair as a skin appendage is important for the secretion and excretion of endogenous substances (Grice and Segre, 2011). In fact, studies have analysed the components of animal hair for forensic investigations (Vincenti *et al.,* 2013), and as an indicator of the internal metabolic processing of medicines (Gratacos-Cubarsi *et al.,* 2006). Hair samples are not only easily to collect but have also been found to become naturally impregnated with secreted and excreted substances produced during infection; a reflection of the internal metabolism. Previous research has identified breast cancer associated lipids excreted on scalp hair (Mistry *et al.,* 2012). In this context, it can be stated that the volatiles that are emitted by hair can be representative of the volatiles that are emitted through the skin (Bernier *et al.,* 1999). Regarding infected dogs, previous research has demonstrated that the hair samples from *Le. infantum* seropositive and seronegative dogs, identified following ELISA showed a clear difference in their volatile emission profiles (Oliveira *et al.,* 2008). More recent research has also demonstrated the same phenomenon when diagnosing infected dogs using PCR (Magalhães-Junior *et al.,* 2014b). Furthermore, this research also identified variations between the volatile profiles of uninfected dogs and infected

dogs with and without clinical signs. The observed variations between the groups within the infected dogs suggested that the volatile profiles may be related to the type of immune response generated during infection.

The possibility of using hair samples to distinguish between the odour profiles of *Leishmania* infected dogs could provide new perspectives regarding the pathophysiology of cVL, as well as the interactions between the parasite, host and vector. This previously observed change in odour profile of infected dogs could contribute to the study of parasite transmission in endemic areas. As hair samples are easy to collect and a non-invasive, this methodology is predominantly acceptable to dog owners and therefore suggests a new methodology for sample collection for disease diagnosis using dog odour.

4.1.4.2 *Leishmania* biomarkers in blood

The chemical analysis of blood as a biological sample is currently the most common way of diagnosing most pathological conditions. Pathogenic microbial species produce various volatile metabolites with the analysis of secondary biomarkers in the blood forming the diagnostic approach (Bos *et al.,* 2013). Biomarkers found in blood are widely used in research not only to understand several aspects related to infectious and non-infectious diseases, such as susceptibility and exposure to infection, but also to identify an infected individual (Bryan, 2016). Blood is also an important source of body odours with many metabolically produced biomarkers being secreted into blood during infection and subsequently emitted to the external environment (Shirasu and Touhara, 2011). As research has demonstrated the ability to detect between the external odour profile of infected and uninfected individuals, it

could also be suggested that such a difference would also be observed through the internal odour analysis of blood

Due to the vector borne nature of most parasitic diseases, and the common ingestion of blood meals by such vectors, many parasites are found predominantly in the blood of an infected individual. The complex interaction between parasites with host immune cells, in addition to the host immune response has been shown to produce biomarkers of infection within the blood (Veras *et al.,* 2018). When focusing specifically on cVL, *Leishmania* specific biomarkers have been identified in the blood of *Le. infantum* infected dogs (Maia and Campino, 2018). *Leishmania infantum* infection has been shown to causes oxidative stress of canine neutrophils leading to an increase in reactive oxygen species and thus reduction of antioxidant compounds (Almeida *et al.,* 2017), Alterations in protein levels in the blood have also been observed, with an elevation of renal and hepatic biochemical parameters also being commonly associated with cVL (Meléndez-Lazo *et al.,* 2018). In addition to protein alterations, a reduction in some inflammatory markers such as butyrylcholinesterase and adenosine deaminase has also been reported in blood samples of infected dogs (Tonin *et al.,* 2016). Therefore that the detection of various biomarkers and volatile metabolites during infection could be used as a potential diagnosis for cVL.

4.1.5 VOC Analyser - principles and applications

4.1.5.1 Technology

Over the past 20 years, work has been undertaken to understand the principles behind odour receptors and the organisation of the olfactory system (Buck, 2005; Grabe and Sachse, 2018;). Only one type of odorant receptor is located on each

olfactory receptor cell which has the capacity to detect only a limited number of different substances. For a more complex odour that is composed of a variety of odorant molecules, several different receptors are activated. This resulting receptor pattern is what determines our impression of the specific odour (Rock *et al.,* 2008).

In 1982, Persaud and Dodd presented the first idea of a VOC Analyser as a device with the capabilities of mimicking the discrimination shown by the mammalian olfactory system when identifying the volatile organic compounds (VOCs) that are present in the headspace of a biological sample that is being analysed. It was reported that a VOC Analyser has the capability to discriminate between a wide variety of different odours, and that this discrimination could be achieved without the use of highly specific receptors (Persaud and Dodd, 1982). Technical limitations of VOC Analysers have been shown however, with the presence of water vapour of high concentrations of single compounds; for example, alcohols, causing a loss of sensitivity, sensor drift between replicates and a relatively short lifespan of some sensors (Cellini *et al.,* 2017).

A VOC Analyser is an instrument that is made of an array of chemical sensors that are ordinarily combined with a pattern recognition system (Bartlett *et al.,* 1997). Currently, the classical VOC Analyser which consists of this array of chemical sensors is the adopted approach to this technology. Common types of chemical sensors currently used are metal oxide and conducting polymer sensors, with metal oxide sensors capable of detecting large numbers of different volatiles (Estakhroyeh *et al.,* 2017). In order to better understand how a VOC Analyser works, an awareness of the mechanisms behind the human olfactory system is essential. When we smell, air samples that contain molecules of odours are moved past curved bony structures

known as turbinates. These structures create a turbulent airflow that carries the mixture of VOCs to the olfactory epithelium, where the ends of the nerve cells that sense odours are present. These olfactory receptor cells are then activated, and the signal is relayed to the brain. The premise behind the VOC Analyser is similar to the mechanism behind the human olfactory system. The technology consists of three functional components; a sample handler, an array of sensors and a signal processing system, which operate in sequence. The turbulent air flow produced by turbinates in the human olfactory system is replicated by an intake of air through a tube by the VOC Analysers sample handler (**Figure 4.2**). The array of sensors is a fundamental part of this technology with each sensor array in the array having a different sensitivity to individual VOCs. These sensors mimic the distinguishability of the nerve cells in our olfactory system. For example, where odour A may produce a low response in one sensor, but higher responses to others, odour B might produce a low reading for sensors that gave a high response to odour A. This variation in sensitivities between different sensors generates different patterns of response to different odours meaning every sample has a unique 'smell'. This distinguishability allows the VOC Analyser to identify unknown odours from the patterns generated by the sensors. Each sensor in the array is designed to have a unique response to the spectrum of different odours that are being tested. The generated pattern of response from all the sensors in the array is what is used to identify/characterise the odour.

Other types of VOC Analysers have been developed which utilise mass spectrometry (Fenaille *et al.,* 2003) or gas chromatography (Wiśniewska *et al.,* 2017) as the detection system. However, the VOC Analyser developed by RoboScientific and used for this project employs the same mechanisms as conventional VOC Analysers

using a chemical sensor array. The chemical sensors used were thin films of semi-conducting polymers deposited onto interdigitated gold structures on a silicon substrate. An air sample is pulled by a vacuum pump through a plastic or stainless-steel tube into a small chamber which houses this electronic sensor array. The odour is then exposed to the sensors by the sample-handling unit. The exposure of each volatile to each sensor produces a change in electrical properties due to the volatile's interaction with the semi-conducting polymer surface. After a few seconds to a few minutes depending on the sensor type, a steady-state condition is reached. During this period, the response of the sensor to the volatiles exposed to it are recorded and delivered to the signal-processing unit. It is the response generated by the sensors that creates data which can be analysed and used to determine whether a difference can be observed between samples.

Figure 4.2: VOC Analyser (Model 307 VOC analyser, RoboScientific Ltd, Leeds, United Kingdom) employing 12 semi-conducting polymer sensors. Hair samples sealed in individual foil bags were analysed using this device. Each bag was pierced by a needle connected to tubing leading into the instrument. An intake of air through a tube by the VOC Analysers sample handler allows for the headspace of the sample to be passed over the sensor array. Each individual VOC present in the sample reacts with individual sensors. The response for each sample was then displayed on a computer screen in real time as the sample was analysed. The specific response pattern was used to identify and characterise the sample's odour.

4.1.5.2 CVL Diagnosis using VOC Analysers

Physicians have valued odour for centuries for diagnostic purposes with a variety of research proving the use of odour in the diagnosis of disease. The VOC Analyser has shown great potential when used in *in vitro* experiments, having the ability to detect a variety of bacteria and fungi and, in some cases, distinguishing between them (Magan *et al.,* 2001). Furthermore, in clinical research, VOC Analyser technology has been shown to be a useful potential diagnostic device for the diagnosis of a variety of different diseases. Electronic noses have been used to analyse sweat,

breath, urine, and stools, with research having shown the ability of the VOC Analyser to distinguish between common bacterial pathogens of the upper respiratory tract (Lewis *et al.,* 2017) as well as proving able to detect mycobacterium tuberculosis in human sputum samples (Teixeira, *et al.,* 2017) and using urine to diagnose urinary tract infections and type 2 diabetes (Roine *et al.,* 2014; Esfahani *et al.,* 2018). Despite the extensive amount of research already undertaken into the use of VOC Analysers, there remain other domains that are relatively unexplored. Many of these involve the potential detection of a change in odour generated by the skin and contained in the sweat and on the hair of infected individuals (Rock *et al.,* 2008). Previous VOC Analyser applications have also shown the potential to analyse blood in order to diagnose and monitor disease. Research has demonstrated the potential of the VOC Analyser to discriminate between pre-dialysis from post-dialysis blood due to the presence of different biomarkers (Fend *et al.,* 2004). The ability of VOC Analysers to distinguish between these unique chemicals which can then be associated to specific diseases, leads to the hypothesis that this technology could potentially be used in the detection and diagnosis of parasitic diseases including cVL.

The production of VOC Analysers which can detect and discriminate between parasite specific VOCs, could play a significant role in the development of early, point of care (POC) diagnostics for a variety of parasitic diseases (Turner and Magan, 2004). The distinct difference already demonstrated between the VOCs on the hair of *Leishmania* infected and uninfected dogs (De Oliveira *et al*, 2008 Magalhães-Junior *et al* 2014b), in addition to the identification of *Leishmania infantum* biomarkers in infected dog blood (Maia and Campino *et al.,* 2018), suggests that VOC Analysers could be used as an alternative tool for the diagnosis of cVL using these sample types. The

use of this technology would achieve most of the ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment free and deliverable to end users) criteria, which has been outlined by the World Health Organisation for all new POC Devices (Staniek *et al.,* 2019). The aim of this part of this study was to determine whether a commercially available VOC Analyser could distinguish between the odours of *Le. infantum* infected and uninfected dogs with a high sensitivity and specificity.

4.1.6 Chapter Aims

In order to disrupt the *Leishmania* transmission cycle, it is important to successfully identify canines infected with *Leishmania* at an early stage of infection and remove them from the population. However, existing in-field methods used for cVL diagnosis are ineffective and inaccurate. Previous research has shown that *Leishmania* infected dogs produce a different odour to those that are uninfected (de Oliveira *et al*., 2008; Magalhães-Junior *et al*., 2014). However, the detection of this odour change as a diagnostic methodology has also not been investigated. Due to previous research demonstrating the ability of VOC Analysers to distinguish between the odour of infected and uninfected individuals with diseases such as TB or cancer, the aim of this chapter was to determine whether the VOC Analyser could distinguish between cVL infected and ininfected dogs, thereby providing preliminary evidence of this novel technology as an in-field cVL diagnostic.

4.2 Methods

4.2.1 Samples

The collection of the dog hair and buffy coat samples in the field was as described in the General Methods Chapter 2.0. As all analysed hair samples from both 2017 (n=55) and 2018 (n=149) were also used for additional GC/MS analysis (Chapter 6.0), before VOC Analyser analysis, the samples were split equally between two foil bags in a cold room set at 4°C. This temperature was required to minimise the loss of volatiles during the transfer.

4.2.2 VOC Analyser System Used

For this study, a VOC Analyser (Model 307 VOC analyser, RoboScientific Ltd, Leeds, United Kingdom) that employed 11 functioning semi-conducting polymer sensors was used. Three different sensor arrays were used in total for the analysis of the hair and buffy coat samples. The type of sample analysed by each sensor array were as follows;

Sensor array 1: analysis of the 2017 dogs and the 2018 buffy coat

Sensor array 2: analysis of the 2018 dogs

Sensor array 3: analysis of the 2018 buffy coat

Each sensor array was a derivation of one another, with 50% of the sensors being identical in each array. Therefore, the arrays had similar characteristics making the readings comparable.

Initial calibration of the device was achieved by two calibration points being automatically set by the sensor unit. The first was a baseline which was obtained when carbon-filtered air was passed over the sensor at a flow rate of 200ml/min. This was

automatically adjusted to zero on the Y axis scale. The second was a reference point which was obtained from sampling the head space of 5ml of a water control which was placed in a plastic vial.

For the analysis of the head space of each hair and buffy coat sample, each foil bag was connected to the VOC Analyser through the insertion of an 18-gauge needle connected to a PTFE tube into the head space of each bag. This was connected to the sample port of the VOC analyser and the head space passed over the sensor surfaces. Each individual sensor used as part of the array was a different type, chosen from a group of polymers that included polypyrrole, polyaniline and polythiophene. Each sensor had semi-selectivity to a different group of volatile chemicals; alcohols, aldehydes, amines, ketones and organic acids etc. Therefore, a digital fingerprint of the VOC mixtures from the samples was generated. The change in the electrical properties of each sensor was measured, recorded and displayed on the VOC analyser data logger screen. Four different parameters were recorded from each sensor response; absorbance, desorption, divergence (maximum response from the baseline) and the area under each response curve (**Figure 4.3**). Each functioning sensor array generated 2 outputs; a positive and a negative response. Therefore, the total number of VOC measurements for each sample was 88 (11 functioning sensors x 4 parameters x 2 outputs). The sampling profile was set at; 2 seconds baseline, 7 seconds absorption followed by a 1 second pause, 5 seconds desorption and 12 seconds flush to bring the sensors back to baseline.

The headspace of each foil bag was sampled 4 times. The first sample was discarded as it could potentially contain volatile carryover from the previous sample. Therefore, data was retained from the next 3 samples for the analysis. The individual

dog hair and buffy coat samples in each experiment were tested randomly, with each sample used once only.

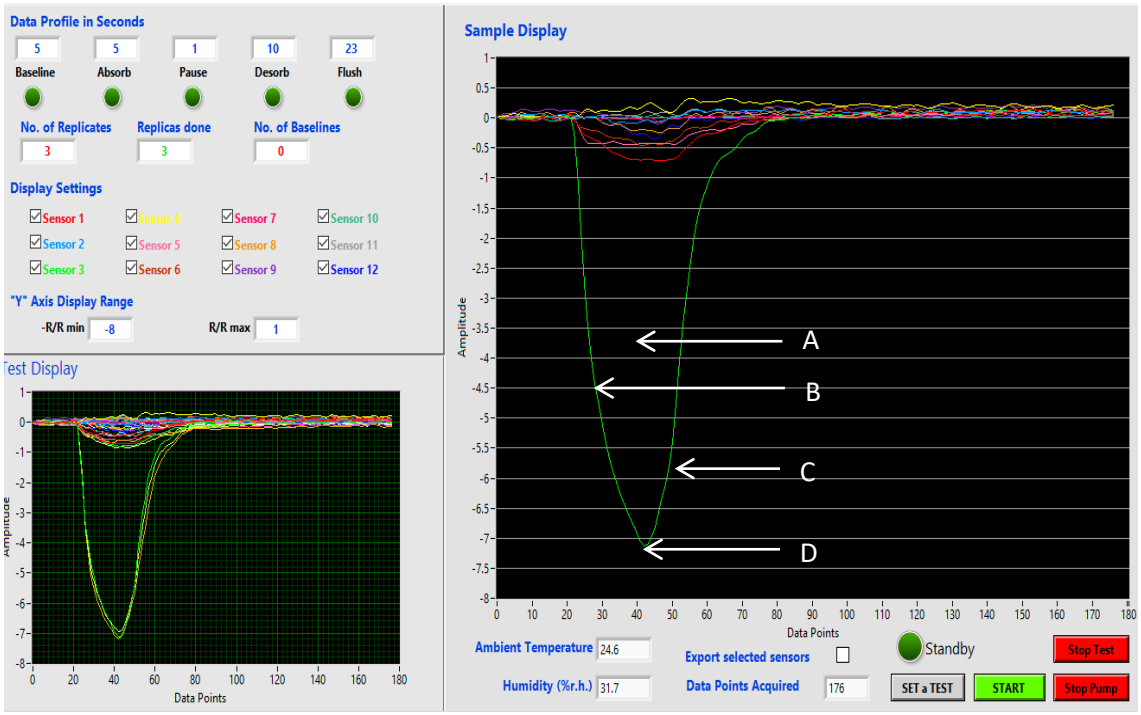


Figure 4.3: VOC Analyser data response for each sensor. Each sensor generated an individual response based on the VOCs present in each sample. Each response is represented by a different coloured line on the graph with each sensor generating a unique area under the curve (A), absorption (B), desorption (C) and divergence (D).

4.2.3 VOC Analyser Analysis

4.2.3.1 Optimisation

Prior to sample analysis of hair collected in the field, differing quantities of water were injected into each bag in order to determine the optimal quantity needed for the discrimination of hair samples collected from two different breeds of dogs (Border Collie n=1 and Cavalier King Charles n=1). The addition of water followed by incubation at 50 degrees produced humidity in the samples which assisted in the release of the VOCs and removed any variability due to different levels of water found

in the collected hair samples. A controlled amount of water keeps the baseline humidity level constant.

Approximately 3 x 1g of hair was collected from each dog and placed in to individual 110mm x 185mm Aluminium grip seal foil bag (Polypouch UK Ltd). The samples were analysed with the VOC Analyser either dry or following the addition of 10µL or 100µL of water. A discriminant function analysis (DFA) within the Excel add-in software XLstat version 3.4 was performed on the data. A multivariable discriminant plot was generated which summarised the classifications of the observations and provided a visual representation of the observations. Two sensor parameters; the divergence and area under the response curve were used to perform the analysis.

4.2.3.2 VOC Analyser Analysis of 2017 and 2018 Dog Hair

Volatile analysis using the VOC Analyser was carried out on all (n=11) of the 2017 infected dog hair samples and a sub-set (n=44) of the uninfected dog hair samples using sensor array 1. The uninfected dogs were randomly selected from groups of dogs which matched the infected dogs for age, sex and whether the dogs were "treated" with other medication (**Table 4.1**). Four uninfected dogs for each category were selected for each infected dog. For the 2018 samples, VOC Analyser analysis was carried out on all the infected dog hair (n=44, including 10 CCZ dogs) and all the uninfected dog hair samples (n=105, including 6 CCZ dogs) using sensor array 2.

Water (10µL) was injected into each foil bag containing individual hair samples with a Hamilton syringe and inflated with 140mL of laboratory air using a diaphragm pump. All hair samples were incubated at 50°C for 15 minutes in an oven, and then allowed to cool for 5 minutes at room temperature prior to the head space analysis.

Samples were heated in order to assist in the release of the volatiles from the hair samples into the headspace.

4.2.3.3 Buffy Coat

In order to determine whether the VOC Analyser could distinguish between the VOCs found in the blood of infected dogs, VOC Analyser analysis on buffy coat samples was performed using sensor arrays 1 and 3. For the analysis, 5 positive dogs were selected at random from the 2017 cohort, along with 5 negative 2017 dogs randomly selected from the previously selected 44 negative dogs (**Table 4.1**).

Buffy coat samples were centrifuged at 14,000 x rpm for 4 minutes, opened and placed into individual foil bags. All bags were inflated with 100mL of laboratory air using a diaphragm pump and incubated at 50^{o}C in an oven for 10 minutes and allowed to cool at room temperature prior to headspace sampling.

Age	Sex	Treated	Positive Dogs	Negative Dogs
0-2years	Female	Yes	014, 150	004, 036, 068, 072, 106, 147, 153, 155
0-2years	Female	No	041, ***132***	018, 029, ***088***, 125, 142, 157, 163, 169
0-2years	Male	Yes	***105***	024, 032, ***057***, 152
0-2years	Male	No	***164***	009, 052, ***115***, 161
3-4years	Female	Yes	123, 146	001, 013, 016, ***043***, 061, 094, 107, 171
3-4years	Female	No	***035***	015, 021, 121, 173
5-8years	Male	Yes	116	082, 099, ***126***, 139
5-8years	Male	No	***026***	045, 070, 103, 149

Table 4.1: List of 2017 dogs selected for volatile organic chemical (VOC) Analyser analysis. The 8 groups which the 2017 positive dogs (n=11) were split into is shown in the table above. A list of the chosen 44 uninfected dogs is also shown. Four dogs were chosen for every one positive dog in order to gain a statistically robust analysis. Dog samples in bold and italic correspond to those samples used for VOC Analyser analysis of the buffy coat.

4.2.4 Data analysis

4.2.4.1 Hair Analysis

In order to test the ability of the VOC Analyser to differentiate between the odours of infected and uninfected dogs in 2017 using sensor array 1 and in 2018 using sensor array 2, two statistical programs were used. The first was MCLUST (Fraley and Raftery 1999), a model-based clustering and classification algorithm embedded in R-cran statistical software (R-cran, R Core Team 2018) and the second was a discriminant function analysis in IBM SPSS Statistics (Version 25). The two different statistical

analyses aimed to identify any significant differences in the VOC analyser response between infected and uninfected dogs in both 2017 and 2018. This would, therefore, allow for an accurate prediction of the infection state of newly sampled dogs in the field.

4.2.4.2 R-cran statistical software analysis

Initial discriminant analysis (DA) using R-cran statistical software was performed on both sets of data from 2017 and 2018. This initial analysis using one class for infected and one class for uninfected dogs indicated that the data was over fitting, therefore the infected and uninfected dog classes were divided into sub-classes using the function MCLUST and the optimal statistical model determined. The optimal number of sub-classes and statistical model for infected and uninfected dogs was obtained by Bayesian information criterion (BIC) (**Table 4.2 and 4.3**). Following the determination of the optimal number of subclasses and model, the discriminant analysis was repeated. In addition, this further analysis also aimed to identify any significant differences in the VOC Analyser variables of 2017 and 2018 infected and uninfected dogs. The relative importance of each variable in discriminating between the infected and uninfected sub-classes was assessed by variable permutation analysis.

The predictability of the clustering model and the robustness of the classification were evaluated within R-cran. This analysis was performed using two types of cross-validation; out-of-sample cross validation (CV) and confounder cross validation (CCV) (**Table 4.8 and 4.9**). For this analysis, the replicates were considered

independent; the three VOC replicates for each dog were considered as coming from three different dogs.

The statistical program was further employed to compare the uninfected dogs collected in 2017 with the uninfected dogs in 2018. This analysis was performed in order to determine whether the negative dogs from both years could be discriminated between, or whether the data was considered equal i.e. there is no significant difference between both sets of dogs. If the negative dogs were considered equal, then further analysis could be carried out comparing individual dogs that were negative in 2017 but became positive in 2018, thereby allowing for a direct control.

In addition, the effect of parasite load and clinical signs on VOC Analyser discrimination was also determined. This analysis was based on the RT-qPCR results and identified clinical signs (Chapter 3, Results **Figure 3.11**). The probability of an infected dog belonging to the established infected clusters was determined. Those positive dogs which were considered to have a high VOC Analyser discrimination when compared to the negative dogs produced the highest probability (discriminatory factor). This discrimination was then compared to the parasite load in order to determine whether parasite load is correlated to VOC Analyser response. Similarly, the identified discrimination was also compared to the presence or absence of clinical signs to determine whether clinical signs influenced the VOC Analyser discrimination. This analysis was performed on the 2018 dogs due to the larger cohort of positive dogs diagnosed during this period.

4.2.4.3 SPSS Statistics

Following the discriminant analysis performed in R-cran statistical software, a discriminant analysis using IBM SPSS Statistics software was also performed on both sets of data from 2017 and 2018. This was done to increase the statistical robustness of the analysis in addition to validating the results gathered from the R-cran MCLUST analysis. Unlike the R-cran statistical analysis, infected and uninfected dogs were not split into sub-classes, instead only two classes were used (uninfected and infected) which were the dependent variables. 88 predictor variables were utilized to predict category membership.

Initial analysis was performed assuming that all dog replicates were considered independent; therefore, each individual VOC replicate for each dog was considered as coming from three independent dogs. Following which, the mean values for each variable was taken for each individual dog; giving an average value for the three dog replicates (n=55), and the analysis repeated.

4.2.4.4 Buffy Coat Analysis

The VOC Analyser analysis of the VOCs emitted by the 10 dog buffy coat samples (5 positive and 5 negative) was performed 4 times for each sample using sensor array 2 and sensor array 3 with the first sensor response removed as with the hair analysis. Using discriminant function analysis (DFA), a multivariable discriminant plot was generated using Excel add-in software (XLstat version 3.4). Two sensor parameters; the divergence and area under the response curve were used to perform the analysis with a multivariable discriminant plot being generated.

4.3 Results

4.3.1 Optimisation

Three lots of hair (3x1g) were collected from two domesticated dogs and analysed using the VOC Analyser following incubation either dry, or after the addition of 10ul or 100ul of water. A clear discrimination can be observed between the two dogs when a volume of 10ul of water was added to each sample due to the large distance shown between samples on the graph (**Figure 4.4**). However, when analysed dry or with 100ul of water, the VOC analyser was unable to effectively discriminate between the two different dogs. The the addition of 10ul of water is therefore required to gain discriminatory results when analysing the hair samples collected in the field.

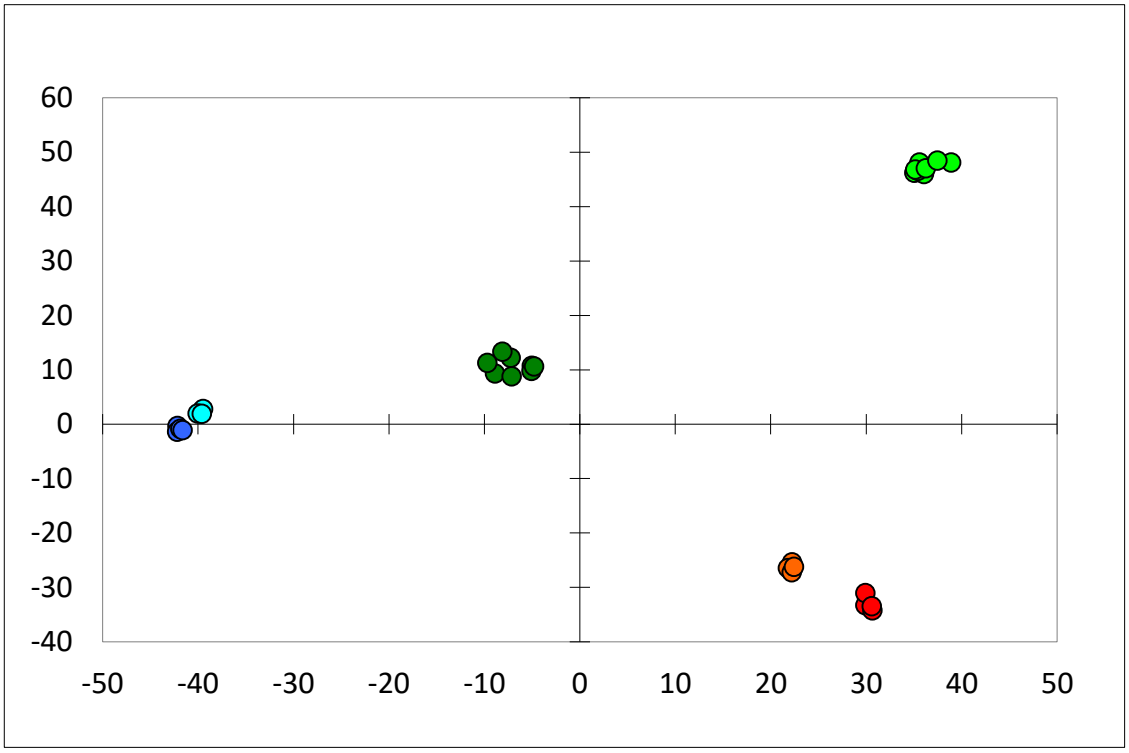


Figure 4.4: Discriminant Function Analysis (DFA) (XLstat version 3.4), using sensor array 1, of dog hair from a Border Collie (BC) and Cavalier King Charles (CKC). Following the addition of 10 μL of water, a clear discrimination can be observed between the CKC and the BC. When analysed dry or with 100 μL of water the DFA was unable to clearly discriminate between the two dogs. Furthermore, the addition of various quantities of water also results in a different spatial discrimination of the samples. The ellipses were added manually. Results were generated by XLstat version 3.4.

4.3.2 R-cran Statistics Model Analysis

The best model for the analysis of 2017 and 2018 infected and uninfected dogs using R-cran statistical software was determined to be EEE, apart from infected 2017 dogs where the best model was VVI (**Table 4.2 and 4.3**). EEE assumes an ellipsoidal co-variances and equal shape, volume and orientation for all the classes, while VVI

assumes diagonal co-variances with orientation parallel to the coordinate axes and variable shape and volume for the classes. This is because Gaussian mixture modelling algorithm utilises different forms of eigen decomposition of the covariance matrices (Fraley and Raftery 2007). Based on these results, the clustering of infected and uninfected dogs in 2017 identified 1 class for uninfected dogs and 3 classes for infected dogs using two different models, while for the 2018 data the clustering identified 2 classes for uninfected dogs and 6 classes for infected dogs both using the same model.

2017 Classes	Model 1	Model 2	Model 3
Uninfected	**EEE 1 class (-90584)**	EEV 1 class (-90584)	EVE 1 class (-90584)
Infected	**VVI 3 classes (-25125)**	VEI 4 classes (-25169)	VEI 3 classes (-25180)

Table 4.2: Outcome of the mixture model analysis showing the top three models for the 2017 uninfected and infected dog data. Bayesian Information Criterion (BIC) values are shown within brackets. The closer the BIC value is to zero, the stronger the evidence for the model

2018 Classes	Model 1	Model 2	Model 3
Uninfected	**EEE 2 classes (-93455)**	EEE 3 classes (-93621)	EEE 4 classes (-93884)
Infected	**EEE 6 classes (-212458)**	EEV 6 classes (-212628)	EVE 6 classes (-212988)

Table 4.3: Outcome of the mixture model analysis showing the top three models for the 2018 uninfected and infected dog data. Bayesian Information Criterion (BIC) values are shown within brackets. The closer the BIC value is to zero, the stronger the evidence for the model. Analysis provided by Dr Luigi Sedda.

4.3.3 Analysis of uninfected vs infected dogs in 2017 and 2018

4.3.3.1 R Statistics Supervised Classification (discriminant analysis).

Once the optimal model and number of classes were decided the training errors, training sensitivity and training specificity were calculated for both the 2017 and 2018 cohort. A discriminant analysis was applied to find the separation plane or planes between the classes. Results presented here show that in both years infected dog odours were significantly different from the uninfected dog odours using the VOC analyser. In the 2017 data (**Table 4.4 and 4.5**), the uninfected and infected dogs were discriminated with 99% specificity and 90% sensitivity prior to division into subclasses. This was improved to 100% for both specificity and sensitivity when the data was divided (1 uninfected dog class and 3 infected dog classes). In 2018 with a larger cohort of dogs (**Table 4.6 and 4.7**), uninfected and infected dogs were discriminated with 89% specificity and 100% sensitivity prior to the data being divided into subclasses. Following this division (2 uninfected dog classes and 6 infected dog classes) the specificity was improved to 95% however, the sensitivity was reduced slightly to 97%. The overall training error was reduced from 2.8% to 0% when the 2017 data was divided into subclasses and from 7.6% to 4.2% when the 2018 data was divided in subclasses. When the classes were not perfectly separated, training errors resulted.

Observed\Predicted	Uninfected	Infected	Specificity (%)	Sensitivity (%)
Uninfected	111	1	99	
Infected	3	28		90
Training error	0.028			

Table 4.4: Confusion matrix for Gaussian mixture model Eigen Decomposition

Table 4.5: Confusion matrix for Gaussian mixture model Eigen Decomposition Discriminant Analysis (EDDA) classification for 1 uninfected dog class and 3 infected dog classes in 2017. Training error is the average error, i.e. the ratio between correctly predicted class members (in the table above 112+31) and the total number of records (143). Analysis provided by Dr Luigi Sedda.

Observed\Predicted	Uninfected	Infected	Specificity (%)	Sensitivity (%)
Uninfected	112	0	100	
Infected	0	31		100
Training error	0			

Observed\Predicted	Uninfected	Infected	Specificity (%)	Sensitivity (%)
Uninfected	280	34	89	
Infected	0	132		100
Training error	0.076			

Table 4.6: Confusion matrix for Gaussian mixture model Eigen Decomposition Discriminant Analysis (EDDA) classification for uninfected and infected dogs in 2018 without subclasses. Training error is the average error, i.e. the ratio between correctly predicted class members (in the table above 280+132) and the total number of records (446). Analysis provided by Dr Luigi Sedda.

Observed\Predicted	Uninfected	Infected	Specificity (%)	Sensitivity (%)
Uninfected	298	16	95	
Infected	3	129		97
Training error	0.042			

Table 4.7: Confusion matrix for Gaussian mixture model Eigen Decomposition Discriminant Analysis (EDDA) classification for 2 uninfected dog classes and 6 infected dog classes in 2018. Training error is the average error, i.e. the ratio between correctly predicted class members (in the table above 298+129) and the total number of records (446). Analysis provided by Dr Luigi Sedda.

4.3.3.2 Cross-validation (CV) and confounder cross validation (CCV)

Cross-validation (CV) and confounder cross validation (CCV) were performed for both the 2017 and 2018 data using R-cran statistical software to validate the chosen model. The number of changed labels that were correctly predicted (i.e. false uninfected predicted as real infected and false infected predicted as real uninfected) was determined either without sub-class division or following division into subclasses.

Prior to division into sub-classes, CV analysis on 10% of the data (leaving the remaining 90% for training) to assess the risk of overfitting, showed a reduced sensitivity of 50% and specificity of 84% for the 2017 data and a sensitivity of 48% and specificity of 96% for the 2018 data (**Table 4.8 and Table 4.9** first line; first two columns). CCV analysis showed a significantly reduced sensitivity of 11% and specificity of 60% for the 2017 data and a sensitivity of 18% and specificity of 67% for the 2018 data (**Table 4.8 and Table 4.9** first line; last two columns). Both analyses demonstrate a reduced ability to correctly identify true uninfected and infected dogs compared to the training set (as reported in **Table 4.4, 4.5, 4.6 and 4.7**).

When the analyses were repeated with sub-classes; either 4 classes for 2017 (1 uninfected dog class and 3 infected dog classes) or 8 classes for 2018 (2 uninfected dog classes and 6 infected dog classes) both CV and CCV improved (**Table 4.8 and Table 4.9** second line). CV analysis showed a sensitivity of 75% and specificity of 80% for the 2017 dogs, However, CCV only showed 60% sensitivity and 70% specificity for the 2017 data set. Analysis of the 2018 dogs however showed a sensitivity of 93% and a specificity of 92% when performing CV analysis, and 74% sensitivity and 84% specificity for the CCV analysis.

Model	CV sensitivity	CV specificity	CVV sensitivity	CVV specificity
2 classes	0.50	0.84	0.11	0.60
4 classes	0.75	0.80	0.60	0.70

Table 4.8: Cross Validation and Confounder Cross Validation analysis of 2017 data 2 groups vs 4 groups. Comparison of sensitivity and specificity analysis of the 2017 data analysed as either 2 classes (1 uninfected and 1 infected) or 4 classes (1 uninfected and 3 infected) as determined in **Table 4.2**. Analysis provided by Dr Luigi Sedda.

Model	CV sensitivity	CV specificity	CVV sensitivity	CVV specificity
2 classes	0.48	0.96	0.18	0.67
8 classes	0.93	0.92	0.74	0.84

Table 4.9: Cross Validation and Confounder Cross Validation analysis of 2018 data 2 groups vs 8 groups. Comparison of sensitivity and specificity analysis of the 2018 data analysed as either 2 classes (1 uninfected and 1 infected) or 8 classes (2 uninfected and 6 infected) as determined in **Table 4.3**. Analysis provided by Dr Luigi Sedda.

4.3.3.3 IBM SPSS Discriminant Analysis

Following the discriminant analysis performed in R-cran statistics, a discriminant analysis using the discriminant procedure in IBM SPSS was performed. Analysis showed that infected and uninfected dogs could be discriminated between with a high sensitivity and specificity. In the 2017 data, when all VOC readings were considered as independent, the uninfected and infected dogs were discriminated with 99.1% specificity and 80.6% sensitivity (**Table 4.10**). This was improved to 100% for

both specificity and sensitivity when the means of the data were analysed (**Table 4.11**). In 2018 with a larger cohort of dogs, uninfected and infected dogs were discriminated with 91.1% specificity and 50% sensitivity prior to the means being analysed (**Table 4.12**). Following which both specificity and sensitivity was improved to 91.4% and 56.8% respectfully (**Table 4.13**). The classification results; actual class by predicted class, for each analysis are shown in the tables below.

A Wilks' Lambda value was determined to demonstrate how well the prediction model fit for each individual analysis, with a *P* value of less than 0.05 indicating a significant difference. For the 2017 data a *P* value of 0.043 was determined for both the independent analysis and the analysis of the means. For the 2018 data a *P* value of 0.000002 was determined when the data was analysed independently and a *P* value of 0.0107 was determined when the means were analysed.

Observed\Predicted 2017	Uninfected	Infected	Specificity (%)	Sensitivity (%)
Uninfected	111	1	99.1	
Infected	6	25		80.6
Wilks' Lambda (Sig.)	0.415 (0.043)			

Table 4.10. Discriminant analysis for uninfected and infected dogs in 2017 (n=143). Summary of number and percent of subjects classified correctly and incorrectly. Here, the Lambda of 0.415 has a significant value (*P*. = 0.043) indicating a significant difference.

Observed\Predicted 2017 Means	Uninfected	Infected	Specificity (%)	Sensitivity (%)
Uninfected	44	0	100	
Infected	0	11		100
Wilks' Lambda (Sig.)	0.113 (0.043)			

Table 4.11. Discriminant analysis for the means of uninfected and infected dogs in 2017 (n=55). Summary of number and percent of subjects classified correctly and incorrectly. Here, the Lambda of 0.113 has a significant value (*P*. = 0.043) indicating a significant difference.

Observed\Predicted 2018	Uninfected	Infected	Specificity (%)	Sensitivity (%)
Uninfected	286	28	91.1	
Infected	66	66		50.0
Wilks' Lambda (Sig.)	0.727 (0.000002)			

Table 4.12. Discriminant analysis for uninfected and infected dogs in 2018 (n=446). Summary of number and percent of subjects classified correctly and incorrectly. Here, the Lambda of 0.727 has a significant value (*P*. = 0.000) indicating a significant difference.

Observed\Predicted 2018 Means	Uninfected	Infected	Specificity (%)	Sensitivity (%)
Uninfected	96	9	91.4	
Infected	19	25		56.8
Wilks' Lambda (Sig.)	0.681 (0.0107)			

Table 4.13. Discriminant analysis for the means of uninfected and infected dogs in 2018 (n=149). Summary of number and percent of subjects classified correctly and incorrectly. Here, the Lambda of 0.681 has a significant value (*P*. = 0.010) indicating a significant difference.

4.3.3.4 Selecting important variables

To compare the importance of each variable in determining the number of sub-classes in both infected and uninfected dogs for 2017 and 2018, a clustering with permuted variable values was performed. In order to perform this analysis, the tested variable values were permuted, while the rest of the variables were kept unchanged and the clustering was performed. The optimal number of sub-classes was then analysed to determine whether they are larger or smaller than the optimal number obtained from the un-permuted variables. The permutation was repeated 999 times and the P-value produced represented the number of times that the optimal number of sub-classes obtained following permutation is larger or smaller than the observed one. A *P* value was then obtained by dividing this number by 1000. A larger *P*-value demonstrates a more important variable. The importance of each variable obtained from the VOC analyser in the clustering is shown in **Table 4.14**.

A		**B**	
Variable	**P Value**	**Variable**	**P Value**
F3.Abs.5	0.93	F3.Abs.17	0.99
F1.Div.20	0.92	F4.Des.21	0.98
F4.Des.8	0.07	F4.Des.9	0.95
F1.Div.5	0.06	F4.Des.4	0.94
F1.Div.19	0.06	F4.Des.6	0.91

Table 4.14: Relative Importance of the first 5 different sensor variables in the contribution to the clustering by R-cran, observed in the 2017 data; A and 2018 data; B. 0.99 P-value means that in 99% of the permutation the number of optimal clusters changed, indicating a strong influence of the variable in the final clustering. Analysis provided by Dr Luigi Sedda.

4.3.3.5 Uninfected 2017 vs uninfected 2018

A discriminant analysis within R-cran demonstrated that although the 2017 and 2018 uninfected dogs shared some multivariate space, both groups were not sufficiently mixed to be considered equal. The uninfected dogs from both years separated with a 79% specificity and 99% sensitivity. Therefore, the 2017 uninfected dogs and their corresponding sample from 2018 when they became infected could not be compared (**Table 4.15**).

Observed\ Predicted	**Uninfected 2017**	**Uninfected 2018**	**Specificity (%)**	**Sensitivity (%)**
Uninfected 2017	61	16	79	
Uninfected 2018	1	313		99
Training error	0.043			

Table 4.15. Confusion matrix for Gaussian mixture model Eigen Decomposition Discriminant Analysis (EDDA) classification for uninfected dogs in 2017 and 2018. The uninfected dogs in both 2017 and 2018 can be discriminated with 70% specificity and 99% sensitivity therefore not considered equal. Training error is the average error, i.e. the ratio between correctly predicted class members (in the table above 61+313) and the total number of records (374). Analysis provided by Dr Luigi Sedda.

4.3.3.6 Parasite load and Clinical Signs

Parasite load had no effect on VOC analyser discrimination for the 2018 dogs upon identification of the dogs which were considered to have a high or low VOC Analyser discrimination based on their cluster probability (discriminatory factor). Those dogs that were considered as having a high or low VOC Analyser discrimination presented with a range of parasite loads as determined by previous RT-qPCR (Chapter 3, **Figure 3.11**). However, although the presence or absence of clinical signs had no effect on a high VOC Analyser discrimination, with high discriminated dogs being both symptomatic and asymptomatic, those dogs which presented with a low VOC Analyser discrimination were all asymptomatic (**Table 4.16**).

Positive Dog Number	**VOC Analyser discrimination**	**Parasite Load parasites mL^{-1} of blood** ***(Chapter 3, Figure 3.11)***	**Clinical signs** ***(Chapter 3, Figure 3.11)***
Dog 176	High	50.91	Symptomatic
Dog 141	High	13.73	Asymptomatic
Dog 178	High	28.32	Asymptomatic
Dog 126	High	853.44	Symptomatic
Dog 134	High	50.53	Asymptomatic
Dog 074	Low	115.93	Asymptomatic
Dog 082	Low	128.00	Asymptomatic
Dog 102	Low	6.92	Asymptomatic
Dog 044	Low	27.89	Asymptomatic
Dog 019	Low	61.59	Asymptomatic

Table 4.16. Parasite load and clinical signs of high and low VOC Analyser discriminated dogs. Dogs which were highly discriminated presented with parasite loads ranging from 13.73 parasites mL^{-1} to 853.44 parasites mL^{-1}. Dogs with a low discrimination had parasite loads ranging from 6.92 parasites mL^{-1}, to 128 parasites mL^{-1}. For the context of this analysis, symptomatic dogs were considered as those which presented with one or more clinical signs.

4.3.4 Analysis of Buffy Coat from Naturally Infected Dogs

Buffy coat samples obtained from a cohort of naturally infected and uninfected dogs in 2017 (**Table 4.1**) were used to determine, in a preliminary experiment, whether the VOC analyser could discriminate between the blood of uninfected and infected dogs. **Figure 4.5** shows the discriminant function analyses (DFA) using two different sensor arrays (sensor array 1 and sensor array 3), of the raw data obtained with the VOC analyser (XLstat version 3.4). Using sensor array 1, the VOC analyser was able to discriminate effectively between infected and uninfected dogs, with the infected dogs forming a tighter cluster than the negative dogs. Sensor array 3 however was unable to differentiate between infected and uninfected dogs.

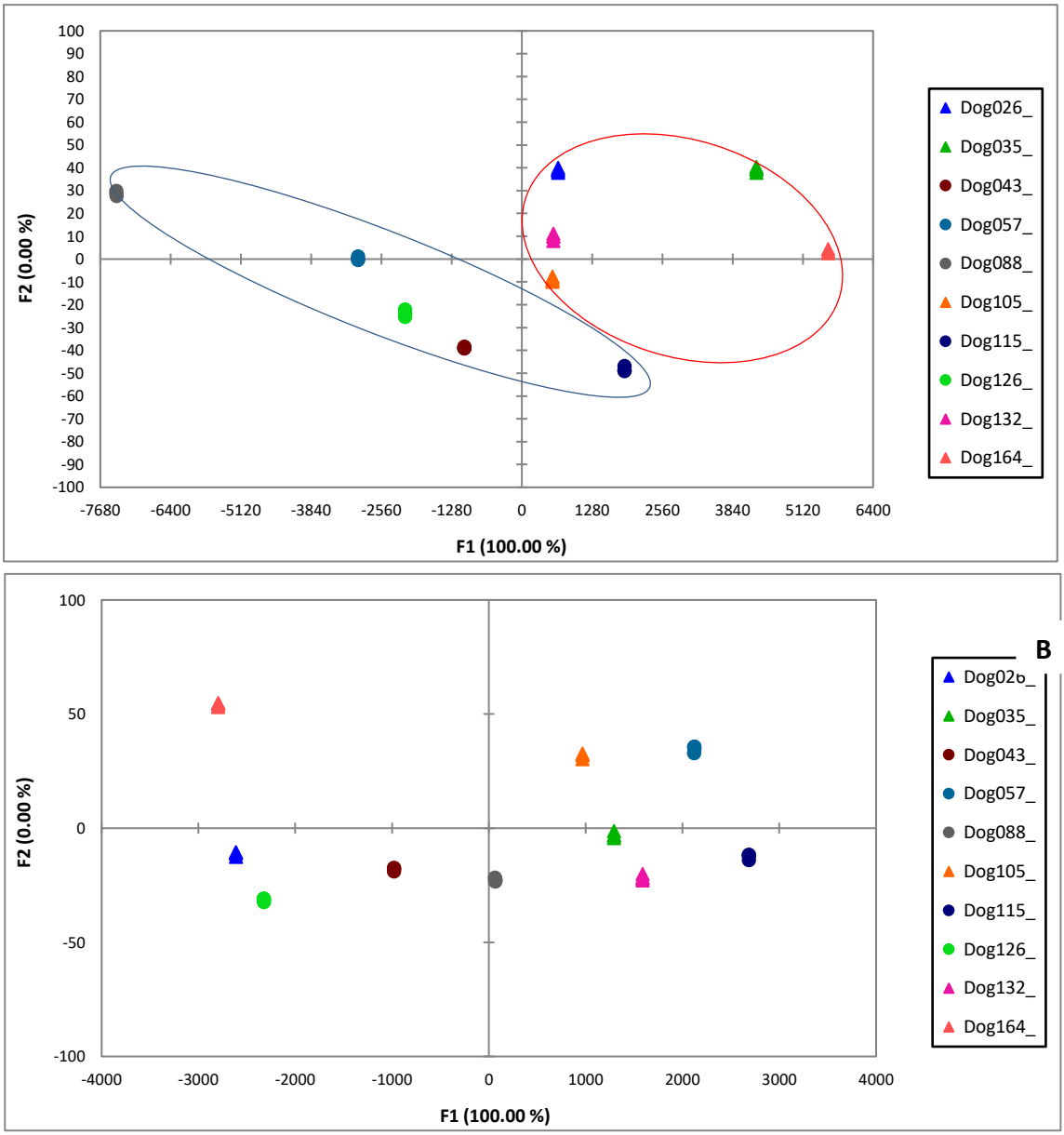


Figure 4.5: DFA with multiple variables using sensor array 1 (A) and sensor array 3 (B) of VOCs released from the buffy coat of dogs naturally infected with *Le. infantum* (Triangles) or from negative controls (Circles). Buffy coat was obtained from each animal 5 months prior the analysis and stored at -20°C. F1: discriminant function 1; F2: discriminant function 2. The numbers in parentheses show the percentages of the data matrix as described by the functions and relevant components. The ellipses were manually included. Results were generated by XLstat version 3.4.

4.4 Discussion

The results presented here show that through the combination of a VOC analyser with clustering analysis, dogs naturally infected with *Leishmania infantum* can be identified by analysis of the odour of their hair with high sensitivity and specificity. Furthermore, these results also showed no correlation between parasite load, clinical signs and VOC Analyser discrimination of the dogs (high medium or low discrimination) This outcome was observed in two data sets; samples collected in 2017 and 2018, using two different statistical analysis programs (R-cran statistical software and IBM SPSS). When the sensitivity and specificity of the data was determined following the calculation of means or division into subclasses, using two different statistical approaches, a very high specificity and sensitivity was calculated for the 2017 data; 100% for all analyses. However, the 2018 larger cohort of dogs demonstrated a variety of results. Following the division into subclasses and analysis using R-cran, a specificity of 95% and sensitivity of 97% was observed. However, using the calculated means and analysis with SPSS, the observed specificity and sensitivity were reduced to 91.4% and 56.8% respectfully demonstrating variability in the results. This variability could be mainly due to the type of statistical approach used.

Despite the relatively low sensitivity demonstrated when performing SPSS analysis on the data, the high sensitivity and specificity observed using R-cran statistics illustrates the potential of the VOC Analyser to accurately identify those dogs that are infected with *Le. infantum* in Brazil. This novel approach could significantly improve cVL diagnosis in Brazil as well as contributing to further research investigating the use of this technology for the effective diagnosis of human VL and other parasitic diseases. However further improvements still need to be made to the VOC analyser sensor

sensitivity, as well as the technologies potential as a point of care diagnostic approach which would include an improvement of the sampling methodology.

4.4.1 Optimisation of VOC analyser technology

Due to the VOC analyser being an innovative technique, with no prior use in the diagnosis of leishmaniasis, optimisation experiments were performed in order to design appropriate methodology to sample the head space of the dog hair. These volatiles were then passed over the sensor head. When 10μl of water was added to each sample, a high discrimination was observed as shown in **Figure 4.4**. However, when analysing the sample either dry or with 100μl of water, the VOC analyser was unable to effectively discriminate between the two different breeds of dog.

Research has shown that water, particularly when in a gaseous form, is not only able to trap volatiles (Laaks *et al.,* 2010), but also break down yeast and bacteria excreta (Rose *et al.,* 2015). The micro-excreta of bacteria and yeast are present on the skin of dogs as well as in dog hair, along with any volatiles that are excreted in sebaceous glands. Therefore, the evaporation of water from dog hair is required to release these volatiles. Additionally, a pilot study by Young *et al.,* (2002), demonstrated that the addition of water to dog hair, followed by incubation, can cause a substantial change in the observed VOCs. Certain volatile groups showed an increase in concentration following the addition of water including, phenylaldehyde, benzaldehyde acetaldehyde, 2methyl butanal and phenol. The importance of the addition of water to each sample was confirmed in optimisation experiments above, because when the dog hair samples were analysed dry, the analysis was unable to discriminate between the two species of dogs. However, results presented here also

demonstrated that the addition of too much water; 100µl, causes a lack of discrimination between the two dog species, as released VOCs are being overpowered by the volatile signature of water. Although the addition of 10µL caused adequate separation, the optimisation experiments only considered three quantities of water. Therefore, further optimisation is required in order to identify the optimal quantity of water needed to generate the greatest VOC Analyser response. In addition, high water content in samples has been found in previous studies to have a significant effect on VOC Analyser response as many of the sensors found on the sensor array respond strongly to water and therefore prevent any differences in the samples from being observed (Mottram and Elmore *et al.,* 2003). This could in turn have implications for the use of the VOC Analyser in the field as any water present on the dog hair could alter the results. This therefore would need to be controlled through further optimisation of the VOC Analyser device or the future development of sensors that are not as sensitive to water.

4.4.2 VOC Analyser diagnosis of dogs

4.4.2.1 Infected vs Uninfected 2017 and 2018

Although the current recommended protocol used for cVL diagnosis is widely adopted throughout Brazil, an observed lack of sensitivity combined with recent research which has demonstrated that the current protocol is not entirely reliable, is of concern (Belo *et al.,* 2017). It is widely recognised that the current protocol shows the same lack of sensitivity and specificity as the protocol which it replaced (Laurenti *et al.,* 2014). Furthermore, the accuracy of the DPP test has been evaluated independently several times, with the most recent evaluation carried out by

Figueiredo *et al.,* (2018) demonstrating a sensitivity of 75% and specificity of 73% when diagnosing asymptomatic dogs. Although the sensitivity increases to 94% when diagnosing symptomatic dogs, the specificity is even lower at 56%. The inaccuracy of the current diagnostics can be demonstrated further with evidence suggesting that the current protocols surrounding the culling of infected dogs need reviewing as this control strategy is deemed as failing (Ribeiro *et al.,* 2018). This current ineffectiveness therefore confirms the need for the development of a new in-field, non-invasive rapid diagnostic technique in order to improve VL control strategies in Brazil (Fraga *et al.,* 2016).

Results presented here demonstrate the VOC analyser as having a higher sensitivity and specificity than the current DPP in-field diagnostic test. Following a discriminant analysis performed using R statistical software, the analysed 2017 dog samples (n= 55) showed a 100% sensitivity and specificity. The VOC Analyser analysis of the 2018 dog samples (n = 149) also determined a 95% specificity and 97% sensitivity when discriminating between infected and uninfected dogs. In addition, further cross validation analysis of the statistical model also confirmed the accuracy of the VOC Analyser. CV analysis demonstrated that the model was able to discriminate between the infected and uninfected dogs of the validation set with 75% sensitivity and 80% specificity for the 2017 dogs and 93% sensitivity and 92% specificity for the 2018 dogs. These results not only provide validation of this model as an appropriate statistical analysis for the VOC Analyser, but also confirm the use of this device as an effective cVL diagnostic technique.

For a more robust analysis, the discriminant analysis was repeated using IBM SPSS. When performing the analysis using the mean variable values for each dog, a

100% sensitivity and specificity was determined for the 2017 data set when using the VOC Analyser. However, in 2018 this analysis showed a decreased 91.4% specificity and 56.8% sensitivity when discriminating between infected and uninfected dogs. This variation in results could be attributed to clustering not being used when the discriminant analysis was performed using IBM SPSS. Furthermore, as the SPSS analysis is a classical statistical approach, whereas the R-cran analysis is a Bayesian statistical approach, the algorithms used for both programmes differ which could influence the results produced.

Although independent VOC Analyser analysis of the 2017 and 2018 dogs demonstrated high specificities and sensitivities, a longitudinal study comparing dogs that were uninfected in 2017 but became infected in 2018 was unable to be performed. This is due to the observation that negative dogs from 2017 could be separated from negative dogs from 2018 with 79% specificity and 99% sensitivity. Therefore, the 2017 and 2018 sets of hair samples are considered as two independent data sets. This statistical difference observed between the uninfected dogs in 2017 and 2018 could be due to several factors. These include type and age of the VOC analyser used with there being a slight variation between the sensor heads used in 2017 and 2018, as well as the time of sample storage in 4^{o}C. It could be suggested that the storage of hair over a long period of time could potentially lead to a change in the relative abundance of bacteria on the hair samples (Lauber *et al.,* 2010), as well as having an effect on hormone concentrations, such as hair cortisol, with hair stored for longer having a lower concentration (Abell *et al.,* 2016). The production of different bacteria and hormone concentrations during storage could untimely have a significant effect on VOC Analyser sensor response, due to different chemicals being found in the

headspace. In fact, research has already identified that VOC Analysers have an ability to detect, with a high sensitivity, different bacterial colonies and hormones produced (Garcia-Cortes *et al.,* 2009). The length of storage of hair therefore could influence the volatiles produced. In order to complete a follow up study, samples would need to be analysed at the same time after sample collection using the same sensor array and VOC Analyser to produce reliable results.

Although the use of the VOC Analyser as a diagnostic tool offers many advantages over the current in-field DPP (cVL) test, further research needs to be undertaken to confirm the practical application and diagnostic capabilities of the device. First, the effect of other canine infections on the VOC analyser response was not determined. Research has shown that the skin dog mite *Demodex canis*, which affects many dogs with weakened immune systems in Brazil, produces similar clinical manifestations as cVL. This ectoparasite lives in the sebaceous glands and hair follicles of dogs and therefore could potentially have a significant effect on the volatiles being produced (Tsai *et al.,* 2011). Second, the ability of the VOC Analyser to detect and distinguish between different *Leishmania* species needs to be determined. All dogs used for this analysis were infected with *Le. infantum*, which is considered as the main causative agent of cVL in Brazil. However, recent studies in Governador Valadares found *Lutzomyia longipalpis* sandflies infected with multiple circulating *Leishmania* parasites including *Le. (Viannia*) spp. and *Le. amazonensis* (Cardoso *et al.,* 2019). Research has also identified dogs infected with *Le. amazonensis* in this region (Valdiva *et al.,* 2016).

4.4.2.2 Buffy Coat Analysis

Preliminary results presented here demonstrate that the blood of *Leishmania infantum* infected and uninfected dogs can be discriminated between using the VOC Analyser and sensor array 1. Furthermore, infected dogs formed a visibly tighter cluster than uninfected dogs suggesting a similar VOC signature being produced following *Leishmania* infection. These results therefore suggest the potential to use blood instead of hair for VOC Analyser analysis of *Leishmania* infected and uninfected dogs. However, when performing the analysis using sensor array 3, no such discrimination was observed. This is interesting as it confirms the importance of selecting the optimal chemical composition of each sensor when designing a sensor array for the specific diagnosis of cVL using a VOC Analyser.

The identification of blood metabolites as a potential diagnostic technique has been demonstrated throughout research. For example, previous studies have identified octane and 1-octen-3-ol as potential biomarkers of liver cancer following the analysis of VOCs in the blood of patients (Xue *et al.,* 2008). Furthermore, research has also identified plasma biomarkers which can be used for the discrimination between individuals with tuberculosis and those who are healthy (La Manna *et al.,* 2018). Regarding parasitological research, a cohort of malaria research has focused on the identification and analysis of malaria blood biomarkers. Studies have demonstrated that blood biomarker identification may facilitate the identification of malaria species, estimation of the parasite load, and determination of the intensity of immune response (Krampa *et al.,* 2017). In addition, a variety of different *Leishmania* blood biomarkers have also been identified (Maia and Campino, 2018). It has been demonstrated that the *Leishmania* parasite itself produces a variety of different metabolites which have previously been found in infected blood (Atan *et al.,* 2018). In

addition, the immune response produced during *Leishmania* infection leads to the production of different metabolites through changes in the metabolic pathways (Akpunarlieva *et al.,* 2017) with lipids, threonine, alanine and lactate found to be the most prominent *Leishmania* metabolites found in infected blood (Tafazzoli *et al.,* 2010). Additionally, research has shown that blood metabolites differ substantially between cellular extracts of different *Leishmania* species (Westrop *et al.,* 2015). This therefore suggests that not only can the VOC Analyser differentiate between the blood of infected and uninfected dogs but could also be used to identify the specific *Leishmania* species present within the blood itself.

4.4.2.3 Effect of parasite load on VOC Analyser diagnosis

The results presented suggested that VOC analyser response was not related to the parasite load in the dog peripheral blood. This can be demonstrated as most infected animals, regardless of parasite load (determined in Chapter 3, **Figure 3.11**), were identified as infected, with a high sensitivity and specificity being produced, particularly following clustering and R statistical analysis. Those dogs that were considered as having a high VOC Analyser discrimination presented with a wide range of parasite loads (13.73 parasites/mL to 853.44 parasites/mL), as did those dogs which were considered to have a low discrimination (6.92 parasites/mL, to 128 parasites/mL) (**Table 4.16**). The results presented here also suggest that there is no correlation between clinical signs and VOC Analyser discrimination, with highly discriminated dogs being both symptomatic and asymptomatic. This observation suggests that the VOC Analyser could facilitate the early diagnosis of asymptomatic dogs with a high

sensitivity, a quality currently lacking with the DPP (cVL) diagnostic (Figueiredo *et al.,* 2018).

Despite these observations, previous parasitological research has demonstrated that an increase in malaria volatile biomarkers is directly correlated with parasite load during infection (Berna *et al.,* 2015). In addition, research regarding the use of volatiles to discriminate between *Leishmania* infected and uninfected dogs also challenge the response of the VOC Analyser. Previous research using gas chromatography mass spectrometry (GC/MS) to analyse the volatile profiles of dogs, has suggested that the odour profiles of symptomatic dogs which therefore present with a higher parasite load, differ to asymptomatic dogs (Magalhães-Junior *et al.,* 2014b). GC/MS is a technique 10,000 times more sensitive than a VOC Analyser. This difference in sensitivity could explain the observed variation in results as a higher sensitivity will detect a broader range of VOCs. However, as their study only analysed 36 dogs, 24 of which were positive for CVL, a far smaller sample size then was analysed here (n = 55 for 2017; n = 149 for 2018) the validity of their study could be undermined.

Despite a large amount of research which has identified an observed difference in the volatiles produced during parasitic infections, the origin of these volatiles is still debated. Regarding *Leishmania*, the demonstrated correlation between parasite load and odour profile of infected dogs, suggests that the volatiles produced during infection may be related to the immune response (Magalhães-Junior *et al.,* 2014b). This is due to research which has not only demonstrated that an immune response produces a variety of VOC that are released externally by an infected individual (Shirasu and Touhara *et al.,* 2011), but also studies demonstrating that a high parasite load is directly correlated with immune response to *Leishmania* (Verma *et al.,* 2010).

The results presented here however suggest an alternative origin for these volatiles as there was no correlation observed between parasite load and VOC Analyser response. Interestingly, recent research has identified a distinct transmissible dysbiotic skin microbiota that is induced by cutaneous leishmaniasis infection (Gimblet *et al.,* 2017). This report also suggested that there is no correlation between parasite load and skin microbiota. It could therefore be suggested that the difference in volatile profiles observed between *Leishmania* infected and uninfected dogs could be related to changes in the skin microbiome. However, due to the variety of contradictory studies available, further research needs to be undertaken in order to confirm the origins of the volatiles produced during cVL infection.

Despite the lack of correlation shown between VOC Analyser response and parasite load, determining the Analysers limit of detection will be important in the future to validate this device as an early diagnostic technique. The results presented here suggest that the change in odour profile occurred soon after infection. This is because infected dogs with very low parasite loads could be differentiated from uninfected dogs. However, the exact quantity of parasites required to generate a VOC Analyser response is unknown. Furthermore, the use of blood parasite load as an indicator for the stage of cVL infection is disputed. This is due to research which has suggested that there is no correlation between the levels of circulating parasites and disease severity (Borja *et al.,* 2016). Therefore, in order to determine the VOC Analysers limit of detection collecting skin samples over blood may be suggested for future research. This is due to increasing evidence which has highlighted the importance of parasites found in the skin regarding the distribution of parasite load and disease severity (Doehl *et al.,* 2017). Aditionally, changes in skin odour profiles

have been shown to occur soon after infection in other disease states such as malaria, where changes in the odour profiles of skin volatiles were affected 6 to 8 days after infection (de Boer *et al.,* 2017).

4.4.3 Summary

The application of VOC Analyser technology could potentially be an important step towards the non-invasive diagnosis of cVL through disease volatile biomarkers. The results presented here suggest the potential for a modified VOC Analyser as a rapid, accurate and non-invasive diagnostic tool for the diagnosis of cVL. As only a small proportion of the sensor variables; 2 out of 88 in 2017 and 5 out of 88 in 2018 (**Table 14**) had a significant effect on the discrimination between infected and uninfected dogs, it can be suggested that the sensitivity and specificity of the device could be further enhanced. This could be achieved through modifications to the sensor head, with the addition of more *Leishmania* specific sensors. Additionally, improving the methods used for hair collection in the field and the odour analysis could provide the opportunity for a longitudinal study which could also enhance the sensitivity and specificity of a future device. Further developments in the portability and simplicity of the VOC Analyser would also improve the reliability and convenience of the device in a field-based setting.

The use of the VOC Analyser as part of the current Ministry of Health protocol for cVL diagnosis in Brazil; either in addition to the DPP cVL or as a replacement, could potentially remove the need for the ELISA confirmatory test that is relatively expensive and time consuming. Due to the rapid, accurate and non-invasive nature of the VOC Analyser which identifies cVL by using a different set of disease markers, a future

device could prove highly effective in the ongoing control of *Leishmania* transmission. Furthermore, the results presented here demonstrate the ability of the VOC Analyser to diagnose not only asymptomatic dogs, but also dogs with low levels of infection, which would be an additional benefit to current cVL control programs. However, further work is required to compare the sensitivity and specificity of a VOC Analyser combined with DPP cVL diagnosis protocol against the current Ministry of Health protocol.

There is a need for the current VOC Analyser technology to be adapted into a simpler and more portable device which would provide accurate and rapid diagnostic tests. This is due to growing demand for improved healthcare techniques that are non-invasive and would speed up POC diagnosis, allowing for faster treatment for diseases as well as an improved prognosis and a reduction in healthcare costs (Wilson, 2018). Continued research into POC diagnostics, as demonstrated here, will help to bring VOC Analyser technology into routine clinical practice. The development of a VOC Analyser POC diagnostic tool for cVL that is based on host odour opens up the opportunity to diagnose not only *Leishmania* in dogs, but also potentially *Leishmania* infections in humans, and other parasitic diseases such as Chagas's disease, malaria and trypanosomiasis.

Chapter 5 - Attraction of sandflies to the odour of *Leishmania infantum* infected dogs

5.1 Introduction

Parasite manipulation of vertebrate hosts is a common occurrence that is likely to have significant consequences for disease epidemiology and ecology (Lafferty and Shaw, 2013) In the case of vector-borne pathogens which move between hosts, such a manipulation includes both the vector and the animal host. Such a manipulation has been demonstrated in research which has shown that vector-borne parasites may enhance their own transmission by directly effecting vector behaviour (Rogers and Bates, 2007). In addition, successful transmission also requires extensive contact between the host and insect vector, usually during blood feeding. Due to the intricate lifestyle of many vector borne diseases which require vertebrate hosts for successful parasite transmission and development, establishing vector attraction to hosts is vital for disease ecology.

Research has demonstrated that blood sucking insects, show an increased attraction to some hosts more than others (Heil, 2016). However, the explanation for this difference in attractiveness is yet to be established. It has been suggested that certain individual hosts are naturally more attractive to insect vectors, with sandflies and mosquitoes having shown a higher degree of attraction to certain individuals, regardless of their infection status (Fernández-Grandon *et al*., 2015; Tavares *et al.,* 2018). However, epidemiological models have shown that vector preference for infected hosts could boost disease transmission during the early stages of disease

epidemics. This suggests that vector attraction to infected hosts may be a result of parasite manipulation (Hosack *et al.,* 2008). Recent research has established parasite alteration of certain traits of their primary vertebrate hosts. It has been suggested that transmission mechanisms are an important factor that influence such vector-borne pathogen manipulations. Such manipulations which influence vector dispersal and attraction, as well as increase the likelihood of parasite acquisition by vectors during vector-host interactions has been demonstrated (Hafer, 2016). Previous research has demonstrated that malaria-infected individuals are more attractive to the mosquito vector (Robinson *et al.,* 2018). This suggests that a vector's choice to feed on infected or uninfected hosts could have an effect on the epidemiology of vector-borne diseases (Gandon *et al.,* 2017). It is thought that vector attractiveness is odour-mediated, with studies not only demonstrating that parasite infected individuals produce a different odour profile to those who are healthy (Magalhães-Junior *et al.,* 2014b), but also showing an increased vector attraction to infected odour profiles (De Moraes *et al.,* 2014; Robinson *et al.,* 2018). Therefore, behavioural bioassays can be utilised to establish vector attraction to specific volatiles and odour profiles. For this reason, such bioassays can be employed in order to determine the role that *Leishmania* infection plays on sandfly vector attraction. By establishing the attractiveness of *Leishmania* infected dogs to sandfly vectors, a further understanding of parasite transmission due to an increase in sandfly biting could be established. Furthermore, an increase in overall attraction to infected hosts could be predicted to optimise transmission.

The identification of *Leishmania* induced changes to host odours which influence vector behaviour could have further potential applied implications (De Oliveira *et al*, 2008; Magalhães-Junior *et al*, 2014b). In addition to establishing a greater

understanding of parasite transmission in *Leishmania* endemic areas, laboratory-based bioassays could also be used to determine the potential of infected dog odour as a sandfly lure, facilitating the development of potential vector control methodologies. The use of vector control to reduce the transmission of disease is considered an effective method (Golding *et al*., 2015). Many of the vector control strategies currently in place involve the use of interior residual spraying as well as insecticide-treated nets. However, as shown throughout mosquito research, the development of vector insecticide resistance is an emerging issue (Chaiphongpachara *et al.,* 2018). Therefore, the use of traps to capture and subsequently reduce the number of adult sandflies is becoming increasingly more popular. Research has proposed the used of synthetic attractants as baits in sandfly traps in order to improve specificity (Andrade *et al*., 2008; Magalhães-Junior *et al.,* 2014). This has stimulated the search for specific volatile compounds that are known to attract *Lutzomyia (Lu.) longipalpis*. The identification of these volatiles or odour profiles would allow for the modification of light traps by either using the volatiles directly as attractants or associating the compound with light. Identifying the attraction of *Lu. longipalpis* to natural odour of dogs infected with *Le. infantum* could prove vital in the identification of new volatiles for sandfly control, and the development of effective odour baited traps.

5.1.1 Sandfly olfaction

In order to identify a specific host or mate, insects must be able to not only recognise a specific blend of odour, but also discriminated this odour from a complex and continuously changing background. Therefore, they have evolved a highly efficient

and sensitive olfactory system in order to detect behaviourally relevant compounds and properly decode olfactory messages to lead to appropriate adapted behaviours (Martin *et al.,* 2011). Insect vectors of disease rely heavily on their olfactory system to locate hosts which is essential for blood feeding and, in turn, parasite transmission. As vector control has previously been demonstrated as an efficient way of reducing canine leishmaniasis (Courtenay *et al.*, 2019), understanding the sensory biology of sandflies and their attractiveness to host odour is becoming increasingly more important.

Various selection pressures have led to a remarkable diversity in the morphology of different insect peripheral olfactory systems. This therefore means that sandflies can detect specific volatile chemicals. Many of the volatiles which can be detected by sandfly olfactory sensory neurons have already been identified. These include aggregation, sex and alarm pheromones and compounds associated with egg-deposition behaviour (Guidobaldi *et al.,* 2014). Previous research has also identified many specific volatiles that female *Lu. longipalpis* use to locate suitable hosts. These include ketones such as 4-methyl-2-pentanone, carboxylic acids such as 2-methyl propanoic acid and aldehydes such as benzaldehyde (Dougherty *et al.,* 1999). As with other insects, sandflies respond weakly to relevant single odour compounds however natural mixtures induce a strong response.

The evolution of the sandfly olfactory system, which has evolved to be highly specific for certain volatiles, could prove important for further research. As parasite manipulation of host odour has been shown previously, understanding the interaction between the sandfly olfactory system and the volatiles released by a *Leishmania* infected host, could further our understanding of *Leishmania* transmission.

5.1.2 Sandfly attraction to host odour

The survival of vector-borne parasites is highly dependent on its ability to pass from one host to another via an insect vector. Parasites that are predominantly found in the circulatory system or superficially in the skin, have been shown to influence vector transmission from one host to another through the manipulation of both the vector and the host animal. Such a manipulation has been demonstrated through parasitic manipulation of the odour of the host animal (Hughes and Libersat, 2019). This phenomenon has been demonstrated for both malarial infections (De Moraes et *al.,* 2014) and leishmaniasis (Tavares et *al.,* 2018). Understanding the influence that parasitic infections have on infected individuals would lead to a greater understanding of parasite transmission and ecology.

Developing an in depth understanding of the chemical and ecological relationship between sandflies and host organisms is vital in gaining knowledge on the vectors biology and could prove important in the development of improved strategies related to leishmaniasis control. Research regarding parasite/vector interactions have identified and investigated compounds which demonstrate an increased attractiveness to vectors of neglected tropical disease, such as *Anopheles gambiae* (malaria), *Aedes aegypti* (yellow fever, dengue fever, chikungunya, Zika virus) and phlebotomine sandflies (leishmaniasis) (Geier *et al.,* 2002; Logan *et al*., 2008; Verhulst *et al.,* 2010; Tavares *et al.,* 2018). Research has demonstrated the importance of odours in the host-seeking behaviour of sandflies with an increased attraction to odours produced by animal baits having been observed in the field (Bongiorno *et al*., 2003). In Brazil, canines play a vital role in the *Leishmania* transmission cycle, being

considered the primary source of infection for the *Lu. longipalpis* sand-fly; the vector of visceral leishmaniasis, in both the domiciliary and pre domiciliary environment (Travi *et al.,* 2018).

Previous research has observed that vector-borne pathogens can manipulate host odours in order to influence vector attraction and transmission, with infected individuals demonstrating an increased attractiveness. As the insect's main orientation mechanism is based primarily on the recognition of different odours, this increase attractiveness is more than likely to be related to different odours that are exhaled by infected animals. This enhanced attraction is extensively shown throughout literature, particularly regarding malaria infection. Research has shown that humans infected with malaria have altered blood components and skin volatiles when carrying the infective parasitic stage these changes have been shown to alter mosquito behaviour, causing an increase in attraction to infected human hosts (Lacroix *et al.*, 2005). Due to the longitudinal nature of the study, it could be suggested that host odour is directly altered by the parasite, with the observed modification of mosquito behaviour being the ultimate effect. This manipulation of host odour causes an increase in parasite transmission to the vector with more mosquitoes feeding on infected hosts and therefore an increase in the transmission of the parasite (Emami *et al.*, 2017). This enhanced attraction has also been demonstrated with mice infected with *Plasmodium chabaudii*; a rodent malaria parasite, having an enhanced attraction of mosquitoes (De Moraes *et al.*, 2014). This enhanced attraction was facilitated not only by an overall elevation of volatiles emitted by infected mice, but also due to specific changes in the quantities of individual compounds. Furthermore, a clear difference in the odour profiles of infected and uninfected mice was persistent throughout the duration of the

infection (De Moraes *et al.,* 2014). An enhanced mosquito attraction to infected humans has also been demonstrated, with individuals infected by malaria parasites in its gametocyte stage attracting roughly twice as many mosquitoes when compared to both uninfected individuals and individuals infected with the asexual stage of the malaria parasite (Lacroix *et al.,* 2005).

More relevant *Leishmania* research has also observed differences between the odour profiles of *Leishmania* infected and uninfected individuals causing an enhanced attraction. Earlier research has demonstrated an increase in the attractiveness of *Lu. Longipalpis* to exhaled compounds from foxes (*Vulpes vulpes*) (Dougherty *et al.,* 1999), as well as alcohols that are found in plants (Magalhães-Junior *et al.,* 2014). In addition, a recent study demonstrated, that hamsters infected with *Le. infantum* emit an odour that enhances the attraction the sandfly vector *Lu. Longipalpis*. Six out of 13 hamsters used in this study became significantly more attractive following infection. (Nevatte *et al.,* 2017). In nature however, due to differences in habitats, potential hosts are not equally exposed to sandfly vectors; hosts which live in a closer proximity to vector breeding sites are more likely to be exposed. Furthermore, host characteristics such as age, size, sex, health status and blood type may also influence host attractiveness to sandfly vectors. Interestingly, the location of the parasite in the skin of an infected host has also been shown to effect host infectiousness. Sandflies have demonstrated a preference to feed on patches of skin which contain larger numbers of parasites thereby increasing their infection load and potential outward transmission (Doehl *et al.,* 2017). This not only highlights the skin as important in the attraction of sandflies and subsequent disease transmission, but also suggests a parasite manipulation of the volatiles excreted in the skin in skin parasite patches.

This attraction phenomenon has also been reported in research investigating the attractiveness of human skin odours that are predominantly produced by skin microbiota, to sand-flies. These bacteria have been proven to have an effect on the attraction of insects, therefore affecting an individual's risk of infection (Smallegange *et al.,* 2011). A recent study identified a transmissible dysbiotic skin microbiota induced by cutaneous leishmaniasis that promotes a skin inflammatory response (Gimblet *et al.,* 2017). Interestingly, skin appendages such as hair also provide an ideal specific environment which aids in microbiota growth (Kong and Segre, 2012) which could prove an important insight into insect attraction to host odour. As previously reported, hair analysis has provided a means of odour profile determination in dogs with cVL using metabolomics techniques and has been considered as a potential tool in the detection of *Le. infantum* markers, which could prove invaluable in the development of diagnostic techniques (Oliveira *et al.,* 2008; Magalhães-Junior *et al.,* 2014b). It could therefore be suggested that the reported distinction between dogs infected with *Leishmania* and those that are uninfected could be linked to a change in skin microbiota.

5.1.3 Behavioural Bioassay

Experiments which demonstrate the behavioural activity of specific compounds or group of compounds are important for establishing factors which may influence insect behaviour. Bioassays which determine a chemical(s) effect on different insects allows for the identification of the type of response they elicit. Well-designed behavioural bioassays therefore, can be highly beneficial when deducing the communicative function relating to a chemical and the insect tested, as well as

furthering our understanding of disease transmission and potential parasite manipulation of host odour (Smallegange and Takken, 2010).

5.1.3.1 Olfactometers

Olfaction is the major sense involved in the host-seeking and oviposition behaviour of insects. It is also known that these olfaction-based activities play an important role in the natural survival of vector borne diseases such as *Leishmania* through periodic travelling between insect vectors and hosts.

Several different types of olfactometers have been developed in order to study the host-seeking behaviour of vectors such as mosquitoes and sand-flies. Briefly a commonly used type of insect olfactometer consists of a glass Y-tube, in which insects are introduced to the long stem of the Y and move up the tube being attracted by samples that are contained at the free ends of the arms (**Figure 5.1**). The principle behind this design of insect olfactometer is to attract insects equally towards the entrances of the forks by a volatile stimulus however, when an insect reached the forks they are influenced unequally by the odours, with each fork containing a different volatile stimulus (Omrani *et al.,* 2010).

It has been proposed that olfactometers could prove beneficial in understanding qualitative manipulation theories, in which insects develop a feeding preference for specific hosts (Lefevre *et al.,* 2006). It has been suggested that this qualitative manipulation could be achieved in two ways. First, preference towards an infected host could be achieved by host infection inducing a sensory bias in the vector through the alteration of the volatiles released during infection. However, although research has demonstrated a change in the volatiles within *Leishmania* infected hosts

(Oliveira *et al.,* 2008; Nevatte *et al*., 2017; Magalhães-Junior *et al.,* 2014b), limit research is available which has confirmed whether this VOC alteration has an effected on sandfly attraction to naturally infected hosts. Second, it has been suggested that certain hosts are naturally more attractive to insect vectors and therefore more likely to become infected (Fernández-Grandon *et al.,* 2015). This phenomenon, however, has currently not been demonstrated in sandflies and their hosts. Previous research, however, has demonstrated a *Leishmania* manipulation of sandfly host-seeking behaviour (Rogers and Bates, 2007). This observed increase in attraction towards a specific host could be achieved by parasitic manipulation of the insect sensory system, increasing attraction towards a specific host and host traits that are correlated with the optimal suitability for the parasite. There are three potential ways in which vector manipulation could occur; (i) infected vectors could feed on a specific host species for the parasite, (ii) infected vectors may feed on hosts with a weakened immune system or (iii) infected vectors may be more attracted to uninfected hosts that do not harbour any potential competitors (Lefevre *et al.,* 2006).

Regardless of the proposed mechanisms of parasite manipulation, be it manipulation of the sandfly or of the host, with the aid of olfactometers to study vector attraction research could be able to provide further information on the physiological principles behind the host-seeking behaviour of sandfly vectors, and further our understanding of *Leishmania* transmission. Olfactometers have the potential to quantify the behavioural responses of both infected and uninfected vectors to volatiles that are emitted by different hosts both infected and uninfected.

5.1.3.2 Sandfly behavioural bioassays

Despite recent research which has identified *Leishmania* specific VOCs in both dogs (e.g. Magalhães-Junior *et al*., 2014b) and humans (Tavares *et al*., 2018), there is limited research which has investigated the role of these volatiles in the attraction of sandflies to *Leishmania* infected dogs, which would prove important in our understanding of disease transmission in Brazil. Female *Lu. longipalpis*, have been shown to be attracted to specific volatiles identified from *Leishmania* infected human patients, with research suggesting that 2-phenylacetaldehyde, 6-methylhept-5-en-2-one and icosane stimulate an attraction response to female sandflies (Tavares *et al.,* 2018). Additionally, *in vivo* experiments demonstrated that the odours of hamsters infected with *Leishmania infantum* are more attractive to female *Lu. longipalpis* sand flies when compared to the odours of uninfected hamsters (O'Shea *et al*., 2002; Nevatte *et al*., 2017).

Bioassays have provided an ideal tool to identify important volatiles related to sandfly attraction. Although in excess of 400 VOCs have been identified as being produced from skin (Dormont *et al.,* 2013), limited research has shown the effect of these volatiles on sandfly attraction to naturally infected hosts. As changes in the skin VOCs of infected dogs in endemic areas has been observed, understanding the role these changes may have on sandfly attraction would prove vital in our understanding of disease transmission and provide further evidence for potential sandfly attractants.

5.1.4 Chapter Aims

Research suggests that vector-borne pathogens may manipulate host odour in order to influence vector attraction (Robinson *et al*., 2018). Such effects could have implications in the development of olfactory lures to improve current vector control,

as well as further our understanding on parasite-host interactions and the mechanisms underpinning parasite transmission. By utilising Y-tube olfactometer behavioural bioassays, I aim to determine whether *Le. infantum* infected dogs are significantly more attractive to *Lu. longipalpis* sandflies, compared to dogs which are uninfected. Such an attraction could imply host odour manipulation by *Leishmania*, aiding its own transmission through increasing sandfly attraction to infected individuals. Additionally, the use of canine host odour in the devlopment of odour-baited traps to improve current vector control methodologies in Brazil is also discussed.

5.2 Methods

5.2.1 Sample collection and Entrainment

Canine hair was collected in the field as described in the general methods section for both 2017 and 2018. Following sample collection, a minimum of 1g of hair was introduced into a portable entrainment system in order to collect released volatiles. Entrainment of VOCs from the hair samples is also described in the general methods section.

5.2.2 Bioassay

5.2.2.1 Sandflies

Bioassays were performed using both male and female *Lu. longipalpis* sand-flies. The sandflies were originally collected in Jacobina, Brazil (40°31’ W, 11°11’S) and routinely fed on a membrane feeding system in order to maintain the colony.

5.2.2.2 Optimisation

In order to perform the bioassay, the ideal dilution factor needed to achieve optimal sandfly response was determined. Three uninfected dogs were chosen at random; dogs 138, 128 and 027 and compared to a hexane control standard. Initial experiments tested the effect of various dilution factors; 1:1, 1:4, 1:8, 1:10 and 1:15, on female sandfly response to dog 138. Once the optimal dilution factor was determined for this specific dog sample, the remaining two dog samples were analysed. The experiment was performed using three separate dogs using this determined dilution factor in order to confirm sandfly responsiveness and investigate

whether sandflies are attracted to dog volatiles over a hexane blank. Following confirmation of the optimal dilution, all further bioassay experiments were carried out using this dilution. A binomial test *P* value <0.05 was considered significant. This value was calculated as the proportion of female sandflies which responded to the test side, out of the total number of that responded.

5.2.2.3 Infected vs uninfected dogs

Initially, 9 infected dogs and 9 uninfected dogs were selected from the VOC Analyser results based on their discrimination following data analysis. As described previously, the VOC analyser discrimination was determined based on a dog's cluster probability (discriminatory factor).

Infected dogs chosen were as follows:

High discrimination: Dogs 141, 176 and 178; medium discrimination: Dogs 105, 140 and 003; low discrimination: Dogs 74, 82 and 102.

Uninfected dogs chosen were as follows:

High discrimination: Dogs 021, 037 and 093; medium discrimination: Dogs 137, 004 and 175; low discrimination: Dogs 181, 130 and 124.

The samples which were compared for the female sandfly bioassays are shown in **Table 5.1**. The initial bioassay comparisons are cross comparisons between infected and uninfected groups to allow for a more robust analysis. The *P*-value was calculated as previously described.

1g of dog hair/2.5 hour entrainment (1:10 dilution) ***(infected dogs in bold)***	**VOC Analyser discrimination of dogs** ***(infected in bold)***
Dog 176 vs Dog 021	**High** vs High
Dog 141 vs Dog 137	**High** vs Medium
Dog 178 vs Dog 181	**High** vs low
Dog 105 vs Dog 037	**Medium** vs High
Dog 140 vs Dog 004	**Medium** vs Medium
Dog 003 vs Dog 130	**Medium** vs Low
Dog 074 vs Dog 093	**Low** vs High
Dog 082 vs Dog 175	**Low** vs Medium
Dog 102 vs Dog 124	**Low** vs Low

Table 5.1: Samples compared during the female sandfly unblinded bioassay. Dog samples which were compared are shown in the left-hand column. Each individual bioassay was performed using 80 female virgin *Lu. longipalpis* sand-flies.

Following the initial analysis, the bioassay was then performed following a blinded experimental protocol. This was done in order to confirm female sandfly response and remove any experimental bias. 6 new pairs of dogs were chosen at random (n = 12 individual dogs). Infected (n = 6) and uninfected (n = 6) dogs were compared against each other with 2 highly discriminated pairs, 2 medium discriminated pairs and 2 low discriminated pairs chosen. The pairs of dogs selected for the blinded trail were chosen based on VOC Analyser discrimination criteria. Samples were randomly labelled A through to M, with the samples to be compared shown in **Table 5.2**. Individual entrained dog samples were diluted to a 1:10 dilution using hexane and the response of 80 female sandflies was recorded.

1g of dog hair/2.5 hour entrainment (1:10 dilution) ***(infected dogs in bold)***	**Dog Number** ***(infected dog in bold)***	**VOC Analyser discrimination of dogs** ***(infected in bold)***
K vs H	**Dog 126** vs Dog 169	**High** vs High
L vs C	**Dog 047** vs Dog 153	**High** vs Medium
M vs G	**Dog 044** vs Dog 005	**Low** vs Low
A vs F	**Dog 080** vs Dog 136	**Medium** vs Medium
J vs D	**Dog 134** vs Dog 070	**High** vs High
B vs E	**Dog 019** vs Dog 043	**Low** vs Low

Table 5.2: Samples compared during the female sandfly blinded bioassay. Dog samples which were compared are shown in the middle column and their assigned letter in order in the left-hand column. Each individual bioassay was performed using 80 female virgin *Lu. longipalpis* sand-flies.

Following the bioassays using female sand-flies, the experiment was repeated using male sand-flies. All 15 dog pairs; 9 pairs from the initial female bioassay (**Table 5.1**) and 6 pairs from the blinded experimental protocol (**Table 5.2**) were re-randomised. Individual bioassays were then performed initially on 9 dog pairs, followed by a blinded protocol using the remaining 6 dog pairs. Entrained dog samples were diluted to a 1:10 dilution using hexane and the response of 80 male sandflies was recorded. The orders in which the bioassays were performed, and the dog samples used for both the blinded and non-blinded experiments are shown in the **Table 5.3 and 5.4**. Again, a binomial test *P* value <0.05 was considered significant. This value was calculated as the proportion of male sandflies which responded to the test side, out of the total number of that responded.

1g of dog hair/2.5 hour entrainment (1:10 dilution) ***(infected dogs in bold)***	**VOC Analyser discrimination of dogs** ***(infected in bold)***
Dog 176 vs Dog 021	**High** vs High
Dog 080 vs Dog 136	**High** vs Medium
Dog 082 vs Dog 175	**High** vs low
Dog 019 vs Dog 043	**Medium** vs High
Dog 074 vs Dog 093	**Medium** vs Medium
Dog 105 vs Dog 037	**Medium** vs Low
Dog 003 vs Dog 130	**Low** vs High
Dog 140 vs Dog 004	**Low** vs Medium
Dog 044 vs Dog 005	**Low** vs Low

Table 5.3: Samples compared during the male sandfly unblinded bioassay. Dogs classified as high, medium or low discrimination based on VOC Analyser analysis.

1g of dog hair/2.5 hour entrainment (1:10 dilution) (infected dogs in bold	**Dog Number (Infected dog in bold)**	**VOC Analyser discrimination of dogs (infected in bold)**
C vs G	**Dog 141** vs Dog 137	**High** vs Medium
E vs A	**Dog 178** vs Dog 181	**High** vs Low
D vs L	**Dog 134** vs Dog 070	**High** vs High
F vs I	**Dog 126** vs Dog 169	**High** vs High
B vs J	**Dog 047** vs Dog 153	**Medium** vs Medium
K vs H	**Dog 102** vs Dog 124	**Low** vs Low

Table 5.4 Samples compared during the male sandfly blinded bioassay. Dogs classified as high, medium or low discrimination based on VOC Analyser analysis.

5.2.3 Methodology

5.2.3.1 Lutzomyia longipalpis

80 female, virgin *Lutzomyia longipalpis* sand flies or 80 male *Lu. longipalpis* sandflies were collected 1-day post emergence and held for 5 to 7 days in a Barraud cage (18x18x18 cm) at 27°C with a 70% relative humidity within a plastic bag, without any excess sugar. One hour prior to the start of the experiment the sand fly cage was moved into the bioassay room (68.0% rH; 27.8°C), the plastic bag removed and the sandflies allowed to acclimatise to the room conditions. The humidity and temperature within the room were kept constant with bioassays performed at the same time of day. This is due to research demonstrating that flight activity is triggered by daily changes in abiotic factors such as temperature and relative humidity but also by changes in light intensity (Rivas *et al.,* 2014).

5.2.3.2 Y-tube olfactometer

The Y-tube olfactometer was formed of three lengths of glass tubing (10mm id, 1.27cm od). Two long arms measuring 10cm were joined at an angle of 65° with a long stem measuring 10 cm being centrally positioned between the two arms in the same plane in order to form the Y shape (**Figure 5.1**).

A gentle flow of bottled, zero grade air was fed through two charcoal traps (flow rate 2 ml/sec) and allowed to flow down to the Y-tube via Teflon tubing (¼" od). Airflow was confirmed using a bubble meter. Prior to reaching the Y-tube, the tubing was divided into two using a brass Swagelok T-junction. A 15cm length of Teflon tubing was connected to each side of the T-junction and these were pushed into a 3cm long section of Teflon tubing. Each of these short sections had a small hole pierced through

the wall, with a 2cm diameter of rolled up grade 1 filter paper inserted into it. Extracted sample (1μl) was injected onto the piece of filter paper. The other end of the short pieces of Teflon tubing were connected to a 40cm long section of Teflon tubing which was connected to the arms of the Y-tube with a brass reducing union (1.27cm to 0.64cm). Glass wool was inserted into the Swagelok connector at the end of each arm to prevent sandflies escaping up the Teflon tubing. All connections and tubing joints were sealed with PTFE ® tape. All Teflon tubing and glassware was thoroughly cleaned 24 hours prior to the experiment. Glassware was cleaned by washing with 10% Teepol solution, followed by being washed in distilled water and then acetone before being baked over night at 225^{o}C. All Teflon tubing was washed with hexane (pesticide grade) and allowed to air dry overnight.

In order to carry out the bioassay experiment, the Y-tube was placed horizontally on a solid bench. Before each replicate, an additional 1ul of extract was injected onto the filter paper contained in the 3cm long section of Teflon tubing. The hole was sealed with PTFE ® tape. To test the attraction of individual sand-flies, each fly was released individually at the base of the Y-tube and walked upwind towards the far end of one of the arms of the Y. A timer was started and the final position of the sandfly within the Y-tube was recorded after three minutes; either the test or control arm or, if it remained in the stem, a “no choice” was recorded. In order to reduce bias in the experimental set up, after 10 replicates, the rolled-up filter paper was removed and replaced, and the test and control ports were swapped round with the Y-tube also being rotated through 180^{o}.

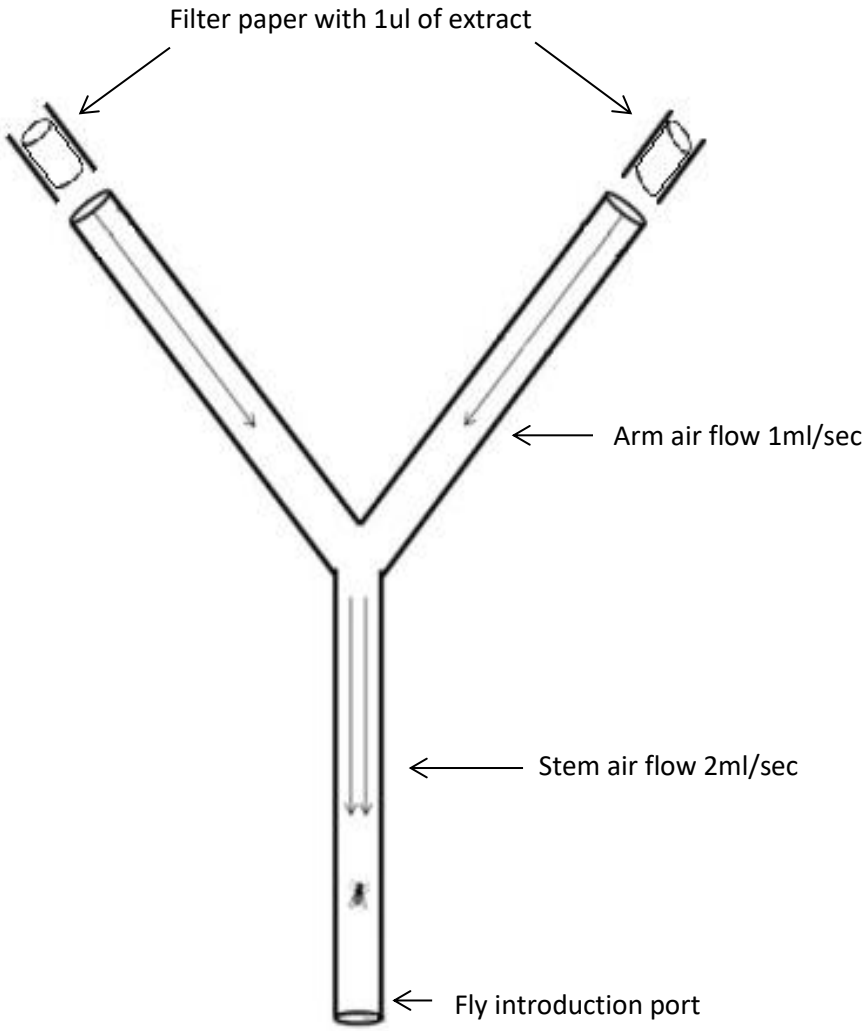


Figure 5.1: Basic Y-tube olfactometer design. Flies were introduced into the lower port and travelled upwind, making a locomotor choice at the Y split. Flies were recorded as having made a definitive choice when they had travelled a minimum of 15 cm up a Y-tube arm (Image adapted from Haselton *et al.,* 2015).

5.3 Results

5.3.1 Optimisation

Following the entrainment of VOCs from canine hair collected in the field (as described in Chapter 2), three uninfected dogs from the 2018 cohort were chosen at random. In order to determine the optimal dilution required to generate the greatest sandfly response, dilutions were performed on eluted volatiles from one of the uninfected dogs (dog #138). Dilutions were as follows; 1:1, 1:4, 1:8, 1:10, 1:15. The optimal dilution was assessed by determining at which dilution female sandflies showed a significant attraction to the entrained dog sample. If there was no definitive choice observed, a response of Undecided was reported. **Table 5.5 and Figure 5.2** show that at a dilution of 1:10, a significant number of female sandflies responded to the dog sample, with 60.0% of all sandflies analysed for that dilution actively choosing the dog odour.

	No. of Female sand-flies			
Dog 138 1g of hair/2.5hr entrainment	**Dog 138**	**Hexane**	**Undecided**	***P*-Value (Binomial Test)**
1:1	17	25	38	0.0579
1:4	21	19	40	0.1194
1:8	31	24	25	0.0691
1:10	48	21	11	0.0005
1:15	30	27	23	0.0973

Table 5.5: Number of female sandflies which responded to Dog #138. Dog #138 was diluted using hexane to 1:1, 1:4, 1:8, 1:10 or 1:15 dilutions. The number of female sandflies attracted to each sample was recorded. 80 sandflies were used per bioassay. $P<0.05$ dog #138 vs hexane was considered significant.

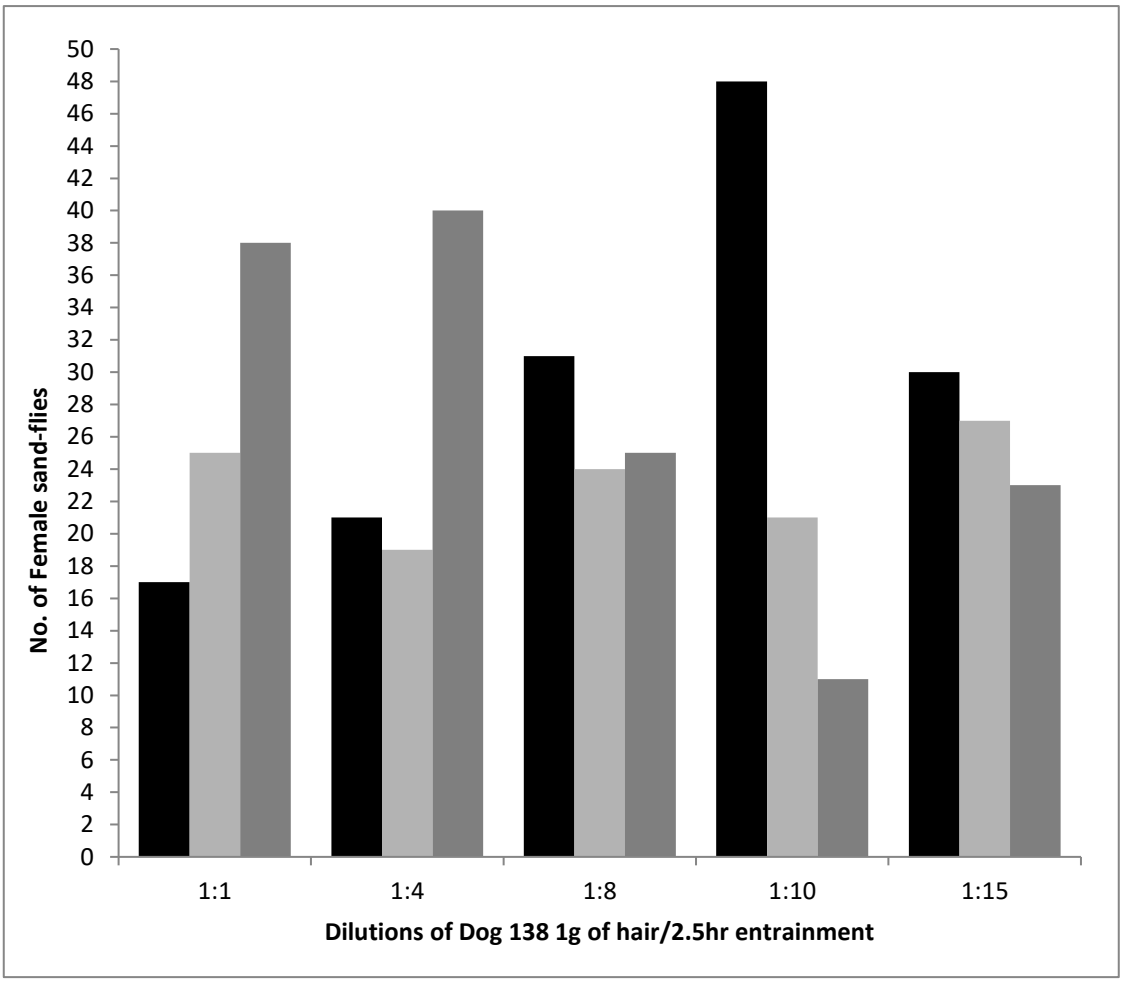


Figure 5.2: Dog #138 bioassay to determine optimal dilution. 5 bioassays were performed in total using 80 female sandflies. The number of female sandflies which responded to the test dog sample (Dog #138) is shown in black. Hexane control; light grey, undecided; Dark grey. * = *P*-value <0.05 which was considered significant.

Following the determination of a 1:10 dilution as being the optimal dilution, the bioassay was repeated using female sandflies for dog #128 and #027 (**Table 5.6 and Figure 5.3**). This experiment was performed in order to confirm the optimal dilution If there was no definitive choice observed, a response of Undecided was reported.

Not only was a 1:10 dilution confirmed as the optimal dilution to provoke sandfly response, female sandflies were also shown to be significantly more attracted to dog volatiles compared to a hexane blank, with 57.9% (n = 139) all the sandflies analysed (n = 240) responding to the dog volatiles over the hexane blank and 66.5% of all sandflies which responded (n=209) also favouring the dog odour.

	No. of Female sand-flies			
Dog dilutions	**Dog Sample**	**Hexane**	**Undecided**	***P*-Value (Binomial test)**
Dog #138 - 1:10 dilution	48	21	11	0.0005
Dog #128 - 1:10 dilution	45	29	6	0.0166
Dog #027 - 1:10 dilution	46	20	14	0.0006

Table 5.6: Number of female sandflies which responded to Dogs #138, #128 and #027. Dogs #138, #128 and #027 were diluted using hexane to a 1:10 dilution. The number of sandflies which responded to each sample was recorded in the table above. 80 sandflies were used per bioassay. $P<0.05$ dog sample vs hexane control was considered significant.

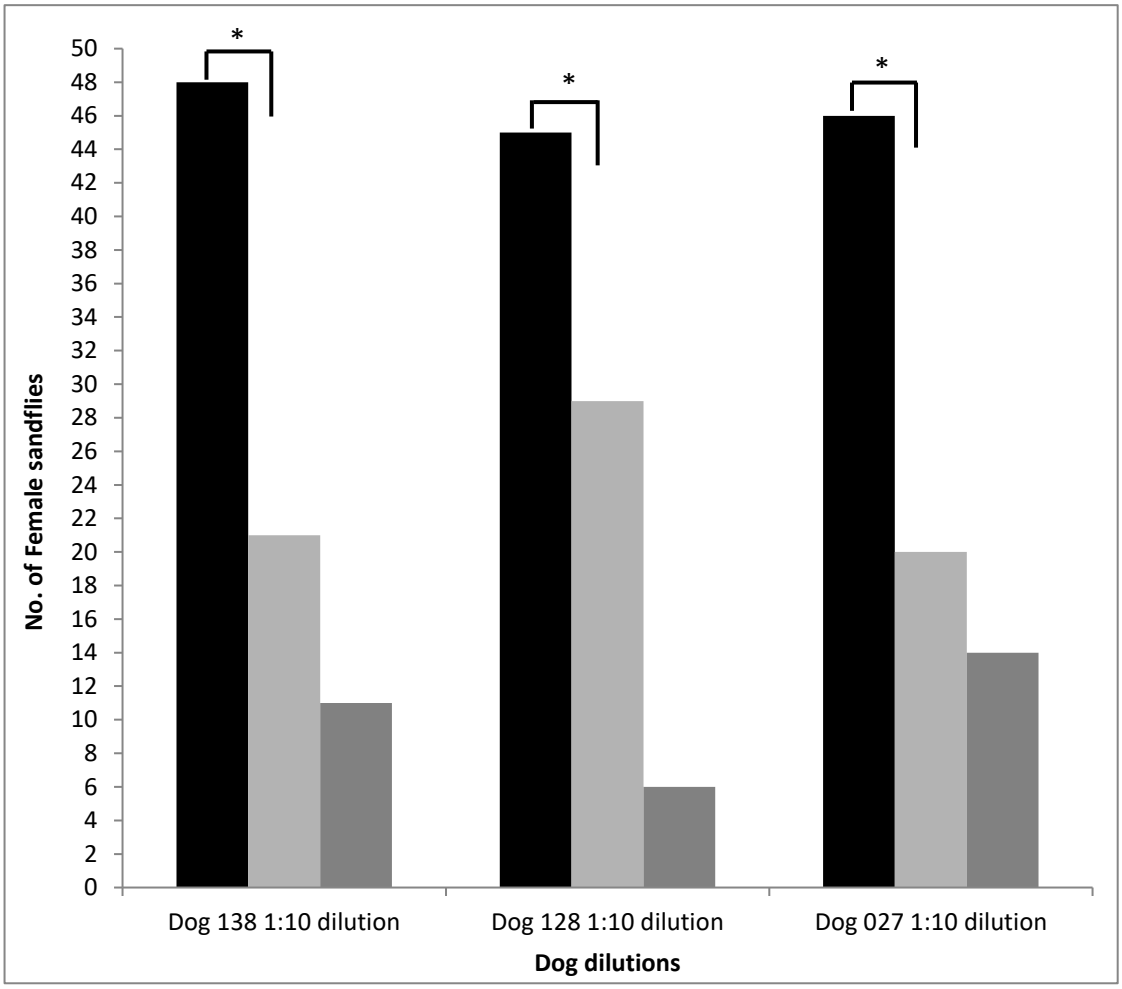


Figure 5.3: Dogs #138, #128 and #027 bioassay to confirm optimal dilution. 3 bioassays were performed using 80 female sandflies. The number of female sandflies which responded to the test dog sample is shown in black. Hexane control; light grey, undecided; Dark grey. * = *P*-value <0.05 which was considered significant.

5.3.2 2018 Dogs Female Sandfly Bioassay using (Unblinded Protocol)

Bioassays were performed following an unblinded experimental protocol using 9 pairs of infected and uninfected dog samples; dogs were diagnosed using PCR. Overall, female *Lu. longipalpis* were shown to respond to both infected and uninfected dog volatile samples (85.4%) with only a minority (14.6% of all sand flies analysed) being recorded as undecided (**Table 5.7**). However, the bioassay response showed a significant attraction (*P*=<0.05) of female sandflies to infected dog odour, with 56.9% (n = 410) of all flies used during the bioassays (n = 720) responding to the infected dog odour (**Figure 5.4**). Furthermore, of those sandflies which responded to the odour profile (n=615), 66.7% responded to the infected dog sample.

The response of 80 female sandflies was recorded to 1:10 diluted dog samples. The pairs of dogs which were analysed were chosen based on VOC Analyser discrimination criteria (**Table 5.1**). If there was no definitive choice observed, a response of Undecided was reported.

1g of dog hair/2.5 hour entrainment (1:10 dilution)	No. of Female Sand-flies			*P*-Value (Binomial test)
	Infected	Uninfected	Undecided	
Dog 176 vs Dog 021	49	21	10	0.0003
Dog 141 vs Dog 137	46	21	13	0.0009
Dog 178 vs Dog 181	51	18	11	0.0000
Dog 105 vs Dog 037	44	24	12	0.0051
Dog 140 vs Dog 004	51	20	9	0.0001
Dog 003 vs Dog 130	42	22	15	0.0062
Dog 074 vs Dog 093	40	24	16	0.0136
Dog 082 vs Dog 175	45	26	9	0.0075
Dog 102 vs Dog 124	42	29	10	0.0237

Table 5.7: Number of female sandflies which responded to infected and uninfected dog odour; Unblinded protocol. All dog samples were diluted using hexane to a 1:10 dilution. Samples were chosen based on VOC Analyser discrimination criteria and a cross comparison performed. The number of sandflies which responded to each sample was recorded in the table above. 80 sandflies were used per bioassay. All infected dogs are in bold. $P<0.05$ Infected vs Uninfected was considered significant.

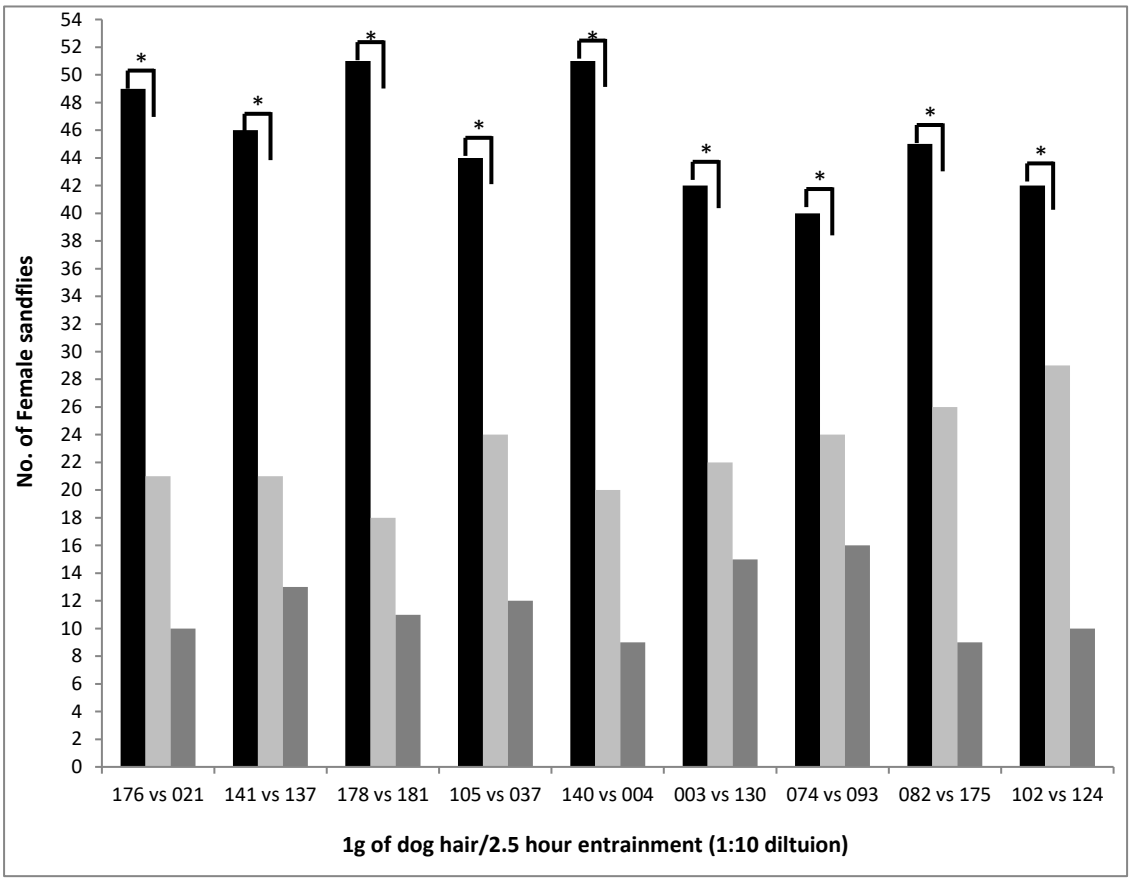


Figure 5.4: Female sandfly behavioural bioassay using an unblinded protocol of 2018 infected vs uninfected dogs. 9 bioassays using an unblinded experimental protocol were performed using 80 female sandflies per bioassay (720 sandflies in total) with each individual sandfly response recorded. Positive dog sample; black, negative dog sample; light grey, undecided; Dark grey. * = *P*-value <0.05 which was considered significant.

5.3.3 2018 Dogs Female Sandfly Bioassay (Blinded Protocol)

Following the initial unblinded experiments, the bioassays were performed following a blinded protocol using 6 pairs of infected and uninfected dog samples (A through to M).

The blinded experiment confirmed that female *Lu. longipalpis* respond to both dog volatile samples (infected and uninfected) with only a minority (10.8% of all sand flies analysed) being recorded as undecided. A significant attraction of female sandflies to infected dog odour (p=<0.05) was also seen following the blinded bioassay, with 57.3% (n = 275) of all flies used during the bioassays (n = 480) responding to the infected dog odour. Furthermore, of those sandflies which responded (n=428), 64.3% responded to the infected dog sample (**Table 5.8 and Figure 5.5**). By performing the experiment blinded, any experimental bias that could have occurred during the conduction of each individual bioassay was removed.

Dogs were diagnosed using PCR. The response of 80 female sandflies was recorded to 1:10 diluted dog samples. If there was no definitive choice observed, a response of Undecided was reported.

	No. of Female Sand-flies			
1g of dog hair/2.5-hour entrainment (1:10 dilution) ***Sample 1 dog in bold***	**Sample 1**	**Sample 2**	**Undecided**	***P*-Value (Binomial test)**
K (Dog 126) vs H (Dog 169)	48	25	7	0.0025
L (Dog 047) vs C (Dog 153)	45	26	9	0.0075
M (Dog 044) vs G (Dog 005)	48	26	6	0.0035
A (Dog 80) vs F (Dog136)	45	25	10	0.0055
J (Dog 134) vs D (Dog 70)	42	29	9	0.0290
B (Dog 019) vs E (Dog 43)	47	22	11	0.0010

Table 5.8: Number of female sandflies which responded to infected and uninfected dog odour. Blinded protocol. All dog samples were diluted using hexane to a 1:10 dilution. Samples were chosen based on VOC Analyser discrimination criteria with pairs of dogs being randomised and assigned a random letter. The corresponding dog which was assigned to each letter and the infection status is shown in **Table 5.4**. The number of sandflies which responded to each sample was recorded in the table above. 80 sandflies were used per bioassay. $P<0.05$ dog sample vs hexane control was considered significant.

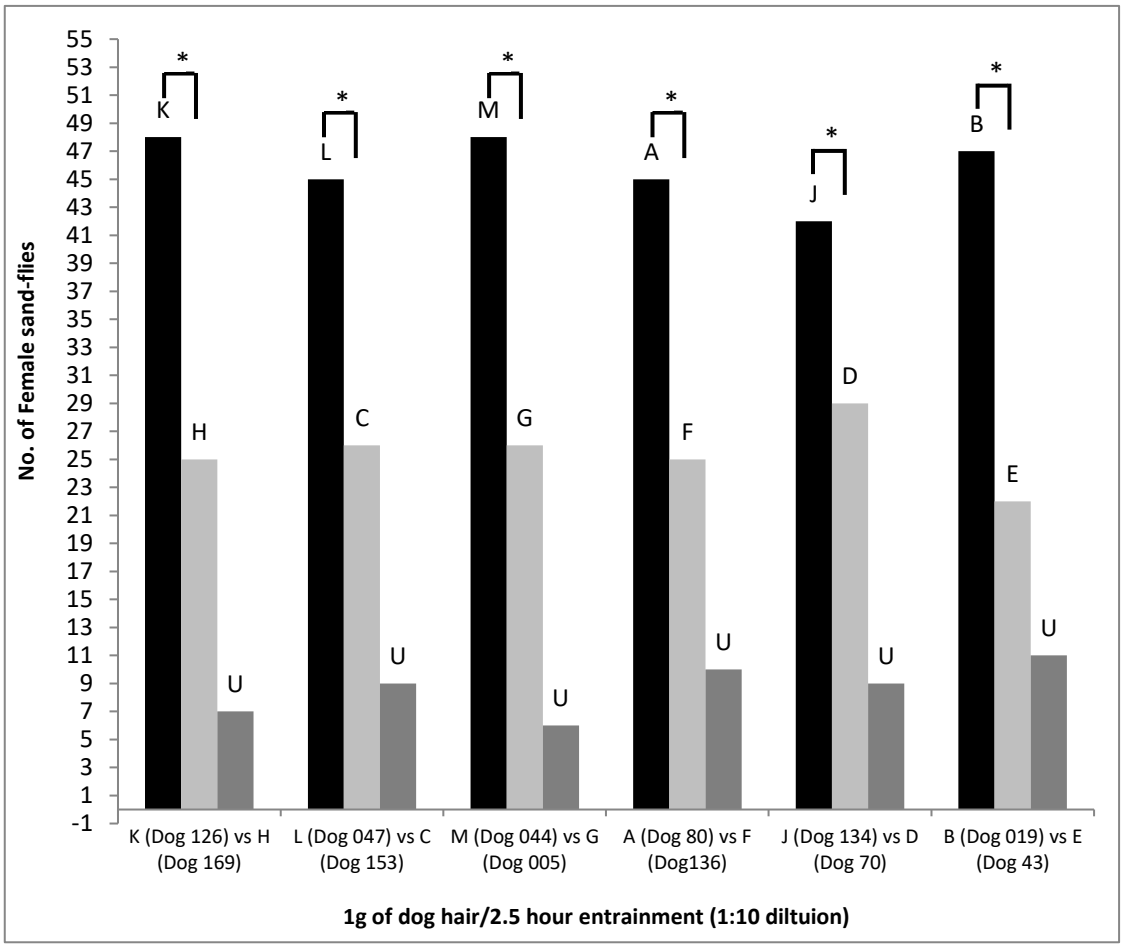


Figure 5.5: Female sandfly behavioural bioassay performed using a blinded protocol of 2018 infected vs uninfected dogs. 6 bioassays performed using a blinded experimental protocol were performed using 80 female sandflies per bioassay (480 sandflies in total) with each individual sandfly response recorded. Sample 1; black, Sample 2; light grey, undecided (U); dark grey. * = *P*-value <0.05 which was considered significant.

5.3.4 2018 Dogs Male Sandfly Bioassay (Unblinded Protocol)

The behavioural bioassay using male sandflies determined that male *Lu. longipalpis* respond to both dog volatile samples (infected and uninfected) with only a minority (9.5% of all sand flies analysed) being recorded as undecided (**Table 5.9**). However, a significant attraction to the infected dog sample was not observed (**Figure 5.6**). Of the males which responded (n = 706), 46.0% (n = 325) responded to the infected dog, and 54.0% (n = 381) responded to the uninfected dog.

Bioassays using an unblinded experimental protocol were performed using 9 pairs of infected and uninfected dog samples; re-randomised following female sandfly bioassays. The response of 80 male sandflies was recorded to 1:10 diluted dog samples. If there was no definitive choice observed, a response of Undecided was reported.

	No. of Male Sand-flies			
1g of dog hair/2.5-hour entrainment (1:10 diltuion) ***infected dog in bold***	**Infected**	**Uninfected**	**Undecided**	***P*-value (Binomial Test)**
Dog 176 vs Dog 021	35	37	8	0.0912
Dog 080 vs Dog 136	35	35	10	0.0950
Dog 082 vs Dog 175	38	31	11	0.0674
Dog 019 vs Dog 043	38	35	7	0.0876
Dog 074 vs Dog 093	36	34	10	0.0924
Dog 105 vs Dog 037	34	38	8	0.0840
Dog 003 vs Dog 130	37	36	7	0.0924
Dog 140 vs Dog 004	37	37	6	0.0924
Dog 044 vs Dog 005	35	38	7	0.0876

Table 5.9: Number of male sandflies which responded to infected and uninfected dog odour; Unblinded protocol. All dog samples were diluted using hexane to a 1:10 dilution. The number of sandflies which responded to each sample was recorded in the table above. 80 sandflies were used per bioassay. All infected dogs are in bold. $P<0.05$ Infected vs Uninfected was considered significant.

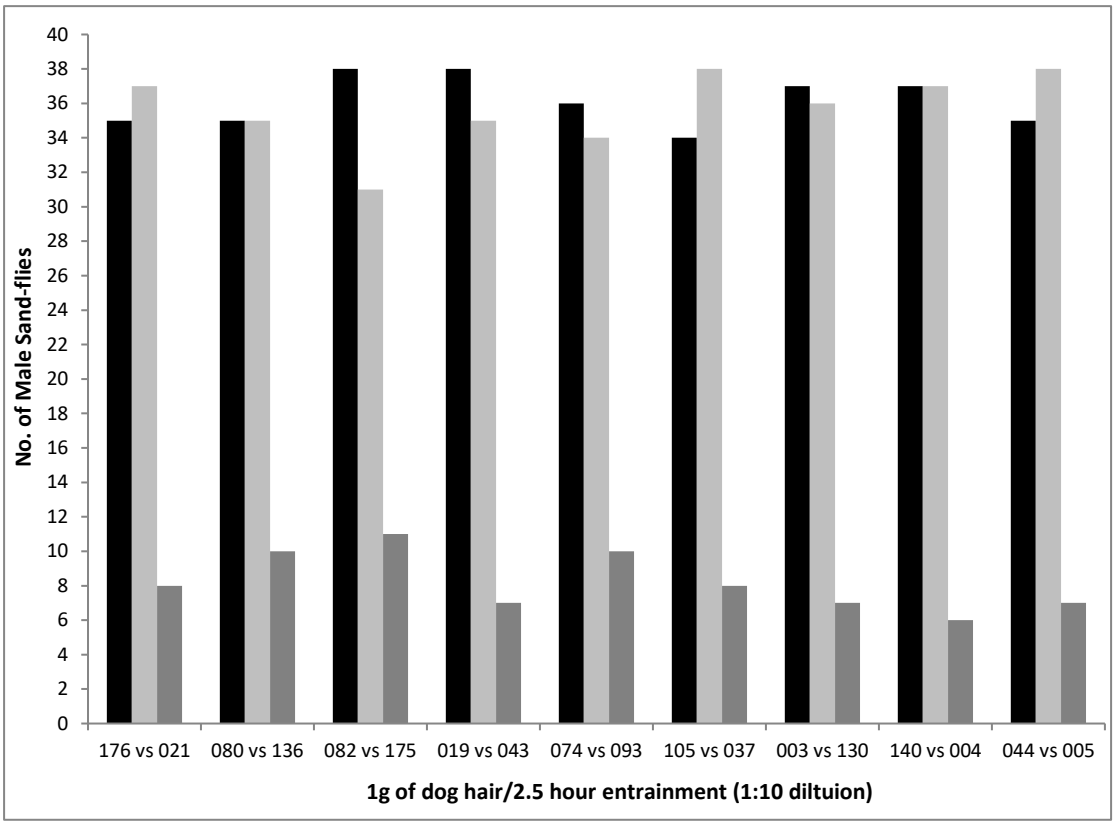


Figure 5.6: Male sandfly behavioural bioassay performed using an unblinded experimental protocol of 2018 infected vs uninfected dogs. 9 bioassays were performed using 80 male sandflies per bioassay (720 sandflies in total) with each individual sandfly response recorded. Positive dog sample; black, negative dog sample; light grey, undecided; dark grey. * = *P*-value <0.05 which was considered significant.

5.3.5 2018 Dogs Male Sandfly Bioassay (Blinded Protocol)

In order to confirm the male sandfly response to dog volatiles and relive any experimental bias, the behavioural bioassay was repeated using a blinded experimental protocol. This involved the remaining 6 dog pairs to be analysed being randomised and then assigned a letter, A through to M.

The blinded bioassay confirmed that although male *Lu. longipalpis* respond to both dog volatile samples (infected and uninfected), there is no significant attraction to either the infected or uninfected sample. Only a minority (10.2% of all sand flies analysed) were recorded as undecided demonstrating that male sandflies respond to dog odour (**Table 5.10**). Unlike female sandflies, males were not significantly attracted to infected dog odour (**Figure 5.7**). Of the flies which responded (n = 431) during the blinded bioassay, 50.2% (n = 216) responded to the infected dog odour and 49.8% (n = 215). Blinding the bioassay removed the possibility of any experimental bias that could have occurred during the conduction of each individual bioassay.

Individual entrained dog samples were diluted to a 1:10 dilution using hexane and the response of 80 male sandflies was recorded. If there was no definitive choice observed, a response of Undecided was reported.

	No. of Male Sand-flies			
1g of dog hair/2.5-hour entrainment (1:10 diltuion) *sample 1 in bold*	**Sample 1**	**Sample 2**	**Undecided**	***P*-Value (Binomial test)**
C (Dog 141) vs G (Dog 137)	34	36	10	0.0924
E (Dog 178) vs A (Dog 181)	36	36	8	0.0937
D (Dog 134) vs L (Dog 070)	38	35	7	0.0876
F (Dog 126) vs I (Dog 169)	34	38	8	0.0840
B (Dog 047) vs J (Dog 153)	35	38	7	0.0860
K (Dog 102) vs H (Dog 124)	39	32	9	0.0671

Table 5.10: Number of male sandflies which responded to infected and uninfected dog odour. Blinded protocol. All dog samples were diluted using hexane to a 1:10 dilution. The dog samples used for the bioassay which followed a blinded experimental protocol were re-randomised and assigned a random letter. The corresponding dog which was assigned to each letter and the infection status is shown in **Table 5.4**. The number of sandflies which responded to each sample was recorded in the table above. 80 sandflies were used per bioassay. $P<0.05$ Sample 1 vs Sample 2 was considered significant.

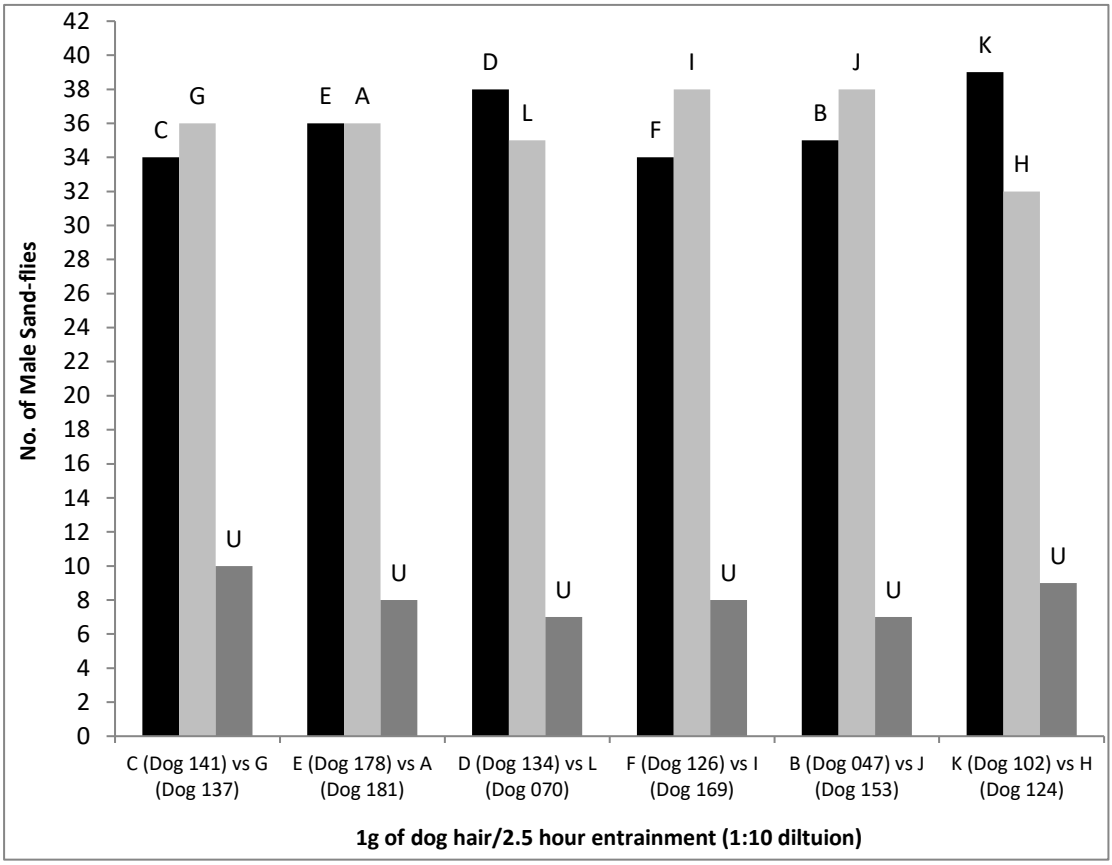


Figure 5.7: Male sandfly behavioural bioassay following a blinded experimental protocol of 2018 infected vs uninfected dogs. 6 bioassays following a blinded experimental protocol were performed using 80 male sandflies per bioassay (480 sandflies in total) with each individual sandfly response recorded. Sample 1; black, Sample 2; light grey, undecided (U); dark grey. * = *P*-value <0.05 which was considered significant.

5.4 Discussion

The results presented here suggest that female *Lu. Longipalpis* sandflies are significantly more attracted to the odour of dogs infected with *Leishmania infantum*. Of the female sandflies which responded to either the infected or uninfected dog (n=1,043), a total of 685 (65.7%) were attracted to the infected dog sample, therefore indicating a significant attraction ($P<0.05$). Over the two bioassays performed 1,043 out of the 1,200 female flies used throughout the study responded to dog odour. This result confirms the ability to obtain a behavioural response to the entrained odour of dog volatiles in a Y-tube olfactometer in the lab, which to our knowledge is a first. Recent research has however described a similar phenomenon using a wind tunnel and individual volatile components of the dog odour (Magalhães-Junior *et al.,* 2019). However, the use of a Y-tube olfactometer requires sandflies to make a locomotive choice between infected and uninfected dogs allowing for a more robust analysis. The male sandfly bioassays however, demonstrated an equal attraction to both dog samples with no significant difference being observed between the infected and uninfected dogs. This observation could impact transmission of *Leishmania*, with male sandflies producing pheromone equally around infected and uninfected dogs. This production of pheromone would therefore facilitate female blood feeding of uninfected dogs and reducing extreme preference for just those dogs that are infected.

5.4.1 Sandfly attraction to hosts

In order to optimise not only the concentration of the dog volatiles to be used, but also to determine female sandfly attractiveness to dog odour over a hexane

control, optimisation bioassay experiments were performed using female *Lu. Longipalpis* sandflies and the volatiles entrained from three separate uninfected dogs. Initial experiments indicated a 1:10 dilution of dog odour with hexane was required in order to provoke a response from the female sand-fly. Subsequent experiments using this dilution when determining the attractiveness of female sandflies to dog odour, indicated a significant attraction of the flies to the uninfected dog (66.5%). This result confirms the importance of the sandfly olfactory system in the response to animal odours in order to gain a blood meal, that is important for oviposition.

This demonstrated attractiveness to dogs is not a surprising result. Blood meal analysis has identified that female *Lu. Longipalpis* sandflies are known to feed on many animals including dogs, rodents, chickens and humans (Sales *et al.,* 2015). However, research in Brazil has shown that *Lu. Longipalpis* is an opportunistic feeder, with studies demonstrating that chicken blood is the most common blood meal source, inducing the greatest egg production. This was followed in descending order by livestock, humans and finally dogs (Guimaraes-E-Silva *et al.,* 2017). Due to the proximity of chickens and dogs in the domestic environment in Brazil and the opportunistic nature of *Lu. Longipalpis*, it is therefore understandable that female sandflies are attracted to dogs as blood meal source despite there being more satisfactory blood meal sources. This increased attraction in dogs could therefore lead to a high incidence of canine leishmaniasis within the domestic environment which in turn could lead to more cases of human VL due to the opportunistic feeding nature of *Lu. longipalpis*.

5.3.2 Female sandfly attraction to infected dogs

Results presented here demonstrate a significant attraction of female *Lu. longipalpis* to the entrained odour of dogs infected with *Le. infantum*. During the initial unblinded behavioural bioassay, 66.7% of female sandflies which responded (n=615) were significantly attracted to the infected dog odour. During the subsequent blinded bioassay, 64.3% of female sandflies which responded (n=428) demonstrated a significant response to the infected dog samples. In addition, the results here also demonstrate that the odour of those dogs which were considered highly discriminant, based on previous VOC Analyser analysis, have the highest quantity of female sandflies responding to the infected dog. This result further implies the importance of infected dog odour in the attraction of female sandflies and therefore the transmission of *Leishmania*.

Several studies have previously identified potential compounds which may have an involvement in the mediation of the differential attractiveness of *Lu. longipalpis* sandflies to *Leishmania* infected and uninfected dogs. An increased attraction of female sandflies to the individual compounds decanal and nonanal,, possible biomarkers for *Le. infantum* infected in dogs has been shown (Magalhães-Junior *et al*., 2014b; Magalhães-Junior *et al.,* 2019). Not only do the results presented here coincide with such research, but this study demonstrated an increased female attraction to the whole volatile profile of an infected dog rather than individual volatiles found in infected dog odour. In nature different blood sources, such as dog hosts, release volatile compounds simultaneously therefore the components released intermingle forming a fluctuating and complex olfactory environment. Therefore, the female sandfly attraction demonstrated here provides a more accurate representation

of the natural interaction between infected dogs and female *Lu. longipalpis*, thus providing a more robust ideology of the transmission of *Leishmania infantum* in Brazil.

This observed response of female sandflies could have implications on the transmission of human visceral leishmaniasis throughout Brazil. Transmission of *Leishmania infantum* from dogs to humans is predominantly through the bite of infected sand-flies. The observed increase in attraction of female sandflies to *Leishmania infantum* infected dog odour and the proximity of dogs to humans in the urban environment in Brazil could lead to an increased transmission of the parasite to humans. Furthermore, the adoption and transportation of dogs in Brazil from areas endemic for cVL has caused the introduction and spread of the disease to areas where the infection was previously not found (Otranto *et al.,* 2009). This movement of dogs throughout Brazil combined with the increased female sandfly attraction to infected dogs further highlights the need for the removal and/or treatment of infected dogs which could be made more efficient by the development of more accurate diagnostics as presented in preceding chapters (Staniek *et al.,* 2019).

Despite this observed attraction and therefore an increased transmission of the disease which ultimately benefits the parasite, the benefit to the sandfly remains unclear. Research has demonstrated that *Leishmania* infection causes severe anaemia and leukopenia in infected hosts and therefore a poorer quality of blood meal (Lafuse *et al.,* 2013). However, it has also been shown that female sandflies that are infected with *Leishmania* are able to withstand infection from bacterial entomopathogens, reducing the mortality of the fly. This suggests that *Leishmania* infection may benefit the sandfly vector, whilst also increasing its potential to establish itself within the sand fly vector (Sant'Anna *et al.,* 2014). This potential benefit to the sandfly could explain

the increase in attraction to infected dogs, providing further evidence of parasite manipulation leading to an increase in parasite transmission.

Despite this observed increase in attraction, research has demonstrated that the host choice behaviour of insect vectors may be influenced by the infection status of the vector itself. Studies have shown that uninfected vectors are more attracted towards infected hosts however, after becoming infected they are more attracted towards uninfected hosts. Research has demonstrated the consequences of these behavioural shifts and the impact such behavioural alterations has on disease epidemiology (Roosien *et al.,* 2013). Such an important modification of vector behaviour seems to be very adaptive for parasite transmission however this hypothesis remains to be investigated thoroughly. Regarding the results shown here, further investigation into the attraction of infected female sandflies to uninfected dogs is vital to further the current understanding surrounding disease transmission and ecology in endemic areas.

5.3.2.1 Parasite Manipulation Inducing Increased Transmission

The longitudinal study performed by Nevatte *et al.,* (2017) combined with the results presented here suggests a parasitic manipulation of the canine metabolism in order to favour the biology of the parasite. Despite these results however, it cannot be discounted that female *Lu. Longipalpis* sandflies are also naturally more attracted to some dogs over others. Research has demonstrated that female mosquitoes show a preference for certain host individuals over others, with differences in VOCs produced by the host being detected by mosquitoes and causing a difference in attraction (Fernández-Grandon *et al.,* 2015). Therefore, a longitudinal follow up study

would need to be undertaken in order to demonstrate an increase in the attractiveness of the odour of the same dog after infection compared to before infection.

5.3.3 Male sandfly attraction to infected dogs

Although results shown here demonstrate a significant attraction of female sandflies to infected dog odour, no such difference was observed in the male sand-flies. Although the males were generally more attracted to dog odour, with only 10% (n=123) of all the males analysed (n=1200) remaining "undecided", infection status appeared to have no influence on attraction. In total, only 541 of the 1077 male sandflies which were responsive were attracted to infected dog odour (50%) compared to 685 of the 1043 females which responded (66%). This is an important observation as it not only suggests a difference between female and male host-seeking behaviour, but also suggests that parasite manipulation is specific for the host-seeking behaviour of female sandflies. Furthermore, this observation also furthers our understanding on *Leishmania* transmission, with the male formation of lekking sites around both infected and uninfected dogs playing a vital role in the attraction of females to both infected dogs to gain a parasite blood meal, and uninfected dogs to continue disease transmission.

There are a few reasons that could be suggested for this observation. First, despite many olfactory morphological features being the same between male and female vectors, differences have been observed. For example, female mosquitoes have been found to possess three/four times more antennal sensilla than males (McIver *et al.,* 1982). Research in Brazil has also demonstrated a difference between

the type and number of sensilla present on male and female *Lu. longipalpis* antennae, with the mean number of sensilla being significantly greater on male sandflies (Fernandes *et al.,* 2008). Such observed differences may reflect function, as only female feed on blood and therefore rely on olfactory stimuli to locate hosts. These differences could therefore potentially explain the difference in attraction observed in the results presented here. Additionally, in nature male *Lu. longipalpis* are attracted to a possible blood source first in order to form a lekking site. Research has demonstrated the attraction of male *Lu. longipalpis* sandflies to octenol and nonanol that are related to skin odour (Magalhães-Junior *et al.,* 2014). Following the formation of lekking sites around a host, female sandflies are then attracted (Kelly & Dye, 1997). This aggregation dynamic and formation of lekking sites around suitable hosts, that is facilitated by the production of host kairomones, may explain the unbiased attraction of male *Lu. Longipalpis* to canine volatiles as demonstrated in this study.

When present together, host kairomones and male pheromones have been found to attract a higher number of female sandflies compared to the host odour or male pheromone alone (Bray and Hamilton 2007). The observed attraction of male sandflies to both infected and uninfected dog odour would therefore facilitate an increase in the copulation of sandflies. This is due to the formation of lekking sights around a larger number of hosts as infection status has no influence on male attraction. However, the relative attraction of infected dog odour compared to male pheromone and uninfected host odour has not been investigated. In order to further understand the importance of the volatiles produced by an infected dog in a natural setting and their role in female sandfly attraction, additional experiments should be undertaken to investigate whether females have a greater attraction to infected dogs

compared to lekking sites formed around uninfected dogs. Uninfected dogs with a lekking site could potentially be more attractive than an infected dog, therefore having a major effect on disease transmission and disease control.

The observed attraction dynamic of the male sandflies may also prove beneficial to the *Leishmania* parasite. As demonstrated here, although female *Lu. longipalpis* sandflies are significantly more attracted to the odour of infected dogs, extreme preference of female sandflies to infected dogs could limit or even stop transmission. It can therefore be suggested that the equal aggregation of male sandflies around both infected and uninfected dogs and the subsequent production of pheromones, would attract infected females to uninfected dogs thereby aiding parasite transmission. Furthermore, recent studies have shown that sequential blood meals promote the replication of *Leishmania* parasites (Serafim *et al.,* 2018). Therefore, the formation of male lekking sites around both infected and uninfected dogs promotes the intake of further blood meals and subsequent parasite replication, further aiding disease transmission.

The demonstrated male attraction and subsequent formation of lekking sites around both infected and uninfected dogs will lead to a combined odour blend of male pheromone with infected and uninfected host kairomones. As male pheromone has already been identified as an effective attractant used when combined with insecticide for sandfly vector control (Brey *et al.,* 2014; Bell *et al.,* 2018), further research into the attraction of male pheromone plus infected dog odour should be undertaken as a possible aditional contribution to vector control.

5.3.4 Odour Baited Sandfly Traps

The results presented here showing an increase in the attraction of female *Lu. longipalpis* to the whole odour profile of *Le. infantum* infected dogs, demonstrates the potential for the development of a novel tool for vector control. Additional evidence regarding sandfly attractiveness to infected dog volatiles further supports such an approach, with female sandflies showing a significant attraction to decanal and nonanal, two VOCs which have previously been identified in the hair of *Le. infantum* infected dogs (Magalhães-Junior *et al*., 2014b; Magalhães-Junior *et al.,* 2019).

The combination of synthetic odours and chemicals with current vector control methods has already been demonstrated. Traps baited with human odour (socks worn for 12 hours) have been proven to attract a significant amount of *Anopheles* mosquitoes (Njiru *et al.,* 2006; Jawara *et al.,* 2009). Previous research has also demonstrated that traps baited with chemical attractants significantly increases the number of female sandflies caught. For example, the use of pheromones baited light traps as a sandfly control method has shown a significant increase in the attraction of female sandflies, which when combined with insecticides, could result in more sandflies being killed and therefore a potentially more effective control programme (Bell *et al.,* 2018). Other studies have also demonstrated an increase in the attraction of certain vectors to specific odour profiles as well as specific target sizes, shapes and other visual stimuli such as light. Research has demonstrated that *Anopheles* mosquitoes are highly attracted to matte black card. Traps which were baited with human odour plus the black card visual stimuli caught significantly more *Anopheles* than traps which were baited with odour alone (Hawkes *et al.,* 2017). These studies provide evidence that suggests a similar trapping methodology could be employed to sandflies. The use of light traps baited with whole dog odour profile, with or without

a visual stimulus, could produce an innovative vector control methodology for the trapping and killing of sandflies in Brazil.

5.3.5 Summary

Existing research has demonstrated that synthetic odour baits have the potential to be used successfully as a lure of sandfly vectors to traps (Bell *et al.,* 2018; Magalhães-Junior *et al.,* 2019). Malaria research which modelled the effect of mass trapping techniques has already demonstrated that such an approach could be used for effective malaria control programmes where odour-baited traps are used together with Insecticide-treated walls or nets (Homan *et al.,* 2016). One such example is the development of the SolarMal Project, a vector control project which developed and tested odour-baited traps for malaria vector control in Western Kenya. This project resulted in a decrease in malaria prevalence, which was explained by the reduction in the densities of *An. funestus* (Hiscox *et al.,* 2012; Homan *et al.,* 2016). Odour-baited traps therefore demonstrate effective disease intervention.

Results here therefore indicate that the use of infected dog odour-baited traps as part of a mass trapping control programme for *Leishmania* vectors, could potentially contribute to vector control and lead to a reduction of disease burden. This is due to the observed attraction of female sandflies to the odour of naturally infected dogs. However, due to the unbiased attraction of male sandflies to dog odour regardless of infection status, further studies must be done in order to further understand the effect of pheromone with regards to infected dog odour and baited sandfly traps. Despite this, odour-baited traps have the potential to be used as a complementary method with other novel intervention strategies for *Leishmania* control.

Chapter 6 - Gas Chromatography-Mass Spectrometry (GC/MS) Analysis of Hair samples

6.1 Introduction

Systems biology is a vital component of biological analysis. It allows for the modelling of different biological systems through the analysis of the components and metabolites that are produced by biological interactions (Breitling, 2010). Metabolomics, as a component of system biology, involves the scientific study of the metabolites produced by an organism, tissue or cell. It has developed rapidly in recent years, being used extensively throughout biomedical research (Daviss, 2005). Such research has emphasised the importance of metabolites as biomarkers for detecting and diagnosing various diseases, with metabolomics analysis allows for the identification of the whole spectrum of metabolites within an organism in various disease conditions in a non-invasive manner (Atan *et al.,* 2018).

A variety of volatile components have been identified as being directly related to different diseases. For example, numerous cancer specific biomarkers, such as chemokine ligand 2 and chemokine receptor type 2 for breast cancer, have been identified (Buonaguro *et al.,* 2019) along with biomarkers such as an increase in neurofilament light chain during neurological disorders as well as biomarkers of tau and amyloid pathology for Parkinson's disease (Parnetti *et al.,* 2019) as well as biomarkers for various parasitic diseases (Ndao 2009). Recent research has shown that volatiles in *Leishmania infantum* infected dog hair can serve as biomarkers that are associated with *Leishmania infantum* infection. The individual VOCs determined have

not only allowed for the discrimination between infected and non-infected dogs (Magalhães-Junior *et al.,* 2014b) but have also been demonstrated to elicit the attraction of female sandflies (Magalhães-Junior *et al.,* 2019; Chapter 5). Therefore, further isolation, quantification and identification of the different biomarkers in the hair of *Leishmania infantum* infected dogs, through metabolomics techniques, could pave the way for the future development of new diagnostic tools (Staniek *et al.,* 2019) and vector control methodologies.

6.1.1 Metabolomics

Metabolites are small endogenous molecules that are produced as end products of biochemical pathways and play a vital role in biological systems. They represent a useful dataset to allow for the understanding of disease phenotypes, with the identification of changes in metabolite levels having been shown to help with the diagnosis of diseases in early stages. The variations in metabolite levels have been applied as a tool for disease prediction with metabolites already having been used to characterise many diseases from a variety of different biological samples. These include, the diagnosis of breast cancer by analysing exhaled air (Herman-Saffar *et al.,* 2018), cholera by analysing stools (Garner *et al.,* 2009), and cardiovascular disease by analysing plasma (Rupérez *et al.,* 2012). The discovery of biomarkers for various diseases and their application in the field of integrative systems biology, are considered as the main areas in which metabolomics analysis is applied. Biomarker identification using metabolomics is a promising approach leading to the diagnosis of disease in early stages, which has led to an increase in effective disease diagnosis. For example, research has demonstrated that the identification of urinary volatile organic

compounds could lead to a non-invasive methodology for the diagnosis of prostate cancer (Gao *et al.,* 2019). Recent research has also demonstrated the non-invasive diagnosis of malaria through the detection of breath and skin VOCS produced by infected individuals (Berna *et al.*, 2015; De Moraes *et al.*, 2018). These studies confirm the advantages of identifying specific biomarkers for the early, non-invasive and accurate diagnosis of disease.

The study of metabolomics could contribute to the development of an innovative way to improve the current cVL diagnostic tests in Brazil. Research has already shown that metabolomics analysis can be used for the diagnosis of canine VL, distinguishing between the hair of *Leishmania* infected and uninfected dogs (Magalhães-Junior *et al.,* 2014b). Hair as a skin appendage is an important biological sample to consider when performing metabolomics analysis. This is due to the bodily functions that hair possesses, which includes the secretion and excretion of substances that are produced by biological endogenous mechanisms (Stenn and Paus, 2001). Hair samples have been used in the forensic and biomedical fields for the detection of a variety of exogenous compounds. Specifically, the analysis of animal hair has shown great potential as an indicator of the internal metabolic processing of different medicines (Gratacos-Cubarsi *et al.,* 2006), the identification of specific drugs in the field of toxicology (Bararpour *et al.,* 2018) and for the profiling of endogenous hormones such as cortisol (Shih *et al.,* 2018). Hair samples allow for easy non-invasive collection with the additional benefit of becoming naturally impregnated with substances that are excreted and secreted by the body during metabolic processes (Stenn and Paus, 2001). This offers chemical stability to the analysis, capturing long-term exposures to disease, unlike plasma or urine (Seymour *et al.,* 2018). Research has demonstrated that pathogenic infections

cause an alteration in substances that are exogenously secreted by an individual (Shirasu and Touhara, 2011). Therefore, there is real potential to detect different diseases through the analysis of hair samples from individuals who are infected. The odour that is emitted during infection could prove to be one of the first clues which will lead to a successful early diagnosis.

6.1.2 Gas chromatography mass spectrometry (GC-MS)

Gas chromatography mass spectrometry (GC/MS) is a complex technique that produces, separates and detects ions from a sample in a gaseous phase (**Figure 6.1**). GC/MS is an effective system used for separating VOCs present in a complex sample based on their chemical properties and by their mass to charge (m/z) ratio (Bindu *et al.,* 2013). GC/MS consists of two functional components; a gas chromatograph and a mass spectrometer. Gas chromatography (GC) (performed by a gas chromatograph) is widely used in many branches of science and over the last 50 years has played an essential role in the isolation and quantification of volatile organic compounds (VOCs) in a mixture (Stashenko and Martinez, 2014). GC is a separation technique, which uses the flow of a carrier gas through a column to separate compounds based on their volatility and their interaction with a liquid stationary phase present inside the column. The column itself is in an oven which controls the temperature of the gas. There are two types of columns used for GC analysis; packed and capillary, however capillary columns are considered more efficient and therefore more widely used. Capillary columns are divided into two types; support coated open tubular (SCOT) or wall-coated open tubular (WCOT) (**Figure 6.2**). To achieve optimum column efficiency, the sample introduced to the column should be small and introduced as a plug of vapour.

The most common method of injection is using a micro-syringe, which injects the sample through a rubber septum and into a flash vaporiser port that is present at the head of the column. For capillary GC, the injector can be either split or splitless. For a split injection, when the sample is vaporised only a proportion of the sample passes onto the column with most exiting through a split outlet. A splitless injection however, results in the entire sample passing onto the column (Pravallika, 2016).

To establish the chemical structure of these GC separated compounds however, an additional spectroscopic step such as mass spectrometry (MS) is required. In coupled GC/MS, a mass spectrometer receives the effluent from the GC. MS measures the m/z ratio of the ions in the sample, presenting the results as a mass spectrum which is a plot of intensity as a function of the m/z ratio. A mass spectrum is often described as a "fingerprint" of the VOC as it provides information on the elemental composition, molecular weight and abundance of each volatile component of the sample. The structural and chemical information provided by the analysis can assist with the identification of compounds through a library fitting. However, any identification obtained by the library tends to be unambiguous with further experiments normally required to confirm identification. Mass spectrometry has proven to be useful across diverse scientific fields and applications including forensic toxicology, proteomics, metabolomics and clinical research (Minkler *et al.,* 2017).

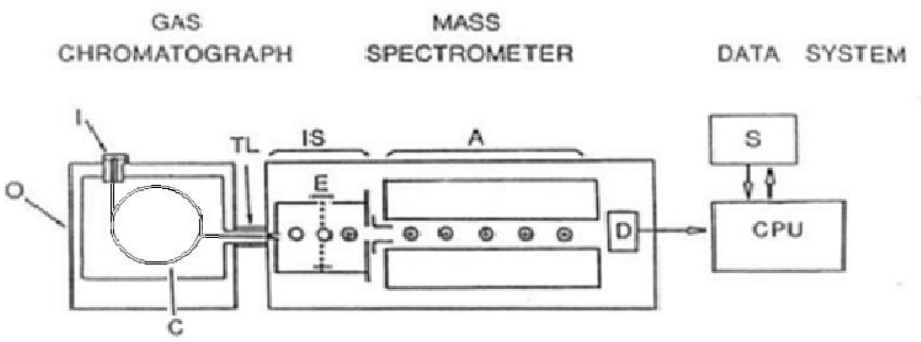


Figure 6.1: Schematic diagram of a GC/MS system. GC/MS is a technique that is comprised of a gas chromatograph (GC) that is coupled to a mass spectrometer (MS). Instrument allows for the separation, identification and quantification of complex mixtures of chemicals. O; Oven, I; Injector, C; Column, TL; Transfer Line, IS; Ion Source, E; Electron Beam, A; Analyser, D; Detector, CPU; Central Processing Unit, S; Data Storage Device (Adapted from Wu *et al*, 2012).

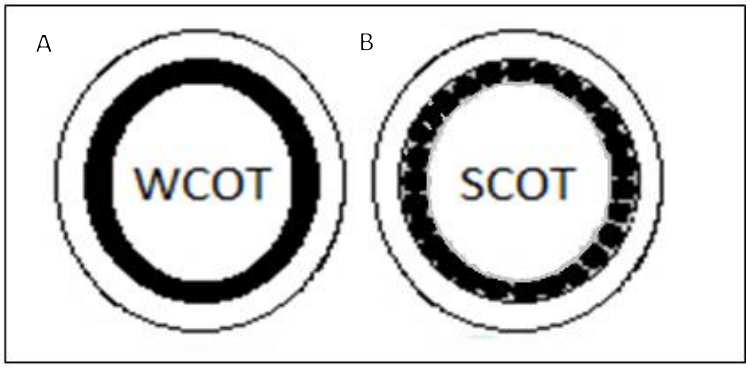


Figure 6.2: Types of Capillary columns. There are two types of Capillary column used for GC analysis. A; Wall coated open tubular column (WCOT). B; support coated open tubular column (SCOT). In SCOT columns, the capillary inner wall is lined with a thin layer of support material, on which the liquid stationary phase is adsorbed. WCOT columns however consist of a capillary tube which has its walls coated with the stationary phase (Adapted from Engewald *et al.,* 2014)

6.1.2.1 Application of GC/MS in Clinical Research

GC/MS has been used extensively for the detection and identification of VOCs which have been found to be emitted in sweat, blood, urine, faeces and breath, the expression levels of which reflect an organism's metabolic state. There is an increasing interest in the evaluation and use of VOCs in the diagnosis of various diseases, with emerging analytical technologies such as GC/MS being at the forefront of this research (Sethi *et al.,* 2013).

GC/MS is considered to be highly sensitive and robust, with the added advantages of having a high chromatographic separation power, reproducible retention times and fast compound identification through the possession of large commercial and public compound libraries (Beale *et al.,* 2018). This technology allows for the simultaneous profiling of several hundred chemically diverse compounds which include most amino and organic acids, sugars and sugar alcohols, fatty acids and aromatic amines (Roessner *et al.,* 2000). Volatile organic metabolites can also be separated and quantified directly by GC–MS, with research demonstrating the successful identification of metabolites for the diagnosis of gastrointestinal diseases such as Crohn's disease (CD) and ulcerative colitis using GC/MS analysis. Specifically, 93% sensitivity and 78 % specificity were demonstrated when distinguishing between patients with CD and healthy controls (Cauchi *et al.,* 2014). Research has also demonstrated the use of GC/MS for the identification and classification of different mycobacterial species. Mycobacteria species-specific VOC profiles which cause serious mammalian zoonotic diseases have been identified. These included *Mycobacterium (M.) tuberculosis* which causes tuberculosis (TB) in dogs, elephants, parrots and humans as well as *Mycobacterium caprae* and *Mycobacterium bovis* which causes TB

in humans and ruminants respectively (Küntzel *et al.,* 2018). For specific disease diagnosis, research has shown the successful use of GC/MS as a TB diagnostic through the identification of high and low abundant biomarkers of *Mycobacterium tuberculosis*. This analysis was performed on human serum samples with a variety of lipid biomarkers specific for *Mycobacterium tuberculosis* identified in the serum of TB infected samples (Joseph *et al.,* 2016).

Metabolomics analysis can be applied to a range of disease states in order to identify disease biomarkers as well as follow disease progression and severity or provide a therapeutic target (Smoleńska and Zdrojewski 2015). However, despite its effectiveness, GC/MS still requires expensive preparations of samples and expert personnel to handle the samples and technology (Saalberg and Wolff, 2016). Another issue is that the method of volatile collection, whether through the collection of breath or indeed headspace entrainment on Tenax of hair samples, can affect the original concentration of VOCs in the sample. Furthermore, the relative stability of the collected compounds, as well as the effect of different storage bags on collected VOCs can influence the results generated by the GC/MS analysis (Nardi-Agmon and Peled, 2017). Therefore, very short sample storage times are needed to minimize the effect on the sample. It was important to recognise and minimise these limitations when the analysis of collected hair was undertaken using GC/MS for this study. Despite these limitations however, the use of GC/MS as an analytical approach to distinguish between the volatile profiles of individuals in various disease states is a highly effective methodology. This technology could therefore be used to validate the results gathered through VOC Analyser analysis of *Leishmania* infected dogs (Staniek *et al.,* 2019; Chapter 4). The identification of *Leishmania* specific volatile compounds using a

GC/MS could also further enhance the development of the VOC Analyser as a *Leishmania* specific diagnostic. These identified compounds could also be utilised in the development of odour baited sandfly traps due to the demonstrated increase in attraction of *Lu. longipalpis* to infected dogs (Chapter 5).

6.1.2.2 GC/MS for the identification of *Leishmania* VOCs

Previous studies have identified 53 volatile and non-volatile potential biomarkers of *Leishmania* infection using GC/MS analysis. These include direct markers (of parasite origin) and indirect markers (from host cells), for both cutaneous and visceral leishmaniasis (Kip *et al.,* 2015). GC/MS specific identification of volatile biomarkers has been exploited in various studies. Recent research identified changes in the composition of both fatty acids and lipids during the transformation of *Le. donovani* from promastigotes to amastigotes inside the macrophages (Messaoud *et al.,* 2017). Furthermore, metabolomics analysis identified various circulating biomarkers that are related to immune activation, inflammation and oxidative stress in *Leishmania* infected dog serum (Solcà *et al.,* 2016). Specific volatile metabolomics analysis also identified a small number of primarily low molecular weight aldehydes, alkanes and esters (Oliveira *et al.,* 2008; Magalhães-Junior *et al.,* 2014b) which were found to be present in the odour of VL infected dogs, with six specific VOCs identified as biomarkers for *Leishmania* infection. Again, this research shows the potential application of biomarkers as the basis for the identification of infected and uninfected individuals.

Regardless of the origin of VOCs, GC/MS analysis has given researchers an insight into the pathogenesis, biology and mechanisms of *Leishmania* infection. The

clear observed change in biomarkers following infection, whether related to the immune system or from another source can be utilised by GC/MS, as the identification of biomarkers which have been previously linked to leishmaniasis would allow for the diagnosis of an infected individual. The use of biomarkers in the diagnosis of leishmaniasis may have several advantages including their use as an additional analysis, but more importantly the relatively non-invasive collection of samples which has been observed (Kip *et al.,* 2015, Staniek *et al.,* 2019). The identification of specific biomarkers in infected individuals which have been linked to a change in odour profile could also provide further information as to the transmission cycle of *Leishmania* via parasitized host interaction with the sandfly vector.

6.1.3 Chapter Aims

Previous research (Oliveira *et al*., 2008; Magalhães-Junior *et al*., 2014b) in addition to the results presented here demonstrate a distinct difference between the odour of *Lesihmania* infected and uninfected dogs. Although a VOC Analyser can distinguish between dog samples with a high sensitivity and specificity (Staniek *et al*., 2019), the device is still a novel approach which requires further research to improve diagnostic potential. By utilising gas chromatography mass spectrometry, I aim to not only confirm the results determined through the VOC Analyser analysis, but also preliminary identify those VOCs which could be suggested as *Leishmania* specific.

6.2 Methods

6.2.1 Sample collection and Headspace Entrainment on Tenax

Dog hair was collected in the field as described in the general methods section in both 2017 and 2018. Following sample collection, a minimum of 1g of hair was introduced into a portable entrainment system in order to collect released volatiles. Methodology for the entrainment of VOCs from the dog hair samples is described in the general methods section (Chapter 2).

6.2.2 GC/MS Set Up

6.2.2.1 Machine specifications

The VOCs present in the 2017 and 2018 entrained samples were analysed by a gas chromatograph (Agilent Technologies 5890B) coupled to a mass spectrometer (Agilent Technologies 5975C) using either a fitted 10µl auto sampler syringe (G4513A model, Agilent/SGE) or manually under the following conditions: 2ml/min carrier gas (H_2) flow with an oven temperature program as follows; 40°C for 3 min, then increased at 10^{o}C/min to 180^{o}C and then held for 5 minutes (total run time, 28 minutes). The injector was a splitless injection with an inlet temperature of 65^{o}C that is held for 0.5mins prior to an increase in temperature to 180^{o}C at 720^{o}C/min. This temperature was held for the remainder of the run. An Agilent premium inlet septum 5183-4757 which was not pre corded was used with the pressure inside the inlet increased from 6psi to 40psi and held for 2 mins. The mass spectrometer consisted of a heated transfer line (280^{o}C) and a MS scan range of 30-400 amu with a scan frequency of 12.5 scans per second. The solvent delay prior to data acquisition was 6.5 minutes. The purpose of the solvent delay was to protect the MS filament from the effects of a high

concentration of solvent vapour at the source and therefore overcome a large solvent peak at the beginning of the analysis.

6.2.2.2 Methodology

GC/MS analysis on each sample of dog hair was carried out using two separate columns;

- HP5-MS Ultra Inert non-polar column; 30m × 0.25mm id × 0.25µM film thickness.
- DB-Wax capillary polar column; 30m × 0.25mm id × 0.25µM film thickness.

The use of two columns allowed for the identification of the broad range of volatiles present on the dog hair samples. The HP5-MS detected non-polar compounds and the DB-Wax detect polar compounds. Hydrocarbon standards were injected randomly throughout the experiment (after approximately every 5 uninfected dog samples) as an external standard and with a hexane blank prior to and immediately following infected dog samples.

6.2.2.3 Preparation of Entrainment Samples Prior to GC/MS Analysis

For all auto sampler injections (2017 HP5-MS analysis), 200µl of each sample was collected from the 500µl of original sample. The sample was then dispensed into small evaporation containers. The 200µl of each sample was reduced to 20µl by blowing nitrogen air into the sample. The quantity of the sample was confirmed by measurement with a 100µl syringe. The 20µl of sample was placed into a glass insert inside an auto injector vial which was sealed with PTFE tape and tin foil to reduce

evaporation. For all manual injections (2017 DB-Wax, 2018 HP5-MS and 2018 DB-Wax analysis) 50μl of dog-hair entrainment sample was collected from the 500μl of original sample. A copper nitrogen line was used to reduce the sample to 5μl. The quantity of the sample was confirmed by measurement with a 100μl syringe. This sample was subsequently manually injected into the GC/MS.

An internal standard (caryophyllene, 10μl of 50ng/μl) was added to each sample. The internal standard was used to improve the precision of the analysis by providing a known concentration of a substance present in each analysed sample. Caryophyllene was used as it does not occur naturally and therefore can provide a signal which can be distinguished from any other analyte present.

A syringe fitted with a long needle was used to collect all extracts from the original sample and rinsed with 1ml of hexane before withdrawing. All evaporation containers were made by cutting down glass ampules to 20mm lengths. This allowed for the remaining sample to be stored for future experiments and reduced the possibility of sample contamination. The cut-down ampules were rinsed with 1-2 ml hexane and then baked in a drying oven at 240^{o}C before use. The residual dog-hair hexane extract ampule was immediately heat-sealed before proceeding with the GC/MS sample injection. The opening of the copper nitrogen line used for blowing-down samples was rinsed daily with hexane before proceeding with the experiment in order to remove any accumulated volatiles on the tubing and to reduce cross contamination between samples. The 10μl injection syringe and external needle surface were rinsed with 10x 10μl of hexane before drawing up injection volume.

6.2.3 GC/MS 2017 Dog Sample Analysis

Following the previous VOC analyser analysis of 55 dogs (44 negatives and 11 positives) from the 2017 cohort, the entrained samples collected from the same cohort of dogs were run in triplicate (i.e. 3 replicates: A, B and C, supplementary material **Tables 9.1A, 9.1B and 9.1C**) on the HP5-MS column using an auto sampler injection. A further replicate D was analysed on the DB-wax column (Supplementary material **Table 9.2**) however these samples were manually injected.

For the HP5-MS analysis, samples were placed in an auto injector rack which formed part of the injection tower (Agilent 7693A Automatic liquid sampler). This allowed for the automated analysis of a maximum of 16 samples with a capacity for one waste bottle and two solvent bottles. The auto sampler injected 5μl of each individual sample into the GC/MS (equivalent of 50ml of original sample) according to the schedules shown in supplementary material **Tables 9.1A, 9.1B and 9.1C**. During the analysis, the room temperature was set at 16^{o}C to reduce hexane evaporation as the samples were stored in the rack for up to 10 hours. Following analysis, the lid on the sample vial was replaced and the samples stored at -20^{o}C prior to replicates B and C. For the 2017 DB-Wax analysis 5μl was manually injected using a 10μl injection syringe following a randomised analysis schedule (Supplementary **Table 9.2**).

6.2.4 GC/MS 2018 Dog Sample Analysis

Following the VOC analyser analysis of all 149 samples collected in 2018 (44 infected and 105 negatives), 60 samples (30 infected and 30 uninfected) were selected and injected manually according to a predetermined randomised schedule (supplementary material **Tables 9.3 and 9.4**). The entrained samples which were

analysed were from the same cohort of dogs which were used for the bioassay analysis (30 dog pairs; 15 infected and 15 negative, Chapter 5 methodology section 3.2) and an additional randomly chosen 30 entrained samples (15 infected and 15 negative). Initially the analysis was done on the HP5-MS column, following which the samples were also run on the DB wax column. All samples were manually injected using a 10µl injection syringe.

6.2.5 Agilent Qualitative Analysis

Post-acquisition, all raw data files were subjected to supervised deconvolution analysis. This is an automated program in the Agilent MassHunter Qualitative Analysis B.07.00 software package (Agilent technologies UK). This analysis resulted in the removal of some samples which had a high level of background noise allowing for a better peak alignment and comparison. The chemical noise can result from a variety of different sources, such as the GC mobile phase or buffers which have a high contribution in the signal.

The samples removed from the analysis because of background noise were:

2017 HP5-MS: 21 samples (1 infected and 20 negatives)

2017 DB-Wax: 20 samples (3 infected and 17 negatives)

2018 HP5-MS: 14 samples (8 infected and 6 negatives)

2018 DB-Wax: 21 samples (13 infected and 8 negatives)

The total samples removed were roughly a third of the data set.

6.2.6 Agilent Quantitative analysis: the “Unknowns Analysis”

Initial analysis identified and quantified all the known and unknown compounds which were present within the dog samples using Agilent MassHunter Workstation- Unknowns Analysis software (Agilent Technologies, UK). This program used a chromatographic deconvolution algorithm to resolve the complexity of the chromatogram where different compounds may have co-eluted or eluted closely together. This method consisted of fitting a linear regression to the single or multi-peaks of the chromatograms. The software can recognise these peaks, remove background noise and determine the spectra of components from the underlying ion chromatograms (Dromey *et al.,* 1976).

The deconvoluted chromatograms containing the reconstructed spectra were then searched in the National Institute of Standards and Technology 2014 library (NIST14) within the Agilent MassHunter Workstation- Unknowns Analysis software. In addition to mass spectral data, this process also utilised the retention times of the compounds to provide very accurate comparison of compounds in different chromatograms. These deconvoluted chromatograms (Agilent termed extracted ion chromatograms (EICs)) were saved as *cef* format files for subsequent statistical analyses and data visualisation in Agilent Mass Profiler Professional (MPP) v15.0 software (Agilent Technologies, UK).

6.2.7 Mass Professional Profiler (MPP) Analysis

6.2.7.1 Data filtering and Identification of VOCs characteristic for cVL infection

For analysis of the GC/MS data files, a project file was created in Agilent Mass Profiler Professional (MPP) containing the data from the four separate analytical experiments i.e. the deconvoluted GC/MS data from the 2017, 2018 HP5-MS and DB-

Wax analyses. For each of the experiments the data files were grouped and a condition of either infected or uninfected was assigned to each sample based on previous PCR diagnosis. The chromatograms were then aligned using a R_T correction function using three compounds present in all the chromatograms; caryophyllene (mw 204.36amu) and nonadecane (mw 268.5amu) and Phenanathrene (mw 178.23amu). The chromatograms were then baselined using the Pareto scaling method. Pareto scaling reduces the influence of intense peaks by reducing their loadings and emphasises weaker peaks which may be more biological relevant by increasing their loadings (Worley and Powers, 2013), further aligning the chromatograms at their baseline.

The compounds (individual VOCs) which were identified in the deconvoluted GC/MS data files were then filtered based on a Relative Frequency (Rel Freq) threshold. For this project, only compounds which were present in 70% of the samples in at least one or more condition (70% Rel Freq threshold) were retained for the analysis. Further filtering of the compounds was also performed using an unpaired T-test analysis in order to identify the compounds which were considered as significantly important for distinguishing between infected and uninfected samples (volatiles characteristic for cVL infection). A cut of value of $P < 0.05$ was considered statistically significant for all experiments apart from the DB-Wax 2018 analysis in which *P*-value <0.5 was set. This analysis used a Benjamini-Hochberg False Discovery Rate set to 5% for multiple testing corrections (Hochberg and Benjamini, 1990). This reduced the rate of incorrectly rejecting the true null hypothesis. Finally, the remaining compounds were automatically identified and annotated by comparison with the NIST14 library entries using the “IDBrowser Identification” facility in the MPP software.

6.2.7.2 CVL identification: Statistical analysis and Visualisation

Following the acquisition of the compounds which were considered as important for distinguishing between infected and uninfected dogs, a principle component analysis (PCA) was performed in order to classify samples belonging to infected or uninfected infection status. The PCA was performed using by centring and scaling the variables which displayed the relationship between the infected and uninfected dogs. PCA is a multivariate analysis technique using an unpaired t-test to detect the grouping trends of the VOCs with respect to the two studied groups (infected and uninfected) and then displays this information as a matrix. This allowed us to predict which group a dog belonged based on their VOC profile. A P value of <0.05 was considered statistically significant for all experiments apart from the DB-Wax 2018 analysis in which a P value of <0.5 was set.

6.2.7.3 Class Prediction Model

Following the PCA analysis, confusion matrices of the separation of the uninfected vs infected dogs in 2017 and 2018 using both the HP5-MS and DB-Wax columns were obtained. The analyses were performed using the class prediction model tool in Agilent Mass Profiler Professional (MPP).

6.3 Results

6.3.1 Qualitative Analysis

6.3.1.1 2017 Dogs

The Agilent MassHunter Qualitative Analysis of the 2017 dogs detected 6,991 VOCs in the DB-Wax column analysis and 11,638 VOCs in the HP5-MS column analysis as being recognisable compounds. These volatiles were chemically identified using Agilent MassHunter Workstation- Unknowns Analysis (Agilent Technologies, UK) which utilised the NIST14 electronic library and individual retention times. Although all these volatile compounds were recognised, only 1,546 VOCs from the DB-Wax analysis and 3,587 VOCs from the HP5-MS analysis were subsequently identified and named in all the chromatograms analysed.

6.3.1.2 2018 Dogs

The Agilent MassHunter Qualitative Analysis of the 2018 dogs detected 5,858 VOCs in the DB-Wax column analysis and 6,273 VOCs in the HP5-MS column analysis and recognisable compounds. As with the 2017 data, these volatiles were chemically identified using Agilent MassHunter Workstation- Unknowns Analysis (Agilent Technologies, UK) which utilised the NIST14 electronic library and individual retention times for each compound. Although all volatiles were recognised, only 1,478 VOCs from the DB-Wax analysis and 1,397 VOCs from the HP5-MS analysis were subsequently identified and named in all the chromatograms which were analysed.

6.3.1.3 Identification of the VOCs characteristic for cVL infection

Agilent Mass Profiler Professional (MPP) analysis identified 5 entities for 2017 HP5-MS, 3 entities for 2017 DB-Wax, 27 entities for 2018 DB-Wax and 4 entities for 2018 HP5-MS which were considered as being significantly important in distinguishing between infected and uninfected dogs. However, due to the diversity and quantity of VOCs found within samples, and the identification only being performed using the libraries inbuilt into the MPP software, some significantly important volatiles remained unnamed. The most significantly important VOCs to differentiate infected from uninfected dogs in both years which were named are shown in **Table 6.1**.

Column	Compound	Concentration Increase/Decrease in Infected dogs
2017 HP5-MS	n-Tridecan-1-ol	Increase
	Silane, trichlorodocosyl-	Decrease
	(-)-cis-Myrtanylamine	Increase
	Octanal, 2-(phenylmethylene)-	Increase
	5-Tridecene, (Z)-	Decrease
2017 DB-Wax	o-Terphenyl	Increase
2018 HP5-MS	Undecane, 2,3-dimethyl-	Increase
2018 DB-Wax	Pyrene	Decrease
	Squalene	Decrease
	Benzene, (1-butylhexyl)-	Decrease
	4- Trifluoroacetoxytetradecane	Decrease
	Biphenyl	Increase
	2-Methylhexacosane	Increase
	Butanoic acid, 2-hexenyl ester, (Z)-	Increase
	Benzene, (1-pentylhexyl)-	Decrease
	Benzene, (1-ethylnonyl)-	Decrease
	Styrene	Increase
	Diethyl Phthalate	Decrease

Table 6.1: Significantly important volatiles identified in the hair samples from dogs in 2017 and 2018. All samples were identified following further filtering using an unpaired t-test significance analysis in Agilent MPP software. $P<0.05$; 2017 HP5-MS/DB-Wax and 2018 HP5-MS. $P<0.5$; 2018 DB-Wax.

6.3.2 CVL identification: Statistical analysis and Visualisation

6.3.2.1 Principle Component Analysis (PCA)

The PCA was performed on the conditions set for the data (infected or uninfected) which allowed for the detection of any similarities between samples, which were discriminated by major trends in the data. The results of the PCA analyses for each of the four different experiments are shown in score graphs of the first and second principle components (PC) (**Figures 6.3 and 6.4**). This pair of PCs were chosen because of the better grouping tendencies which were obtained. The score graphs illustrated in figures 3 and 4 of the samples from both 2017 and 2018 using the two different columns (HP5-MS and DB-Wax) illustrates segregation between infected and uninfected dogs.

Visual analysis of the PCA score graphs suggests that most of the samples from infected dogs displayed a degree of tighter clustering than the uninfected dogs. However, when performing the analysis of the 2017 dogs with the HP5-MS column, the uninfected dogs tend to show tighter clustering with the infected dogs showing a higher degree of dispersion.

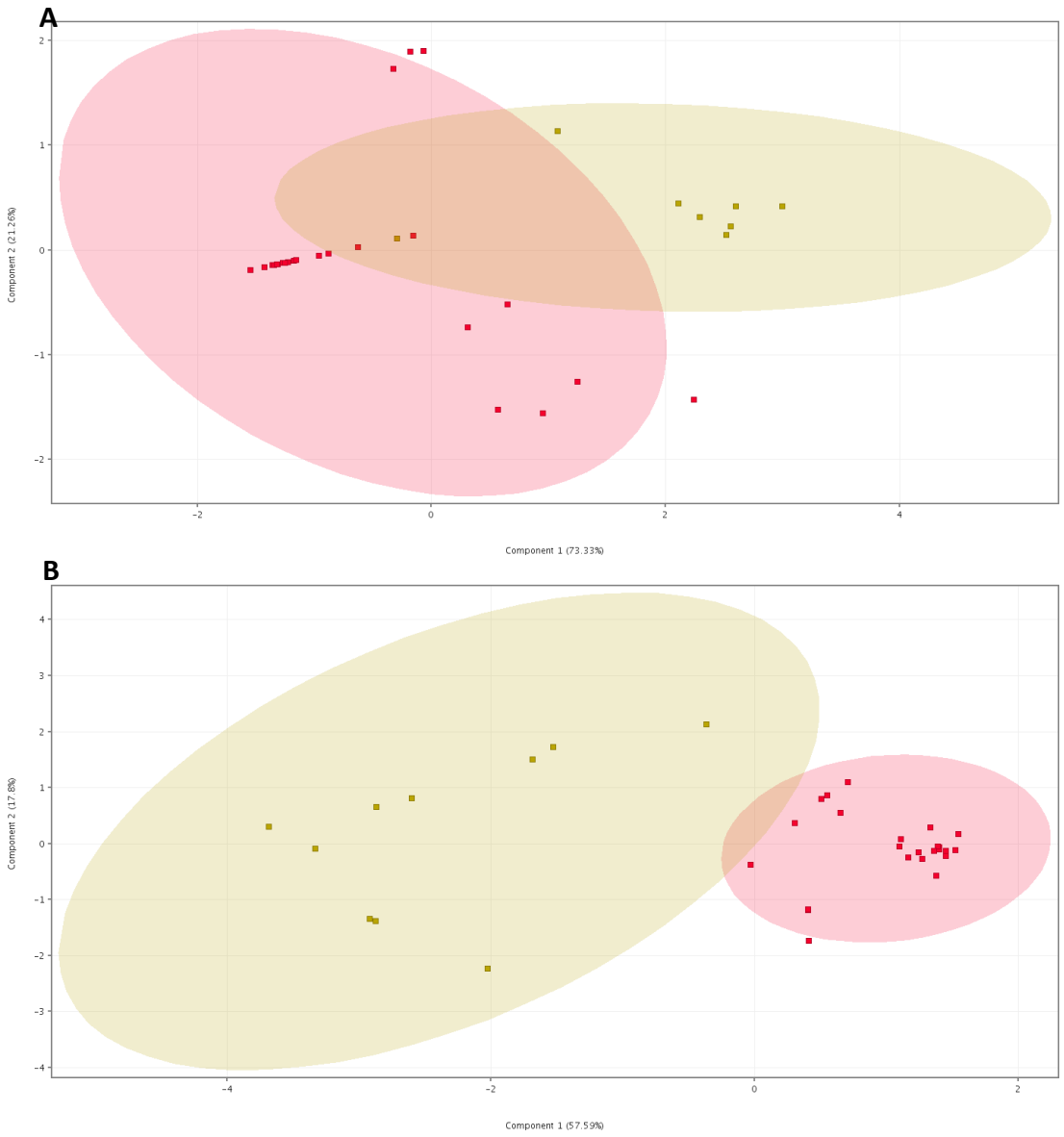


Figure 6.3: PCA score plots of dog hair volatile samples from the GC/MS analyses of 2017 dogs on A) DB-Wax column and B) HP5-MS column. Uninfected dogs are shown in red solid and infected dogs in yellow. The numbers next to each positive dog represent the individual dog analysed (and replicate A, B or C for HP5-MS experiment). The corresponding parasite loads are shown in supplementary Tables 1A, 1B, 1C and 2. PCA preformed using Agilent Mass Profiler Professional (MPP) v15.0 software (Agilent Technologies, UK). A) PC1=73.33%, PC2=21.26%; B) PC1=57.59%, PC2=17.8%. The solid colour ellipses were produced by MPP.

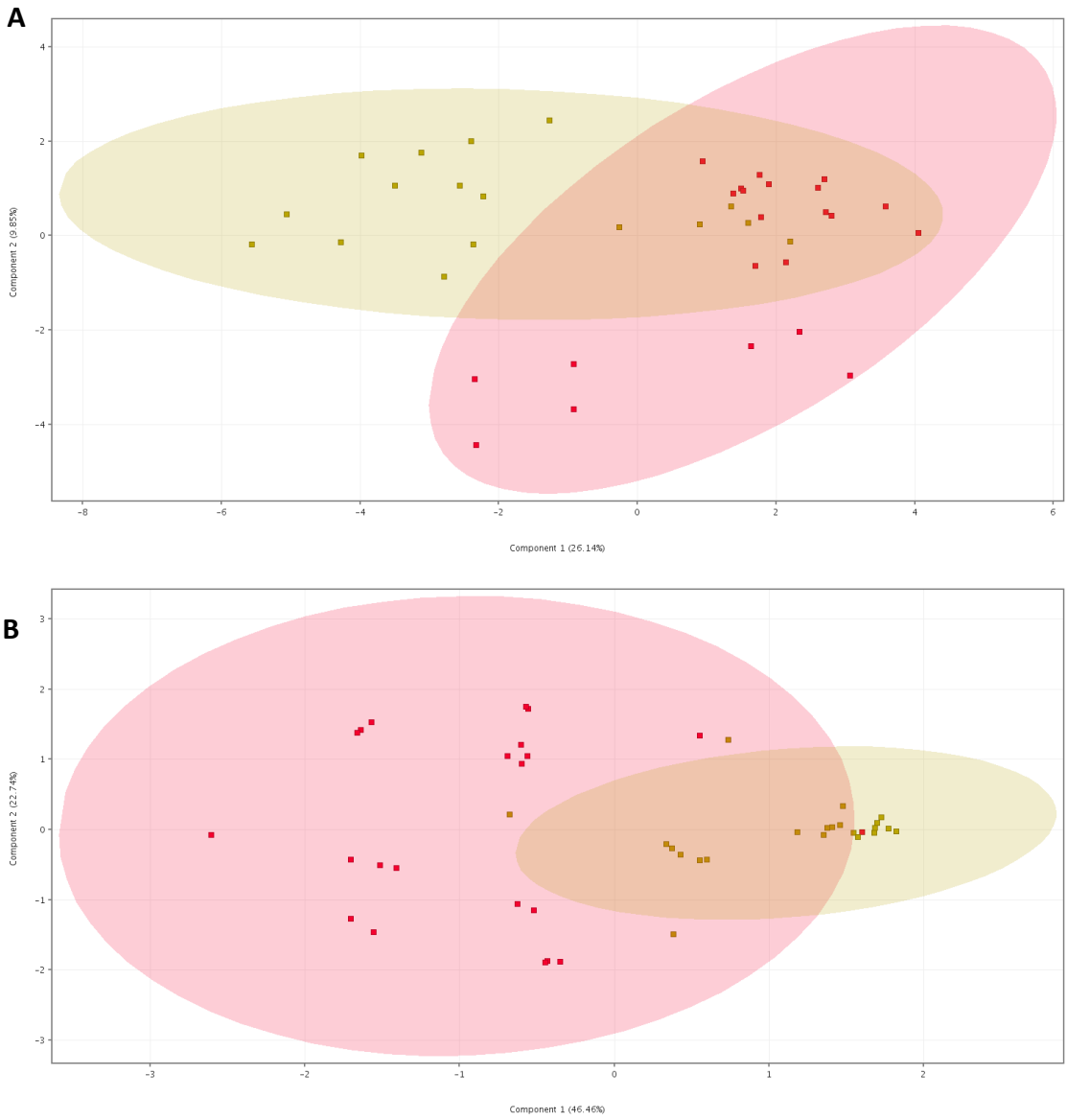


Figure 6.4: PCA score plots of dog hair volatile samples from the GC/MS analyses of 2018 dogs on A) DB-Wax column and B) HP5-MS column. Uninfected dogs are shown by the solid red ellipse and infected dogs by the yellow ellipse. The numbers next to each positive dog represent the individual dog analysed. The corresponding parasite loads are shown in supplementary Tables 3 and 4. PCA preformed using Agilent Mass Profiler Professional (MPP) v15.0 software (Agilent Technologies, UK). A) PC1=73.33%, PC2=21.26%; B) PC1=57.59%, PC2=17.8%. The solid colour ellipses were produced by MPP.

6.3.2.2 Class Prediction Model

For the 2017 dogs (**Table 6.2**), HP5-MS non-polar column analysis shows a distinct segregation with a specificity of 95.8% and sensitivity of 100% being determined. However, although showing some separation, the 2017 DB-Wax analysis showed less specificity (66.7%) and sensitivity (87.5%) with some samples being misclassified.

For the 2018 dogs (**Table 6.3**), the HP5-MS non-polar column analysis showed a high segregation with a specificity of 91.7% and sensitivity of 95.5% being determined. The 2018 DB-Wax analysis also showed a relatively high specificity (90.9%) but a slightly reduced sensitivity (82.4%).

2017 DB-Wax				
Observed\Predicted	**Uninfected**	**Infected**	**Specificity (%)**	**Sensitivity (%)**
Uninfected	18	9	66.7	
Infected	1	7		87.5
2017 HP5-MS				
Observed\Predicted	**Uninfected**	**Infected**	**Specificity (%)**	**Sensitivity (%)**
Uninfected	23	1	95.8	
Infected	0	10		100

Table 6.2: 2017 Confusion matrixes for the Principle Component Analyses (PCA's) using Agilent Mass Professional Profiler software (MPP) v15.0. Class prediction of the 2017 infected/uninfected dogs using both the DB-Wax and HP5-MS columns.

2018 DB-Wax				
Observed\Predicted	**Uninfected**	**Infected**	**Specificity (%)**	**Sensitivity (%)**
Uninfected	20	2	90.9	
Infected	3	14		82.4
2018 HP5-MS				
Observed\Predicted	**Uninfected**	**Infected**	**Specificity (%)**	**Sensitivity (%)**
Uninfected	22	2	91.7	
Infected	1	21		95.5

Table 6.3: 2018 Confusion matrixes for the Principle Component Analyses (PCA's) using Agilent Mass Professional Profiler software (MPP) v15.0. Class prediction of the 2018 infected/uninfected dogs using both the DB-Wax and HP5-MS columns.

6.4 Discussion

The PCA performed using Agilent MPP software demonstrates the ability of the GC/MS to identify and discriminate between *Leishmania* infected and uninfected dogs. However, unsurprisingly the type of column used had a significant effect on the discrimination capabilities of this diagnostic technique, with samples analysed using the HP-MS non-polar column for both years discriminating between infected and uninfected dogs with a higher sensitivity and specificity. This can be demonstrated by the confusion matrices performed on the PCA data for the HP5-MS column which demonstrated 100% sensitivity for the 2017 dogs and 95.5% sensitivity for the 2018 dogs. These results further validate the capabilities of the VOC Analyser to distinguish between infected and uninfected dogs (Staniek *et al.,* 2019), with GC/MS analysis also identifying a differentiation between dogs. In addition, as with the VOC Analyser analysis, the origin of the observed change in volatiles following *Leishmania* infection is still under debate as parasite load also appears to have no effect on GC/MS distribution of the infected dogs. This result further suggests evidence that the change in odour profile is not related to an immune response, contradicting previous cVL research (Magalhães-Junior *et al.,* 2014b)

GC/MS analysis of the dog samples also provided the structural and chemical information of specific volatiles which were up or downregulated in positive dogs. This allowed for a GC/MS library search to preliminary identify those volatiles which could be considered as being *Leishmania* specific. This is an important observation, not only as a contribution to the further development of a VOC Analyser as a new and minimally invasive diagnostic technique, but also for the future development of vector control methodologies based on odour baited sandfly traps. Both observations are likely to

reinforce the clinical diagnosis and prognosis of cVL currently performed by classical techniques as well as to improve clinical decisions which currently consist of the removal of infected dogs by culling.

6.4.1 GC/MS discrimination between infected and uninfected dogs

Volatile organic compounds (VOCs) have previously been used to characterise a range of disease conditions. These include the analysis of various parasitic diseases such as the diagnosis of malaria through the odour of socks worn by children (Guest *et al.,* 2019), but also cVL through the analysis of VOCs found in hair (Magalhães-Junior *et al.,* 2014; Staniek *et al*., 2019). Through this research, particularly the identification of parasite specific biomarkers and volatiles, metabolomics has offered the potential for the development of promising laboratory tools for the analysis of parasitic diagnostic biomarkers. This analysis may be used to not only assess disease susceptibility, but also to diagnose an infected individual and evaluate the therapeutic response to treatment. To this end, the use of GC/MS in the diagnosis of cVL shows great promise as shown in previous work by Oliveira *et al.,* (2008) and Magalhães-Junior *et al.,* (2014b), but also the results demonstrated here.

The results presented here demonstrate the use of GC/MS technology as a non-invasive and sensitive diagnostic technique. Despite a small amount of overlap following PCA analysis, confusion matrices demonstrated the ability of the GC/MS fitted with a HP5-MS non-polar column to distinguish between 2017 infected and uninfected dogs with 95.8% specificity and 100% sensitivity and 2018 infected and uninfected dogs with 91.7% specificity and 95.5% sensitivity. However, analysis using the DB-Wax polar column demonstrated a lower sensitive and specificity when

distinguishing between 2017 dogs (66.7% specificity and 87.5% sensitivity) and 2018 dogs (90.9% specificity and 82.4% sensitivity). These results are however not surprising. Although different types of volatile compounds are found in different biological media, many of the molecules found in sweat, which are excreted onto hair samples, are non-polar with the outer layer of skin also predominantly consisting of non-polar molecules (Dormont *et al.,* 2013). Therefore, the use of a non-polar GC column such as the HP5-MS that is made using the least selective non-polar stationary phase would be expected to produce more accurate results due to the general chemical principle of like dissolving like. In order to improve the potential sensitivity of the analysis however, various other biological samples should be considered for GC/MS analysis. Although sweat contains predominantly non-polar molecules, research has demonstrated other biological samples as containing both polar and non-polar compounds thereby allowing for the utilisation of both columns in a simultaneous statistical analysis to generate a higher sensitivity. For example, a variety of polar and non-polar volatiles have been identified in the breath of individuals in certain disease states such as cancer (Amor *et al.,* 2019) and respiratory tract infections (Christ-Crain and Muller, 2007). Specific *Leishmania* research has demonstrated an increase in the levels of certain chemokines in the skin cells of dogs naturally infected with *Le. infantum* (Menezes-Souza *et al.,* 2012). A variety of both polar and non-polar interactions contribute to the production of chemokines production and their receptor binding; therefore, the use of skin scrapings or aspirates could be suggested as a biological sample a more robust GC/MS analysis. The results presented here can be further validated by recent research which also demonstrated a distinctive differentiation between the VOC profiles of *Leishmania* infected and

uninfected dogs (Magalhães-Junior *et al.,* 2014b). However, this study not only demonstrated more overlap between infected and uninfected dogs following partial least squares discriminant analysis (PLS-DA), but in addition only 36 dogs were used for the analysis. It could therefore be suggested that the data present here provides a more robust analysis and accurate representation of the discrimination between the volatile profiles of cVL infected and uninfected.

Further analysis of the results presented her demonstrate a high level of dispersion between individual hair samples, particularly within the uninfected dog groups (**Figure 6.3 and 6.4**). This variation is to be expected due to hair not being a homogeneous matrix as well as several intrinsic factors which can influence the analysis. The colour and texture of the hair as well as the use of products to prevent ectoparasites and for cosmetic purposes, the hair growth rate and the anatomical origin of the sample may also interfere with the dispersion results (Vincenti *et al.,* 2013). Therefore, in order to relieve bias in this work, the dorsal area of all dogs sampled was standardised. Furthermore, due to the study consisting of a variety of genders, species and age, the colour and texture of the hair are less of a contributing factor towards bias.

Many mechanisms have been identified which occur during *Leishmania* infection in dogs. These responses range from internal immune responses (Pinelli *et al.,* 1994), to alteration of the microbiome on the skin of infected dogs (Gimblet *et al.,* 2017). Therefore, the use of hair to diagnose the pathological changes which occur during cVL infection is an interesting metabolomic approach to standardising the biomarkers of the endogenous metabolism in cVL. This is due to not only the ease of sample collection, but also the nature of hair follicles which secrete and excrete

substances that are produced endogenously (Harkey, 1993). Therefore, regardless of the mechanism behind the change in the volatiles produced by infected and uninfected dogs, hair follicles will represent the biological changes which occur during cVL infection.

6.4.2 *Leishmania* specific compounds

Following further filtering in Agilent MPP Software using an unpaired t-test significance analysis, a total of 6 VOCs for 2017 (5 for HP5-MS; 1 for DB Wax) and 12 VOCs for 2018 (1 for HP5-MS; 11 for DB Wax) whose expression profiles varied comparatively according to the infected or uninfected infection status of the dogs evaluated were preliminary identified. Although the identity of the compound can be suggested (**Table 4**), GC/MS analysis can only provide structural and chemical information which can assist with the identification of the compounds. Therefore, the identification of the compounds obtained from the library fitting is ambiguous with further experiments normally required in order to achieve the exact identification. However, despite the exact identity of the compounds being unknown, due to the HP5-MS column providing the best differentiation between infected and uninfected dogs in both 2017 and 2018 it can be suggested that 5 VOCs for 2017 dogs and 1 VOC for 2018 dogs are significant for differentiating between infected and uninfected dogs. Of the 5 volatiles identified in 2017, 3 were found to have an increase in concentration in infected dogs in addition to singular volatile for the 2018 dogs (**Table 6.4**). This therefore suggests these volatiles as potential biomarkers for *Le. infantum* infection in dogs which could be utilised for the further development of the VOC Analyser diagnostic technique (Staniek *et al.,* 2019). Previous research has also demonstrated

an increase in sandfly attractiveness to cVL specific volatiles (Magalhães-Junior *et al.,* 2019) in addition to the results presented in Chapter 5, suggested that these volatiles could lead to the development of synthetic odorant traps to improve current vector control in Brazil. However, in order to further our understanding of the role that these volatiles have in sandfly attractiveness, further research should be undertaken to demonstrate the sandfly behavioural response when exposed to these individual chemicals.

As shown in **Table 6.1**, the volatiles identified as being *Leishmania* specific differ depending on the column used with all the chemicals found in the HP5-MS analysis differing to those identified following the DB-Wax analysis. This is not surprising however, due to the polar and non-polar nature of the different columns having differing sensitivities to different volatiles. What is surprising however is the difference between the groups of volatiles which were identified as being up or downregulated in the 2017 dog volatiles compared to the 2018 dog volatiles when using the same column. Although the exact reason for this difference is unknown, it could be suggested that the storage time of the samples could have had an effect. The 2017 hair samples were stored for a longer period compared to the 2018 samples prior to entrainment. Previous research has demonstrated the production of different bacteria and hormone concentrations during different storage times (Lauber *et al.,* 2010). Furthermore, the 2018 analyses used a larger data set with a larger number of positive dogs being analysed. Therefore, it could be suggested that these analyses were more robust, providing a greater representation of the volatiles that are considered as being *Leishmania* specific as they were identified in a larger number of positive dogs. The volatiles identified here also differed from those identified by

Magalhães-Junior *et al.,* (2014b) who suggested 6 volatiles as being *Leishmania* specific; octan*al.,* nonan*al.,* β-hydroxyethyl phenyl ether, decan*al.,* heptadecane and 2-ethylhexyl-salicylate. Although the exact reason for this difference in identified volatiles is unknown, it could be suggested that the use of two different extraction techniques; Solid phase micro extension (SPME) (Magalhães-Junior *et al.,* 2014b) and the headspace entrainment on Tenax methodology used here, could be a contributing factor. There are a few suggested disadvantages identified for SPME including a lack of absorption of light volatiles (e.g. acetaldehydes) when using certain SPME fibres, in addition to the preferential absorption of heavier volatiles. Additionally, polar volatiles have been shown to be present in higher levels in SPME extracts, a further disadvantage of this methodology as the molecules that are found on hair samples are predominantly non-polar. Further research has also demonstrated that headspace entrainment on Tenax allows for the extraction of higher amounts of most volatiles, in particular low-boiling compounds. (Elmore *et al.,* 2001). This evidence not only provides an explanation for the observed differences between the two studies, but also suggests headspace entrainment as being a more efficient entrainment methodology for the analysis of *Leishmania* infected hair samples.

The identification of these biomarkers as potential *Le. infantum* specific volatiles could have a major impact on the development of the VOC Analyser technology as a non-invasive diagnostic device for cVL. The use of VOC Analysers for the diagnosis of specific diseases present in animal hosts has already been achieved (Fend *et al.,* 2005). Through the development of specific sensor arrays and improvements made in the design of new sensors, VOC Analysers have been adapted for specific host types as well as chemical classes of volatiles which have been identified as being closely

associated with certain diseases (Wilson, 2016). A significant amount of progress in the development of VOC Analysers for animal disease detection, combined with metabolomics, has already proven effective in the veterinary medicine field. Important biomarkers and aroma profiles have been discovered for major infectious diseases of wildlife, fish and livestock (Moore *et al.,* 2007). Results presented in this report along with Staniek *et al.,* (2019) demonstrate a high sensitivity and specificity when using a

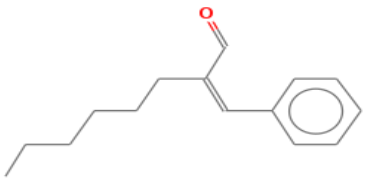
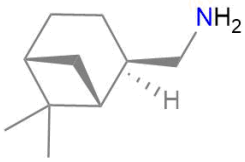
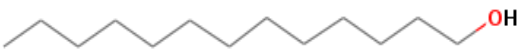
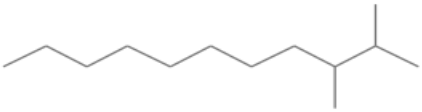
Name	Structure
2-(phenylmethylene)-octanal	
(-)-cis-myrtanylamine	
n-tridecan-1-ol	
2,3-dimethyl-undecane	

Table 6.4: VOCs identified as potential biomarkers for *Le. infantum* infection in Dogs. In the 2017 samples, 3 volatiles were found to have an increase in concentration in infected dogs; 2-(phenylmethylene)-octanal (-)-cis-myrtanylamine and n-tridecan-1-ol. In the 2018 samples, 1 volatile was found to have an increased concentration in infected dogs; 2,3-dimethyl-undecane.

VOC Analyser to diagnose *Leishmania* infected dogs. For the purpose of this study, a random array of electronic chemical sensors, which had partial specificity, was utilised. The individual sensors used for the VOC Analyser diagnosis had semi-selectivity to different groups of volatile chemicals such as alcohols, aldehydes, ketones and organic acids. However, following analysis only a small percentage of the sensor variables (2/88 in 2017 and 3/88 in 2018) contributed significantly to the separation of infected and uninfected dogs (Staniek *et al.,* 2019). This therefore suggests that there is the potential to improve the sensitivity and specificity of the VOC Analyser device. This could be achieved by modifying the sensor array through the addition of new sensors that are highly sensitive to the individual volatiles identified here as being *Leishmania* specific VOCs. This will therefore lead to the development of a *Leishmania* specific sensor array. The development of a specific sensor array made using the known compounds identified here could increase the sensitivity and specificity of the VOC Analyser device whilst maintaining the non-invasive qualities of this technique.

6.4.3 Summary

The results presented here suggest that the VOCs that are emitted in the hair of infected dogs can be used as biomarkers of *Le. infantum* infection. Furthermore, the specific volatiles identified could be used to further develop methodologies for the diagnosis of *Leishmania* in dogs through the modification of a VOC Analyser. The results presented here identified 4 VOCs which may prove important in the further development of VOC Analyser technology as a diagnostic tool for cVL in Brazil through the development of a *Leishmania* specific sensor array (Staniek *et al.,* 2019). Increasing the specificity of such a device through the addition of adapted sensors based on these

results, could improve the current *Leishmania* control methods in Brazil which rely on the use of an accurate diagnostic device in order to achieve effective culling. Additionally, it can be suggested that these identified volatiles may be attractive to sandfly vectors due to previous research which has shown an increase in sandfly attraction to *Leishmania* specific volatiles identified through the SPME/GC/MS analysis of infected canine hair samples (Magalhães-Junior *et al.,* 2019). The results presented here could lead to the development of synthetic odorants that are attractive to sand-flies, as well as developing improved entomological traps and other instruments which contribute to the control of VL in Brazil.

Chapter 7 – Discussion

Despite recent advances in modern medicine, infectious diseases continue to devastate developing countries. Visceral leishmaniasis is still a problem in Brazil, with approximately 3,500 cases reported annually and a peak lethality rate of 20% reported from 2011 to 2016 (Luz *et al.,* 2018). In addition to the early diagnosis of *Leishmania* infected individuals and case management, current control methods employed in Brazil in order to control the spread of leishmaniasis include the diagnosis and subsequent culling of infected dogs as well as methods of vector control in order to reduce sandfly numbers and disease transmission (Boelaert *et al.,* 2018).

Despite the use of dog-based strategies for the control and prevention of VL in Brazil, including dog culling and vaccinations, there is still no satisfactory dog control strategy currently available which aids in a reduction of disease transmission (Ribeiro *et al.,* 2018). Dogs have been found to respond poorly to anti-leishmanial therapy, with the current commercial vaccine used in Brazil having a protective efficacy of 71% (Regina-Silva *et al.,* 2016). Furthermore, despite hundreds of thousands of dogs being culled every year in Brazil, the prevalence of canine leishmaniasis is still very high in several endemic areas due, in part, to inaccurate diagnostics (Dantas-Torres *et al.,* 2019). New diagnostic tests that are rapid, affordable and highly accurate therefore need to be developed in order to help control the continual spread of infection and reduce disease transmission and incidence throughout Brazil. The effective use of volatile analysis as a potential diagnostic tool for cVL has already been demonstrated. Research has observed that different VOCs are produced by *Leishmania* infected and uninfected dogs, with multivariate analysis able to group these dogs based on the

VOCs produced (Magalhães-Junior *et al.,* 2014). These results combined with research which has demonstrated the effective use of VOC Analysers in the diagnosis of diseases such as tuberculosis (Teixeira *et al.,* 2017) and lung cancer (Tirzite *et al.,* 2018) through the analysis of changes in VOC profiles, suggest the potential use of VOC Analysers as a non-invasive diagnostic for cVL.

In addition to the development of new diagnostic approaches to improve current *leishmaniasis* control methods in Brazil, a reduction in the number of female sandflies will also reduce disease transmission. Sandfly control is an important component of many of the anti-*Leishmania* programs currently in place in Brazil. These currently include the spraying of aggregations sites with insecticides such as DDT and the use of deltamethrin insecticide-impregnated collars (Coulibaly *et al.,* 2018; Leite *et al.,* 2018). Although it has been suggested that the use of insecticides may be effective in reducing the transmission of *Leishmania*, it has been demonstrated that insecticide resistance may result in the failure to control sandfly population numbers. Spraying of aggregation sites that are near reported human cases of VL has not proved effective in the reduction of disease prevalence (Amora *et al.,* 2009). Research has also demonstrated a 36.3% rate of resistance of *Lu. longipalpis* to deltamethrin in Brazil (Alexander *et al.,* 2009). Therefore, the development of new, more effective vector control methods which result in a reduction in the number of female sandflies will not only have an impact on disease transmission but also help with a reduction in the current reliance on insecticides and subsequent development of insecticide resistance. Recent research has demonstrated the use of pheromone lures as a potential sandfly control program in Brazil. *Lutzomyia longipalpis* showed a significant increase in attraction to chicken sheds which were sprayed with a combination of pheromone and

insecticide. Interestingly, sheds which were treated with insecticide alone resulted in fewer males being attracted and a significant decrease in female sandfly attraction (Bell *et al.,* 2018). Therefore, the successful development vector control methodologies which utilise a combination of sandfly lures and insecticide could be suggested. This approachcould have a major impact on the transmission of leishmaniasis throughout Brazil, resulting in an increase of the numbers of sandflies caught and killed.

7.1 CVL in Field Diagnostics

Canine visceral leishmaniasis, caused by *Leishmania infantum*, is considered a zoonosis that is potentially fatal to both dogs and humans. Dogs are the main reservoir host and source of human infection in Brazil (Roque and Jansen *et al.,* 2014). The ability of dogs to acts as a reservoir host for the *Leishmania* parasite is of major concern not only due to the rapid spread of cVL among dogs, but also the close proximity of dogs to humans in the domestic environment (Baneth *et al.,* 2008; Woodroffe *et al.,* 2012). The increasing awareness that the control of human VL is highly dependent on the effective control of cVL has prompted an increase in research regarding *Leishmania* infection in dogs. However, there is still no cVL diagnostic technique that is 100% specific and sensitive (Pessoa-e-Silva *et al.,* 2019). The cVL diagnostic protocol adopted by the Brazilian Ministry of Health in 2012 included the use of the invasive, in-field and rapid Dual-Path Platform (DPP) test, which has demonstrated a low sensitivity of 56% when diagnosing symptomatic dogs and 75% when diagnosing asymptomatic dogs (Coura-Vital *et al.,* 2014; Almeida *et al.,* 2017b; Figueiredo *et al.,* 2018). The development of new in-field diagnostic tests that are cheap, non-invasive and reliable

is essential to improve diagnosis of cVL in the field, which will subsequently help reduce disease transmission.

The use of point of care diagnosis of cVL has many advantages over current laboratory-based techniques. First, this type of analysis would provide a faster access to test results enabling clinicians to make rapid clinical decisions as well as suggest more appropriate interventions and treatments due to early stage diagnosis (Kazmierczak, 2011). Second, point of care analysis also minimises time-dependent changes in disease state by giving an accurate diagnosis in the field rather than waiting for laboratory results. This is particularly important for the diagnosis of cVL due to positive dogs currently remaining in the environment for a few months following sampling due to the transport of blood samples to laboratories in order to perform diagnosis. Third, the adaptation of current diagnostic tests to provide point of care testing could facilitate large-scale screening programs (Duthie *et al.,* 2018). Finally, point of care analyses predominantly require a smaller volume of sample then that which is needed for testing in a laboratory (John and Price, 2014). Although new rapid diagnostic tests could deliver a faster, less invasive and accurate disease diagnosis, with all new diagnostic tests there are many barriers which need to be overcome following their introduction. New diagnostics are subjected to uncertainties concerning coverage decisions, slow and maladapted regulatory approval processes, ethical and social issues, as well as resistance among physicians to new diagnostic methods, all of which combine to hinder innovation and slow the distribution of new diagnostic tests into mainstream clinical use (Ivanov, 2013). These hurdles are an important aspect to consider when developing a new diagnostic and could have a significant effect on the introduction of a new, non-invasive in-field diagnostic for cVL.

The use of VOC Analysers as a point of care diagnostic tool for cVL is a novel idea. Results presented here demonstrate that the VOC Analyser can distinguish between cVL infected and uninfected dogs with a 97-100% sensitivity and 95-100% specificity (Staniek *et al.,* 2019). These results were further confirmed through a more sensitive GC/MS analysis which demonstrated 96-100% sensitivity and 92-96% specificity. Although this study did not compare the VOC Analyser with the current Brazilian diagnostic protocol in the field, results presented here suggests that this method is more reliable than the current in-field DPP diagnostic test. This suggests the potential use of a VOC Analyser as a non-invasive, accurate, point of care cVL diagnostic technique which could be adopted by the Brazilian Ministry of Health. The non-invasive nature of the VOC Analyser will result in both a higher rate of dog owner compliance and an increase in dog welfare, thereby allowing for a larger number of dogs to be sampled in the field (Lefort *et al.,* 2019).

Despite recent developments the novel nature of VOC Analyser technology means that further investigative research, particularly on sensor development and design, still needs to be undertaken in order to improve the accuracy of the diagnostic (Teixeira *et al.,* 2017; Wilson *et al*., 2018). Recent research has demonstrated the importance of sensor design in significantly increasing the accuracy of VOC Analyser lung cancer screening (Li *et al.,* 2017). In order to improve the accuracy of the VOC Analyser in the diagnosis of cVL, the development of a sensor array which contains a specific sensor array that is sensitive for *Leishmania* specific volatiles would be highly beneficial (Brekelmans *et al.,* 2016). GC/MS analysis using the HP5-MS non-polar column of the hair samples collected in 2017 and 2018 provided information on the structure and chemical identity of 4 volatiles which had an increase in concentration

in *Le. infantum* infected dogs. A preliminary library search suggested the identify of these volatiles as; 2-(phenylmethylene)-octanal (-)-cis-myrtanylamine, n-tridecan-1-ol and 2,3-dimethyl-undecane. Therefore, developing a sensor array that is highly specific for these volatiles could improve the specificity of the VOC Analyser leading to the development of a cVL specific diagnostic test. The methodology used for hair collection could also be an important factor to consider in the future development of a VOC Analyser diagnostic tool. The collection of hair samples here which was performed in the dorsal region of all dogs could prove a disadvantage, as recent research has demonstrated that there are distinct differences between the microbial communities which originate from different regions of the body (Torres *et al.,* 2017). Therefore, in order to increase the accuracy of the VOC Analyser as a cVL diagnostic tool, further research identifying the optimum location for hair collection should be considered.

However, despite the limitations, the high sensitivity, specificity and non-invasive nature of the VOC Analyser, along with the potential to develop a more specific *Leishmania* sensor array strongly supports the use of a VOC Analyser as a new, rapid, point of care diagnostic tool for cVL in Brazil.

7.2 Vector Control

In addition to canine control strategies, vector control methods are also recommended by the Brazilian Ministry of Health for the prevention and control of VL. Sandflies play a vital role in the maintenance of VL with transmission occurs through a cycle between the sandfly species *Lu. longipalpis,* which transmits *Le. infantum* through dog reservoir hosts. Due to the ineffectiveness of current dog vaccines

(Regina-Silva *et al.,* 2016), expensive treatment and ineffective culling strategies (Sousa-Paula *et al.,* 2019), more cost-effective and efficient approaches to sandfly control should be considered in order to reduce the transmission of *Leishmania.* The use of insecticides, which involves the indoor residual spraying (IRS) of households and aggregations sites that are within 200m of reported cases of human VL as well as the use of insecticide-impregnated dog-collars, are currently recommended in Brazil. However, the long-term sustainability and success of these methods is threatened by the development of sandfly insecticide resistance (Dhiman and Yadav, 2016) with little evidence suggesting that IRS is reducing disease burden (Quinnell and Courtenay, 2009). Therefore, new complementary and novel approaches to vector control must be explored.

Using an appropriate parasite/vector combination, the results presented here demonstrate that female *Lu. longipalpis* sandflies are significantly more attracted to the whole odour profile of *Leishmania infantum* infected dogs. A total of 685 (65.7%) of all female sandflies which responded (n=1043) showed an increased attraction to infected dog odour. This increase in attraction confirms previous research which identified an increase in female attraction to individual VOCs of *Leishmania* infected dogs (Magalhães-Junior *et al.,* 2019) as well as previous results which demonstrated an increase in attraction of female sandflies to the whole odour profile of infected golden hamsters (Nevatte *et al.,* 2017). These results further confirm the differentiation observed between infected and uninfected dogs following VOC Analyser and GC/MS Analysis. This increased attraction to the whole odour profile of infected dogs could also further our understanding of diseases epidemiology due to the required dog/sandfly interaction necessary for *Leishmania* transmission in Brazil.

The results also demonstrated that male *Lu. longipalpis* are equally attracted to both infected and uninfected dog odour, with only 10% (n=123) of all the males analysed (n=1200) remaining "undecided" and infection status having no influence on attraction. This is an important observation which furthers our understanding on *Leishmania* transmission in Brazil. The formation of male lekking sites equally around infected and uninfected dogs, not only facilitates the attraction of female sandflies to infected dogs to gain a parasite blood meal but will also result in female attraction to uninfected dogs and continued *Leishmania* transmission. This heterogeneous distribution of female sandflies between hosts as a result of leks is a known phenomenon (Lane *et al.,* 1990). Despite this observation however, the relative attraction of female sandflies to infected dog odour compared to uninfected dog odour plus a lekking site remains to be explored. Understanding this relative attraction could prove highly significant as uninfected dogs with lekking sites may prove more attractive than infected dog odour. This would potentially influence disease transmission if fewer female sandflies gained a parasite blood meal. Despite the further work that needs to be undertaken, the results presented here not only suggest that infected dog odour profile could have a major effect on *Leishmania* transmission, but also provides evidence to facilitate the development of novel vector control strategies.

It has previously been suggested that the development of odour-baited traps combined with insecticides would improve capture and kill vector methodologies (Matowo *et al.,* 2013). Research has demonstrated that natural human odour-baited traps have shown potential as affordable and effective vector control methodologies for luring and killing mosquitoes. Additional research has also demonstrated an

increase in attraction to the whole odour profiles of cows, humans and pigs for both teste flies and *Anopheles* mosquitoes (Njiru *et al.,* 2006; Jawara *et al.,* 2009; Rayaisse *et al*., 2010). These results are in concordance with the results presented here, demonstrating an increase in sandfly attraction to the whole odour profile of infected dogs. This provides evidence to suggest the use of the whole dog odour profile of infected dogs in the development of new vector control methods such as those seen using human odour baited traps for mosquitoes. However, further studies which implement and evaluate the use of dog odour baited traps for sandfly control in diverse eco-systems throughout Brazil are needed in order to develop this methodology for the incorporation into integrated vector management strategies for sandfly control (World Health Organisation, 2012). In addition, the development of a cost effective and reproducible lure based on the odour profile of infected dogs should be explored. The structural and chemical information of potential *Leishmania* specific volatiles provided by the GC/MS analysis could contribute to this further research. Despite the additional research required, the observed increase in attraction to infected dogs demonstrates a potential novel approach for the development of a novel tool to use in vector control. The use of infected dog odour as a lure could be a widespread methodology used throughout Brazil.

7.3 Conclusion

In conclusion, the research presented here demonstrates that *Le. infantum* infected dogs have a different odour profile to those dogs which were uninfected. This

differentiation has been demonstrated not only through GC/MS analysis, which confirms and improves upon previous research due to a larger sample size and improved instrument sensitivity (Magalhães-Junior *et al.,* 2014b), but also through the use of two new approaches; sandfly behavioural bioassays and a VOC Analyser. This observed difference in odour profile could not only lead to the development of an innovative diagnostic technique (Staniek *et al.,* 2019), but also lead to the development of novel sandfly vector control methodologies utilising odour baited traps previously been shown for other vector borne diseases (Abong'o *et al.,* 2018; Matowo *et al.,* 2013).

The results obtained during this study, demonstrate the potential use of a VOC Analyser to accurately and rapidly identify dogs infected with *Le. infantum*. The use of dog hair is not only a non-invasive sampling method, thereby benefiting animal welfare and improving owner compliance, but also provides further knowledge of the interactions that are occurring between host animal and parasite. However, in order to improve the sensitivity of the device further improvements to the sensor array, portability of the device and sampling methodology could be suggested. The structural and chemical information of potential *Leishmania* specific volatiles provided by the GC/MS analysis could contribute to further sensor array development. Despite these suggested improvements, the VOC Analyser demonstrates potential not only as a future in-field diagnostic device for cVL, modifying the current Brazilian Ministry of Health protocol, but also as an effective diagnostic for VL in humans and other parasitic diseases.

With respect to sandfly behaviour and vector control, the results presented here confirm previous research which demonstrated an increased attraction to golden

hamsters infected with *Le. infantum* (Nevatte *et al.,* 2017). However, the use of an appropriate parasite-vector combination here furthers our understanding of the natural transmission of *Leishmania*. This observed attraction also suggests the use of whole dog odour profile as a means of vector control throughout Brazil, using odour baited traps. Combining such traps with current insecticide-based methodologies in Brazil, could lead to a significant reduction in the number of female sandflies (Coulibaly *et al.,* 2018). Furthermore, through GC/MS analysis, the structural and chemical information of specific VOCs that are emitted in infected dog hair have the potential to be employed as chemical attractants for odour baited traps which could contribute to vector control.

7.4 Future work

- The development of a new in-field diagnostic test for the accurate diagnosis of *Leishmania* infected dogs in Brazil is essential for the success of the current control methods. Although the results here demonstrate the ability of a VOC Analyser to discriminate between infected and uninfected dogs, future improvement to the device need to be made. The current device used was a bench top version of the technology. In order to make the device suitable for the in-filed diagnosis of dogs a portable, hand-held device should be developed. Additionally, to improve diagnostic sensitivity and specificity, a *Leishmania* specific sensor array should be developed using, in part, the GC/MS identification of *Leishmania* specific volatiles identified here.
- It is also important to follow up the current investigation with further in-field studies including the comparison of the sensitivity and specificity of the VOC

Analyser diagnostic test when combined with DPP cVL diagnostic against the current protocol of the DPP cVL combined with an ELISA.

- Furthermore, the ability of the VOC Analyser to distinguish between different *Leishmania* species is also essential for the future development of this device. Previous work has identified dogs infected with *Le. amazonensis* and *Le. braziliensis* in Brazil. Although this study only demonstrated the diagnosis of *Le. infantum* infection, results presented in the supplementary material (**Table 9.5**) demonstrate the ability of the VOC Analyser to distinguish between different *Leishmania* species in vitro. This suggests the ability of a VOC Analyser to distinguish between dogs naturally infected with other *Leishmania* species in Brazil.
- Further proposed research also involves the identification of the attraction of *Lu. longipalpis* female sandflies to the individual volatiles identified as being *Leishmania* specific through GC/MS analysis (2-(phenylmethylene)-octanal, (-)-cis-myrtanylamine, n-tridecan-1-ol and, 2,3-dimethyl-undecane). This determination of sandfly behaviour could further research into chemical attractants which can be combined with baited light traps.
- In order for the proposed vector control methods to have an immediate impact on sandfly populations, field-based studies should be undertaken in which either whole dog odour or individual volatiles identified as being *Leishmania* specific is incorporated into disease control activities that are already undertaken (such as insecticide residual spraying).
- Performing a longitudinal study or a controlled comparison between different Brazilian municipalities which currently employ different vector control

methodologies will need to be undertaken in order to test the ability of dog volatiles to attract and reduce the numbers of female sandflies as well as help in the refinement of odour-based vector control strategies.

- Identifying sandfly visual stimuli could also lead to the development of dog odour baited traps that are combined with a specific coloured material. By exploiting sandfly behavioural responses to visual stimuli, further research could develop new innovative methodologies which could revolutionise vector surveillance and disease control. There is also the potential through this further work to develop a practical way of delivering killing agents such as insecticides on to targets which have been designed to lure sandflies.

Chapter 8 - References

A

- Abbehusen, M.M.C., Almeida, V.A., Solcà, M.S., Pereira, L.S., Costa, D.J., Gil-Santana, L., Bozza, P.T., Fraga, D.B.M., Veras, P.S.T., dos-Santos, W.L.C., Andrade, B.B., Brodskyn, C.I. 2017. Clinical and immunopathological findings during long term follow-up in *Leishmania infantum* experimentally infected dogs. Scientific Reports 7, Article number: 15914 (2017).
- Abel, G. 2015. Current status and future prospects of point-of-care testing around the globe. Expert Review of Molecular Diagnostics. Volume 15, 2015 - Issue 7.
- Abell, J.G., Stalder, T., Ferrie, J.E., Shipley, M.J., Kirschbaum, C., Kivimäki, M., Kumari, M. 2016. Assessing cortisol from hair samples in a large observational cohort: The Whitehall II study. Psychoneuroendocrinology. 2016 Nov; 73: 148–156.
- Abong'o, B., Yu, X., Donnelly, M.J., Geier, M., Gibson, G., Gimnig, J., ter Kuile, F., Lobo, N.F., Ochomo, E., Munga, S., Ombok, M., Samuels, A., Torr, S.J., Hawkes, F.M. 2018. Host Decoy Trap (HDT) with cattle odour is highly effective for collection of exophagic malaria vectors. Parasites & Vectors201811:533
- Akhoundi, M., Downing, T., Votýpka, J., Kuhls, K., Lukeš, J., Cannet, A., Ravel, C., Marty, P., Delaunay, P., Kasbari, M., Granouillac, B., Gradoni, L., Sereno, D. 2017. *Leishmania* infections: Molecular targets and diagnosis. Molecular Aspects of Medicine. Volume 57, October 2017. 1-29

- Akhoundi, M., Kuhls, K., Cannet, A., Votýpka, J., Marty, P., Delaunay, P., Sereno, D. 2016. A Historical Overview of the Classification, Evolution, and Dispersion of *Leishmania* Parasites and Sandflies. PLoS Negl Trop Dis. 2016 Mar 3;10(3):e0004349.
- Akpunarlieva, S., Weidt, S., Lamasudin, D., Naula, C., Henderson, D., Barrett, M., Burgess, K., Burchmore, R. 2016. Integration of proteomics and metabolomics to elucidate metabolic adaptation in Leishmania. Journal of Proteomics 155 (2017) 85–98.
- Albuquerque, A., Campino, L., Cardoso, L., Cortes, S. 2017. Evaluation of four molecular methods to detect *Leishmania* infection in dogs. Parasites & Vectorsvolume 10, Article number: 57 (2017).
- Alexander, B., Maroli, M. 2003. Control of *Phlebotomine* sandflies. Med Vet Entomol. 2003 Mar;17(1):1-18.
- Alexander, B., Barros, V.C., Souza, S.F., Barros, S.S., Teodoro, L.P., Soares, Z.R., Gontijo, N.F., Reithinger, R. 2009. Susceptibility to chemical insecticides of two Brazilian populations of the visceral leishmaniasis vector *Lutzomyia longipalpis* (Diptera: Psychodidae). Tropical Medicine and International Health. volume 14 no 10 pp 1272–1277 october 2009.
- Almeida, A.B.P.F., Sousa, V.R.F., Gasparetto, N.D., da Silva, G.F.R., Figueiredo, F.B., Dutra, V., Nakazato, L., Madeira, M.F. 2013. Canine visceral leishmaniasis: diagnostic approaches based on polymerase chain reaction employing different biological samples. Diagnostic Microbiology and Infectious Disease 76 (2013) 321–324.

- Almeida, B.F., Silva, K.L., Chiku, V.M., Leal, A.A., Venturin, G., Narciso, L. 2017. The effects of increased heme oxygenase-1 on the lymphoproliferative response in dogs with visceral leishmaniasis. Immunobiology 222, 693–703.
- Almeida, P.S., Sciamarelli, A., Batista, P.M., Ferreira, A.D., Nascimento, J., Raizer, J., Andrade Filho, J.D., Gurgel-Gonçalves, R. 2013. Predicting the geographic distribution of *Lutzomyia longipalpis* (Diptera: Psychodidae) and visceral leishmaniasis in the state of Mato Grosso do Sul, Brazil. Mem Inst Oswaldo Cruz. 2013 Dec; 108(8):992-6.
- Almeida, S., Carla, L.G., Elaynne, C.S., Viana, K., Viana, K. 2017b. Dual-path platform (DPP) and enzyme-linked immunosorbent assay (ELISA): Change the sequence of the tests does not change the number of positive dogs for canine visceral leishmaniasis. African journal of microbiology research 11(3):106-109.
- Alvar, J., Canavate, C., Molina, R., Moreno, J., Nieto, J. 2004. Canine leishmaniasis. Adv Parasitol 57: 1-88.
- Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M. 2012. Leishmaniasis Worldwide and Global Estimates of Its Incidence. PLoS One. 2012; 7(5): e35671.
- Amor, R.E., Nakhleh, M.K., Barash, O., Haick, H. 2019. Breath analysis of cancer in the present and the future. European Respiratory Review 2019 28: 190002.
- Amóra, S.S.A., Bevilaqua, C.M.L., Feijó, F.M.C., Alves, N.D., Maciel, M.d.V. 2009. Control of *Phlebotomine* (Diptera: Psychodidae) leishmaniasis vectors. Neotrop Entomol. 2009, 38: 303-310.
- Anderson, R.M., May, R.M. 1991. Infectious diseases of humans: dynamics and control. Oxford: New York: Oxford University Press; 1991.

- Andrade, A.J., Andrade, M.R., DiasII, E.S., PintoII, M.C., Eiras, A.E. 2008. Are light traps baited with kairomones effective in the capture of *Lutzomyia longipalpis* and *Lutzomyia* intermedia? An evaluation of synthetic human odor as an attractant for *Phlebotomine* sand flies (Diptera: Psychodidae: Phlebotominae). Mem. Inst. Oswaldo Cruz vol.103 no.4 Rio de Janeiro June 2008.
- Andrade, H.M., Reis, A.B., dos Santos, S.L., Volpini, A.C., Marques, J.M., Romanha, A.J. 2006. Use of PCR-RFLP to identify *Leishmania* species in naturally infected dogs. Vet. Parasitol., 140 (2006), pp. 231-238
- Antonia, A.L., Wang, L., Ko, D.C. 2018. A real-time PCR assay for quantification of parasite burden in murine models of leishmaniasis. PeerJ. 2018 Nov 9;6:e5905.
- Araki, A.S., Vigoder, F.M., Bauzer, L.G., Ferreira, G.E., Souza, N.A., Araújo, I.B., Hamilton, J.G., Brazil, R.P., Peixoto, A.A. 2009. Molecular and behavioral differentiation among Brazilian populations of *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae). PLoS Negl Trop Dis. 2009; 3(1):e365.
- Araújo-Santos, T., Andrade, B. B., Gil-Santana, L., Luz, N. F., Dos-Santos, P. L., de-Oliveira, F. A. 2017 Anti-parasite therapy drives changes in human visceral leishmaniasis-associated inflammatory balance. Sci. Rep. 7:4334. doi: 10.1038/s41598-017-04595-8.
- Aronson, N., Herwaldt, B.L., Libman, M., Pearson, R., Lopez-Velez, R., Weina, P., Carvalho, E.M., Ephros, M., Jeronimo, S., Magill, A. 2016. Diagnosis and Treatment of Leishmaniasis: Clinical Practice Guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical

Medicine and Hygiene (ASTMH), Clinical Infectious Diseases, Volume 63, Issue 12, 15 December 2016, Pages e202–e264

- Arshak, K., Moore, E., Lyons, G.M., Harris, J., Clifford, S. 2004. A review of gas sensors employed in electronic nose applications. Sensor Review. Volume 24 Number 2 2004 pp. 181-198.
- Ashford, R.W. 2003. When Is a Reservoir Not a Reservoir? Emerg Infect Dis. 2003 Nov; 9(11): 1495–1496.
- Ashley, D.L., Bonin, M.A., Cardinali, F.L., McCraw, J.M., Wooten, J.V. 1996. Measurement of volatile organic compounds in human blood. Environ Health Perspect. 1996 Oct; 104(Suppl 5): 871–877.
- Aslan, H., Oliveira, F., Meneses, C., Castrovinci, P., Gomes, R., Teixeira, C., Derenge, C.A., Orandle, M., Gradoni, L., Oliva, G., Fischer, L., Valenzuela, J.G., Kamhawi, S. 2016. New Insights Into the Transmissibility of *Leishmania infantum* From Dogs to Sand Flies: Experimental Vector-Transmission Reveals Persistent Parasite Depots at Bite Sites. J Infect Dis. 2016 Jun 1; 213(11): 1752–1761.
- Atan, N.A.D., Koushkie, M., Ahmadi, N.A., Rezaei-Tavirani, M. 2018. Metabolomics-based studies in the field of Leishmania/leishmaniasis. Alexandria Journal of Medicine. Volume 54, Issue 4, December 2018, Pages 383-390
- Aulakh, J.S., Malik, A.K., Kaur, V., Schmitt-Kopplin, P. 2005. A Review on Solid Phase Micro Extraction—High Performance Liquid Chromatography (SPME-HPLC) Analysis of Pesticides. Critical Reviews in Analytical Chemistry. Volume 35, 2005 - Issue 1

- Azevedo, A.C.R., Monteiro, F.A., Cabello, P.H., Souza, N.A., Rosa-Freitas, M.G., Rangel, E.F. 2000. Studies on Populations of *Lutzomyia longipalpis* (Lutz & Neiva, 1912) (Diptera: Psychodidae: Phlebotominae) in Brazil Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. 95(3): 305-322.

B

- Bach, J.P., Gold, M., Mengel, D., Hattesohl, A., Lubbe, D., Schmid, S., Tackenberg, B., Rieke, J., Maddula, S., Baumbach, J.I., Nell, C., Boeselt, T., Michelis, J., Alferink, J., Heneka, M., Oertel, W., Jessen, F., Janciauskiene, S., Vogelmeier, C., Dodel, R., Koczulla, A.R. 2015. Measuring Compounds in Exhaled Air to Detect Alzheimer's Disease and Parkinson's Disease. PLoS One. 2015 Jul 13;10(7):e0132227.
- Bahrami, F., Harandi, A.M., Rafati, S. 2018. Biomarkers of Cutaneous Leishmaniasis. Front Cell Infect Microbiol. 2018; 8: 222.
- Baietto, M., Wilson, A.D., Bassi, D., Ferrini, F. 2010. Evaluation of three electronic noses for detecting incipient wood decay. Sensors. 2010;10:1062–1092.
- Baneth, G., Koutinas, A.F., Solano-Gallego, L., Bourdeau, P., Ferrer, L. 2008. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. Trends Parasitol. 2008 Jul;24(7):324-3
- Banjara, M.R., Das, M.L., Gurung, C.K., Singh, V.K., Joshi, A.B., Matlashewski, G., Kroeger, A., Olliaro, P.

- Bararpour, N., Sporkert, F., Augsburger, M., Thomas, A. 2018. Omics in Forensic Toxicology a Bridge Towards Forensic Medicine. P5 Medicine and Justice pp 475-485.
- Barata, R.A., Peixoto, J.C., Tanure, A., Gomes, M.E., Apolinário, E.C., Bodevan, E.C., de Araújo, H.S., Dias, E.S., Pinheiro Ada, C. 2013. Epidemiology of visceral leishmaniasis in a re-emerging focus of intense transmission in Minas Gerais State, Brazil. Biomed Res Int. 2013.
- Barbosa, D.S., Belo, V.S., Rangel, M.E.S., Werneck, G.L. 2014. Spatial analysis for identification of priority areas for surveillance and control in a visceral leishmaniasis endemic area in Brazil. Acta Tropica. Volume 131, March 2014, Pages 56-62
- Bari, A. 2006. Chronology of cutaneous leishmaniasis: An overview of the history of the disease. Journal of Pakistan Association of Dermatologists 2006; 16: 24-27.
- Barreaux, P., Barreaux, A.M.G., Sternberg, E.D., Suh, E., Waite, J.L., Whitehead, S.A., Thomas, M.B. 2017. Priorities for broadening the malaria vector control tool kit. Trends Parasitol. 2017 Oct; 33(10): 763–774.
- Bartlett, P.N., Elliot, J.M., Gardner, J.W. 1997. Application of, and developments in, machine olfaction. Ann. Chim. 87:33-44.
- Bates, P.A., Rogers, M.E. 2004. New insights into the developmental biology and transmission mechanisms of Leishmania. Curr Mol Med. 2004 Sep; 4(6):601-9.
- Baum, M., de Castro, E.A., Pinto, M.C., Goulart, T.M., Baura, W., Klisiowicz, D.R., Costa-Ribeiroa, M.C.V. 2015. Molecular detection of the blood meal source of

sand flies (Diptera: Psychodidae) in a transmission area of American cutaneous leishmaniasis, Paraná State, Brazil. Acta Tropica. Volume 143, March 2015, Pages 8-12.

- Beale, D.J., Pinu, F.R., Kouremenos, K.A., Poojary, M.M., Narayana, V.K., Boughton, B.A., Kanojia, K., Dayalan, S., Jones, O.A.H., Dias, D.A. 2018. Review of recent developments in GC–MS approaches to metabolomics-based research. Metabolomics. November 2018, 14:152.
- Beati, L., Cáceres, A.G., Lee, J.A., Munstermann, L.E. 2004. Systematic relationships among *Lutzomyia* sand flies (Diptera: Psychodidae) of Peru and Colombia based on the analysis of 12S and 28S ribosomal DNA sequences. International Journal for Parasitology Volume 34, Issue 2, February 2004, Pages 225–234.
- Becker, S., Franco, J.R., Simarro, P.P., Stich, A., Abel, P.M., Steverding, D. 2004. Real-time PCR for detection of Trypanosoma brucei in human blood samples. Diagn Microbiol Infect Dis. 2004 Nov;50(3):193-9.
- Bejarano, E.E., Uribe, S., Rojas, W., Velez, I.D. 2001. Presence of *Lutzomyia* evansi, a vector of American visceral leishmaniasis, in an urban area of the Colombian Caribbean coast. Trans R Soc Trop Med Hyg 95: 27–28.
- Bell, M.J., Sedda, L., Gonzalez, M.A., de Souza, C.F., Dilger, E., Brazil, R.P., Courtenay, O., Hamilton, J.G.C. 2018. Attraction of *Lutzomyia longipalpis* to synthetic sex-aggregation pheromone: Effect of release rate and proximity of adjacent pheromone sources. PLoS Negl Trop Dis 12(12):.
- Belo, VS., Gregório, EA., Teixeira-Neto, RG., da Rocha Lima, A.C.V.M., Pereira, A.A.S., Marcelino, A.P., Paz, G.F., da Silva, E.S. 2017. Reliability of techniques

used in the diagnosis of canine visceral leishmaniasis by the national control program in Brazil: A survey in an area of recent transmission. Prev Vet Med. 2017 Oct 1;146:10-15.

- Berdoy, M., Webster, J.P., Macdonald, D.W. 2000. Fatal attraction in rats infected with Toxoplasma gondii. Proc. R. Soc. Lond. B (2000) 267, 1591^1594.
- Berna, A.Z., McCarthy, J.S., Wang, R.X., Saliba, K.J., Bravo, F.G., Cassells, J., Padovan, B., Trowell, S.C. 2015. Analysis of Breath Specimens for Biomarkers of *Plasmodium* falciparum Infection. J Infect Dis. 2015 Oct 1; 212(7): 1120–1128.
- Bernabei, M., Pennazza, G., Santonico, M., Corsi, C., Roscioni, C., Paolesse, R., Di Natale, C., D'Amico, A. 2008. A preliminary study on the possibility to diagnose urinary tract cancers by an electronic nose. Sensors and Actuators B: Chemical Volume 131, Issue 1, 14 April 2008, Pages 1-4.
- Bevilacqua, P.D., Paixao, H.H., Modena, C.M., Castro, M.C.P.S. 2001. Urbanization of visceral leishmaniose in Belo Horizonte, Brazil. Arq Bras Med Vet Zoot. 2001; 53:1-8.
- Bezerra, J.M.T., de Araújo, V.E.M., Barbosa, D.S., Martins-Melo, F.R., Werneck, G.L., Carneiro, M. 2018. Burden of leishmaniasis in Brazil and federated units, 1990-2016: Findings from Global Burden of Disease Study 2016. PLoS Negl Trop Dis. 2018 Sep 6;12(9):e0006697.
- Bi, K., Chen, Y., Zhao, S., Kuang, Y., Wu, C.H.J. 2018. Current Visceral Leishmaniasis Research: A Research Review to Inspire Future Study. BioMed Research International. Volume 2018, Article ID 9872095.

- Bindu, M.R.L.H., Parameswari, S.A., Gopinath, C. 2013. A review on GC/MS and method development and validation. International Journal of Pharmaceutical Quality Assurance 4(3):42-51.
- Biron, D.G., Ponton, F., Marché, L., Galeotti, N., Renault, L., Demey-Thomas, E. 2006. Suicide of crickets harbouring hairworms: a proteomics investigation. Insect Mol. Biol. 15, 731–742.
- Boelaert, M., Burza, S., Romero, G. 2018. Control and Public Health Aspects. In: Bruschi F., Gradoni L. (eds) The Leishmaniases: Old Neglected Tropical Diseases. Springer, Cham.
- Bongiorno, G., Habluetzel, A., Khoury, C., Maroli, M. 2003. Host preferences of phlebotominae sand flies at a hypoendemic focus of canine leishmaniasis in central Italy. Acta Trop 88: 109-116.
- Bos, L.D.J., Sterk, P.J., Schultz, M.J. 2013. Volatile Metabolites of Pathogens: A Systematic Review. PLoS Pathog. 2013 May; 9(5): e1003311.
- Bray, D.P., Hamilton, J.G.C. 2007. Host odor synergizes attraction of virgin female *Lutzomyia longipalpis* (Diptera: Psychodidae). J. Med. Entomol. 44: 779–787.
- Bray, D.P., Alves, G.B., Dorval, M.E., Brazil, R.P., Hamilton, J.G.C. 2010. Synthetic sex pheromone attracts the leishmaniasis vector *Lutzomyia longipalpis* to experimental chicken sheds treated with insecticide. Parasites & Vectorsvolume 3, Article number: 16 (2010).
- Bray, D.P., Carter, V., Alves, G.B., Brazil, R.P., Bandi, K.K. & Hamilton, J.G.C 2014. Synthetic sex pheromone in a long-lasting lure attracts the visceral

leishmaniasis vector, *Lutzomyia* longipalpis, for up to 12weeks in Brazil. PLoS Neglected Tropical Diseases,8, e2723.

- Bray, R.S. 1982. The zoonotic potential of reservoirs of leishmaniasis in the Old World. Ecol Dis 1: 257–267.
- Brazil, R.P. 2013. The dispersion of *Lutzomyia longipalpis* in urban areas. Rev. Soc. Bras. Med. Trop. vol.46 no.3 Uberaba May/June 2013.
- Breitling, R. 2010. What is Systems Biology? Front Physiol. 2010; 1: 9.
- Brereton, R. 2009. Chemometrics for pattern recognition. John Wiley & Sons, Ltd
- Brekelmans, M.P., Fens, N., Brinkman, P., Bos, L.D., Sterk, P.J., Tak, P.P., Gerlag, D.M. 2016. Smelling the Diagnosis: The Electronic Nose as Diagnostic Tool in Inflammatory Arthritis. A Case-Reference Study. PLoS ONE 11(3).
- Brinkac, L., Clarke, T.H., Singh, H., Greco, C., Gomez, A., Torralba, M.G., Frank, B., Nelson, K.E. 2018. Spatial and Environmental Variation of the Human Hair Microbiota. Scientific Reportsvolume 8, Article number: 9017 (2018).
- Broza, Y.Y., Zuri, L., Haick, H. 2014. Combined Volatolomics for Monitoring of Human Body Chemistry. Scientific Reports 4, Article number: 4611.
- Bruins, M., Rahim, Z., Bos, A., van de Sande, W.W., Endtz, H.P., van Belkum, A. 2013. Diagnosis of active tuberculosis by e-nose analysis of exhaled air. Tuberculosis (Edinb). 2013 Mar;93(2):232-8.
- Bryan, J. 2016. The current state of clinical application of serum biomarkers for canine lymphoma. Front. Vet. Sci. 3:87.
- Buck, L.B. 2005. Unraveling the sense of smell (Nobel lecture). Angew Chem Int Ed Engl. 2005 Sep 26;44(38):6128-40.

- Buckingham-Jeffery, E., Hill, E.M., Datta, S., Dilger, E., Courtenay, O. 2019. Spatio-temporal modelling of *Leishmania infantum* infection among domestic dogs: a simulation study and sensitivity analysis applied to rural Brazil. Parasites & Vectorsvolume 12, Article number: 215 (2019).
- Buonaguro, F.M., Caposio, P., Tornesello, M.L., Re, V.D., Franco, R. 2019. Cancer Diagnostic and Predictive Biomarkers 2018. BioMed Research International. Volume 2019, Article ID 3879015, 3 pages.
- Bursle, E., Robson, J. 2016. Non-culture methods for detecting infection. Aust Prescr. 2016 Oct; 39(5): 171–175.
- Burton, A. 2001. Sharing needles may produce artificial *Leishmania* cycle. Lancet, Infect. Dis., 1 (2001), p. 4.
- Burza, S., Croft, S.L., Boelaert, M. 2018. Leishmaniasis. Lancet 2018; 392: 951–70.

C

- Caliendo, A.M., Gilbert, D.N., Ginocchio, C.C., Hanson, K.E., May, L., Quinn, T.C., Tenover, F.C., Alland, D., Blaschke, A.J., Bonomo, R.A., Carroll, K.C., Ferraro, M.J., Hirschhorn, L.R., Joseph, W.P., Karchmer, T., MacIntyre, A.T., Reller, L.B., Jackson, A.F. 2013. Infectious Diseases Society of America (IDSA). Better tests, better care: improved diagnostics for infectious diseases. Clin Infect Dis. 2013 Dec;57.
- Camargo, L.B.I., Langoni, H. 2006. Impact of leishmaniasis on public health. J. Venom. Anim. Toxins incl. Trop. Dis vol.12 no.4 Botucatu 2006.

- Campos, J.H.F., Costa, F.A.L. 2014. PARTICIPATION OF TICKS IN THE INFECTIOUS CYCLE OF CANINE VISCERAL LEISHMANIASIS, IN TERESINA, PIAUÍ, BRAZIL. Rev Inst Med Trop Sao Paulo. 2014 Jul-Aug; 56(4): 297–300.
- Cao, W.Q., Duan, Y.X. 2007. Current status of methods and techniques for breath analysis. Crit Rev Anal Chem 2007;37:3–13
- Cao, Y., Li, C., Li, L. 2019. Clinical Application of Volatile Organic Compound–Based Exhaled Breath Tests for Cancer Diagnosis. JAMA Oncol.
- Cardoso, M.S., Bento, G.A., de Almeida, L.V., de Castro, J.C., Reis-Cunha, J.L., Barbosa, V de E., de Souza, C.F., Brazil, R.P., Valdivia, H.O., Bartholomeu, D.C. Detection of multiple circulating *Leishmania* species in *Lutzomyia longipalpis* in the city of Governador Valadares, southeastern Brazil. PLoS ONE 14(2): e0211831.
- Carson, C., Quinnell, R.J., Holden, J., Garcez, L.M., Deborggraeve, S. 2010. Comparison of *Leishmania* OligoC-TesT PCR with conventional and real-time PCR for Diagnosis of canine *Leishmania* infection. J Clin Microbiol 48: 33.
- Castro-Júnior, J.G., Freire, M.L., Campos, S.P.S., Scopel, K.K.G., Porrozzi, R., Da Silva, E.D., Colombo, F.A., da Silveira, R.C.V., Marques, M.J., Coimbra, E.S. 2014. Evidence of *Leishmania* (Leishmania) *infantum* in Dogs from Juiz de Fora, Minas Gerais State, Brazil, Based on Immunochromatographic Dual-Path Platform (DPP®) and PCR assays. Rev. Inst. Med. Trop. Sao Paulo. 56(3):225-229, May-June, 2014.
- Cattet, M., Stenhouse, G.B., Janz, D.M., Kapronczai, L., Anne, Erlenbach. J., Jansen, HT., Nelson, O.L., Robbins, C.T., Boulanger, J. 2017. The quantification of reproductive hormones in the hair of captive adult brown bears and their

application as indicators of sex and reproductive state. Conserv Physiol. 2017 Jun 1;5(1):cox032.

- Cauchi, M., Fowler, D., Walton, C., Bai, H. 2014. Application of gas chromatography mass spectrometry (GC/MS) in conjunction with multivariate classification for the diagnosis of gastrointestinal disease. November 2014Metabolomics 10(6):1113-1120.
- Cavalcantia, M.P., Felinto de Brito, M.E., de Souza, W.V., Gomes, Y.M., Abath, F.G.C. 2008. The development of a real-time PCR assay for the quantification of *Leishmania infantum* DNA in canine blood. The Veterinary Journal. Volume 182, Issue 2, November 2009, Pages 356-358.
- Cazzola, M., Segreti, A., Capuano, R., Bergamini, A., Martinelli, E., Calzetta, L. 2015. Analysis of exhaled breath fingerprints and volatile organic compounds in COPD. COPD Research and Practice20151:7.
- Ceccarelli, M., Galluzzi, L., Migliazzo, A., Magnani, M. 2014. Detection and Characterization of *Leishmania* (Leishmania) and *Leishmania* (Viannia) by SYBR Green-Based Real-Time PCR and High Resolution Melt Analysis Targeting Kinetoplast Minicircle DNA. PLoS ONE 9(2).
- Cellini, A., Blasioli, S., Biondi, E., Bertaccini, A., Braschi, I., Spinelli, F. 2017. Potential Applications and Limitations of Electronic Nose Devices for Plant Disease Diagnosis. Sensors (Basel). 2017 Nov; 17(11): 2596.
- Chaiphongpachara, T., Bunyuen, P., Chansukh, K.K. 2018. Development of a More Effective Mosquito Trapping Box for Vector Control. The Scientific World Journal. Volume 2018, Article ID 6241703, 8 pages.

- Chan, D.K., Leggett, C.L., Wang, K.K. 2016. Diagnosing gastrointestinal illnesses using fecal headspace volatile organic compounds. World J Gastroenterol. 2016 Jan 28; 22(4): 1639–1649.
- Chang, J.E., Lee, D.S., Ban, S.W., Oh, J., Jung, M.Y., Kim, S.H., Park, S.J., Persaud, K., Jheon, S. 2018. Analysis of volatile organic compounds in exhaled breath for lung cancer diagnosis using a sensor system. Sensors and Actuators B: Chemical Volume 255, Part 1, February 2018, Pages 800-807.
- Chargui, N., Haouas, N., Slama, D., Gorcii, M., Jaouadi, K., Essabbah-Aguir, N., Mezhoud, H., Babba, H. 2013. Transmission of visceral leishmaniasis in a previously non-endemic region of Tunisia: Detection of *Leishmania* DNA in Phlebotomus perniciosus. Journal of Vector Ecology, Volume 38, Issue 1 June 2013. Pages 1–5.
- Chappuis, F., Rijal, S., Soto, A., Menten, J., Boelaert, M. 2006. A meta-analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. Br Med J 333: 723–726.
- Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S., Peeling, R.W., Alvar, J., Boelaert, M. 2007. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nature Reviews Microbiology , S7-S16 (November 2007).
- Chen, S., Mahadevan, V., Zieve, L. Volatile fatty acids in the breath of patients with cirrhosis of the liver. J Lab Clin Med 1970; 75: 622-627.
- Chen, S., Wang, Y., Choi, S. 2013. Applications and Technology of Electronic Nose for Clinical Diagnosis. Open Journal of Applied Biosensor, 2013, 2, 39-50.

- Christ-Crain, M., Müller, B. 2007. Biomarkers in respiratory tract infections: diagnostic guides to antibiotic prescription, prognostic markers and mediators. European Respiratory Journal 2007 30: 556-573.
- Chulay, J.D, Bryceson, A.D. 1983. Quantitation of amastigotes of *Leishmania* donovani in smears of splenic aspirates from patients with visceral leishmaniasis. Am J Trop Med Hyg. 1983 May; 32(3):475-9.
- Ciaramella, P., Oliva, G., Luna, R.D., Gradoni, L., Ambrosio, R., Cortese, L., Scalone, A., Persechino, A. 1997. A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by *Leishmania* infantum. Vet Rec. 1997, 141 (21): 539-543.
- Citypopulation.de. 2017. GOVERNADOR VALADARES. Municipality in Southeast Region. Source: Instituto Brasileiro de Geografia e Estatistica (web). https://www.citypopulation.de/php/brazil-regiaosudeste-admin.php?adm2id=3127701. [Accessed 16/08/18].
- Claborn, D.M. 2010. The Biology and Control of Leishmaniasis Vectors. J Glob Infect Dis. 2010 May-Aug; 2(2): 127–134.
- Clarke, W. 2017. Mass spectrometry in the clinical laboratory: determining the need and avoiding pitfalls. Academic Press, Mass Spectrometry for the Clinical Laboratory 2017, Pages 1-15
- Coelho, E.A.F., Ramírez, L., Costa, M.A.F., Coelho, V.T.S., Martins, V.T., Chávez-Fumagalli, M.A., Oliveira, D.M., Tavares, C.A.P., Bonay, P. 2009. Specific Serodiagnosis of Canine Visceral Leishmaniasis Using *Leishmania* Species Ribosomal Protein Extracts.Clin Vaccine Immunol December 2009 vol. 16 no. 12 1774-1780

- Coelho, M.V., Falcão, A.R. 1966. Epidemiological aspects of calazar in Minas Gerais Brazilian Journal of Medicine.
- Colombo, F.A., Odorizzi, R.M., Laurenti, M.D., Galati, E.A., Canavez, F., Pereira-Chioccola, V.L. 2011. Detection of *Leishmania* (Leishmania) *infantum* RNA in fleas and ticks collected from naturally infected dogs. Parasitol Res. 2011 Aug;109(2):267-74.
- Conti, R.V., Moura Lane, V.F., Montebello, L., Pinto Junior, V.L. 2016. Visceral leishmaniasis epidemiologic evolution in timeframes, based on demographic changes and scientific achievements in Brazil. J Vector Borne Dis. 2016;53:99–104.
- Correa, R., Coronado, L.M., Garrido, A.C., Durant-Archibold, A.A., Spadafora, C. 2017. Volatile organic compounds associated with *Plasmodium* falciparum infection in vitro. Parasit Vectors. 2017; 10: 215.
- Costa, C.H., Tapety, C.M., Werneck, G.L. 2007. Control of visceral leishmaniasis in urban areas: randomized factorial intervention trial. Revista da Sociedade Brasileira de Medicina Tropical 2007;40(4):415-9
- Costa, L.N.G., Borba, A.S., Castagna, C.L., Carvalho Filho, E.B., Marson, F.A.L., Sa Junior, F.F., Angerami, R.N., Levy, C.E. 2015. Evaluation of PCR in the diagnosis of canine leishmaniasis in two different epidemiological regions: Campinas (SP) and Teresina (PI), Brazil. Epidemiol. Infect. (2015), 143, 1088–1095.
- Costa, P.L., Dantas-Torres, F., da Silva, F.J., Guimarães, V.C.F.V, Gaudêncio, K., Brandão-Filho, S.P. 2013. Ecology of *Lutzomyia longipalpis* in an area of visceral

leishmaniasis transmission in north-eastern Brazil. Acta Tropica. Volume 126, Issue 2, May 2013, Pages 99–102.

- Costa Lima Junior, M.S., Zorzenon, D.C.R., Dorval, M.E.C., Pontes, E.R.J.C. 2013. Sensitivity of PCR and real-time PCR for the diagnosis of human visceral leishmaniasis using peripheral blood. Asian Pac J Trop Dis. 2013 Feb; 3(1): 10–15.
- Cota, G.F., de Sousa, M.R., Demarqui, F.N., Rabello, A. 2012. The diagnostic accuracy of serologic and molecular methods for detecting visceral leishmaniasis in HIV infected patients: meta-analysis. PLoS Negl Trop Dis. 2012;6(5):e1665. doi: 10.1371/journal.pntd.0001665. Epub 2012 May 29.
- Coulibaly, C.A., Traore, B., Dicko, A., Samake, S., Sissoko, I., Anderson, J.M., Valenzuela, J., Traore, S.F., Faye, O., Kamhawi, S., Oliveira, F., Doumbia, S. 2018. Impact of insecticide-treated bednets and indoor residual spraying in controlling populations of Phlebotomus duboscqi, the vector of *Leishmania* major in Central Mali. Parasit Vectors. 2018; 11: 345.
- Coura-Vital, W., Ker, H.G., Roatt, B.M., Aguiar-Soares, R.D.O., Leal, G.G.A., Moreira, N.D., Oliveira, L.A.M., Machado, E.M.M., Morais, M.H.F., Corrêa-Oliveira, R., Carneiro, M., Reis, A.B. 2014. Evaluation of Change in Canine Diagnosis Protocol Adopted by the Visceral Leishmaniasis Control Program in Brazil and a New Proposal for Diagnosis. PLoS ONE 9(3): e91009.
- Costa, C.H., Stewart, J.M., Gomes, R.B., Garcez, L.M., Ramos, P.K. 2002. Asymptomatic human carriers of *Leishmania* chagasi. Am J Trop Med Hyg 66: 334–337.

- Costa, D.N.C.C., Codeço, C.T., Silva, M.A., Werneck, G.L. 2013. Culling Dogs in Scenarios of Imperfect Control: Realistic Impact on the Prevalence of Canine Visceral Leishmaniasis. PLoS Negl Trop Dis 7(8): e2355.
- Coura-Vital, W., Marques, M.J., Veloso, V.M., Roatt, B.M., Aguiar-Soares, R.D., Reis, L.E. 2011. Prevalence and factors associated with *Leishmania infantum* infection of dogs from an urban area of Brazil as identified by molecular methods. PLoS Negl Trop Dis. 2011;5(8):e1291.
- Courtenay, O., Quinnell, R.J., Garcez, L.M., Dye, C. 2002. Low infectiousness of a wildlife host of *Leishmania* infantum: The crab-eating fox is not important for transmission. Parasitology, vol. 125, no. 5, pp. 407–414, 2002
- Courtenay, O., Peters, N.C., Rogers, M.E., Bern, C. 2017. Combining epidemiology with basic biology of sand flies, parasites, and hosts to inform leishmaniasis transmission dynamics and control. PLoS Pathog. 2017 Oct; 13(10): e1006571.
- Courtenay, O., Dilger, E., Calvo-Bado, L.A., Kravar-Garde, L., Carter, V., Bell, M.J., Alves, G.B., Goncalves, R., Makhdoomi, M.M., González, M.A., Nunes, C.M., Bray, D.P., Brazil, R.P., Hamilton. J.G.C. 2019. Sand fly synthetic sex-aggregation pheromone co-located with insecticide reduces canine *Leishmania infantum* infection incidence: a stratified cluster randomised trial. bioRxiv 712174; doi: https://doi.org/10.1101/712174.
- Cruz, I., Chicharro, C., Nieto, J., Bailo, B., Canarate, C., Figueras, M.C. 2006. Comparison of new diagnostic tools for management of pediatric Mediterranean visceral leishmaniasis. J Clin Microbiol; 44: 2343-7.

- Cruz, I., Morales, M.A., Noguer, I., Rodríguez, A., Alvar, J. 2002. *Leishmania* in discarded syringes from intravenous drug users. Lancet. 2002 Mar 30;359(9312):1124-5.
- Cuervo, M.R., Asedegbega, N.E., Díaz, E., Vega, A., Ordóñez, S., Castillejos-López, E., Rodriguez-Ramos, I. 2008. Effect of Carbon Nanofiber Functionalization on the Adsorption Properties of Volatile Organic Compounds. Journal of chromatography. A. 1188. 264-73.

D

- D'Ávila, D.A., Galvão, L.M.C., Sousa, G.R., Britto, C., Moreira, O.C., Chiari, E. 2018. Monitoring the parasite load in chronic Chagas disease patients: comparison between blood culture and quantitative real time PCR. PLoS One. 2018 Nov 29;13(11):e0208133. doi: 10.1371/journal.pone.0208133. PMID: 30496249; PMCID: PMC6264508.
- Da Silva, K.R., 1 Ramos de Mendonça, V.R., Silva, K.M., do Nascimento, L.F.M., Mendes-Sousa, A.F., de Pinho, F.A., Barral-Netto, M., Barr*al.,* A.M.P., Pires e Cruz, M.S. 2017. Scoring clinical signs can help diagnose canine visceral leishmaniasis in a highly endemic area in Brazil. Mem Inst Oswaldo Cruz. 2017 Jan; 112(1): 53–63.
- Da Silva Solcà, M., Bastos, L.A., Guedes, C.E.S., Bordoni, M., et al. 2014. Evaluating the Accuracy of Molecular Diagnostic Testing for Canine Visceral Leishmaniasis Using Latent Class Analysis. PLoS ONE 9(7).

- Da Silveira, N.L., Sobrinho, L.S.V., Martins, C.O., Machado, R.Z., Marcondes, M., de Lima, V.M.F. 2011. Use of crude, FML and rK39 antigens in ELISA to detect anti-*Leishmania* spp. antibodies in Felis catus. Vet Parasitol. 2011;177:374–7.
- Dantas-Torres, F. 2005. Increasing case-fatality rate of visceral leishmaniasis in Brazil. REVISA. 2005;1:260–263.
- Dantas-Torres, F. 2007. The role of dogs as reservoirs of *Leishmania* parasites, with emphasis on *Leishmania* (Leishmania) *infantum* and *Leishmania* (Viannia) braziliensis. Veterinary Parasitology 149 (2007) 139–146.
- Dantas-Torres, F. 2009. Canine leishmaniosis in South America. Parasit Vectors. 2009; 2(Suppl 1): S1.
- Dantas-Torres, F., Brandao-Filho, S.P. 2006. Visceral leishmaniasis in Brazil: revisiting paradigms of epidemiology and control. Rev.Inst. Med. Trop. Sao Paulo 48, 151–156.
- Dantas-Torres, F., Brito, M.E.F., Branda˜o-Filho, S.P., 2006. Seroepidemiological survey on canine leishmaniasis among dogs from an urban area of Brazil. Vet. Parasitol. 140, 54–60.
- Dantas-Torres, F., Miró, G., Bowman, D.D., Gradoni, L., Otranto, D. 2019. Culling Dogs for Zoonotic Visceral Leishmaniasis Control: The Wind of Change. Trends Parasitol. 2019 Feb;35(2):97-101.
- Dantas-Torres, F., Solano-Gallego, L., Baneth, G., Ribeiro, V.M., de Paiva-Cavalcanti, M., Otranto, D. 2012. Canine leishmaniosis in the Old and New Worlds: unveiled similarities and differences. Trends in Parasitology, December 2012, Vol. 28, No. 12.

- Dantas-Torres, F., da Silva Sales, K.G., Gomes da Silva, L., Otranto, D., Figueredo, L.A. 2017. Leishmania-FAST15: A rapid, sensitive and low-cost real-time PCR assay for the detection of *Leishmania infantum* and *Leishmania* braziliensis kinetoplast DNA in canine blood samples. Mol Cell Probes 31: 65–69.
- DATASUS. 2019 American Cutaneous Leishmaniasis—cases confirmed in Minas Gerais notified to SINAN Ministério da Saúde: Secretaria de Vigilância em Saúde; [accessed 11 Apr. 19] [Available from: http://tabnet.datasus.gov.br/cgi/deftohtm.exe?sinannet/cnv/ltamg.def.
- Daughaday, W.H. 1968. The adenohypophysis. In Textbook of Endocrinology, 4th ed.; Williams, R.H., Ed.; W.B. Saunders: Philadelphia, PA, USA; pp. 27-84.
- Daviss, B. 2005. Growing pains for metabolomics. Scientist. 2005;19:25–28.
- Dawkins, R. 1999. The Extended Phenotype. Oxford: Oxford University Press.
- De Boer, J.G., Robinson, A., Powers, S.J., Burgers, S.L.G.E., Caulfield, J.C., Birkett, M.A., Smallegange, R.C., van Genderen, P.J.J., Bousema, T., Sauerwein, R.W., Pickett, J.A., Takken, W., Logan, J.G 2017. Odours of *Plasmodium* falciparum-infected participants influence mosquito-host interactions. Scientific Reports 7, Article number: 9283 (2017).
- De Carvalho, F.L.N., Riboldi, E.O., Bello, G.L., Ramos, R.R., Barcellos, R.B., Gehlen, M., Halon, M.L., Romão, P.R.T., Dallegrave, E., Rossetti, M.L.R. 2018. Canine visceral leishmaniasis diagnosis: a comparative performance of serological and molecular tests in symptomatic and asymptomatic dogs. Epidemiology and Infection. Volume 146, Issue 5 pp. 571-576

- De Freitas, E., Melo, M.N., da Costa-Val, A.P., Michalick, M.S. 2006. Transmission of *Leishmania infantum* via blood transfusion in dogs: potential for infection and importance of clinical factors. Vet Parasitol. 2006 Apr 15; 137(1-2):159-67.
- De Lima Cunha, J.C., de Oliveira Lima, J.W., Pompey, M.M. 2006. Domestic transmission of cutaneous leishmaniasis and the association between human and canine leishmaniasis, during an epidemic in Serra de Baturité, State of Ceará, Brazil. Rev. bras. epidemiol. vol.9 no.4 São Paulo Dec. 2006.
- De Lima, V.M., Fattori, K.R., Michelin, A.F., da Silveira, N.L., Vasconcelos, R.O. 2010. Comparison between ELISA using total antigen and immunochromatography with antigen rK39 in the diagnosis of canine visceral leishmaniasis. Vet Parasitol. 2010 Oct 29;173(3-4):330-3.
- De Moraes, C.M., Stanczyk, N.M., Betz, H.S., Pulido, H., Sim, D.G., Read, A.F., Meschera, M.C. 2014. Malaria-induced changes in host odors enhance mosquito attraction. Proc Natl Acad Sci U S A. 2014 Jul 29; 111(30): 11079–11084.
- De Moraes, C.M., Wanjiku, C., Stanczyk, N.M., Pulido, H., Sims, J.W., Betz, H.S., Read, A.F., Torto, B., Mescher, M.C. 2018. Volatile biomarkers of symptomatic and asymptomatic malaria infection in humans. Proceedings of the National Academy of Sciences May 2018, 115 (22) 5780-5785.
- De Morais, R.C., da Costa Oliveira, C.N., de Albuquerque, S.C., Mendonça Trajano Silva, L.A., Pessoa-E-Silva, R., Alves da Cruz, H.L., de Brito, M.E., de Paiva Cavalcanti M. 2016. Real-time PCR for *Leishmania* species identification:

Evaluation and comparison with classical techniques. Exp Parasitol. 2016 Jun;165:43-50.

- De Oliveira, L.S., Rodrigues, F.M., de Oliveira, F.S., Mesquita, P.R.R, Leal, D.C., Alcântara, A.C., Souza, B.M., Franke, C.R., Pereira, P.A.P., de Andrade, J.B. 2008. Headspace solid phase microextraction/gas chromatography–mass spectrometry combined to chemometric analysis for volatile organic compounds determination in canine hair: A new tool to detect dog contamination by visceral leishmaniasis. Journal of Chromatography B. Volume 875, Issue 2, 15 November 2008, Pages 392–398.
- Deane, L.M., Deane, M.P. 1955. Observac¸o˜es preliminares da importaˆncia comparativa do homem, do ca˜o e da raposa (Lycalopex vetulus) como reservato´rios de *Leishmania* donovani, em a´rea endeˆmica do calazar, no Ceara´. Hospital (Rio J) 48, 79–98.
- Desjeux, P. 1996. Leishmaniasis. Public health aspects and control. Clinical Dermatology 1996;14:417-23.
- Desjeux, P. 2004. Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis 27: 305–318.
- Dhiman, R.C., Yadav, R.S. 2016. Insecticide resistance in *Phlebotomine* sandflies in Southeast Asia with emphasis on the Indian subcontinent. Infectious Diseases of Povertyvolume 5, Article number: 106 (2016).
- Di Natale, C., Macagnano, A., Martinelli, E., Paolesse, R., D'Arcangelo, G., Roscioni, C., Finazzi-Agro`, A. D'Amico, A. 2003. Lung cancer identification by the analysis of breath by means of an array of non-selective gas sensors. Biosensors and Bioelectronics 18 (2003) 1209/1218.

- Di Natale, C., Mantini, A., Macagnano, A., Antuzzi, D., Paolesse, R., D'Amico, A. 1999. Electronic nose analysis of urine samples containing blood. Physiol. Meas. 20 (1999) 377–384.
- Doehl, J.S.P., Bright, Z., Dey, S., Davies, H., Magson, J., Brown, N., Romano, A., Dalton, J.E., Pinto, A.I., Pitchford, J.W., Kaye, P.M. 2017. Skin parasite landscape determines host infectiousness in visceral leishmaniasis. Nature Communications 8, Article number: 57 (2017).
- Domanovic, D., Giesecke, J. 2012 How to define an area where transmission of arthropod-born disease is occurring? Euro Surveill 17(20): pii = 20171. pmid:22642947.
- Dormond, L., Jaton, K., de Vallière, S., Genton, B., Greub, G. 2015. Malaria real-time PCR: correlation with clinical presentation. New Microbes New Infect. 2015 May; 5: 10–12.
- Dormont, L., Bessière, J., Cohuet, A. 2013. Human Skin Volatiles: A Review. J Chem Ecol. 2013, 39, 569–578. pmid:23615881.
- Dos Santos, S.O., Arias, J.R., de Paiva, H.M., Furlan, M.B., Ferreira, W.F. 2003. The presence of *Lutzomyia longipalpis* in a focus of American visceral leishmaniasis where the only proven vector is *Lutzomyia* cruzi. Corumba, Mato Grosso do Sul State. Rev Soc Bras Med Trop 36: 633–634.
- Dromey, R.G., Stefik, M.J., Rindfleisch, T.C., Duffield, A.M. 1976. "Extraction of Mass Spectra Free of Background and Neighboring Component Contributions from Gas Chromatography/Mass Spectrometry Data Anal. Chem. 1976, 48 (9), 1368-1375.

- Duffy, M.J. 2017. Clinical use of tumor biomarkers: An overview. Klin. Biochem. Metab., 25 (46), 2017, No. 4, p. 157–161.
- Dujardin, J. C., Campino, L., Cañavate, C., Dedet, J. P., Gradoni, L., Soteriadou, K., Mazeris, A., Ozbel, Y., Boelaert, M. 2008. Spread of vector-borne diseases and neglect of Leishmaniasis, Europe. Emerging Infectious Diseases 14, 1013–1018.
- Duncan, A. Malaria host manipulation increases probability of mosquitoes feeding on humans. Peer Community In Evolutionary Biology, 100057 (2018).
- Duprey, Z.H., Steurer, F.J., Rooney, J.A., Kirchhoff, L.V., Jackson, J.E., Rowton, E.D., Schantz, P.M. 2006. Canine Visceral Leishmaniasis, United States and Canada, 2000–2003. Emerg Infect Dis. 2006 Mar; 12(3): 440–446.
- Duthie, M.S., Lison, A., Courtenay, O. 2018. Advances toward Diagnostic Tools for Managing Zoonotic Visceral Leishmaniasis. Trends in Parasitology. Volume 34, Issue 10, October 2018, Pages 881-890.
- Dutta, R., Hines, E.L., Gardner, J.W., Boilot, P. 2002. Bacteria classification using Cyranose 320 electronic nose. BioMedical Engineering OnLine20021:4.
- Dye, C. 1996. The logic of visceral leishmaniasis control. Am J Trop Med Hyg. 1996 Aug; 55(2):125-30.

E

- El-Salam, N.M.A., Ayaz, S., Ullah, R. 2014. PCR and Microscopic Identification of Isolated *Leishmania* tropica from Clinical Samples of Cutaneous Leishmaniasis in Human Population of Kohat Region in Khyber Pakhtunkhwa. BioMed Research International, vol. 2014, Article ID 861831, 5 pages, 2014.

- Elmore, J.S., Papantoniou, E., Mottram, D.S. 2001. A comparison of headspace entrainment on Tenax with solid phase microextraction for the analysis of the aroma volatiles of cooked beef. Adv Exp Med Biol. 2001;488:125-32.
- Emami, S.N., Lindberg, B.G., Hua, S., Hill, S.R., Mozuraitis, R., Lehmann, P., Birgersson, G., Borg-Karlson, A.K., Ignell, R., Faye, I. 2017. A key malaria metabolite modulates vector blood seeking, feeding, and susceptibility to infection. Science. 2017 Mar 10;355(6329):1076-1080.
- Engewald, W., Dettmer-Wilde, K., Rotzsche, H. 2014. Columns and Stationary Phases. Practical Gas Chromatography pp 59-116.
- Ertabaklar, H., Çalışkan, O.S., Boduç, E., Ertuğ, S. 2015. Comparison of direct microscopy, culture and polymerase chain reaction methods for the diagnosis of cutaneous leishmaniasis. Mikrobiyol Bul. 2015 Jan;49(1):77-84.
- Esch, K.J., Petersen, C.A. 2013. Transmission and Epidemiology of Zoonotic Protozoal Diseases of Companion Animals. Clinical microbiology reviews 26(1):58-85.
- Esfahani, S., Wicaksono, A., Mozdiak, E., Arasaradnam, R.P., Covington, J.A. 2018. Non-Invasive Diagnosis of Diabetes by Volatile Organic Compounds in Urine Using FAIMS and Fox4000 Electronic Nose. Biosensors (Basel).
- Espir, T.T., Guerreiro, T.S., Naiff, M.F., Figueira, L.P., Soares, F.V., Silva, S.S., Franco, A.M.R. 2016. Evaluation of different diagnostic methods of American Cutaneous Leishmaniasis in the Brazilian Amazon. Experimental Parasitology. Volume 167, August 2016, Pages 1-6.
- Estakhroyeh, H.R., Rashedi, E., Mehran, M. 2017. Design and Construction of Electronic Nose for Multi-purpose Applications by Sensor Array Arrangement

Using IBGSA. Journal of Intelligent & Robotic Systems. October 2018, Volume 92, Issue 2, pp 205–221

- Eunice, G.A.B., Ovallos, F.G., Lawer, P., Leger, N., Depaquit, J. 2017. An illustrated guide for characters and terminology used in descriptions of Phlebotominae (Diptera, Psychodidae). July 2017Parasite 24(7).
- Evans, T.G., Vasconcelos, I.A., Lima, J.W., Teixeira, J.M., McAullife, I.T., Lopes, U.G., Pearson, R.D., Vasconcelos, A.W. Canine visceral leishmaniasis in northeast Brazil: assessment of serodiagnostic methods. Am J Trop Med Hyg. 1990 Feb; 42(2):118-23

F

- Faraj, C., Yukich, J., Adlaoui, E.B., Wahabi, R., Mnzava, A.P., Kaddaf, M., Idrissi, A.L.E., Ameur, B., Kleinschmidt, I. 2016. Effectiveness and Cost of Insecticide-Treated Bed Nets and Indoor Residual Spraying for the Control of Cutaneous Leishmaniasis: A Cluster-Randomized Control Trial in Morocco. Am J Trop Med Hyg. 2016 Mar 2; 94(3): 679–685.
- Feliciangeli, M.D., Rodriguez, N., De Guglielmo, Z., Rodriguez, A. 1999. The re-emergence of American visceral leishmaniasis in an old focus in Venezuela. II. Vectors and parasites. Parasite 6: 113–120.
- Fenaille, F., Visani, P., Fumeaux, R., Milo, C., Guy, P.A. 2003. Comparison of mass spectrometry-based electronic nose and solid phase microextraction gas chromatography-mass spectrometry technique to assess infant formula oxidation. J Agric Food Chem. 2003 Apr 23;51(9):2790-6.

- Fend, R., Bessant, C., Williams, A.J., Woodman, A.C. 2004. Monitoring haemodialysis using electronic nose and chemometrics. Biosensors and Bioelectronics. Volume 19, Issue 12, 15 July 2004, Pages 1581-1590.
- Fend, R., Geddes, R., Lesellier, S., Vordermeier, H.M., Corner, L.A.L., Gormley, E., Costello, E., Hewinson, R.G., Marlin, D.J., Woodman, A.C., Chambers, A.M. 2005. Use of an Electronic Nose To Diagnose Mycobacterium bovis Infection in Badgers and Cattle. J Clin Microbiol. 2005 Apr; 43(4): 1745–1751.
- Fernandes, F.F., Bahia, A.C., Pinto, L.C., Pimenta, P.F.P. 2008. Fine Structure and Distribution Pattern of Antennal Sensilla of *Lutzomyia longipalpis* (Diptera: Psychodidae) Sand Flies. Journal of Medical Entomology 45(6):982-90.
- Ferreira, S.A., Leite, R.S., Ituassu, L.T., Melo, M.N., Andrade, A.S.R. 2009. COMPARISON OF CLINICAL SAMPLES FOR VISCERAL LEISHMANIASIS DIAGNOSIS IN ASYMPTOMATIC DOGS BY PCRHYBRIDIZATION. 2009 International Nuclear Atlantic Conference - INAC 2009
- Ferroglio, E., Maroli, M., Gastaldo, S., Mignone, W., Rossi, L. 2005. Canine leishmaniasis, Italy. Emerging Infect. Dis. 11 (10): 1618–20.
- Figueiredo, F.B., Bello de Vasconcelos, T.C., Madeira, M.F., Menezes, R.C., Maia-Elkhoury, A.N.S., Marcelino, A.P., Werneck, G.L. 2018. Validation of the Dual-path Platform chromatographic immunoassay (DPP® cVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. Mem Inst Oswaldo Cruz. 2018; 113(11): e180260.
- Figueiredo, F.B., Bonna, I.C.F., Nascimento, L.D., Costa, T., Baptista, C., Pacheco, T.M.V. 2009. Serological evaluation for detection of anti-*Leishmania*

antibodies in dogs and cats in the district of Santa Rita de Cássia, municipality of Barra Mansa, State of Rio de Janeiro. Rev Soc Bras Med Trop. 2009;42:141–5.

- Figueiredo, F.B., MadeiraI, M.F., NascimentoII, L.D., Abrantes, T.R., Mouta-confortI, E., PassosII, S.R.L., Schubach, T.M.P. 2010. Canine visceral leishmaniasis: study of methods for the detection of IgG in serum and eluate samples. Rev. Inst. Med. trop. S. Paulo vol.52 no.4.
- Fisa, R., Gállego, M., Riera, C., Aisa, M.J., Valls, D., Serra, T., de Colmenares, M., Castillejo, S., Portús, M. 1997. Serologic diagnosis of canine leishmaniasis by dot-ELISA. J Vet Diagn Invest 9:50-55 (1997).
- Francino, O., Altet, L., Sánchez-Robert, E., Rodriguez, A., Solano-Gallego, L., Alberola, J., Ferrer, L., Sánchez, A., Roura, X. 2006. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. Veterinary Parasitology. Volume 137, Issues 3–4, 30 April 2006, Pages 214-221.
- Franco, A.O., Davies, C.R., Mylne, A., Dedet, J.P., Gllego, M., Ballart, C., Gramiccia, M., Gradoni, L., Molina, R., Glvez, R., Morillas-Mrquez, F., Barn-Lpez, S., Pires, C.A., Afonso, M.O., Ready, P.D., Cox, J. 2011. Predicting the distribution of canine leishmaniasis in western Europe based on environmental variables. Parasitology, 138 (14). pp. 1-14.

G

- Gao, Q., Su, X., Annabi, M.H., Schreiter, B.R., Prince, T., Ackerman, A., Morgas, S., Mata, V., Williams, H., Lee, W.Y. 2019. Application of Urinary Volatile

Organic Compounds (VOCs) for the Diagnosis of Prostate Cancer. Clin Genitourin Cancer. 2019 Jun;17(3):183-190.

- García, N., Moreno, I., Alvarez, J., de la Cruz, M.L., Navarro, A., Pérez-Sancho, M., García-Seco, T., Rodríguez-Bertos, A., Conty, M.L., Toraño, A., Prieto, A., Domínguez, L., Domínguez, M. 2014. Evidence of *Leishmania infantum* Infection in Rabbits (Oryctolagus cuniculus) in a Natural Area in Madrid, Spain. BioMed Research International, vol. 2014, Article ID 318254, 5 pages, 2014.
- Garcia-Cortes, A., Marti, J., Sayago, I., Santos, J.P., Gutierrez, J., Horrillo, M.C. 2009. Detection of stress through sweat analysis with an electronic nose. 2009 Spanish Conference on Electron Devices, Santiago de Compostela, 2009, pp. 338-341.
- Garlapati, R.B., Abbasi, I., Warburg, A., Poché, D., Poché, R. 2012. Identification of bloodmeals in wild caught blood fed Phlebotomus argentipes (Diptera: Psychodidae) using cytochrome b PCR and reverse line blotting in Bihar, India. J Med Entomol 49: 515–521.
- Garner, C., Smith, S., Bardhan, P., Ratcliffe, N., Probert, C. 2009. A pilot study of faecal volatile organic compounds in faeces from cholera patients in Bangladesh to determine their utility in disease diagnosis. Trans R Soc Trop Med Hyg 103:1171–1173.
- Geier, M., Bosh, O., Steib, B., Rose, A., Boeckh, J. 2002. Odour-guides host finding mosquitoes: identification of new attractants on human skin. In: Proceedings of the international conference on urban pests, 4. 2002. p. 37–46.

- Georgiadou, S.P., Makaritsis, K.P., Dalekos, G.N. 2015. Leishmaniasis revisited: Current aspects on epidemiology, diagnosis and treatment. J Transl Int Med. 2015 Apr-Jun; 3(2): 43–50.
- Gharbi, M., Mhadhbi, M., Rejeb, A., Jaouadi, K., Rouatbi, M., Darghouth, M.A. 2015. "Leishmaniosis (*Leishmania infantum* infection) in dogs," Revue Scientifique et Technique de l'OIE, vol. 34, no. 2, pp. 613–626, 2015.
- Ghatei, M.A., Hatam, G., Hossini, S.M.H, Sarkari, B. 2009. Performance of Latex Agglutination Test (KAtex) In Diagnosis of Visceral Leishmaniasis in Iran. Iran.J.Immunol. VOL.6 NO.4 December 2009.
- Ghorbani, M., Farhoudi, R. 2018. Leishmaniasis in humans: drug or vaccine therapy? Drug Des Devel Ther. 2018; 12: 25–40.
- Gikunoo, E., Abera, A., Woldesenbet, E. 2014. Novel carbon nanofibers grown on glass microballoons immunosensor: a tool for early diagnosis of malaria. Sensors. 2014;14:14686–14699.
- Goldberg, E.M., Blendis, L.M., Sandler, S. A gas chromatographic—mass spectrometric study of profiles of volatile metabolites in hepatic encephalopathy. J Chromatogr 1981; 226: 291-299
- Golding, I.M. 1965. An unusual cause of bromhidrosis. Pediatrics 36, 791-792.
- Golding, N., Wilson, A.L., Moyes, C.L., Cano, J., Pigott, D.M., Velayudhan, R., Brooker, S.J., Smith, D..L, Hay, S.I., Lindsay, S.W. 2015. Integrating vector control across diseases. BMC Med. 2015 Oct 1;13:249.
- Gomes, A.H., Ferreira, I.M., Lima, M.L., Cunha, E.A., Garcia, A.S., Araújo, M.F., Pereira-Chioccola, V.L. 2007. PCR identification of *Leishmania* in diagnosis and control of canine Leishmaniasis. Vet Parasitol. 2007 Mar 31;144(3-4):234-41.

- González, C., Cabrera, O.L., Munstermann, L.E., Ferro, C. 2006. Distribution of vectors of *Leishmania infantum* (Kinetoplastida: Trypanosomatidae) in Colombia. Biomedical. 2006; 26: 64-72.
- Goullé, J.P., Chèze, M., Pépin, G. 2003. Determination of endogenous levels of GHB in human hair. Are there possibilities for the identification of GHB administration through hair analysis in cases of drug-facilitated sexual assault? J Anal Toxicol. 2003 Nov-Dec;27(8):574-80.
- Grabe, V., Sachse, S. 2018. Fundamental principles of the olfactory code. Biosystems Volume 164, February 2018, Pages 94-101
- Gratacos-Cubarsi, M., Castellari, M., Valero, A., Garcia-Regueiro, J. 2006. Hair analysis for veterinary drug monitoring in livestock production. J Chromatogr B 834:14–25.
- Greer, C.E., Peterson, S.L., Kiviat, N.B., Manos, M.M., 1991. PCR amplification from paraffin-embedded tissues. Effects of fixative and fixation time. Am. J. Clin. Pathol. 95, 117–124.
- Grice, E.A., Segre, J.A. 2011. The skin microbiome. Nat Rev Microbiol. 2011 Apr; 9(4): 244–253.
- Grimaldi, G., Teva, A., Ferreira, A.L., dos Santos, C.B., de-Souza Pinto, I., de-Azevedo, C.T., Falqueto, A. 2012. Evaluation of a novel chromatographic immunoassay based on Dual-Path Platform technology (DPP® CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. Transactions of The Royal Society of Tropical Medicine and Hygiene, Volume 106, Issue 1, 1.
- Grimaldi, G., Teva, A., Santos, C.B., Ferreira, A.L., Falqueto, A. 2012b. The effect of removing potentially infectious dogs on the numbers of canine *Leishmania*

infantum infections in an endemic area with high transmission rates. Am J Trop Med Hyg. 2012;86(6):966-71.

- Guest, C., Pinder, M., Doggett, M., Squires, C., Affara, M., Kandeh, B., Dewhirst, S., Morant, S.V., D'Alessandro, U., Logan, J.G., Lindsay, S.W. 2019. Trained dogs identify people with malaria parasites by their odour. The Lancet Infectious Diseases. CORRESPONDENCE| VOLUME 19, ISSUE 6, P578-580, JUNE 01, 2019
- Guimarães, A., Raimundo, J.M., Santos, H.D., Machado, R.Z., Baldani C.D. 2016. Serosurvey for canine visceral leishmaniasis in rural and urban areas of the Brazilian Legal Amazon. Braz J Infect Dis. 2016.
- Guimarães, V.C.F.V., Pruzinova, K., Sadlova, J., Volfova, V., Myskova, J., Filho, S.P.B., Volf, P. 2016. *Lutzomyia* migonei is a permissive vector competent for *Leishmania* infantum. Parasit Vectors. 2016; 9: 159.
- Guimarães-E-Silva, A.S., Silva, S.O., Ribeiro da Silva, R.C., Pinheiro, V.C.S., Rebêlo, J.M.M., Melo, M.N. *Leishmania* infection and blood food sources of *Phlebotomine*s in an area of Brazil endemic for visceral and tegumentary leishmaniasis. PLoS One. 2017 Aug 24;12(8):e0179052.

H

- Hadjipetrou, A., Anyfantakis, D., Gkogkou, A., Palla, K., Lagoudaki, E., Milonaki, T., Kastanakis, S. 2014. Visceral leishmaniasis in a psoriatic arthritis patient treated with methotrexate. Leishmania Infezioni in Medicina, n. 3, 230-235, 2014.

- Hafer, N. 2016. Conflicts over host manipulation between different parasites and pathogens: Investigating the ecological and medical consequences. Bioessays. 2016 Oct; 38(10): 1027–1037.
- Haldar, A.K., Sen, P., Roy, S. 2011. Use of Antimony in the Treatment of Leishmaniasis: Current Status and Future Directions. Molecular Biology International
- Volume 2011, Article ID 571242, 23 pages.
- Hamad, I., Forestier, C.L., Peeters, M., Delaporte, E., Raoult, D., Bittar, F. 2015. Wild Gorillas as a Potential Reservoir of *Leishmania* major. J Infect Dis. 2015 Jan 15; 211(2): 267–273.
- Hamilton, J. G. C., Hurd, H. 2002. In The Behavioual Ecology of Parasites (ed Lewis, E.E., Campbell, J. F., Sukhdeo, M. V. K.) Ch. 13, 384 (CAB Internation*al.,* 2002).
- Hamilton, J., Maingon, R., Alexander, B., Ward, R. & Brazil, R. 2005. Analysis of the sex pheromone extract of individual male *Lutzomyia longipalpis* sandflies from six regions in Brazil. Medical and Veterinary Entomology,19, 480–488.
- Han, S., Wu, W.P., Chen, K., Osman, I., Kiyim, K., Zhao, J., Hou, Y.Y., Wang, Y., Wang, L.Y., Zheng, C.J. 2018. Epidemiological survey of sheep as potential hosts for *Leishmania* in China. BMC Veterinary Researchvolume 14, Article number: 378 (2018).
- Handman, E., Bullen, D.V.R. 2002. Interaction of *Leishmania* with the host macrophage. Trends in Parasitology. Volume 18, Issue 8, 1 August 2002, Pages 332-334.
- Harkey, M. 1993. Anatomy and physiology of hair. Forensic Sci Int 63:9–18.

- Hart, D.T. 1989. Leishmaniasis: The Current Status and New Strategies for Control. Series A: Life Sciences Vol. 163.
- Harsányi, G. 2000. Polymer films in sensor applications: a review of present uses and future possibilities. Sensor Review, Vol. 20 No.2, pp.98-105.
- Haselton, A.T., Acevedo, A., Kuruvilla, J., Werner, E., Kiernan, J., Dhar, P. 2015. Repellency of α-pinene against the house fly, Musca domestica. Phytochemistry. Volume 117, September 2015, Pages 469-475
- Hassani, K., Olivier, M. 2013. Immunomodulatory impact of leishmania-induced macrophage exosomes: a comparative proteomic and functional analysis. PLoS Negl Trop Dis. 2013; 7(5):e2185.
- Hawkes, F.M., Dabiré, R.K., Sawadogo, S.P., Torr, S.J., Gibson, G. 2017. Exploiting *Anopheles* responses to therm*al.,* odour and visual stimuli to improve surveillance and control of malaria. Scientific Reportsvolume 7, Article number: 17283 (2017).
- Heil, M. 2016. Host Manipulation by Parasites: Cases, Patterns, and Remaining Doubts. Front. Ecol.Evol.4:80.
- Henderson G (1993) Mechanisms of drug incorporation into hair. Forensic Sci Int 63:19–29.
- Herman-Saffar, O., Boger, Z., Libson, S., Lieberman, D., Gonen, R., Zeiri, Y. 2018. Early non-invasive detection of breast cancer using exhaled breath and urine analysis. Computers in Biology and Medicine. Volume 96, 1 May 2018, Pages 227-232
- Hide, M., Bucheton, B., Kamhawi, S., Bras-Goncalves, R., Sundar, S., Lemesre, J.L. & Banuls, A.L. (2007) Understanding Human Leishmaniasis: The need or an

Integrated Approach. Encyclopedia of Infectious Diseases: Modern Methodologies (ed. by M. Tibayrenc), pp. 87.

- Hiscox, A., Maire, N., Kiche, I. 2012. The SolarMar Project: innovative mosquito trapping technology for malaria control. *Malaria J*. 2012; 11:045.
- Hochberg, Y., Benjamini, Y. 1990. More powerful procedures for multiple significance testing.Stat Med. 1990 Jul; 9(7):811-8.
- Holmes, J.C., Bethel, W.M. 1972. Modification of intermediate host behavior by parasites. Behavioral Aspects of Parasite Transmission, eds E. U. Canning and C. A. Wright (London: Academic Press), 123–149.
- Homan, T., Hiscox, A., Mweresa, C.K., Masiga, D., Mukabana, W.R., Oria, P., Maire, N., Pasquale, A.D. 2016. The eff ect of mass mosquito trapping on malaria transmission and disease burden (SolarMal): a stepped-wedge cluster-randomised trial. Lancet 2016; 388: 1193–201.
- Hosack, G.R., Rossignol, P.A., van den Driessche, P. 2008. The control of vector-borne disease epidemics. J. Theor. Biol., vol. 255, no. 1, pp. 16–25, Nov. 2008.
- Hotez, P.J. 2008. Stigma: the stealth weapon of the NTD. PLoS Negl Trop Dis. 2008 Apr 30;2(4).
- Hughes, D.P., Brodeur, J., Thomas, F. 2012. Host Manipulation By Parasites. (Oxford University Press, 2012).
- Hughes, D.P., Libersat, F. 2019. Parasite manipulation of host behaviour. Curr. Biol., 29 (2019), pp. PR45-47
- Hurd, H. 2003. Review Manipulation of medically important insect vectors by their parasites. Annu Rev Entomol. 2003; 48():141-61.

I

- Iniesta, L., Fernandez-Barredo, S., Bulle, B., Gomez, M.T., Piarroux, R., Gallego, M., Alunda, J.M., Portus., M. 2002. Diagnostic Techniques To Detect Cryptic Leishmaniasis in Dogs. CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Sept. 2002, p. 1137–1141
- Islam, M.Z., Itoh, M., Shamsuzzaman, S.M., Mirza, R., Kimura, E. 2002. Diagnosis of Visceral Leishmaniasis by Enzyme-Linked Immunosorbent Assay Using Urine Samples. Clin Diagn Lab Immunol. 2002 Jul; 9(4): 789–794.
- Ivanov, A. 2013. Barriers to the Introduction of New Medical Diagnostic Tests. Laboratory Medicine, Volume 44, Issue 4, November 2013, Pages e132–e136.

J

- Jadoon, S., Karim, S., Akram, M.R., Khan, A.K. 2015. Recent Developments in Sweat Analysis and Its Applications. International Journal of Analytical Chemistry Volume 2015.
- Jawara, M., Smallegange, R.C., Jeffries, D., Nwakanma, D.C., Awolola, T.S., Knols, B.G.J., Takken, W., Conway, D.J. 2009. Optimizing Odor-Baited Trap Methods for Collecting Mosquitoes during the Malaria Season in The Gambia. PLoS ONE 4(12): e8167.
- Janeš, D., Klun, I., Vidan-Jeras, B., Jeras, M., Kreft, S. 2010. Infuence of MHC on odour perception of 43 chemicals and body odour. Central European Journal of Biology. June 2010, Volume 5, Issue 3, pp 324–330.
- Jaouadi, K., Bettaieb, J., Bennour, A., Salem, S., Ghawar, W.,Rjeibi, M.R., Khabouchi, N., Gonzalez, J.P., Diouani, M.F., Salah, A.B. 2017. Blood Meal

Analysis of *Phlebotomine* Sandflies (Diptera: Psychodidae: Phlebotominae) for *Leishmania* spp. Identification and Vertebrate Blood Origin, Central Tunisia, 2015–2016. The American Journal of Tropical Medicine and Hygiene. Volume 98, Issue 1.

- John, A.S., Price, C.P. 2014. Existing and Emerging Technologies for Point-of-Care Testing. Clin Biochem Rev. 2014 Aug; 35(3): 155–167.
- Joseph, A., Jain, A, Pachauri, M., Kumar, A. 2016. GCMS Based Detection of Lipid Biomarkers of Mycobacterium tuberculosis in the Serum Specimen. J. of Respiratory Res. 2016 June 2(2): 47-55.

K

- Kamau, E., Alemayehu, S., Feghali, K.C., Saunders, D., Ockenhouse, C.F. 2013. Multiplex RT-qPCR for Detection and Absolute Quantification of Malaria. PLoS ONE 8(8): e71539.
- Kazmierczak, S.C. 2011. Point-of-Care Testing Quality: Some Positives but Also Some Negatives. Clinical Chemistry 57:9. 1219–1220 (2011).
- Kelly, M., Su, C.Y., Schaber, C., Crowley, J.R., Hsu, F.F., Carlson, J.R., Odom, A.R. 2015. Malaria Parasites Produce Volatile Mosquito Attractants. mBio 6(2):e00235-15.
- Killick-Kendrick, R. 1999. The biology and control of *Phlebotomine* sand flies. Clin Dermatol. 1999;17:279–289.
- Kimblin, N., Peters, N., Debrabant, A., Secundino, N., Egen, J., Lawyer, P., Fay, M.P., Kamhawi, S., Sacks, D. 2008. Quantification of the infectious dose of

Leishmania major transmitted to the skin by single sand flies. Proc Natl Acad Sci U S A. 2008 Jul 22; 105(29):10125-30.

- Kip, A.E., Balasegaram, M., Beijnen, J.H., Schellens, J.H., De Vries, P.J., Dorlo, T.P. 2015. Systematic review of biomarkers to monitor therapeutic response in leishmaniasis. Antimicrob. Agents Chemother. 59, 1–14. doi: 10.1128/AAC.04298-14.
- Kong, H.H., Segre, J.A. 2012. Skin Microbiome: Looking Back to Move Forward. J Invest Dermatol. 2012; 132, 933–939. pmid:22189793.
- Krampa, F.D., Aniweh, Y., Awandare, G.A., Kanyong, P. 2017. Recent Progress in the Development of Diagnostic Tests for Malaria. Diagnostics (Basel). 2017 Sep 19;7(3):54.
- Küntzel, A., Oertel, P., Fischer, S., Bergmann, A., Trefz, P., Schubert, J. 2018. Comparative analysis of volatile organic compounds for the classification and identification of mycobacterial species. PLoS ONE 13(3): e0194348.

L

- La Manna, M.P.L., Orlando, V., Donni, P.L., Sireci, G., Carlo, P.D., Cascio, A., Dieli, F., Caccamo, N. 2018. Identification of plasma biomarkers for discrimination between tuberculosis infection/disease and pulmonary non tuberculosis disease. PLoS ONE 13(3).
- Lacroix, R., Mukabana, W.R., Gouagna, L.C., Koella, J.C. 2005. Malaria Infection Increases Attractiveness of Humans to Mosquitoes. PLoS Biol. 2005 Sep; 3(9): e298.

- Lafferty, K.D. 1999. The evolution of trophic transmission. Parasitol. Today 15, 111–115.
- Lafferty, K.D., Shaw, J.C. 2013. Comparing mechanisms of host manipulation across host and parasite taxa. J Exp Biol. 2013 Jan 1;216(Pt 1):56-66.
- Lafuse, W.P., Story, R., Mahylis, J., Gupta, G., Varikuti, S., Steinkamp, H., Oghumu, S., Satoskar, A.R. 2013. *Leishmania* donovani Infection Induces Anemia in Hamsters by Differentially Altering Erythropoiesis in Bone Marrow and Spleen. PLoS ONE 8(3): e59509.
- Lai, S.Y., Deffenderfer, O.F., Hanson, W., Phillips, M.P., Thaler, E.R. 2002. Identification of upper respiratory bacterial pathogens with the electronic nose. Laryngoscope. 2002 Jun;112(6):975-9.
- Lainson, R., Ward, R.D., Shaw, J.J. 1977. *Leishmania* in phlebotomid sandflies: VI. Importance of hindgut development in distinguishing between parasites of the *Leishmania* mexicana and L. braziliensis complexes. Proc R Soc Lond [Biol] 1977;199:309–320
- Landier, J., Parker, D.M., Thu, A.M., Carrara, V.I., Lwin, K.M., Bonnington, C.A., Pukrittayakamee, S., Delmas, G., Nosten, F.H. 2016. The role of early detection and treatment in malaria elimination. Malaria Journal 2016 15:363.
- Lane, R.P., Pile, M.M., Amerasinghe, F.P. 1990. Anthropophagy and aggregation behaviour of the sandfly Phlebotomus argentipes in Sri Lanka. Med Vet Entomol. 1990;4(1):79–88.
- Lappas, N.T., Lappas, C.M. 2016. Chapter 9 - Sample Preparation. Forensic Toxicology. Principles and Concepts 2016, Pages 143-160

- Laranjeira, D.F., Matta, V.L., Tomokane, T.Y., Marcondes, M., Corbet, C.E., Laurenti, M.D. 2014. Serological and infection statuses of dogs from a visceral leishmaniasis-endemic area. Rev Saude Publica. 2014 Aug; 48(4):563-71.
- Lauber, C.L., Zhou, N., Gordon, J.I., Knight, R., Fierer, N. 2010. Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. FEMS Microbiol Lett. 2010 Jun; 307(1): 80–86.
- Laurenti, M.D., de Santana Leandro, M.V. Jr., Tomokane, T.Y., De Lucca, H.R., Aschar, M., Souza, C.S., Silva, R.M., Marcondes, M., da Matta, V.L. 2014. Comparative evaluation of the DPP(®) CVL rapid test for canine serodiagnosis in area of visceral leishmaniasis. Vet Parasitol. 2014 Oct 15;205(3-4):444-50.
- Laurenti, M.D., Rossi, C.N., da Matta, V.L., Tomokane, T.Y., Corbett, C.E., Secundino, N.F., Pimenta, P.F., Marcondes, M. 2013. Asymptomatic dogs are highly competent to transmit *Leishmania* (Leishmania) *infantum* chagasi to the natural vector. Vet Parasitol. 2013 Sep 23;196(3-4):296-300.
- Lefèvre, T., Koella, J.C., Renaud, F., Hurd, H., Biron, D.G., Thomas, F. 2006. New Prospects for Research on Manipulation of Insect Vectors by Pathogens. PLoS Pathog 2(7): e72.
- Lefort, M.C., Cruickshank, R.H., Descovich, K., Adams, N.J., Barun, A., Emami-Khoyi, A., Ridden, J., Smith, V.R., Sprague, R., Waterhouse, B.R., Boyer, S. 2019. Blood, sweat and tears: a review of non-invasive DNA sampling. BIORXIV, doi: https://doi.org/10.1101/385120.
- Leite, B.M.M., Solcà, M.S., Santos, L.C.S., Coelho, L.B., Amorim, L.D.A.F., Donato, L.E., Passos, S.M.S., Almeida, A.O., Veras, P.S.T., Fraga, D.B.M. 2018. The mass use of deltamethrin collars to control and prevent canine visceral

leishmaniasis: A field effectiveness study in a highly endemic area. PLoS Negl Trop Dis 12(5): e0006496.

- Leontides, L.S., Saridomichelakis, M.N., Billinis, C., Kontos, V., Koutinas, A.F., Galatos, A.D., 2002. A cross-sectional study of *Leishmania* spp. Infection in clinically healthy dogs with polymerase chain reaction and serology in Greece. Vet Parasitol; 109(1-2): 19-27.
- Lerner, E.A., O'Luga, A., Reddy, V.B. 2007. Maxadilan, a PAC1 receptor agonist from sand flies. Peptides. 2007 Sep; 28(9): 1651–1654.
- Levine, R.M., Fong, F. 1957. Malignant degeneration in varicose ulcers of lower extremities. Can. Med. J. 76, 961-962.
- Lewis, D.J. 1971. Phlebotomid Sandflies. Bull World Health Organ. 1971; 44(4): 535–551.
- Lewis, J.M., Savage, R.S., Beeching, N.J., Beadsworth, M.B.J., Feasey, N., Covington, J.A. Identifying volatile metabolite signatures for the diagnosis of bacterial respiratory tract infection using electronic nose technology: A pilot study. PLoS ONE 12(12): e0188879.
- Li, W., Liu, H., Xie, D., He, Z., Pi, X. 2017. Lung Cancer Screening Based on Type-different Sensor Arrays. Scientific Reportsvolume 7, Article number: 1969 (2017).
- Liddell, K. 1976. Smell as a diagnostic marker. Postgrad. Med. J. 52, 136–138.
- Liddell, K., White, J.E. 1975. The smell of cancer. Brit. J. Dermatol. 92, 215.
- Lima, I.D., Lima, A.L.M., Mendes-Aguiar, C.O., Coutinho, J.F.V., Wilson, M.E., Pearson, R.D., Queiroz, J.W., Jeronimo, S.M.B. 2018. Changing demographics

of visceral leishmaniasis in northeast Brazil: Lessons for the future. PLoS Negl Trop Dis 12(3): e0006164.

- Lindh, J.M., Goswami, P., Blackburn, R.S., Arnold, S.E.J., Vale, G.A., Lehane, M.J., Torr, S.J. 2012. PLoS Negl Trop Dis. 2012 May; 6(5): e1661.
- Loesche, M., Gardner, S.E., Kalan, L., Horwinski, J., Zheng, Q., Hodkinson, B.P., Tyldsley, A.S., Franciscus, C.L., Hillis, S.L., Mehta, S. 2016. Temporal stability in chronic wound microbiota is associated with poor healing. J. Invest. Dermatol. 2016.
- Logan, J.G., Birkett, M.A., Clark, J.S., Powers, S., Se*al.,* N.J., Wadhams, L.J. 2008 Identification of human-derived volatile chemicals that interfere with attraction of Aedes aegypti mosquitoes. J Chem Ecol. 2008; 34, 308–322. pmid:18306972.
- Loha, E., Deressa, W., Gari, T., Balkew, M., Kenea, O., Solomon, T., Hailu, A., Robberstad, B., Assegid, M., Overgaard, H.J., Lindtjørn, B. 2019. Long-lasting insecticidal nets and indoor residual spraying may not be sufficient to eliminate malaria in a low malaria incidence area: results from a cluster randomized controlled trial in Ethiopia. Malar J. 2019 Apr 18;18(1):141.
- Lopes, E.G., Sevá, A.P., Ferreira, F., Nunes, C.M., Keid, L.B., Hiramoto, R.M., Ferreira, H.L., Oliveira, T.M.F.S., Bigotto, M.F.D., Galvis-Ovallos, F., Galati, E.A.B., Soares, R.M. 2017. Serological and molecular diagnostic tests for canine visceral leishmaniasis in Brazilian endemic area: one out of five seronegative dogs are infected. Epidemiol Infect. 2017 Sep;145(12):2436-2444.
- Louradour, I., Monteiro, C.C., Inbar, E., Ghosh, K., Merkhofer, R., Lawyer, P., Paun, A., Smelkinson, M., Secundino, N., Lewis, M., Erram, D., Zurek, L., Sacks,

D. 2017. The midgut microbiota plays an essential role in sand fly vector competence for Leishmania major. Cell Microbiol. 2017 Oct;19(10):10.1111/cmi.12755.

- Lubes, G., Goodarzi, M. 2018. GC–MS based metabolomics used for the identification of cancer volatile organic compounds as biomarkers. Journal of Pharmaceutical and Biomedical Analysis. Volume 147, 5 January 2018, Pages 313-322.
- Lukes, J., Mauricio, I.L., Schönian, G., Dujardin, J.C., Soteriadou, K., Dedet, J.P., Kuhls, K., Miles, M.A. 2007. Evolutionary and geographical history of the *Leishmania* donovani complex with a revision of current taxonomy. *Proc Natl Acad Sci U S A. 2007 May 29; 104(22):9375-80.*
- Luppi, M.M., Malta, M.C.C., Silva, T.M.A. 2008. Visceral leishmaniasis in captive wild canids in Brazil. Veterinary Parasitology, vol. 155, no. 1-2, pp. 146–151, 2008.
- Luz, J.G.G., Naves, D.B., de Carvalho, A.G., Meira, G.A., Dias, J.V.L., FonteS, C.J.F. 2018. Visceral leishmaniasis in a Brazilian endemic area: an overview of occurrence, HIV coinfection and lethality. Rev Inst Med Trop Sao Paulo. 2018; 60: e12.

M

- MacMorris-Adix, M. 2009. Leishmaniasis: A review of the disease and the debate over the origin and dispersal of the causaitive parasite *Leishmania* Macalester Reviews in Biogeography. Volume 1 Article 2.
- Macedo-Silva, V.P., Martins, D.R., De Queiroz, P.V., Pinheiro, M.P., Freire, C.C., Queiroz, J.W., Dupnik, K.M., Pearson, R.D., Wilson, M.E., Jeronimo, S.M.,

Ximenes, M.de.F. 2014. Feeding preferences of *Lutzomyia longipalpis* (Diptera: Psychodidae), the sand fly vector, for *Leishmania infantum* (Kinetoplastida: Trypanosomatidae). J Med Entomol. 2014 Jan;51(1):237-44.

- Maciel de Arruda, M., Figueiredo, F.B., Marcelino, A.P., Barbosa, J.R., Werneck, G.L., Noronha, E.F., Romero, G.A.S. 2016. Sensitivity and specificity of parallel or serial serological testing for detection of canine *Leishmania* infection. Mem. Inst. Oswaldo Cruz vol.111 no.3 Rio de Janeiro Mar. 2016 Epub Feb 19, 2016.
- Magalhães-Junior, J.T., Barrouin-Melo, S.M., Correa, A.G., da Rocha Silva, F.B., Machado, V.E., Govone, J.S., Pinto, M.C. 2014. A laboratory evaluation of alcohols as attractants for the sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae). Parasites and Vectors, 7, 60.
- Magalhães-Junior, J.T., Mesquita, P.R.R., dos Santos Oliveira, W.F., Oliveira, F.S., Franke, C.R., Rodrigues, F.M., de Andrade, J.B., Barrouin-Melo, S.M. 2014b. Identification of biomarkers in the hair of dogs: new diagnostic possibilities in the study and control of visceral leishmaniasis. Anal Bioanal Chem (2014) 406:6691–6700.
- Magalhães-Junior, J.T., Oliva-Filho, A.A., Novais, H.O., Mesquita, P.R.R., Rodrigues, F.M., Pinto, M.C., Barrouin-Melo, S.M. 2019. Attraction of the sandfly *Lutzomyia longipalpis* to possible biomarker compounds from dogs infected with *Leishmania* infantum. Medical and Veterinary Entomology. Volume33, Issue2. June 2019. Pages 322-325.
- Magan, N., Pavlou, A., Chrysanthakis, I. 2001. Milk-sense: a volatile sensing system recognises spoilage bacteria and yeasts in milk. Sens. Actuators B. 2001;72:28–34

- Magno da Silva, S., Ribeiro, V.M., Ribeiro, R.R., Tafuri, W.L., Melo, M.N., Michalick, M.S.M. 2009. First report of vertical transmission of *Leishmania* (Leishmania) *infantum* in a naturally infected bitch from Brazil. Veterinary Parasitology. Volume 166, Issues 1–2, 3 December 2009, Pages 159-162.
- Maia, C., Cardoso, L. 2015. Spread of *Leishmania infantum* in Europe with dog travelling. Vet Parasitol. 2015 Sep 30;213(1-2):2-11.
- Maia, C., Altet, L., Serrano, L., Cristóvão, J.M., Tabar, M.D., Francino, O., Cardoso, L., Campino, L., Roura, X. 2016. Molecular detection of *Leishmania* infantum, filariae and Wolbachia spp. in dogs from southern Portugal. Parasites & Vectorsvolume 9, Article number: 170 (2016).
- Maia, C., Campino, L. 2018. Biomarkers Associated With *Leishmania infantum* Exposure, Infection, and Disease in Dogs. Front Cell Infect Microbiol. 2018; 8: 302.
- Maingon, R.D., Ward, R.D., Hamilton, J.G., Bauzer, L.G., Peixoto, A.A. 2008. The *Lutzomyia longipalpis* species complex: does population sub-structure matter to *Leishmania* transmission? Trends Parasitol. 2008 Jan; 24(1):12-7.
- Malaquias, L.C., Carmo Romualdo, R., Anjos, J.B.Jr., Giunchetti, R.C., Corrêa-Oliveira, R., Reis, A.B. 2007. Serological screening confirms the re-emergence of canine leishmaniosis in urban and rural areas in Governador Valadares, Vale do Rio Doce, Minas Gerais, Brazil. Parasitol Res. 2007 Jan;100(2):233-9. Epub 2006 Aug 29.
- Mancianti, F., Gramiccia, M., Gradoni, L., Pieri, S. 1988. Studies on canine leishmaniasis control. 1. Evolution of infection of different clinical forms of

canine leishmaniasis following antimonial treatment. Trans R Soc Trop Med Hyg. 1988;82(4).

- Mandy, M., Cornelia, F., Malgorzata, L., Oliver, S., Achim, S., Dorothee, S. 2012. Volatile organic compounds (VOCs) in exhaled breath of patients with breast cancer in a clinical setting. Ginekol Pol 83: 730–736
- Mangabeira Filho, O. 1969. Classification and biology of the Phlebotomus of Ceará. Rev Bras Malariol Doencas Trop. 1969 Jan-Mar; 21(1):1-25.
- Mangold, K.A., Manson, R.U., Koay, E.S.C., Stephens, L., Regner, M., Thomson, Jr, R.B., Peterson, L.R., Kaul, K.L. 2005. Real-Time PCR for Detection and Identification of *Plasmodium* spp. J Clin Microbiol. 2005 May; 43(5): 2435–2440.
- Manna, L., Reale, S., Vitale, F., Gravino, A.E. 2009. Evidence for a relationship between *Leishmania* load and clinical manifestations. Res Vet Sci. 2009 Aug; 87(1):76-8.
- Manna, L., Corso, R., Galiero, G., Cerrone, A., Muzj, P., Gravino, A.E. 2015. Long-term follow-up of dogs with leishmaniosis treated with meglumine antimoniate plus allopurinol versus miltefosine plus allopurinol. Parasites & Vectors, vol. 8, no. 1, article no. 289, 2015.
- Marcondes, M., Day, M.J. 2019. Current status and management of canine leishmaniasis in Latin America. Res Vet Sci. 2019 Apr; 123:261-272.
- Marcussi, L.M., Skraba, C.M., Perles, T.F., Pedroso, R.B., Lonardoni, M.V.C., Silveira, T.G.V. 2013. EVALUATION OF SPECIFIC PRIMERS FOR SPECIES IDENTIFICATION OF *Leishmania* (V.) braziliensis. Rev Patol Trop Vol. 42 (1): 64-71.

- Marcussi, V.M., Marcussi, L.M., Barbosa-Tessmann, I.P., Lonardoni, M.V., Silveira, T.G. 2008. *Leishmania* (Viannia) braziliensis: New primers for identification using polymerase chain reaction. Exp Parasitol 120: 300-305, 2008.
- Marinho, C.R.F., Lima, M.R.D., Grisotto, M.G., Alvarez, J.M. 1999. Influence of Acute-Phase Parasite Load on Pathology, Parasitism, and Activation of the Immune System at the Late Chronic Phase of Chagas' Disease. Infect Immun. 1999 Jan; 67(1): 308–318.
- MaroliI, M., Feliciangeli M.D., Bichaud, L., Charrel, R.N., Gradoni, L. 2013. *Phlebotomine* sandflies and the spreading of leishmaniases and other diseases of public health Concern. Medical and Veterinary Entomology (2012), doi: 10.1111.
- Mary, C., Faraut, F., Lascombe, L., Dumon, H. 2004. Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. J Clin Microbiol 42: 5249–5255.
- Marzochi, M.C., Fagundes, A., Andrade, M.V., Souza, M.B., Madeira, M.de.F., Mouta-Confort, E., Schubach, A.O., Marzochi, K.B. 2009. Visceral leishmaniasis in Rio de Janeiro, Brazil: eco-epidemiological aspects and control. Rev Soc Bras Med Trop. 2009 Sep-Oct; 42(5):570-80.
- Masmoudi, A., Hariz, W., Marrekchi, S., Amouri, M., Turki, H. 2013. Old World cutaneous leishmaniasis: diagnosis and treatment. J Dermatol Case Rep. 2013 Jun 30; 7(2): 31–41.
- Matheson, R. 1950. Medical Entomology, 2nd Edition. Constable and Company Ltd. London.

- Matowo, N.S., Moore, J., Mapua, S., Madumla, E.P., Moshi, I.R., Kaindoa, E.W., Mwangungulu, S.P., Kavishe, D.R., Sumaye, R.D., Lwetoijera, D.W., Okumu, F.O. 2013. Using a new odour-baited device to explore options for luring and killing outdoor-biting malaria vectors: a report on design and field evaluation of the Mosquito Landing Box. Parasit Vectors. 2013 May 4;6:137.
- Mazzone, P.J., Hammel, J., Dweik, R., Na, J., Czich, C., Laskowski, D., Mekhail, T. 2007. Diagnosis of lung cancer by the analysis of exhaled breath with a colorimetric sensor array. Thorax. 2007 Jul; 62(7): 565–568.
- McIver, S.B. 1982. Sensilla of mosquitoes (Diptera: Culicidae). J Med Entomol 19:489–535.
- McKenna, M., Attipa, C., Tasker, S., Augusto, M. 2019. Leishmaniosis in a dog with no travel history outside of the UK Veterinary Record 184, 441.
- McPhatter, L., Gerry, A.C. 2017. Effect of CO2 concentration on mosquito collection rate using odor-baited suction traps. Journal of Vector Ecology. Volume 42, Issue1 June 2017, Pages 44-50.
- Meléndez-Lazo, A., Ordeix, L., Planellas, M., Pastor, J., Solano-Gallego, L. 2018. Clinicopathological findings in sick dogs naturally infected with *Leishmania* infantum: comparison of five different clinical classification systems. Res. Vet. Sci. 117, 18–27.
- Mendonca, I.L., Batista, J.F., Ribeiro, I.M.M., Rocha, F.S.B., Silva, S.O., Melo, M.N. 2017. *Leishmania infantum* in domestic cats from the municipality of Teresina, state of Piauí, Brazil. Parasitology Open, 3, E1. doi:10.1017/pao.2017.1

- Mendonça, I.L., Batista, J.F., Schallig, H., Cruz, M.D.S.P.E., Alonso, D.P., Ribolla, P.E.M., Costa, D.L., Costa, C.H.N. 2017. The performance of serological tests for *Leishmania infantum* infection screening in dogs depends on the prevalence of the disease. Rev Inst Med Trop Sao Paulo. 2017 Jun 1;59:e39.
- Menezes-Souza, D., Guerra-Sa, R., Carneiro, C., Vitoriano- Souza, J., Giunchetti, R., Teixeira-Carvalho, A., 2012. Higher expression of CCL2, CCL4, CCL5, CCL21, and CXCL8 Chemokines in the skin associated with parasite density in Canine Visceral Leishmaniasis. PLoS Negl. Trop. Dis.
- Messaoud, H.B.B., Guichard, M., Lawton, P., Delton, I., Azzouz-Maache, S. 2017 Changes in Lipid and Fatty Acid Composition During Intramacrophagic Transformation of *Leishmania* donovani Complex Promastigotes into Amastigotes. Lipids. 2017 May;52(5):433-441.
- Mettler, M., Grimm, F., Capelli, G., Camp, H., Deplazes, P. 2005. Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic *Leishmania* infections in dogs. J Clin Microbiol. 2005 Nov;43(11):5515-9.
- Miekisch, W., Schubert, J.K., Vagts, D.A., Geiger, K. 2001. Analysis of Volatile Disease Markers in Blood. Clinical Chemistry 47:6. 1053–1060.
- Meléndez-Lazo, A., Ordeix, L., Planellas, M., Pastor, J., Solano-Gallego, L. 2018. Clinicopathological findings in sick dogs naturally infected with *Leishmania* infantum: comparison of five different clinical classification systems. Res. Vet. Sci. 117, 18–27.

- Millán, J., Ferroglio, E., Solano-Gallego, L. 2014. Role of wildlife in the epidemiology of *Leishmania infantum* infection in Europe. Parasitol Res. 2014 Jun;113(6):2005-14.
- Millán, J., Travaini, A., Zanet, S., López-Bao, J.V., Trisciuoglio, A., Ferroglio, E., Rodríguez, A. 2016. Detection of *Leishmania* DNA in wild foxes and associated ticks in Patagonia, Argentina, 2000 km south of its known distribution area. Parasit Vectors. 2016; 9: 241.
- Miki, Y., Swensen, J., Shattuck-Eidens, D., Futre*al.,* P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W., al. et. 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 07 Oct 1994: Vol. 266, Issue 5182, pp. 66-71.
- Ministério da Saúde. 2011. Esclarecimento sobre substituição do protocolo diagnóstico da leihsmaniose visceral canina; Nota técnica conjunta n° 01/2011 - CGDT-CGLAB/DEVIT/SVS/MS.
- Ministério da Saúde. 2016. Letalidade de Leishmaniose Visceral. Brasil, Grandes Regiões e Unidades Federadas. 2000 a 2016. http://portalarquivos.saude.gov.br/images/pdf/2017/setembro/14/LV-Letalidade.pdf. Accessed: 08 August 2019.
- Minkler, P.E., Stoll, M.S.K., Ingalls, S.T., Hoppel, C.L. 2017. Appropriate Use of Mass Spectrometry in Clinical and Metabolic Research. Special Issues. Volume 15, Issue 1, pg 22–27, 34
- Miranda de Araújo, V.E., Pinheiro, L.C., Almeida, M.C.M., Carvalho de Menezes, F., Morais, M.H.F., Reis, I.A., Assunção, R.M., Carneiro, M. 2013. Relative Risk

of Visceral Leishmaniasis in Brazil: A Spatial Analysis in Urban Area. PLoS Negl Trop Dis. 7(11).

- Miro, G., Checa, R., Montoya, A., Hernández, L., Dado, D., Gálvez, R. 2012. Current situation of *Leishmania infantum* infection in shelter dogs in northern Spain. Parasites & Vectors, 5:60.
- Mistry, D.A.H., Haklani, J., French, P.W. 2012. Identification of Breast Cancer-Associated Lipids in Scalp Hair. Breast Cancer (Auckl). 2012; 6: 113–123.
- Mitruka, B.M. 1975. Presumptive diagnosis of infectious diseases. New York: John Wiley and Sons, 1975:349–74.
- Mochalski, P., King, J., Haas, M., Unterkofler, K., Amann, A., Mayer, G. 2014. Blood and breath profiles of volatile organic compounds in patients with end-stage renal disease. BMC Nephrology201415:43.
- Mohamed, E.I., Linder, R., Periello, G., Di Daniele, N., Poppl, S.J., De Lorenzo, A. 2002. Predicting Type 2 diabetes using an electronic nose-based artificial neural network analysis. Diabetes, Nutr. Metab. 2002, 15, 215.
- Mohammadiha, A., Mohebali, M., Haghighi, A., Mahdian, R., Abadi, A.R., Zarei, Z., Yeganeh, F., Kazemi, B., Taghipour, N., Akhoundi, B. 2013. Comparison of real-time PCR and conventional PCR with two DNA targets for detection of *Leishmania* (Leishmania) *infantum* infection in human and dog blood samples. Exp Parasitol. 2013 Jan; 133(1):89-94.
- Mol, J.P.S., Soave, S.A., Turchetti, A.P. 2015. Transmissibility of *Leishmania infantum* from maned wolves (Chrysocyon brachyurus) and bush dogs (Speothos venaticus) to *Lutzomyia* longipalpis. Veterinary Parasitology, vol. 212, no. 3-4, pp. 86–91, 2015.

- Mombaerts., P. 2004. Genes and ligands for odorant, vomeronasal and taste receptors. Nat Rev Neurosci. 2004 Apr;5(4):263-78.
- Monteiro, E.M., da Silva, J.C., da Costa, R.T., Costa, D.C., Barata, R.A., de Paula, E.V., Machado-Coelho, G.L., Rocha, M.F., Fortes-Dias, C.L., Dias, E.S. 2005. Visceral leishmaniasis: a study on *Phlebotomine* sand flies and canine infection in Montes Claros, State of Minas Gerais. Rev Soc Bras Med Trop. 2005 Mar-Apr; 38(2):147-52.
- Monteiro de Andrade, H., Reis, A.B., Lopes dos Santos, S., Volpini, A.C., Marques, M.J., Romanha, A.J. 2006. Use of PCR–RFLP to identify *Leishmania* species in naturally-infected dogs. Veterinary Parasitology. Volume 140, Issues 3–4, 10 September 2006, Pages 231–238.
- Moore, J. 2002. Parasites and the Behaviour of Animals. New York: Oxford University Press.
- Moore, J. 1993. Parasites and the behaviour of biting flies. J. Parasitol. 79, 1–16.
- Moore, R.E., Kirwan, J., Doherty, M.K., Whitfield, P.D. 2007. Biomarker Discovery in Animal Health and Disease: The Application of Post-Genomic Technologies. Biomark Insights. 2007; 2: 185–196.
- Moreira, M.A.B., Luvizotto, M.C.R., Garcia, J.F., Corbett, C.E.P., Laurenti, M.D. 2007. Comparison of parasitologic*al.,* immunological and molecular methods for the diagnosis of leishmaniasis in dogs with different clinical signs. Vet Parasitol 2007;145:245–52.
- Moreno, J., Alvar, J. 2002. Canine leishmaniasis: epidemiological risk and the experimental model. Trends Parasitol. 18, 399–405.

- Mottram, D.S., Elmore, J.S. 2003. SENSORY EVALUATION/Aroma. Encyclopedia of Food Sciences and Nutrition (Second Edition) 2003, Pages 5174-5180.
- Moura, A.S., Lopes, H.M., Mourão, M.V., Morais, M.H. 2013. Performance of a rapid diagnostic test for the detection of visceral leishmaniasis in a large urban setting. Rev Soc Bras Med Trop. 2013 Sep-Oct; 46(5):589-93.
- Mozos, E., Pérez, J., Day, M.J., Lucena, R., Ginel, P.J. 1999. Leishmaniosis and Generalized Demodicosis in Three Dogs: a Clinicopathological and Immunohistochemical Study. Journal of Comparative Pathology Volume 120, Issue 3, April 1999, Pages 257-268.

N

- Nagle Jr, H.T., Gutierrez-Osuna, R., Schiffman, S. 1998. The How and Why of Electronic Noses. IEEE Spectrum, September 1998, Volume 35, Number 9, pp. 22-34.
- Nakhleh, M.K., Am*al.,* H., Jeries, R., Broza, Y.Y., Aboud, M., Gharra, A., Ivgi, H., Khatib, S., Badarneh, S., Har-Shai, L., Haick, H., et al. 2017. Diagnosis and classification of 17 diseases from 1404 subjects via pattern analysis of exhaled molecules. ACS Nano, 11, 112−125 DOI: 10.1021/acsnano.6b04930
- Nardi-Agmon, I., Peled, N. 2017. Exhaled breath analysis for the early detection of lung cancer: recent developments and future prospects. Lung Cancer: Targets and Therapy. 17 May 2017 Volume 2017:8 Pages 31—38.
- Naucke, T.J., Amelung, S., Lorentz, S. 2016. First report of transmission of canine leishmaniosis through bite wounds from a naturally infected dog in Germany. Parasites & Vectors 2016 9:256.

- Ndao M. 2009. Diagnosis of parasitic diseases: old and new approaches. Interdiscip Perspect Infect Dis. 2009;2009:278246.
- Nevatte, T.M., Ward, R.D., Sedda, L., Hamilton, J.G.C. 2017. After infection with *Leishmania* infantum, Golden Hamsters (Mesocricetus auratus) become more attractive to female sand flies (*Lutzomyia* longipalpis). Scientific Reportsvolume 7, Article number: 6104 (2017).
- Nicolle, C. 1908. Reproduction experimentale du kala-azar chez le chien. Origine canine probable de cette affection. Bull. Soc. Path. Exot. 1, 188-190.
- Nicolle, C., Comte, C. 1908. “Origine canine du Kala-azar,” Bulletin de la Société de Pathologie Exotique, vol. 1, pp. 299–301, 1908.
- Njiru, B.N., Mukabana, W.R., Takken, W., Knols, B.G. 2006. Trapping of the malaria vector *Anopheles* gambiae with odour-baited MM-X traps in semi-field conditions in western Kenya. Malar J 5: 39.
- Nordström, T., Akre, O., Aly, M., Grönberg, H., Eklund, M. 2018. Prostate-specific antigen (PSA) density in the diagnostic algorithm of prostate cancer. Prostate Cancer and Prostatic Diseasesvolume 21, pages57–63 (2018).
- Novotny, M.V., Soini, H.A., Koyama, S., Wiesler, D., Bruce, K.E., Penn, D.J. 2007. Chemical identification of MHC-influenced volatile compounds in mouse urine. I: Quantitative Proportions of Major Chemosignals. J Chem Ecol. 2007 Feb;33(2):417-34. Epub 2006 Dec 27.
- Ntais, P., Sifaki-Pistola, D., Christodoulou, V., Messaritakis, I., Pratlong, F., Poupalos, G., Antoniou, M. 2013. Leishmaniases in Greece. Am J Trop Med Hyg. 2013 Nov 6; 89(5): 906–915.

- Nunes, C.M., Lima, V.M., Paula, H.B., Perri, S.H., Andrade, A.M., Dias, F.E., Burattini, M.N. 2008. Dog culling and replacement in an area endemic for visceral leishmaniasis in Brazil. Vet Parasitol. 2008 May 6;153(1-2):19-23. doi: 10.1016/j.vetpar.2008.01.005. Epub 2008 Jan 17.
- Nunes, C.M., Pires, M.M., da Silva, K.M., Assis, F.D., Goncalves Filho, J. 2010. Relationship between dog culling and incidence of human visceral leishmaniasis in an endemic area. Vet Parasitol 170: 131–133.
- Nunes, J.B., Coura-Vit*al.,* W., Colombo, F.A., Baêta, F.J.M., Pinheiro, A.C., Roatt, B.M., Reis, L.E.S., Reis, A.B., Marques, M.J. 2018. Comparative analysis of real-time PCR assays in the detection of canine visceral leishmaniasis. Parasitol Res. 2018 Oct;117(10):3341-3346.
- Nunes de Carvalho, F.L., Riboldi, E.O., Bello, G.L., Ramos, R.R., Barcellos, R.B., Gehlen, M., Halon, M.L., Romão, P.R.T., Dallegrave, E., Rossetti, M.L.R. 2018. Canine visceral leishmaniasis diagnosis: a comparative performance of serological and molecular tests in symptomatic and asymptomatic dogs. Epidemiology and Infection 146, 571–576.
- Nyambura, L.W., Jarmalavicius, S., Walden, P. 2018. Impact of *Leishmania* donovani infection on the HLA I self-peptide repertoire of human macrophages. PLoS ONE 13(7): e0200297.

O

- O.I.E. Leishmaniosis. In: Manual of diagnostic tests and vaccines for terrestrial animals (chap.2.1.11) 6th edition. World Organisation for Animal Health,

France. 2018. p.1–12. http://www.oie.int/standard-setting/terrestrial-manual/access-online/ Accessed 10 Apr 2019.

- O'Shea, B., Rebollar-Tellez, E., Ward, R.D., Hamilton, J.G.C., El Naiem, D., Polwart, A. 2002. Enhanced sandfly attraction to Leishmania-infected hosts. Transactions of the Royal Society of Tropical Medicine and Hygiene. (2002) 96, 117-l 18.
- Oliveira, C.C.G., Lacerdac, H.G., Martins, D.R.M., Barbosac, J.D.A., Monteiro, G.R., Queiroz, J.W., Sousa, J.M.A., Ximenes, M.F.F.M., Jeronimo, S.M.B. 2004. Changing epidemiology of American cutaneous Leishmaniasis (ACL) in Brazil: a disease of the urban–rural interface. Acta Tropica Volume 90, Issue 2, Pages 155–162.
- Oliveira, T.N., Guedes, P.E., Souza, G.B., Carvalho, F.S., Carlos, R.S.A., Albuquerque, G.R., Munhoz, A.D., Silva, F.L. 2016. Diagnosis and epidemiology of canine leishmaniasis in southeastern Bahia, Brazil. Genet Mol Res. 2016 Sep 2;15(3).
- Oliveira, V.V.G., Alves, L.C., Junior, V.A.S. 2015. Transmission routes of visceral leishmaniasis in mammals. Cienc. Rural vol.45 no.9 Santa Maria Sept. 2015 Epub June 30, 2015.
- Omrania, S.M., Vatandoosta, H., Oshaghia, M.A., Shokric, F., Guerind, P.M., Ershadia, M.R.Y., Rassia, Y., Tirgaria, S. 2010. Fabrication of an olfactometer for mosquito behavioural studies. J Vector Borne Dis 47, March 2010, pp. 17–25.
- Orlovskis, Z., Canale, M.C., Thole, V. Hogenhout, S.A. 2015. Insect-borne plant pathogenic bacteria: Getting a ride goes beyond physical contact. Current Opinion in Insect Science. Volume 9, June 2015, Pages 16-23.

- Oshaghi, M.A., Ravasan, N.M., Hide, M., Javadian, E.A., Rassi, Y., Sedaghat, M.M., 2009. Development of species-specific PCR and PCR-restriction fragment length polymorphism assays for L. infantum/L. donovani discrimination. Exp Parasitol; 122(1): 61-65. PMid:19545519.
- Osman, O.F., Oskam, L., Zijlstra, E.E., Kroon, N.C., Schoone, G.J., Khalil, E.T., El-Hassan, A.M., Kager, P.A. 1997. Evaluation of PCR for diagnosis of visceral leishmaniasis. J Clin Microbiol. 1997 Oct;35(10):2454-7.
- Otranto, D., Dantas-Torres, F. 2013. The prevention of canine leishmaniasis and its impact on public health. Trends in Parasitology. Volume 29, Issue 7, July 2013, Pages 339-345.
- Otranto, D., Paradies, P., de Caprariis, D., Stanneck, D., Testini, G., Grimm, F., Deplazes, P., Capelli, G. 2009. Toward Diagnosing *Leishmania infantum* Infection in Asymptomatic Dogs in an Area Where Leishmaniasis Is Endemic. Clinical and Vaccine Immunology, Mar. 2009, p. 337–343.
- Owens, S.D., Oakley, D.A., Marryott, K., Hatchett, W., Walton, R., Nolan, T.J., Newton, A., Steurer, F., Schantz, P. 2001. Transmission of visceral leishmaniasis through blood transfusions from infected English Foxhounds to anemic dogs. Journal of the American Veterinary Medical Association. October 15, 2001, Vol. 219, No. 8, Pages 1076-1083

P

- Pace, D. 2014. Leishmaniasis. J Infect 69 S10-18.
- Paiva-Cavalcanti, M., de Morais, R.C.S., Pessoa-e-Silva, R., Trajano-Silva, L.A.M., Gonçalves-de-Albuquerque, S.C., Tavares, D.H.C., Brelaz-de-Castro, M.C.A.,

Silva, R.F., Pereira, V.R.A. 2015. Leishmaniases diagnosis: an update on the use of immunological and molecular tools. Cell & Biosciencevolume 5, Article number: 31 (2015).

- Paltrinieri, S., Gradoni, L., Roura, X., Zatelli, A., Zini, E. 2016. Laboratory tests for diagnosing and monitoring canine leishmaniasis. Vet. Clin. Pathol. 45, 552–578.
- Parnetti, L., Gaetani, L., Eusebi, P., Paciotti, S., Hansson, O., El-Agnaf, O., Mollenhauer, B., Blennow, K., Calabresi, P. 2019. CSF and blood biomarkers for Parkinson's disease. Lancet Neurol 2019; 18: 573–86.
- Papadogiannakis, E., Koutinas, A. 2015. Cutaneous immune mechanisms in canine leishmaniosis due to *Leishmania* infantum. Vet. Immunol. Immunopathol. 163, 94–102.
- Pavlou, A.K., Magan, N., Jones, J.M., Brown, J., Klatser, P., Turner, A.P. 2004. Detection of Mycobacterium tuberculosis (TB) *in vitro* and in situ using an electronic nose in combination with a neural network system. Biosens Bioelectron. 2004 Oct 15;20(3):538-44.
- Pavlou, A.K., Magan, N., McNulty, C., Jones, J.M., Sharp, D., Brown, J., Turner, A.P.F. 2002. Use of an electronic nose system for diagnoses of urinary tract infections. Biosensors and Bioelectronics. Volume 17, Issue 10, October 2002, Pages 893–899.
- Pavlou, A.K., Turner, A.P.F. 2000. Sniffing out the Truth: Clinical Diagnosis Using the Electronic Nose. Clin Chem Lab Med 2000; 38(2):99–112.

- Paz, G.F., Rugani, J.M.N., Marcelino, A.P., Gontijo, C.M.F. 2018. Implications of the use of serological and molecular methods to detect infection by *Leishmania* spp. in urban pet dogs. Acta Tropica Volume 182, June 2018, Pages 198-201.
- Pearce, T.C., Schiffman, S.S., Nagle, H.T., Gardner, J.W. 2003. Handbook of Machine Olfaction, Wiley-VCH, Weinheim.
- Pedrosa, C.M.S., Ximenes, R.A.A., de Almeida, W.A.P., da Rocha, E.M.M. 2013. Validity of the polymerase chain reaction in the diagnosis of clinically suspected cases of American visceral leishmaniasis. The Brazilian Journal of Infectious Diseases Volume 17, Issue 3, May–June 2013, Pages 319-323.
- Penaforte, K.M., Belo, V.S., Teixeira-Neto, R.G., Ribeiro, R.A., de Oliveira, R.B., Schettini, D.A., da Silva, E.S. 2013. *Leishmania* infection in a population of dogs: an epidemiological investigation relating to visceral leishmaniasis control. Rev Bras Parasitol Vet. 2013 Oct-Dec; 22(4):592-6.
- Peng, G., Hakim, M., Broza, Y.Y. 2010. Detection of lung, breast, colorect*al.,* and prostate cancers from exhaled breath using a single array of nanosensors. Br J Cancer. 2010;103(4):542–551.
- Penn, D. & Potts, W. K. 1998. Chemical signals and parasite mediated sexual selection. Trends in Ecolopv and Evolution, 13,391-396.
- Pereira, M.R., Rocha-Silva, F., Graciele-Melo, C., Lafuente, C.R., Magalhães, T., Caligiorne, R.B. 2014. Comparison between Conventional and Real-Time PCR Assays for Diagnosis of Visceral Leishmaniasis. Biomed Res Int. 2014.
- Persaud, K.C., Dodd, G. 1982. Analysis of discrimination mechanisms in the mammalian olfactory system using a model nose. Nature 1982, 299, 352.

- Persichetti, M.F., Solano-Gallego, L., Vullo, A., Masucci, M., Marty, P., Delaunay, P., Vitale, F., Pennisi, M.G. 2017. Diagnostic performance of ELISA, IFAT and Western blot for the detection of anti-*Leishmania infantum* antibodies in cats using a Bayesian analysis without a gold standard. Parasites & Vectors 2017 10:119.
- Pessoa-e-Silva, R., Vaitkevicius-Antão, V., Santos de Andrade, T.A., de Oliveira Silva, A.C., de Oliveira, G.A., Trajano-Silva, L.A.M., Nakasone, E.K.N., de Paiva-Cavalcanti, M. 2019. The diagnosis of canine visceral leishmaniasis in Brazil: Confronting old problems. Experimental Parasitology. Volume 199, April 2019, Pages 9-16.
- Petersen, C.A. 2009. New Means of Canine Leishmaniasis Transmission in North America: The Possibility of Transmission to Humans Still Unknown. Interdiscip Perspect Infect Dis. 2009.
- Petersen, C.A. Barr, S.C. 2009. Canine Leishmaniasis in North America: Emerging or Newly Recognized? Vet Clin North Am Small Anim Pract. 2009 Nov; 39(6): 1065–vi.
- Phillips, M., Cataneo, R.N., Ditkoff, B.A., Fisher, P., Greenberg, J., Gunawardena, R., Won, C.S.K., Rahbari-Oskoui, F., Wong, C. 2003. Volatile markers of breast cancer in the breath Breast J., 9 (2003), p. 184.
- Picado, A., Singh, S.P., Rij*al.,* S., Sundar, S., Ostyn, B., Chappuis, F. 2010. Longlasting insecticidal nets for prevention of *Leishmania* donovani in India and Nepal: paired cluster randomised trial. BMJ 2010;341:c6760

- Pinart, M., Sinclair, D., Firooz, A., Enk, C., Vélez, I.D., Esterhuizen, T.M., Tristan, M., Alvar, J., González, U. 2015. Vector and reservoir control for preventing leishmaniasis. Cochrane Database Syst Rev. 2015 Aug; 2015(8): CD008736.
- Pinelli, E., Killick-Kendrick, R., Wagenaar, J., Bernadina, W., del Re*al.,* G., Ruitenberg, J. 1994. Cellular and humoral immune responses in dogs experimentally and naturally infected with *Leishmania* infantum. Infect Immun. 1994 Jan; 62(1): 229–235.
- Pisarski, K. 2019. The Global Burden of Disease of Zoonotic Parasitic Diseases: Top 5 Contenders for Priority Consideration. Trop. Med. Infect. Dis. 2019, 4(1), 44.
- Plucinski, M.M., Candrinho, B., Dimene, M., Colborn, J., Lu, A., Nace, D., Zulliger, R., Rogier, E. 2019. Assessing Performance of HRP2 Antigen Detection for Malaria Diagnosis in Mozambique. J Clin Microbiol 57:e00875-19.
- Podaliri M.V., Iannetti, L., Paganico, D., Iannino, F., Ferri, N. 2011. Methods of Control of the *Leishmania infantum* Dog Reservoir: State of the Art. Vet Med Int. 2011; 2011:215964.
- Ponte-Sucre, A., Gamarro, F., Dujardin, J.C., Barrett, M.P., López-Vélez, R., García-Hernández, R., Pountain, A.W., Mwenechanya, R., Papadopoulou, B. 2017. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. PLoS Negl Trop Dis. 2017 Dec; 11(12): e0006052.
- Portes, A., Giestal-de-Araujo, E., Fagundes, A., Pandolfo, P., de Sá, G.A., Lira, M.L.F. 2016. *Leishmania* amazonensis infection induces behavioral alterations and modulates cytokine and neurotrophin production in the murine cerebral cortex. J Neuroimmunol. 2016; 301:65–73.

- Pravallika, S. 2016. Gas Chromatography a Mini Review. Research and Reviews Journal of Pharmaceutical Analysis. Volume 5, Issue 2.
- Pulkki-Brännström, A.M., Wolff, C., Brännström, N., Skordis-Worrall, J. 2012. Cost and cost effectiveness of long-lasting insecticide-treated bed nets - a model-based analysis. Cost Eff Resour Alloc. 2012; 10: 5.

Q

- Quaresma, P.F., Murta, S.M., Ferreira, E.C., da Rocha-Lima, A.C., Xavier, A.A., Gontijo, C.M. 2009. Molecular diagnosis of canine visceral leishmaniasis: identification of *Leishmania* species by PCR-RFLP and quantification of parasite DNA by real-time PCR. Acta Tropica. 2009, 111(3):289-294.
- Queiroz, N.M., da Silveira, R., Fau - de Noronha, A.C.F., Jr, de Noronha, A, Jr., Fau – Oliveira, T.M.F.S., Oliveira, T., Fau – Machado, R.Z., Machado, R., Fau - Starke-Buzetti, W.A., Starke-Buzetti, W.A. 2011. Detection of *Leishmania* (L.) chagasi in canine skin. Vet Parasitol 178(1873–2550 (Electronic)):1–8
- Quinnell, R.J., Courtenay, O. 2009. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology. 2009 Dec; 136(14):1915-34.
- Quinnell, R.J., Carson, C., Reithinger, R., Garcez, L.M., Courtenay, O. 2013. Evaluation of rK39 Rapid Diagnostic Tests for Canine Visceral Leishmaniasis: Longitudinal Study and Meta-Analysis. PLoS Negl Trop Dis 7(1).

R

- Rasti, S., Ghorbanzadeh, B., Kheirandish, F., Mousavi, S.G., Pirozmand, A., Hooshyar, H., Abani, B. 2016. Comparison of Molecular, Microscopic, and

Culture Methods for Diagnosis of Cutaneous Leishmaniasis. JournalofClinicalLaboratoryAnalysis30:(2016).

- Rayaisse, J.B., Tirados, I., Kaba, D., Dewhirst, S.Y., Logan, J.G., Diarrassouba, A., Salou, E., Omolo, M.O., Solano, P., Lehane, M.J., Pickett, J.A., Vale, G.A., Torr, S.J., Esterhuizen, J. 2010. Prospects for the Development of Odour Baits to Control the Tsetse Flies Glossina tachinoides and G. palpalis s.l. PLoS Negl Trop Dis 4(3): e632.
- Ready, P.D. 2014. Epidemiology of visceral leishmaniasis. Clin Epidemiol. 2014; 6: 147–154.
- Reale, S., Maxia, L., Vitale, F., Glorioso, N.S., Caracappa, S., Vesco, G. 1999. Detection of *Leishmania infantum* in Dogs by PCR with Lymph Node Aspirates and Blood. J Clin Microbiol. 1999 Sep; 37(9): 2931–2935.
- Rebêlo, J.M.M., Rodrigues, B.L., Bandeira, M.C.A., Moraes, J.L.P., Fonteles, R.S., Pereira, S.R.F. 2016. Detection of *Leishmania* amazonensis and *Leishmania* braziliensis in Culicoides (Diptera, Ceratopogonidae) in an endemic area of cutaneous leishmaniasis in the Brazilian Amazonia. Journal of Vector Ecology. Volume41, Issue2. December 2016. Pages 303-308.
- Regina-Silva, S., Fortes-Dias, C.L., Michalsky, E.M., França-Silva, J.C. 2014. Evaluation of parasitological examination, kDNA polymerase chain reaction and rK39-based immunochromatography for the diagnosis of visceral leishmaniasis in seropositive dogs from the screening-culling program in Brazil. Revista da Sociedade Brasileira de Medicina Tropical 47(4):462-468.
- Regina-Silva, S., Feres, A.M., França-Silva, J.C., Dias, E.S., Michalsky, É.M., de Andrade, H.M. 2016. Field randomized trial to evaluate the efficacy of the

Leish-Tec® vaccine against canine visceral leishmaniasis in an endemic area of Brazil. Vaccine. (2016) 34:2233–9. doi: 10.1016/j.vaccine.2016.03.019

- Reguera, R.M., Morán, M., Pérez-Pertejo, Y., García-Estrada, C., Balaña-Fouce, R. 2016. Current status on prevention and treatment of canine leishmaniasis. Veterinary Parasitology, vol. 227, pp. 98–114, 2016
- Reithinger, R., Lambson, B.E., Barker, D.C., Davies, C.R. 2000. Use of PCR to detect *Leishmania* (Viannia) spp. in dog blood and bone marrow. J Clin Microbiol. 2000 Feb; 38(2):748-51.
- Reithinger, R., Espinoza, J.C., Courtenay, O., Davies, C.R. 2003. Evaluation of PCR as a diagnostic mass-screening tool to detect *Leishmania* (Viannia) spp. in domestic dogs (Canis familiaris). J Cli Microbiol. 2003;41(4):1486-93.
- Reithinger, R and Dujardin, J.C. 2007. Molecular Diagnosis of Leishmaniasis: Current Status and Future Applications. J. Clin. Microbiol. January 2007 vol. 45 no. 1 21-25
- Reithinger, R, Dujardin, J.C., Louzir, H., Pirmez, C, Alexander, B, Brooker, S. 2007. Cutaneous Leishmaniasis. The Lancet Infectious Diseases. Volume 7, Issue 9, September 2007, Pages 581–596.
- Reithinger, R., Quinnell, R.J., Alexander, B., Davies, C.R. 2002. Rapid Detection of *Leishmania infantum* Infection in Dogs: Comparative Study Using an Immunochromatographic Dipstick Test, Enzyme-Linked Immunosorbent Assay, and PCR. J. Clin. Microbiol. July 2002 vol. 40 no. 7.
- Ribeiro, V.M., da Silva, S.M., Menz, I., Tabanez, P., dos Santos Nogueira, F., Werkhaüser, M., da Fonseca, A.L.S., Dantas-Torres, F. 2013. Control of visceral leishmaniasis in Brazil: recommendations from Brasileish. Parasitol Vectors 6:8

- Riley, W.A., Johannsen, O.A. 1938. Medical Entomology. 2nd Edition. McGraw-Hill Book Company, Inc.
- Rittig, M.H., Bogdan, C. 2000. Leishmania-host-cell interaction: complexities and alternative views. Parasitology, 16 (2000), pp. 292-297.
- Rivas, G.B.S., Araujo de Souza, N., Peixoto, A.A., Bruno, R.V. 2014. Effects of temperature and photoperiod on daily activity rhythms of *Lutzomyia longipalpis* (Diptera: Psychodidae). Parasites & Vectors20147:278.
- Roberts, M.T.M. 2006. Current understandings on the immunology of leishmaniasis and recent developments in prevention and treatment. British Medical Bulletin 2006;75, 76:115-30.
- Robinson, A., Busula, A.O., Voets, M.A., Beshir, K.B., Caulfield, J.C., Powers, S.J., Verhulst, N.O., Winskill, P., Muwanguzi, J., Birkett, M.A., Smallegange, R.C., Masiga, D.K., Mukabana, W.R., Sauerwein, R.W., Sutherland, C.J., Bousema, T., Pickett, J.A., Takken, W., Logan, J.G., de Boer, J.G. 2018. Plasmodium-associated changes in human odor attract mosquitoes. Proc Natl Acad Sci U S A. 2018 May 1;115(18):E4209-E4218.
- Rock, F., Barsan, N., Weimar, U. 2008. Electronic Nose: Current Status and Future Trends. Chem. Rev. 2008, 108, 705−725.
- Rodriguez, A.E., Estévez, J.O., Nevot, M.C., Barrios, A., Florin-Christensen, M. 2017. Leishmania. Parasitic Protozoa of Farm Animals and Pets. pp 289-311.
- Rodríguez-Cortés, A., Carrillo, E., Martorell, S., Todolí, F., Ojeda, A., Martínez-Flórez, A. 2016. Compartmentalized immune response in leishmaniasis: changing patterns throughout the disease. PLoS ONE 11:e0155224.

- Roessner, U., Wagner, C., Kopka, J. 2000. Technical advance: simultaneous analysis of metabolites in potato tuber by gas chromatography–mass spectrometry, Plant J , 2000, vol. 23 (pg. 131-42)
- Rogers, M.E., Bates, P.A. 2007. *Leishmania* Manipulation of Sand Fly Feeding Behavior Results in Enhanced Transmission. PLoS Pathog 3(6).
- Rogers, M.E., Chance, M.L., Bates, P.A. The role of promastigote secretory gel in the origin and transmission of the infective stage of *Leishmania* mexicana by the sandfly *Lutzomyia* longipalpis. Parasitology. 2002;124:495–507.
- Roine, A., Saviauk, T., Kumpulainen, P., Karjalainen, M., Tuokko, A., Aittoniemi, J., Vuento, R., Lekkala, J., Lehtimäki, T., Tammela, T.L., Oksala, N.K. 2014. Rapid and accurate detection of urinary pathogens by mobile IMS-based electronic nose: a proof-of-principle study. PLoS One. 2014 Dec 19;9(12):e114279.
- Romero, G.A.S., Boelaert, M. 2010. Control of Visceral Leishmaniasis in Latin America—A Systematic Review. PLoS Negl Trop Dis. 2010 Jan; 4(1): e584.
- Roosien, B.K., Gomulkiewicz, R., Ingwell, L.L., Bosque-Pérez, N.A., Rajabaskar, D., S. D. Eigenbrode. 2013. Conditional vector preference aids the spread of plant pathogens: results from a model. Environ. Entomol., vol. 42, no. 6, pp. 1299–1308, Dec. 2013.
- Roque, A.L.R., Jansen, A.M. 2014. Wild and synanthropic reservoirs of *Leishmania* species in the Americas. Int J Parasitol Parasites Wildl. 2014 Dec; 3(3): 251–262.
- Romero, G.A., Boelaert, M. 2010. Control of visceral leishmaniasis in latin america-a systematic review. PLoS Negl Trop Dis. 2010 Jan 19; 4(1):e584.

- Roque, A.L.R., Jansen, A.M. 2014. Wild and synanthropic reservoirs of *Leishmania* species in the Americas. International Journal for Parasitology: Parasites and Wildlife, vol. 3, no. 3, pp. 251–262.
- Rose, C., Parker, A., Jefferson, B., Cartmell, E. 2015. The Characterization of Feces and Urine: A Review of the Literature to Inform Advanced Treatment Technology. Crit Rev Environ Sci Technol. 2015 Sep 2; 45(17): 1827–1879.
- Rutledge LC, Gupta RK. 2009. Moth flies and sand flies. In Medical and Veterinary Entomology, Mullen R, Durden LA (Eds). 2nd Edition, Academic Press, London, pp, 153-168.

S

- Saalberg, Y., Wolff, M. 2016. VOC breath biomarkers in lung cancer. Clin Chim Acta. 2016;459:5–9.
- Sacks, D.L., Perkins, P.V. 1985. Development of infective stage *Leishmania* promastigotes within *Phlebotomine* sandflies. Am J Trop Med Hyg. 1985;34:456–459.
- Saha, S., Mond*al.,* S., Banerjee, A., Ghose, J., Bhowmick, S., Ali, N. 2006. Immune responses in kala-azar. Indian J. Med. Res. 123:245–266.
- Sakumura, Y., Koyama, Y., Tokutake, H., Hida, T., Sato, K., Itoh, T., Akamatsu, T., Shin, W. 2017. Diagnosis by Volatile Organic Compounds in Exhaled Breath from Lung Cancer Patients Using Support Vector Machine Algorithm. Sensors (Basel). 2017 Feb 4;17(2):287.
- Salgado, V.R., de Queiroz, A.T.L., Sanabani, S.S., de Oliveira, C.I., Carvalho, E.M., Costa, J.M.L., Barral-Netto, M., Barr*al.,* A. 2016. The microbiological signature

of human cutaneous leishmaniasis lesions exhibits restricted bacterial diversity compared to healthy skin. Mem Inst Oswaldo Cruz. 2016 Apr; 111(4): 241–251.

- Salomón, O.D., Basmajdian, Y., Fernández, M.S., Santini, M.S. 2011. *Lutzomyia longipalpis* in Uruguay: the first report and the potential of visceral leishmaniasis transmission. Mem Inst Oswaldo Cruz. 2011 May;106(3):381-2.
- Salomón, O.D., Feliciangeli, M.D., Quintana, M.G., dos Santos Afonso, M.M., Rangel, E.F. 2015. *Lutzomyia longipalpis* urbanisation and control. Mem Inst Oswaldo Cruz. 2015 Nov; 110(7): 831–846.
- Salomón, O.D., Sinagra, A., Nevot, M.C., Barberian, G., Paulin, P., Estevez, J.O., Riarte, A., Estevez, J. 2008. First visceral leishmaniasis focus in Argentina. Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. 103(1): 109-111.
- Sánchez-García, L., Wilkins-Rodriguez, A., Salaiza-Suazo, N., Morales-Montor, J., Becker, I. 2018. Dihydrotestosterone enhances growth and infectivity of *Leishmania* Mexicana. Parasite Immunol. 2018 Mar;40(3). doi: 10.1111/pim.12512. Epub 2018 Jan 18.
- Sant'Anna, M.R.V., Diaz-Albiter, H., Aguiar-Martins, K., Al Salem, W.S., Cavalcante, R.R., Dillon, V.M., Bates, P.A., Genta, F.A., Dillon, R.J. 2014. Colonisation resistance in the sand fly gut: *Leishmania* protects *Lutzomyia longipalpis* from bacterial infection. Parasites & Vectors20147:329.
- Sarkari, B., Ashrafmansouri, M., Hatam, G.R., Habibi, P., Khabisi, S.A. 2014. Performance of an ELISA and Indirect Immunofluorescence Assay in Serological Diagnosis of Zoonotic Cutaneous Leishmaniasis in Iran. Interdiscip Perspect Infect Dis. 2014; 2014: 505134.

- Saviauk, T., Kiiski, J.P., Nieminen, M.K., Tamminen, N.N., Roine, A.N., Kumpulainen, P.S., Hokkinen, L.J., Karjalainen, M.T., Vuento, R.E., Aittoniemi, J.J., Lehtimäki, T.J., Oksala, N.K. 2018. Electronic Nose in the Detection of Wound Infection Bacteria from Bacterial Cultures: A Proof-of-Principle Study. Eur Surg Res. 2018;59(1-2):1-11.
- Savoia, D. 2015. Recent updates and perspectives on leishmaniasis. J Infect Dev Ctries 2015; 9(6):588-596.
- Scalone, A., De Luna, R., Oliva, G., Baldi, L., Satta, G., Vesco, G., Mignone, W., Turilli, C., Mondesire, R.R., Simpson, D., Donoghue, A.R., Frank, G.R., Gradoni, L. 2002. Evaluation of the *Leishmania* recombinant K39 antigen as a diagnostic marker for canine leishmaniasis and validation of a standardized enzyme-linked immunosorbent assay. Vet Parasitol. 2002 Apr 2;104(4):275-85.
- Schmidt, G.D., Roberts, L.S. 1996. Foundations of Parasitology, 5th edition. Times Mirror Higher Education Group, Inc.
- Schriefer, A., Guimarães, L.H., Machado, P.R., Lessa, M., Lessa, H.A., Lago, E., Ritt, G., Góes-Neto, A., Schriefer, A.L., Riley, L.W., Carvalho, E.M. 2009. Geographic clustering of leishmaniasis in northeastern Brazil. Emerg Infect Dis. 2009 Jun; 15(6):871-6.
- Sharma, U., Singh, S. 2008. Insect vectors of Leishmania: distribution, physiology and their control. J Vector Borne Dis 45, December 2008, pp. 255–272.
- Shih, C.L., Wu, H.Y., Liao, P.M., Hsu, J.Y., Tsao, C.Y., Zgoda, V.G., Liao, P.C. 2018. Profiling and comparison of toxicant metabolites in hair and urine using a mass

spectrometry-based metabolomic data processing method. Analytica Chimica Acta.

- Shirasu, M., Touhara, K. 2011. The scent of disease: volatile organic compounds of the human body related to disease and disorder. The Journal of Biochemistry, Volume 150, Issue 3, 1 September 2011, Pages 257–266.
- Seblova, V., Sadlova, J., Vojtkova, B., Votypka, J., Carpenter, S., Bates, P.A., Volf, P. 2015. The Biting Midge Culicoides sonorensis (Diptera: Ceratopogonidae) Is Capable of Developing Late Stage Infections of *Leishmania* enriettii. PLoS Negl Trop Dis. 2015 Sep; 9(9): e0004060.
- Serafim, T.D., Coutinho-Abreu, I.V., Oliveira, F., Meneses, C., Kamhawi, S., Valenzuela, J.G. 2018. Sequential blood meals promote *Leishmania* replication and reverse metacyclogenesis augmenting vector infectivity. Nature Microbiologyvolume 3, pages548–555 (2018).
- Sethi, S., Nanda, R., Chakraborty, T. 2013. Clinical Application of Volatile Organic Compound Analysis for Detecting Infectious Diseases. Clin. Microbiol. Rev. July 2013 vol. 26 no. 3 462-475.
- Sevá, A.P., Ovallos, F.G., Amaku, M., Carrillo, E., Moreno, J., Galati, E.A.B., Lopes, E.G., Soares, R.M., Ferreira, F. 2016. Canine-Based Strategies for Prevention and Control of Visceral Leishmaniasis in Brazil. PLOS ONE 11(9).
- Seymour, J.V., Tu, S., He, X., Zhang, H., Han, T.L., Baker, P.N., Sulek, K. 2018. Metabolomic profiling of maternal hair suggests rapid development of intrahepatic cholestasis of pregnancy. Metabolomics (2018) 14:79.

- Shirasu, M., Touhara, K. 2011. The scent of disease: volatile organic compounds of the human body related to disease and disorder. J Biochem. 2011 Sep;150(3):257-66.
- Silva, D.A., Madeira, M.F., Teixeira, A.C., de Souza, C.M., Figueiredo, F.B. 2011. Laboratory tests performed on *Leishmania* seroreactive dogs euthanized by the leishmaniasis control program. Vet Parasitol. 2011 Jun 30;179(1-3):257-61.
- Silva, F.L., Oliveira, R.G., Silva, T.M., Xavier, M.N., Nascimento, E.F., Santos, R.L. 2009. Venereal transmission of canine visceral leishmaniasis. Vet Parasitol. 2009 Mar 9;160(1-2):55-9. doi: 10.1016/j.vetpar.2008.10.079. Epub 2008 Nov 1.
- Silva, R.C., Richini-Pereira, V.B., Kikuti, M., Marson, P.M., Langoni, H. 2017. Detection of *Leishmania* (L.) *infantum* in stray dogs by molecular techniques with sensitive species-specific primers. Veterinary Quarterly, vol. 37, no. 1, pp. 23–30, 2017.
- Singh, N.S., Phillips-Singh, D. 2010. A Study on Genitalia of Phlebotominae Sand Flies (Phlebotomidae: Diptera) in Northern India: A New Tool for Detection of Species. Journal of Entomology. Volume 7 (4): 235-239, 2010.
- Singh, O.P., Singh, B., Chakravarty, J., Sundar, S. 2016. Current challenges in treatment options for visceral leishmaniasis in India: a public health perspective. Infectious Diseases of Povertyvolume 5, Article number: 19 (2016).
- Siriyasatien, P., Chusri, S., Kraivichian, K., Jariyapan, N., Hortiwakul, T., Silpapojakul, K., Pym, A.M., Phumee, A. 2016. Early detection of novel

Leishmania species DNA in the saliva of two HIV-infected patients. BMC Infect Dis. 2016; 16: 89.

- Smallegange, R.C., Takken, W. 2010. Host-seeking behaviour of mosquitoes: responses to olfactory stimuli in the laboratory. Olfaction in vector-host interactions / Takken, W., Knols, B.G.J., Wageningen: Wageningen Academic Publishers (Ecology and control of vector-borne diseases) p. 143 - 180.
- Smallegange, R.C., Verhulst, N.O., Takken, W. 2011. Sweaty skin: an invitation to bite? Trends Parasitol, 2011; 30, 1–6.
- Smallegange, R.C., Gemert, G.J., Vegte-Bolmer, M., Gezan, S., Takken, W., Sauerwein, R.W., Logan, J.G. 2013. Malaria-infected Mosquitoes Express Enhanced Attraction to Human Odor. PLoS ONE 8(5): e63602.
- Smith, K., Thompson, G.F., Koster, H.D. 1969. Sweat in schizophrenic patients: Identification of the odorous substance. Science, 166, 398-399.
- Smoleńska, Ż., Zdrojewski Z. 2015. Metabolomics and its potential in diagnosis, prognosis and treatment of rheumatic diseases. Reumatologia. 2015;53(3):152-6.
- Sobrinho, L.S.V., Rossi, C.N., Vides, J.P., Braga, E.T., Gomes, A.A.D., de Lima, V.M.F. 2012. Coinfection of *Leishmania chagasi* with *Toxoplasma gondii*, Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV) in cats from an endemic area of zoonotic visceral leishmaniasis. Vet Parasitol. 2012;187:302–6.
- Solano-Gallego, L., Koutinas, A., Miro, G., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G. 2009. Directions for the diagnosis, clinical

staging, treatment and prevention of canine leishmaniosis. Vet Parasitol. 2009, 165 (1-2): 1-18.

- Solano-Gallego, L., Miró, G., Koutinas, A., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G. 2011. LeishVet guidelines for the practical management of canine leishmaniosis. Parasites & Vectors 2011 4:86.
- Solano-Gallego, L., Villanueva-Saz, S., Carbonell, M., Trotta, M., Furlanello, T., Natale, A. 2014. Serological diagnosis of canine leishmaniosis: comparison of three commercial ELISA tests (Leiscan, ID Screen and *Leishmania* 96), a rapid test (Speed Leish K) and an in-house IFAT. Parasit Vectors. 2014;7:111.
- Solano-Gallego, L., Cardoso, L., Pennisi, M.G., Miró, G., Ferrer, L., Baneth, G. 2017. Diagnostic Challenges in the Era of Canine *Leishmania infantum* Vaccines. Trends in Parasitology, Volume 33, Issue 9, P706-717.
- Solcà, M da S., Bastos, L.A., Guedes, C.E.S. 2014. Evaluating the Accuracy of Molecular Diagnostic Testing for Canine Visceral Leishmaniasis Using Latent Class Analysis. Traub-Csekö YM, ed. PLoS ONE. 2014;9(7).
- Solcà, M da S., Bruno, B., Andrade, B.B., Abbehusen, M.M.C., Teixeira, C.R., Khouri, R. 2016. Circulating biomarkers of immune activation, oxidative stress and inflammation characterize severe canine visceral leishmaniasis. Sci. Rep. 6:32619. doi: 10.1038/srep32619
- Sousa-Paula, L.C., Silva, L.G.D., Sales, K.G.D.S., Dantas-Torres, F. 2019. Failure of the dog culling strategy in controlling human visceral leishmaniasis in Brazil: A screening coverage issue?. PLoS Negl Trop Dis. 2019;13(6):e0007553. Published 2019 Jun 26.

- Souza, N.A., Brazil, R.P., Araki, A.S. 2017. The current status of the *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae) species complex. Mem Inst Oswaldo Cruz. 2017 Mar; 112(3): 161–174.
- Souza N,A., Leite R,S., de Oliveira Silva, S., Penna M,G., Vilela L,F,F., Melo, M.N., de Andrade, A.S.R. 2019. Detection of mixed *Leishmania* infections in dogs from an endemic area in southeastern Brazil. Acta Trop. 2019 May;193:12-17.
- Spanakos, G., Piperaki, E.T., Menounos, P.G., Tegos, N., Flemetakis, A., Vakalis, N.C. 2008. Detection and species identification of Old World *Leishmania* in clinical samples using a PCR-based method. Transactions of the Royal Society of Tropical Medicine and Hygiene (2008) 102, 46—53.
- Srivastava, S., Mishra, J., Gupta, A.K., Singh, A., Shankar, P., Singh, S. 2017. Laboratory confirmed miltefosine resistant cases of visceral leishmaniasis from India. Parasites & Vectorsvolume 10, Article number: 49 (2017).
- Stanczyk, N.M., Mescher, M.C., De Moraes, C.M. 2017. Effects of malaria infection on mosquito olfaction and behavior: extrapolating data to the field. Current Opinion in Insect Science. Volume 20, April 2017, Pages 7-12.
- Staniek, M.E., Sedda, L., Gibson, T.D., de Souza, C.F., Costa, E.M., Dillon, R.J., Hamilton, J.G.C. 2019. eNose analysis of volatile chemicals from dogs naturally infected with *Leishmania infantum* in Brazil. PLoS Negl Trop Dis 13(8): e0007599.
- Stashenko, E., Martínez, J.R. 2014. Gas Chromatography-Mass Spectrometry. Advances in Gas Chromatography, Xinghua Guo, IntechOpen, DOI: 10.5772/57492.

- Stenn, K., Paus, R. 2001. Controls of hair follicle cycling. Physiol Rev 81:449–494.
- Stitzel, S.E., Aernecke, M.J., Walt, D.R. 2011. Artificial Noses. Annual Review of Biomedical Engineering. Vol.13:1-552.
- Sujatha, G., Dhivya, N, Ayyadurai, K., Thyagarajan, D. 2012. Advances In Electronic - Nose Technologies. International Journal of Engineering Research and Applications (IJERA). Vol. 2, Issue4, July-August 2012, pp.1541-1546.
- Sundar, S., Chakravarty, J. 2010. Liposomal Amphotericin B and Leishmaniasis: Dose and Response. J Glob Infect Dis. 2010 May-Aug; 2(2): 159–166.
- Sundar, S., More, D.K., Singh, M.K., Singh, V.P., Sharma, S., Makharia, A., Kumar, P.C.K., Murray, H.W. 2000. Failure of Pentavalent Antimony in Visceral Leishmaniasis in India: Report from the Center of the Indian Epidemic. Clinical Infectious Diseases, Volume 31, Issue 4, October 2000, Pages 1104–1107.
- Sundar S., Rai, M. 2002. Laboratory Diagnosis of Visceral Leishmaniasis. Clinical Vaccine Immunology, vol. 9 no. 5951-958.
- Sunyoto, T., Potet, J., Boelaert, M. 2018. Why miltefosine—a life-saving drug for leishmaniasis-is unavailable to people who need it the most. BMJ Glob. Health, 33 (2018), p. 709.

T

- Tafazzoli, M., Fathi, F., Darvizeh, F., Zamani, Z., Pourfallah, F. 2010. A metabonomic study on samples of cutaneous leishmaniasis and its correlation with the selenium level in the blood serum. J Mol Biomark Diagn, 1. p. 101.

- Tarassenko, L., Denham, M. 2006. Sensory Processing. Cognitive Systems - Information Processing Meets Brain Science 2006, Pages 85-104.
- Tavares, D.D.S., Salgado, V.R., Miranda, J.C., Mesquita, P.R.R., Rodrigues, F.M., Barral-Netto, M., de Andrade, J.B., Barr*al.,* A. 2018. Attraction of *Phlebotomine* sandflies to volatiles from skin odors of individuals residing in an endemic area of tegumentary leishmaniasis. PLoS One. 2018 Sep 24;13(9)
- Teixeira, R.C., Rodríguez, M., Romero, N.J., Bruins, M., Gómez, R., Yntema, J.B., Abente, G.C., Gerritsen, J.W., Wiegerinck, W., Bejerano, D.P., Magis-Escurra, C. 2017. The potential of a portable, point-of-care electronic nose to diagnose tuberculosis. Journal of Infection. Volume 75, Issue 5, November 2017, Pages 441-447.
- Thriumani, R., Zakaria, A., Jeffree, A.I., Hishamuddin, N.A., Omar, M.I., Adom, A.H., Shakaff, A.Y.M., Kamarudin, L.M., Yusuf, N., Hashim, Y.Z.H.Y., Helmy, K.M. 2014. Cancer Detection Using an Electronic Nose: A Preliminary Study on Detection and Discrimination of Cancerous Cells. 2014 IEEE Conference on Biomedical Engineering and Sciences, 8 - 10 December 2014.
- Tirzïte, M., Bukovskis, M., Strazda, G., Jurka, N., Taivans, I. 2018. Detection of lung cancer with electronic nose and logistic regression analysis. J Breath Res. 2018 Nov 20;13(1):016006.
- Tonin, A., Calado, A., Bottari, N., Dalenogare, D., Thomé, G., Duarte, T., 2016. Novel markers of inflammatory response and hepatic dysfunction in canine leishmaniasis. Comp. Immunol. Microbiol. Infect. Dis
- Torrecilha, R., Utsunomiya, Y., Bosco, A., Almeida, B., Pereira, P., Narciso, L. 2016. Correlations between peripheral parasite load and common clinical and

laboratory alterations in dogs with visceral leishmaniasis. Prev. Vet. Med. 132, 83–87.

- Torres, S., Clayton, J.B., Danzeisen, J.L., Ward, T., Huang, H., Knights, D., Johnson, T.J. 2017. Diverse bacterial communities exist on canine skin and are impacted by cohabitation and time. PeerJ. 2017; 5: e3075.
- Travi, B.L., Cordeiro-da-Silva, A., Dantas-Torres, F., Miró, G. 2018. Canine visceral leishmaniasis: Diagnosis and management of the reservoir living among us. PLoS Negl Trop Dis 12(1).
- Truppel, J.H., Otomura, F., Teodoro, U., Massafera, R., da Costa-Ribeiro, M.C.V., Catarino, C.M., Dalagrana, L., Ferreira, M.E.M.C., Thomaz-Soccol, V. 2014. Can Equids Be a Reservoir of *Leishmania* braziliensis in Endemic Areas? PLoS ONE 9(4).
- Tsai, Y.J., Chung, W.C., Wang, L.C., Ju, Y.T., Hong, C.L., Tsai, Y.Y., Li, Y.H., Wu, Y.L. 2011. The Dog Mite, Demodex canis: Prevalence, Fungal Co-Infection, Reactions to Light, and Hair Follicle Apoptosis. J Insect Sci. 2011; 11: 76.
- Turner, A.P.F., Magan, N. 2004. Electronic noses and disease diagnostics. Nature Reviews Microbiology 2, 161-166.

U

- University of East London. University of East London Life Sciences Department. (http://homepages.uel.ac.uk/D.P.Humber/akhter/cont.htm#fly) (accessed 08/02/18)

- Uribe, S. 1999. The status of the *Lutzomyia longipalpis* species complex and possible Implications for *Leishmania* transmission. Mem Inst Oswaldo Cruz. 1999; 94: 729-34.

V

- Valdivia, H.O., Almeida, L.V., Roatt, B.M., Reis-Cunha1, J.L., Pereira, A.A.S., Gontijo, C., Fujiwara, R.T., Reis, A.B., Sanders, M.J., Cotton, J.A., Bartholomeu, D.C. 2017. Comparative genomics of canineisolated *Leishmania* (Leishmania) amazonensis from an endemic focus of visceral leishmaniasis in Governador Valadares, southeastern Brazil. Scientific Reports 7:40804.
- Vamvakidis, C., Koutinas, A., Kanakoudis, G., Georgiadis, G., Saridomichelakis, M. 2000. Masticatory and skeletal muscle myositis in canine leishmaniasis (*Leishmania* infantum). Vet. Rec. 146, 698–703.
- Vantaux, A., Yao, F., Hien, D.F., Guissou, E., Yameogo, B.K., Gouagna, L.C., Fontenille, D., Renaud, F., Simard, F., Costantini, C., Thomas, F., Mouline, F., Roche, B., Cohuet, A., Dabiré, K.R., Lefèvre, T. 2018. Field evidence for manipulation of mosquito host selection by the human malaria parasite, *Plasmodium* falciparum. bioRxiv, 207183 ver. 6 peer-reviewed and recommended by PCI Evol Biol (2018). DOI: 10.1101/207183
- Varani, S., Ortalli, M., Attard, L., Vanino, E., Gaibani, P., Vocale, C. 2017. Serological and molecular tools to diagnose visceral leishmaniasis: 2-years' experience of a single center in Northern Italy. PLoS ONE 12(8).
- Veras, P.S.T., Fraga, D.B.M., Solcà, M.S., Guedes, C.E.S. 2014. New Advances in the Diagnosis of Canine Visceral Leishmaniasis. David M. Claborn (Ed.),

Leishmaniasis – Trends in Epidemiology, Diagnosis and Treatment, InTech (2014), pp. 239-273.

- Veras, P.S.T., Ramos, P.I.P., de Menezes, J.P.B. 2018. In Search of Biomarkers for Pathogenesis and Control of Leishmaniasis by Global Analyses of Leishmania-Infected Macrophages. Front Cell Infect Microbiol. 2018 Sep 19;8:326.
- Verde, F.A.L., Verde, F.A.A.L., Neto, A.S., Almeida, P.C., Verde, E.M.L. 2011. Hormonal Disturbances in Visceral Leishmaniasis (Kala-Azar). Am J Trop Med Hyg. 2011 May 5; 84(5): 668–673.
- Verhulst, N.O., Takken, W., Dicke, M., Schraa, G., Smallegange, R.C. 2010. Chemical ecology of interactions between human skin microbiota and mosquitoes. Microb Ecol. 2010; 74, 1–9.
- Verma, S., Kumar, R., Katara, G.K., Singh, L.C., Negi, N.S., Ramesh, V., Salotra, P. 2010. Quantification of parasite load in clinical samples of leishmaniasis patients: IL-10 level correlates with parasite load in visceral leishmaniasis. PLoS One. 2010 Apr 9;5(4):e10107.
- Vieira, J.B., Coelho, G.E. 1998. Visceral leishmaniasis or kala-azar: the epidemiological and control aspects. Rev Soc Bras Med Trop. 1998; 31 Suppl 2():85-92.
- Vincenti, M., Salomone, A., Gerace, E., Pirro, V. 2013. Application of mass spectrometry to hair analysis for forensic toxicological investigations. Mass Spectrom Rev 32:312–332

W

- Wasserberg, G., Kirsch, P., Rowton, E.D. 2014. Orientation of colonized sand flies Phlebotomus papatasi, P. duboscqi, and *Lutzomyia longipalpis* (Diptera: Psychodidae) to diverse honeys using a 3-chamber in-line olfactometer. J Vector Ecol. 2014;39(1):94–102.
- Webster, J.P. 2007. The Effect of Toxoplasma gondii on Animal Behavior: Playing Cat and Mouse. Schizophr Bull. 2007 May; 33(3): 752–756.
- Weese, J.S. 2013. The canine and feline skin microbiome in health and disease. Vet Dermatol. 2013 Feb;24(1):137-45.e31.
- Werneck, G.L. 2014. Visceral leishmaniasis in Brazil: rationale and concerns related to reservoir control. Rev Saude Publica. 2014 Oct; 48(5): 851–856.
- Werneck, G.L., Costa, C.H.N., Carvalho, F.A.A., Pires e Cruz, M.S., Maguire, J.H., Castro, M.C. 2014b. Effectiveness of insecticide spraying and culling of dogs on the incidence of *Leishmania infantum* infection in humans: a cluster randomized trial in Teresina, Brazil. PLoS Neglected Tropical Diseases 2014;8(10):e3172.
- Wesley, M., Moraes, A., Rosa, A.S., Carvalho, J.L., Shiroma, T., Vit*al.,* T., Dias, N., de Carvalho, B., Rabello, D.A., Borges, T.K.S., Dallago, B., Nitz, N., Hagström, L., Hecht, M., 2019. Correlation of Parasite Burden, kDNA Integration, Autoreactive Antibodies, and Cytokine Pattern in the Pathophysiology of Chagas Disease. Front. Microbiol. 10:1856.
- Westrop, G.D., Williams, R.A.M., Wang, L., Zhang, T., Watson, D.G., Silva, A.M., Coombs, G.H. 2015. Metabolomic Analyses of *Leishmania* Reveal Multiple Species Differences and Large Differences in Amino Acid Metabolism. PLoS ONE 10(9): e0136891.

- Wilson, A.D. 2011. Future applications of electronic-nose technologies in healthcare and biomedicine. In Wide spectra of quality control, Akyar I (ed). InTech; 267–290.
- Wilson, A.D. 2016. Recent progress in the design and clinical development of electronic-nose technologies. Nanobiosens. Dis. Diagn. 2016, 5, 15–27.
- Wilson, A.D. 2018. Applications of Electronic-Nose Technologies for Noninvasive Early Detection of Plant, Animal and Human Diseases. Chemosensors 2018, 6, 45.
- Wilson, A.D., Baietto, M. 2011. Advances in Electronic-Nose Technologies Developed for Biomedical Applications. Sensors 2011, 11, 1105-1176.
- Wiśniewska, P., Śliwińska, M., Dymerski, T., Wardencki, W., Namieśnik, J. 2017. Comparison of an Electronic Nose Based on Ultrafast Gas Chromatography, Comprehensive Two-Dimensional Gas Chromatography, and Sensory Evaluation for an Analysis of Type of Whisky. Journal of Chemistry, vol. 2017, Article ID 2710104, 13 pages, 2017.
- Woodroffe, R., Prager, K.C., Munson, L., Conrad, P.A., Dubovi, E.J., 2012. Contact with domestic dogs increases pathogen exposure in endangered African wild dogs (Lycaon pictus). PLoS One 7(1): e30099.
- World Health Organisation. 2012. Handbook for Integrated Vector Management. WHO. Press. Geneva, Switzerland: WHO; 2012.
- World Health Organisation. 2016. Epidemiological situation. [Online]. http://www.who.int/leishmaniasis/burden/en/. Accessed 10/01/2017.

- World Health Organisation. 2017. WHO Model List of Essential Medicines. [Online]. https://apps.who.int/iris/bitstream/handle/10665/273826/EML-20-eng.pdf?ua=1. Accessed 18/04/2019.
- World Health Organisation. 2019. Leishmaniasis. [Online]. https://www.who.int/news-room/fact-sheets/detail/leishmaniasis. Accessed 13/08/19.
- Worley, B., Powers, R. 2013. Multivariate Analysis in Metabolomics. Current Metabolomics, 2013, 1, 92-107.
- Wu, S., Lyu, G., Lou, R. 2012. Applications of Chromatography Hyphenated Techniques in the Field of Lignin Pyrolysis. Applications of Gas Chromatography. Chapter · March 2012.

X

- Xue, R., Dong, L., Zhang, S., Deng, C., Liu, T., Wang, J., Shen, X. 2008. Investigation of volatile biomarkers in liver cancer blood using solid-phase microextraction and gas chromatography/mass spectrometry. Rapid Commun Mass Spectrom. 2008 Apr;22(8):1181-6.

Y

- Yang, S., Rothman, R.E. 2004. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect Dis. 2004 Jun; 4(6):337-48.

- Yasur-Landau, D., Jaffe, C.L., David, L., Baneth, G. Allopurinol resistance in *Leishmania infantum* from dogs with disease relapse. PLOS Neglected Tropical Diseases, vol. 10, no. 1, Article ID e0004341, 2016.
- Yin, P., Lehmann, R., Xu, G. 2015. Effects of pre-analytical processes on blood samples used in metabolomics studies. Anal Bioanal Chem. 2015; 407(17): 4879–4892.
- Young DG, Duncan MA. Guide to the identification and geographic distribution of *Lutzomyia* sand flies in Mexico, the West Indies, Central and South America. J Biol Sci. 1994;14:79–94.
- Young, L., Pollien, P., Roberts, D., Cline, J. 2002. Compounds Responsible for the Odor of Dog Hair Coat. WSAVA 2002 CONGRESS.

Z

- Zhang, J., Puvenna, V., Janigro. D. 2016. Biomarkers of Traumatic Brain Injury and Their Relationship to Pathology. Translational Research in Traumatic Brain Injury. Boca Raton (FL): CRC Press/Taylor and Francis Group; 2016.
- Zetola, N.M., Modongo, C., Matsiri, O., Tamuhla, T., Mbongwe, B., Matlhagela, K., Sepako, E., Catini, A., Sirugo, G., Martinelli, E., Paolesse, R., Di Natale, C. 2018. Diagnosis of pulmonary tuberculosis and assessment of treatment response through analyses of volatile compound patterns in exhaled breath samples. J Infect. 2017 Apr;74(4):367-376.
- Zeyrek, F.Y., Töz, S., Yuksel, F., Turgay, N., Ozbel, Y. The Comparison of Kinetoplastid DNA PCR and Parasitological Methods in the Diagnosis of Cutaneous Leishmaniasis Using Clinical Samples of Suspected Patients in

Turkey. International journal of infectious diseases. August 2018 Volume 73, Supplement, Page 317.

Chapter 9 - Supplementary Material

Rep. A		PCR result	No. of parasites per ml of blood	GC/MS file name	Day	Run Time (hr:mins) 12 mins to reinject
1	hydrocarbons			hyd_001A	1	0:40
2	Blank			blk_001A	1	1:20
3	dog 014	+	103.41	dog_014A	1	2:00
4	Blank			blk_002A	1	2:40
5	057	-	0	dog_057A	1	3:20
6	045	-	0	dog_045A	1	4:00
7	152	-	0	dog_152A	1	4:40
8	161	-	0	dog_161A	1	5:20
9	155	-	0	dog_155A	1	6:00
10	hydrocarbons			hyd_002A	1	6:40
11	009	-	0	dog_009A	1	7:20
12	107	-	0	dog_107A	1	8:00
13	004	-	0	dog_004A	1	8:40
14	126	-	0	dog_126A	1	9:20
15	Blank			blk_003A	1	10:00
16	116	+	5.06	dog_116A	1	10:40
17	Blank			blk_004A	1	11:20
18	hydrocarbons			hyd_003A	1	12:00
19	088	-	0	dog_088A	1	12:40
20	115	-	0	dog_115A	1	13:20
21	103	-	0	dog_103A	1	14:00
22	018	-	0	dog_018A	1	14:40
23	Blank			blk_005A	1	15:20
24	105	+	1.03	dog_105A	1	16:00
25	Blank			blk_006A	1	16:40
26	hydrocarbons			hyd_004A	1	17:20
27	001	-	0	dog_001A	1	18:00
28	149	-	0	dog_149A	1	18:40
29	016	-	0	dog_016A	1	19:20
30	147	-	0	dog_147A	1	20:00
31	072	-	0	dog_072A	1	20:40
32	hydrocarbons			hyd_005A	1	21:20
33	Blank			blk_007A	1	22:00
34	150	+	0.44	dog_150A	1	22:40
35	Blank			blk_008A	1	23:20
36	070	-	0	dog_070A	1	24:00
37	052	-	0	dog_052A	2	0:40
38	hydrocarbons			hyd_006A	2	1:20

39	Blank			blk_009A	2	2:00
40	164	+	0.73	dog_164A	2	2:40
41	Blank			blk_010A	2	3:20
42	157	-	0	dog_157A	2	4:00
43	blank			blk_011A	2	4:40
44	123	+	33.74	dog_123A	2	5:20
45	blank			blk_012A	2	6:00
46	hydrocarbons			hyd_007A	2	6:40
47	121	-	0	dog_121A	2	7:20
48	blank			blk_013A	2	8:00
49	026	+	8.89	dog_026A	2	8:40
50	blank			blk_014A	2	9:20
51	035	+	12.82	dog_035A	2	10:00
52	blank			blk_015A	2	10:40
53	hydrocarbons			hyd_008A	2	11:20
54	173	-	0	dog_173A	2	12:00
55	021	-	0	dog_021A	2	12:40
56	082	-	0	dog_082A	2	13:20
57	094	-	0	dog_094A	2	14:00
56	099	-	0	dog_099A	2	14:40
59	hydrocarbons			hyd_009A	2	15:20
60	163	-	0	dog_163A	2	16:00
61	153	-	0	dog_153A	2	16:40
62	169	-	0	dog_169A	2	17:20
63	171	-	0	dog_171A	2	18:00
64	013	-	0	dog_013A	2	18:40
65	hydrocarbons			hyd_010A	2	19:20
66	106	-	0	dog_106A	2	20:00
67	036	-	0	dog_036A	2	20:40
68	blank			blk_016A	2	21:20
69	132	+	32.73	dog_132A	2	22:00
70	blank			blk_017A	2	22:40
71	hydrocarbons			hyd_011A	2	23:20
72	032	-	0	dog_032A	3	0:40
73	061	-	0	dog_061A	3	1:20
74	068	-	0	dog_068A	3	2:00
75	blank			blk_018A	3	2:40
76	146	+	0.41	dog_146A	3	3:20
77	blank			blk_019A	3	4:00
78	hydrocarbons			hyd_012A	3	4:40
79	142	-	0	dog_142A	3	5:20
80	015	-	0	dog_015A	3	6:00
81	blank			blk_020A	3	6:40
82	041	+	2.14	dog_041A	3	7:20
83	blank			blk_021A	3	8:00
84	hydrocarbons			hyd_013A	3	8:40

85	139	-	0	dog_139A	3	9:20
86	125	-	0	dog_125A	3	10:00
87	043	-	0	dog_043A	3	10:40
88	029	-	0	dog_029A	3	11:20
89	024	-	0	dog_024A	3	12:00
90	hydrocarbons			hyd_014A	3	12:40
91	Blank			blk_022A	3	13:20

Rep. B		**PCR result**	**No. of parasites per ml of blood**	**GC/MS file name**	**Day**	**Run Time (hr:mins) 12 mins to reinject**
1	hydrocarbons			hyd_001B	1	0:40
2	70	-	0	dog_070B	1	1:20
3	155	-	0	dog_155B	1	2:00
4	173	-	0	dog_173B	1	2:40
5	169	-	0	dog_169B	1	3:20
6	Blank			blk_001B	1	4:00
7	35	+	12.82	dog_035B	1	4:40
8	Blank			blk_002B	1	5:20
9	hydrocarbons			hyd_002B	1	6:00
10	29	-	0	dog_029B	1	6:40
11	82	-	0	dog_082B	1	7:20
12	9	-	0	dog_009B	1	8:00
13	Blank			blk_003B	1	8:40
14	123	+	33.74	dog_123B	1	9:20
15	Blank			blk_004B	1	10:00
16	106	-	0	dog_106B	1	10:40
17	hydrocarbons			hyd_003B	1	11:20
18	Blank			blk_005B	1	12:00
19	14	+	103.41	dog_014B	1	12:40
20	Blank			blk_006B	1	13:20
21	150	+	0.44	dog_150B	1	14:00
22	Blank			blk_007B	1	14:40
23	152	-	0	dog_152B	1	15:20
24	52	-	0	dog_052B	1	16:00
25	hydrocarbons			hyd_004B	1	16:40
26	163	-	0	dog_163B	1	17:20
27	43	-	0	dog_043B	1	18:00

28	13	-	0	dog_013B	1	18:40
29	Blank			blk_008B	1	19:20
30	146	+	0.41	dog_146B	1	20:00
31	Blank			blk_009B	1	20:40
32	hydrocarbons			hyd_005B	1	21:20
33	103	-	0	dog_103B	1	22:00
34	142	-	0	dog_142B	1	22:40
35	126	-	0	dog_126B	1	23:20
36	88	-	0	dog_088B	1	24:00
37	99	-	0	dog_099B	2	0:40
38	hydrocarbons			hyd_006B	2	1:20
39	15	-	0	dog_015B	2	2:00
40	121	-	0	dog_121B	2	2:40
41	57	-	0	dog_057B	2	3:20
42	Blank			blk_010B	2	4:00
43	164	+	0.73	dog_164B	2	4:40
44	Blank			blk_011B	2	5:20
45	1	-	0	dog_001B	2	6:00
46	hydrocarbons			hyd_007B	2	6:40
47	147	-	0	dog_147B	2	7:20
48	Blank			blk_012B	2	8:00
49	132	+	32.73	dog_132B	2	8:40
50	Blank			blk_013B	2	9:20
51	139	-	0	dog_139B	2	10:00
52	125	-	0	dog_125B	2	10:40
53	hydrocarbons			hyd_008B	2	11:20
54	Blank			blk_014B	2	12:00
55	26	+	8.89	dog_026B	2	12:40
56	Blank			blk_015B	2	13:20
57	161	-	0	dog_161B	2	14:00
58	24	-	0	dog_024B	2	14:40
59	hydrocarbons			hyd_009B	2	15:20
60	72	-	0	dog_072B	2	16:00
61	21	-	0	dog_021B	2	16:40
62	18	-	0	dog_018B	2	17:20
63	94	-	0	dog_094B	2	18:00
64	hydrocarbons			hyd_010B	2	18:40
65	Blank			blk_016B	2	19:20
66	41	+	2.14	dog_041B	2	20:00

67	Blank			blk_017B	2	20:40
68	68	-	0	dog_068B	2	21:20
69	36	-	0	dog_036B	2	22:00
70	hydrocarbons			hyd_011B	2	22:40
71	153	-	0	dog_153B	2	23:20
72	115	-	0	dog_115B	2	24:00
73	4	-	0	dog_004B	3	0:40
74	157	-	0	dog_157B	3	1:20
75	Blank			blk_018B	3	2:00
76	116	+	5.06	dog_116B	3	2:40
77	Blank			blk_019B	3	3:20
78	hydrocarbons			hyd_012B	3	4:00
79	32	-	0	dog_032B	3	4:40
80	149	-	0	dog_149B	3	5:20
81	45	-	0	dog_045B	3	6:00
82	107	-	0	dog_107B	3	6:40
83	16	-	0	dog_016B	3	7:20
84	hydrocarbons			hyd_013B	3	8:00
85	171	-	0	dog_171B	3	8:40
86	Blank			blk_020B	3	9:20
87	105	+	1.03	dog_105B	3	10:00
88	Blank			blk_021B	3	10:40
89	61	-	0	dog_061B	3	11:20
90	hydrocarbons			hyd_014B	3	12:00
91	Blank			blk_022B	3	12:40

Rep. C		**PCR result**	**No. of parasites per ml of blood**	**GC/MS file name**	**Day**	**Run Time (hr:mins) 12 mins to reinject**
1	hydrocarbons			hyd_001C	1	0:40
2	126	-	0	dog_126C	1	1:20
3	Blank			blk_001C	1	2:00
4	105	+	1.029	dog_105C	1	2:40
5	Blank			blk_002C	1	3:20
6	4	-	0	dog_004C	1	4:00
7	Blank			blk_003C	1	4:40
8	26	+	8.893	dog_026C	1	5:20
9	Blank			blk_004C	1	6:00

10	hydrocarbons			hyd_002C	1	6:40
11	1	-	0	dog_001C	1	7:20
12	163	-	0	dog_163C	1	8:00
13	169	-	0	dog_169C	1	8:40
14	121	-	0	dog_121C	1	9:20
15	149	-	0	dog_149C	1	10:00
16	16	-	0	dog_016C	1	10:40
17	107	-	0	dog_107C	1	11:20
18	hydrocarbons			hyd_003C	1	12:00
19	32	-	0	dog_032C	1	12:40
20	Blank			blk_005C	1	13:20
21	146	+	0.413	dog_146C	1	14:00
22	Blank			blk_006C	1	14:40
23	29	-	0	dog_029C	1	15:20
24	hydrocarbons			hyd_004C	1	16:00
25	Blank			blk_007C	1	16:40
26	35	+	12.82	dog_035C	1	17:20
27	Blank			blk_008C	1	18:00
28	41	+	2.139	dog_041C	1	18:40
29	Blank			blk_009C	1	19:20
30	106	-	0	dog_106C	1	20:00
31	hydrocarbons			hyd_005C	1	20:40
32	Blank			blk_010C	1	21:20
33	150	+	0.442	dog_150C	1	22:00
34	Blank			blk_011C	1	22:40
35	139	-	0	dog_139C	1	23:20
36	52	-	0	dog_052C	1	24:00
37	36	-	0	dog_036C	2	0:40
38	57	-	0	dog_057C	2	1:20
39	hydrocarbons			hyd_006C	2	2:00
40	15	-	0	dog_015C	2	2:40
41	Blank			blk_012C	2	3:20
42	116	+	5.06	dog_116C	2	4:00
43	Blank			blk_013C	2	4:40
44	61	-	0	dog_061C	2	5:20
45	hydrocarbons			hyd_007C	2	6:00
46	9	-	0	dog_009C	2	6:40
47	18	-	0	dog_018C	2	7:20

48	68	-	0	dog_068C	2	8:00
49	24	-	0	dog_024C	2	8:40
50	153	-	0	dog_153C	2	9:20
51	hydrocarbons			hyd_008C	2	10:00
52	Blank			blk_014C	2	10:40
53	14	+	103.409	dog_014C	2	11:20
54	Blank			blk_015C	2	12:00
55	155	-	0	dog_155C	2	12:40
56	21	-	0	dog_021C	2	13:20
57	171	-	0	dog_171C	2	14:00
58	hydrocarbons			hyd_009C	2	14:40
59	103	-	0	dog_103C	2	15:20
60	152	-	0	dog_152C	2	16:00
61	115	-	0	dog_115C	2	16:40
62	72	-	0	dog_072C	2	17:20
63	157	-	0	dog_157C	2	18:00
64	hydrocarbons			hyd_010C	2	18:40
65	43	-	0	dog_043C	2	19:20
66	82	-	0	dog_082C	2	20:00
67	173	-	0	dog_173C	2	20:40
68	88	-	0	dog_088C	2	21:20
69	hydrocarbons			hyd_011C	2	22:00
70	Blank			blk_016C	2	22:40
71	123	+	33.739	dog_123C	2	23:20
72	Blank			blk_017C	2	24:00
73	70	-	0	dog_070C	3	0:40
74	147	-	0	dog_147C	3	1:20
75	142	-	0	dog_142C	3	2:00
76	hydrocarbons			hyd_012C	3	2:40
77	Blank			blk_018C	3	3:20
78	164	+	0.731	dog_164C	3	4:00
79	Blank			blk_019C	3	4:40
80	125	-	0	dog_125C	3	5:20
81	Blank			blk_020C	3	6:00
82	132	+	32.726	dog_132C	3	6:40
83	Blank			blk_021C	3	7:20
84	hydrocarbons			hyd_013C	3	8:00
85	161	-	0	dog_161C	3	8:40

86	45	-	0	dog_045C	3	9:20
87	94	-	0	dog_094C	3	10:00
88	99	-	0	dog_099C	3	10:40
89	13	-	0	dog_013C	3	11:20
90	hydrocarbons			hyd_014C	3	12:00
91	Blank			blk_022C	3	12:40

Supplementary Tables 9.1A, 9.1B and 9.1C. HP5-MS GC/MS run schedule for 2017 cohort of dogs. For the HP5-MS analysis the samples were run in triplicate (replicate A, B and C) using the automatic injection function on the GC/MS. Hexane blanks were run before and after each positive dog samples, with hydrocarbon standards injected randomly throughout the analysis.

Rep. D		PCR result	No. of parasites per ml of blood	GC/MS file name	Run Time (hr:mins) 12mins to reinject
1	hydrocarbons			hyd_001DB	0:40
2	Blank			blk_001DB	1:20
3	dog 014	+	103.41	dog_014DB	2:00
4	Blank			blk_002DB	2:40
5	045	-	0	dog_045DB	4:00
6	152	-	0	dog_152DB	4:40
7	155	-	0	dog_155DB	6:00
8	hydrocarbons			hyd_002DB	6:40
9	009	-	0	dog_009DB	7:20
10	107	-	0	dog_107DB	8:00
11	004	-	0	dog_004DB	8:40
12	126	-	0	dog_126DB	9:20
13	Blank			blk_004DB	11:20
14	hydrocarbons			hyd_003DB	12:00
15	088	-	0	dog_088DB	12:40
16	115	-	0	dog_115DB	13:20
17	103	-	0	dog_103DB	14:00
18	018	-	0	dog_018DB	14:40
19	Blank			blk_005DB	15:20
20	105	+	1.03	dog_105DB	16:00
21	Blank			blk_006DB	16:40
22	hydrocarbons			hyd_004DB	17:20
23	001	-	0	dog_001DB	18:00
24	149	-	0	dog_149DB	18:40
25	016	-	0	dog_016DB	19:20
26	147	-	0	dog_147DB	20:00
27	hydrocarbons			hyd_005DB	21:20
28	Blank			blk_007DB	22:00
29	150	+	0.44	dog_150DB	22:40
30	Blank			blk_008DB	23:20
31	070	-	0	dog_070DB	24:00
32	052	-	0	dog_052DB	0:40
33	hydrocarbons			hyd_006DB	1:20
34	Blank			blk_009DB	2:00
35	164	+	0.73	dog_164DB	2:40
36	Blank			blk_010DB	3:20
37	157	-	0	dog_157DB	4:00
38	blank			blk_011DB	4:40
39	123	+	33.74	dog_123DB	5:20
40	blank			blk_012DB	6:00
41	hydrocarbons			hyd_007DB	6:40
42	121	-	0	dog_121DB	7:20
43	blank			blk_013DB	8:00

44	026	+	8.89	dog_026DB	8:40
45	blank			blk_014DB	9:20
46	035	+	12.82	dog_035DB	10:00
47	blank			blk_015DB	10:40
48	hydrocarbons			hyd_008DB	11:20
49	021	-	0	dog_021DB	12:40
50	082	-	0	dog_082DB	13:20
51	094	-	0	dog_094DB	14:00
52	099	-	0	dog_099DB	14:40
53	hydrocarbons			hyd_009DB	15:20
54	163	-	0	dog_163DB	16:00
55	153	-	0	dog_153DB	16:40
56	171	-	0	dog_171DB	18:00
57	013	-	0	dog_013DB	18:40
58	hydrocarbons			hyd_010DB	19:20
59	106	-	0	dog_106DB	20:00
60	036	-	0	dog_036DB	20:40
61	blank			blk_016DB	21:20
62	132	+	32.73	dog_132DB	22:00
63	blank			blk_017DB	22:40
64	hydrocarbons			hyd_011DB	23:20
65	032	-	0	dog_032DB	0:40
66	061	-	0	dog_061DB	1:20
67	blank			blk_018DB	2:40
68	146	+	0.41	dog_146DB	3:20
69	blank			blk_019DB	4:00
70	hydrocarbons			hyd_012DB	4:40
71	142	-	0	dog_142DB	5:20
72	blank			blk_020DB	6:40
73	041	+	2.14	dog_041DB	7:20
74	blank			blk_021DB	8:00
75	hydrocarbons			hyd_013DB	8:40
76	139	-	0	dog_139DB	9:20
77	125	-	0	dog_125DB	10:00
78	043	-	0	dog_043DB	10:40
79	029	-	0	dog_029DB	11:20
80	024	-	0	dog_024DB	12:00
81	hydrocarbons			hyd_014DB	12:40
82	Blank			blk_022DB	13:20

Supplementary Table 9.2. DB Wax GC/MS run schedule for 2017 cohort of dogs. HP5-MS replicate A was repeated using the DB-wax column (replicate D) however these samples were manually injected. Hexane blanks were run before and after each positive dog samples, with hydrocarbon standards injected

Rep. HP5-MS 2018		PCR result	No. of parasites per ml of blood	GC/MS file name
1	hydrocarbons			hyd2018_001HP5
2	Blank			blk2018_001HP5
3	dog 017	+	28.10	dog2018_017HP5
4	Blank			blk2018_002HP5
5	dog 169	-	0	dog2018_169HP5
6	Blank			blk2018_003HP5
7	dog 082	+	128.00	dog2018_082HP5
8	Blank			blk2018_004HP5
9	hydrocarbons			hyd2018_002HP5
10	dog 153	-	0	dog2018_153HP5
11	Blank			blk2018_005HP5
12	dog 007	+	38.70	dog2018_007HP5
13	Blank			blk2018_006HP5
14	dog 096	+	5.63	dog2018_096HP5
15	Blank			blk2018_007HP5
16	hydrocarbons			hyd2018_003HP5
17	dog 124	-	0	dog2018_124HP5
18	Blank			blk2018_008HP5
19	dog 003	+	233.68	dog2018_003HP5
20	Blank			blk2018_009HP5
21	dog 009	-	0	dog2018_009HP5
22	hydrocarbons			hyd2018_004HP5
23	dog 028	-	0	dog2018_028HP5
24	dog 110	-	0	dog2018_110HP5
25	dog 070	-	0	dog2018_070HP5
26	Blank			blk2018_010HP5
27	dog 176	+	50.91	dog2018_176HP5
28	Blank			blk2018_011HP5
29	dog 112	+	49.38	dog2018_112HP5
30	Blank			blk2018_012HP5
31	hydrocarbons			hyd2018_005HP5
32	dog 032	-	0	dog2018_032HP5
33	Blank			blk2018_013HP5
34	dog 105	+	8.50	dog2018_105HP5
35	Blank			blk2018_014HP5
36	dog 117	+	36.41	dog2018_117HP5
37	Blank			blk2018_015HP5
38	dog 062	+	24.50	dog2018_062HP5
39	Blank			blk2018_016HP5
40	dog 074	+	115.93	dog2018_074HP5

41	Blank			blk2018_017HP5
42	dog 125	-	0	dog2018_125HP5
43	hydrocarbons			hyd2018_006HP5
44	Blank			blk2018_018HP5
45	dog 114	+	27.68	dog2018_114HP5
46	Blank			blk2018_019HP5
47	dog 059	+	25.45	dog2018_059HP5
48	Blank			blk2018_020HP5
49	dog 019	+	61.59	dog2018_019HP5
50	Blank			blk2018_021HP5
51	dog 130	-	0	dog2018_130HP5
52	Blank			blk2018_022HP5
53	dog 054	+	27.68	dog2018_054HP5
54	Blank			blk2018_023HP5
55	dog 075	+	31.50	dog2018_075HP5
56	Blank			blk2018_024HP5
57	dog 068	+	27.47	dog2018_068HP5
58	Blank			blk2018_025HP5
59	dog 034	+	15.16	dog2018_034HP5
60	Blank			blk2018_026HP5
61	hydrocarbons			hyd2018_007HP5
62	dog 004	-	0	dog2018_004HP5
63	dog 043	-	0	dog2018_043HP5
64	Blank			blk2018_027HP5
65	dog 134	+	50.53	dog2018_134HP5
66	Blank			blk2018_028HP5
67	dog 127	+	36.41	dog2018_127HP5
68	Blank			blk2018_029HP5
69	dog 047	+	1.30	dog2018_047HP5
70	Blank			blk2018_030HP5
71	dog 140	+	40.51	dog2018_140HP5
72	Blank			blk2018_031HP5
73	dog 023	-	0	dog2018_023HP5
74	dog 131	-	0	dog2018_131HP5
75	hydrocarbons			hyd2018_008HP5
76	Blank			blk2018_032HP5
77	dog 102	+	6.92	dog2018_102HP5
78	Blank			blk2018_033HP5
79	dog 136	-	0	dog2018_136HP5
80	Blank			blk2018_034HP5
81	dog 178	+	28.32	dog2018_178HP5
82	Blank			blk2018_035HP5
83	hydrocarbons			hyd2018_009HP5
84	dog 045	-	0	dog2018_045HP5
85	Blank			blk2018_036HP5
86	dog 077	+	18.48	dog2018_077HP5

87	Blank			blk2018_037HP5
88	dog 142	-	0	dog2018_142HP5
89	dog 021	-	0	dog2018_021HP5
90	Blank			blk2018_038HP5
91	dog 080	+	21.69	dog2018_080HP5
92	Blank			blk2018_039HP5
93	dog 016	-	0	dog2018_016HP5
94	dog 010	-	0	dog2018_010HP5
95	hydrocarbons			hyd2018_010HP5
96	dog 175	-	0	dog2018_175HP5
97	blank			blk2018_040HP5
98	dog 126	+	853.44	dog2018_126HP5
99	blank			blk2018_041HP5
100	dog 137	-	0	dog2018_137HP5
101	blank			blk2018_042HP5
102	dog 141	+	13.73	dog2018_141HP5
103	blank			blk2018_043HP5
104	dog 103	+	41.76	dog2018_103HP5
105	blank			blk2018_044HP5
106	dog 093	-	0	dog2018_093HP5
107	hydrocarbons			hyd2018_011HP5
108	dog 005	-	0	dog2018_005HP5
109	dog 172	-	0	dog2018_172HP5
110	dog 181	-	0	dog2018_181HP5
111	dog 037	-	0	dog2018_037HP5
112	hydrocarbons			hyd2018_012HP5
113	blank			blk2018_045HP5
114	dog 044	+	27.89	dog2018_044HP5
115	blank			blk2018_046HP5
116	dog 118	-	0	dog2018_118HP5
117	dog 090	-	0	dog2018_090HP5
118	dog 042	-	0	dog2018_042HP5

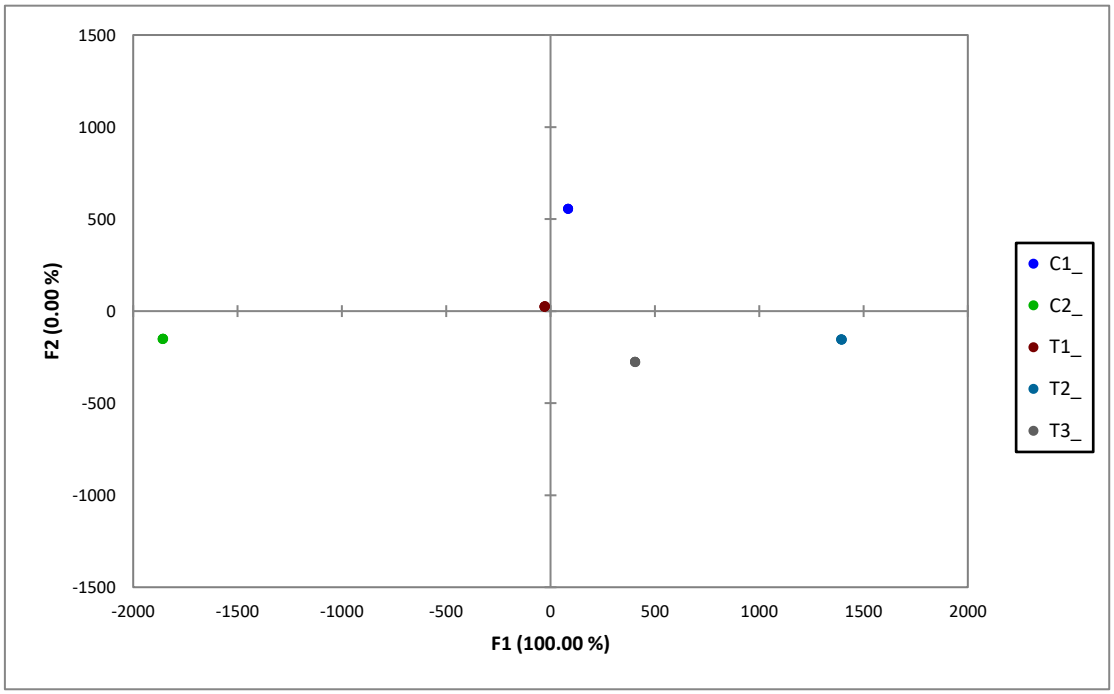
Supplementary Table 9.3. HP5-MS GC/MS run schedule for 2018 cohort of dogs. For the HP5-MS analysis the samples were manually injected into the GC/MS following the schedule above. Hexane blanks were run before and after each positive dog samples, with hydrocarbon standards injected randomly throughout the analysis.

Rep. DB-Wax 2018		PCR result	No. of parasites per ml of blood	GC/MS file name
1	hydrocarbons			hyd2018_001 DB
2	Blank			blk2018_001 DB
3	dog 017	+	28.10	dog2018_017 DB
4	Blank			blk2018_002 DB
5	dog 169	-	0	dog2018_169 DB
6	Blank			blk2018_003 DB
7	dog 082	+	128.00	dog2018_082 DB
8	Blank			blk2018_004DB
9	hydrocarbons			hyd2018_002 DB
10	dog 153	-	0	dog2018_153 DB
11	Blank			blk2018_005 DB
12	dog 007	+	38.70	dog2018_007 DB
13	Blank			blk2018_006 DB
14	dog 096	+	5.63	dog2018_096 DB
15	Blank			blk2018_007 DB
16	hydrocarbons			hyd2018_003 DB
17	dog 124	-	0	dog2018_124 DB
18	Blank			blk2018_008 DB
19	dog 003	+	233.68	dog2018_003 DB
20	Blank			blk2018_009 DB
21	dog 009	-	0	dog2018_009 DB
22	hydrocarbons			hyd2018_004 DB
23	dog 028	-	0	dog2018_028 DB
24	dog 110	-	0	dog2018_110 DB
25	dog 070	-	0	dog2018_070 DB
26	Blank			blk2018_010 DB
27	dog 176	+	50.91	dog2018_176 DB
28	Blank			blk2018_011 DB
29	dog 112	+	49.38	dog2018_112 DB
30	Blank			blk2018_012 DB
31	hydrocarbons			hyd2018_005 DB
32	dog 032	-	0	dog2018_032 DB
33	Blank			blk2018_013 DB
34	dog 105	+	8.50	dog2018_105 DB
35	Blank			blk2018_014 DB
36	dog 117	+	36.41	dog2018_117 DB
37	Blank			blk2018_015 DB
38	dog 062	+	24.50	dog2018_062 DB
39	blank			blk2018_016 DB
40	dog 074	+	115.93	dog2018_074 DB

41	blank			blk2018_017 DB
42	dog 125	-	0	dog2018_125 DB
43	hydrocarbons			hyd2018_006 DB
44	blank			blk2018_018 DB
45	dog 114	+	27.68	dog2018_114 DB
46	blank			blk2018_019 DB
47	dog 059	+	25.45	dog2018_059 DB
48	blank			blk2018_020 DB
49	dog 019	+	61.59	dog2018_019 DB
50	blank			blk2018_021 DB
51	dog 130	-	0	dog2018_130 DB
52	blank			blk2018_022 DB
53	dog 054	+	27.68	dog2018_054 DB
54	blank			blk2018_023 DB
55	dog 075	+	31.50	dog2018_075 DB
56	Blank			blk2018_024 DB
57	dog 068	+	27.47	dog2018_068 DB
58	Blank			blk2018_025 DB
59	dog 034	+	15.16	dog2018_034 DB
60	blank			blk2018_026 DB
61	hydrocarbons			hyd2018_007 DB
62	dog 004	-	0	dog2018_004 DB
63	dog 043	-	0	dog2018_043 DB
64	blank			blk2018_027 DB
65	dog 134	+	50.53	dog2018_134 DB
66	blank			blk2018_028 DB
67	dog 127	+	36.41	dog2018_127 DB
68	blank			blk2018_029 DB
69	dog 047	+	1.30	dog2018_047 DB
70	blank			blk2018_030 DB
71	dog 140	+	40.51	dog2018_140 DB
72	blank			blk2018_031 DB
73	dog 023	-	0	dog2018_023 DB
74	dog 131	-	0	dog2018_131 DB
75	hydrocarbons			hyd2018_008 DB
76	blank			blk2018_032 DB
77	dog 102	+	6.92	dog2018_102 DB
78	blank			blk2018_033 DB
79	dog 136	-	0	dog2018_136 DB
80	blank			blk2018_034 DB
81	dog 178	+	28.32	dog2018_178 DB
82	blank			blk2018_035 DB
83	hydrocarbons			hyd2018_009 DB
84	dog 045	-	0	dog2018_045 DB
85	blank			blk2018_036 DB
86	dog 077	+	18.48	dog2018_077 DB

87	blank			blk2018_037 DB
88	dog 142	-	0	dog2018_142 DB
89	dog 021	-	0	dog2018_021 DB
90	blank			blk2018_038 DB
91	dog 080	+	21.69	dog2018_080 DB
92	blank			blk2018_039 DB
93	dog 016	-	0	dog2018_016 DB
94	dog 010	-	0	dog2018_010 DB
95	hydrocarbons			hyd2018_010 DB
96	175	-	0	dog2018_175 DB
97	blank			blk2018_040 DB
98	dog 126	+	853.44	dog2018_126 DB
99	blank			blk2018_041 DB
100	dog 137	-	0	dog2018_137 DB
101	blank			blk2018_042 DB
102	dog 141	+	13.73	dog2018_141 DB
103	blank			blk2018_043 DB
104	dog 103	+	41.76	dog2018_103 DB
105	blank			blk2018_044 DB
106	dog 093	-	0	dog2018_093 DB
107	hydrocarbons			hyd2018_011 DB
108	dog 005	-	0	dog2018_005 DB
109	dog 172	-	0	dog2018_172 DB
110	dog 181	-	0	dog2018_181 DB
111	dog 037	-	0	dog2018_037 DB
112	hydrocarbons			hyd2018_012 DB
113	blank			blk2018_045 DB
114	dog 044	+	27.89	dog2018_044 DB
115	blank			blk2018_046 DB
116	dog 118	-	0	dog2018_118 DB
117	dog 090	-	0	dog2018_090 DB
118	dog 042	-	0	dog2018_042 DB

Supplementary Table 9.4. DB-Wax GC/MS run schedule for 2018 cohort of dogs. For the DB-Wax analysis the samples were manually injected into the GC/MS following the schedule above. Hexane blanks were run before and after each positive dog samples, with hydrocarbon standards injected randomly throughout the analysis.



Supplementary Figure 9.5: DFA with multiple variables of the VOCs released from in vitro cultures of *Leishmania* and the growth media controls. 5ml of each sample was collected and stored in a 50mL glass vial prior to VOC Analyser analysis. Analysis was performed according to the methodology outlined in Chapter 4, with the headspace of the samples passed over the sensor head. C1: HOMEM medium; C2: Medium 199; T1: $[10]^6$ *Le. infantum*/mL: T2: $[10]^6$ *Le. mexicana*/mL; T3: $[10]^6$ *Le. tarantolae*/mL; F1: discriminant function 1; F2: discriminant function 2. The numbers in parentheses show the percentages of the data matrix as described by the functions and relevant components. Results were generated by XLstat version 3.4.

Appendix

Appendix 1: Staniek, M.E., Sedda, L., Gibson, T.D., de Souza, C.F., Costa, E.M., Dillon, R.J., Hamilton, J.G.C. 2019. eNose analysis of volatile chemicals from dogs naturally infected with *Leishmania infantum* in Brazil. PLoS Negl Trop Dis 13(8): e0007599. https://doi.org/10.1371/journal.pntd.0007599

Appendix 2: Field work documentation, licence and letters for research equipment UK export and Brazilian import

Division of Biomedical and Life Sciences | Lancaster University

28 de Março de 2018

A QUEM POSSA INTERESSAR

Esta carta confirma que os itens listados abaixo são equipamentos serem utilizados conforme descritos no o projeto financiado pelo Wellcome Trust (Reino Unido). Este projeto é uma colaboração entre a universidade de Lancaster (Reino Unido) e o Instituto Fiocruz (Rio de Janeiro, Brasil):

- 200 tubos de EDTA
- 1 Kit de DNA extracao
- 400 sacos de papel aluminio
- 200 almofadas de fibra de carbono

A Srta. Monica Staniek está a transportar o equipamento a serem utilizados única e exclusivamente para fins de pesquisa. Estes equipamentos não possuem valor comercial e não representam qualquer risco químico ou biológico.

Nossos agradecimentos,
Dr. Gordon Hamilton
Tel: 0044 1524 592326
Email: j.g.hamilton@lancaster.ac.uk

Division of Biomedical and Life Sciences | Lancaster University

28th March 2018

To Whom It May Concern:

This is to confirm that the items listed below are equipment to be used as described in a project funded by the Wellcome Trust (United Kingdom). This project is a collaboration between the University of Lancaster (United Kingdom) and the Fiocruz Institute (Rio de Janeiro, Brazil):

- 200 EDTA tubes
- 1 DNA extraction kit
- 400 foil bags
- 200 carbon fibre pads

Miss Monica Staniek is transporting these to be used solely for research purposes. These items of equipment are of no commercial value and present no chemical or biological risk.

Yours sincerely
Dr. Gordon Hamilton
Tel: 0044 1524 592326
Email: j.g.hamilton@lancaster.ac.uk

Appendix 3: Dog Health Questionnaires and Consent Forms

HOUSE ID:	NAME:	ADDRESS: GPS:	INVESTIGATOR:

MICROCHIP BARCODE/BIOPSY BARCODE	NAME OF DOG:	ORIGIN OF DOG:	Leishmania treatment? 1.Y ☐ 0.N ☐
Sample date:	AGE (MONTHS): Confident? 1.Y ☐ 0.N ☐ SEX 1.M ☐ 2.F ☐	1. Brought in from street ☐ 2. Born in house ☐ 3. Born in town ☐ 4. Born out of town ☐ 5. Unknown ☐	Leishmania vaccination ☐ Date: Collars Scalibor ☐ Other ☐ Advantix ☐ Citronella ☐ Anti-parasite ☐

CLINICAL SYMPTOMS	0	1	2	3		0	1	2	3
Dermatitis					Lesions				
Alopecia					Conjunctivitis				
Hyperkeratitis					Lunettes				
Long nails					Uveitis				

BODY SCORE	
1. Very, very thin	
2. Thin	
3. Normal	
4. Fat	

ECTOPARASITES	0	1	2	3
Fleas				
Lice				
Ticks				

Observations/Treatment info:

Ministério da Saúde
FIOCRUZ
Fundação Oswaldo Cruz
Instituto Oswaldo Cruz

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Título do projeto: O Papel do odor de cães infectados por *Leishmania infantum* na atração de *Lutzomyia longipalpis,* e sua utilização como base para diagnóstico precoce da leishmaniose viscer*al.,* Governador Valadares, Minas Gerais, Brasil.

Nome do pesquisador principal: Reginaldo Peçanha Brazil

Razão Social da instituição da CEUA que aprovou: Instituto Oswaldo Cruz - CIAEP nº 01.0234.2014

Objetivos do estudo:

O objetivo do presente trabalho é utilizar cães da cidade de Governador Valadares, Minas Gerais para determinar se o odor de cães infectados com *Leishmania infantum* é mais atraente para os flebotomíneos da espécie *Lutzomyia longipalpis* do que odor de cães não infectados pelo parasita, e determinar se as alterações no odor podem estar relacionadas com o grau de parasitemia do cão, em busca de um diagnóstico precoce da doença nos cães.

Procedimentos a serem realizados com os animais:

Após a contenção física do anim*al.,* será realizado a coleta de sangue periférico das veias radi*al.,* coleta de pelos e implante de um microchip para a identificação do cão. Os procedimento de coleta de sangue e pelos serão realizados por três vezes ao longo de dois anos.

Os animais serão mantidos junto com o proprietário durante todo o período de coleta. As analises serão realizadas após todo o período de coleta e comparadas, o resultado será disponibilizado posteriormente para o proprietário e para a Centro de Controle de Zoonoses do Município de Governador Valadares-MG.

Potenciais riscos para os animais:

Os procedimentos adotados não apresentam riscos para os cães, além de todo o procedimento ser acompanhado pelo Médico Veterinário responsável.

Cronograma:

Composto de três momentos de coletas de sangue periférico e pelos, e no momento da primeira coleta será instalado um microchip para identificar o cão. Serão coletados amostras de sangue até completar o número máximo de 300 cães. As coletas terão início apenas depois que for estabelecido a autorização da CEUA, e terminara quando completar coleta de sangue de 300 cães.

Benefícios:

Em inúmeros focos de leishmanioses descritos no mundo, observa-se uma grande variedade de animais mamíferos incriminados como possíveis reservatórios ou hospedeiros acidentas. Entre os animais domésticos descritos envolvidos no ciclo de transmissão de diferentes espécies de *Leishmania,* o cão *(Canis familiaris)* apresenta papel de destaque, devido sua participação enquanto hospedeiro doméstico,

Há evidências de que os parasitas são capazes de manipular seus hospedeiros de maneiras que melhoram suas perspectivas de transmissão para novos hospedeiros. Um exemplo são parasitas de insetos que modificam o habito alimentar do inseto para sangue para terem mais chances de o parasita ser transmitido. É de conhecimento na medicina que durante centenas de anos parasitas e outras formas de infecções podem modificar o odor dos acometidos, é tanto que para isso a medicina utiliza-se do cheiro (ou mesmo gosto) de urina para diagnosticar doenças. O estudo sugerido pretende detectar alterações no odor de cães ou identificar biomarcadores de odor específicos utilizando métodos analíticos modernos.

O projeto aqui proposto é uma sequência de outros estudos que vem sendo desenvolvido em laboratório, onde foi observada uma mudança significativa na atratividade de *Lutzomyia longipalpis* por hamsters infectados com *Leishmania infantum*. Estas alterações observadas na atratividade foram resultados de alterações apenas no odor dos hospedeiros. Embora hamsters não são os hospedeiros naturais de *Leishmania infantum* é possível que o mesmo efeito possa ocorrer em hospedeiros naturais como os cães. De fato, é descrito que insetos vetores se alimentam mais frequentemente em cães com alta parasitemia de *Leishmania infantum,* quando comparados a cães não infectados e cães infectados têm um perfil de odor diferente em comparação com cães não infectados.

Portanto, é provável que os cães infectados com *Leishmania infantum* são mais atraentes do que os cães não infectados, e é possível que a alteração no odor seja detectável antes que as alterações clinicas de leishmaniose visceral possam ser observadas.

O projeto proposto tem objetivo de investigar esse fenômeno, em uma combinação entre reservatório (cães) / vetor natural (*Lutzomyia longipalpis*), por meio de modernas metodologias de química analítica, bioquímica, comportamental e estatística.

O projeto proposto irá investigar a possibilidade de que um equipamento eletrônico de mão que possa ser capaz de distinguir entre cães infectados (em um estágio em que os sintomas de infecção não são visíveis) e cães não infectados, analisando o odor dos cães. Uma ferramenta de diagnóstico confiável, preciso, rápido e não invasiva seria extremamente útil para reduzir a carga real e potencial dessa doença.

Esclarecimentos ao proprietário sobre a participação do animal neste projeto:

Sua autorização para a inclusão de seu animal neste estudo é voluntária.

A confidencialidade dos seus dados pessoais serão preservadas.

Os membros da CEUA ou as autoridades regulatórias poderão solicitar suas informações e, nesse caso, elas serão dirigidas especificamente para fins de inspeções regulares.

A médica veterinária responsável pelo(s) seu(s) animal(is) será a Dra. Erika Moutinho Costa, inscrito(a) no CRMV-RJ sob o no 7.996. Além dela, e a equipe do Pesquisador Princip*al.,* Dr. Reginaldo Peçanha Brazil, também se responsabilizará pelo bem-estar do(s) seu(s) animal(is) durante a coleta do sangue periférico. Caso necessário, após a coleta, você poderá entrar em contato com a equipe pelo contato:

Equipe: Dr. Reginaldo Brazil, Dra. Erika Moutinho Costa, Dr. Cristian Ferreira de Souza, Monica Staniek.

Endereço: Av. Brasil 4365 - Manguinhos – Rio de Janeiro/RJ – CEP: 21040-900

Telefone: (21) 25621468

DECLARAÇÃO DE CONSENTIMENTO

Fui devidamente esclarecido(a) sobre todos os procedimentos deste estudo, seus riscos e benefícios ao(s) animal(is) pelo(s) qual(is) sou responsável. Fui também informado que posso retirar meu(s) animal(is) do estudo a qualquer momento. Ao assinar este Termo de Consentimento, declaro que autorizo a participação do(s) meu(s) animal(is), identificado(s) a seguir, neste projeto.

Este documento será assinado em duas vias, sendo que uma via ficará comigo e a outra com o pesquisador.

_____

(Cidade), dd/mm/aaaa

_____ _____

Assinatura do Responsável Assinatura do Pesquisador

Responsável

Nome:

Documento de Identidade:
(quando aplicável)

Identificação do(s) animal(is)
(repetir tantas vezes quantos forem os animais)

Nome: Número de identificação:

Espécie: Raça:

Appendix 4: Record of a risk assessment for the field work to be undertaken in Brazil

Record of a risk assessment

Task:
Field work in Brazil is to determine the *Leishmania* infection status of individual dogs and to monitor progression of the infection over time. The involves the collection of blood samples from domesticated dogs this work will be done by a qualified experienced Brazilian vet in accordance with local and UK ethical approvals.

DNA will be extracted from the blood samples in a laboratory in FioCruz, Rio and returned to the UK.

The odour analysis will be done by collecting hair samples from the dorsal region of each individual dog using disposable razors a minimum of 2g of hair being collected.

Department	BLS	Assessment ID	
Assessor	Monica Staniek + Gordon Hamilton	Date of assessment	20/07/2017
Authorised by		Review date	

Step 1 **List significant hazards**	**Step 2** **who might be harmed**	**Step 3** **determine appropriate controls**	**Step 4** **make it happen**
Handling of dog blood potentially infected with *Leishmania infantum* and other pathogens.	Main researcher (Monica Staniek)	I will be wearing PPE; Lab coats and gloves. Any skin abrasions/wounds to be covered with plaster before handling the EDTA tubes either in the field or in the lab. Correct procedures for the handling of biological samples will be implemented. EDTA tubes containing collected blood samples will be handled using	Details of this risk assessment, scheme of work, correct handling of canines and safety procedures to be made clear to all team members prior to work commencing.

		gloves and sealed until DNA extraction occurs. DNA extraction from the blood samples will be done in a fully appropriately equipped lab in FioCruz, Rio. Risk of contamination will be reduced by restricting the DNA extraction to an extraction hood. I have received previous training and authorisation to work with *L. infantum* cultures inn LU and so is competent to work with media likely to contain pathogenic material.	
Dog Bite	All team members working within close proximity to dogs	All dogs will be muzzled and restrained by the vet and dog owner.	Details of this risk assessment, scheme of work, correct handling of canines and safety procedures to be made clear to all team members prior to work commencing.
Handling of sharps (BIC Razors)	Main researcher (Monica Staniek)	Correct handling of razor. All sharps will be disposed of in a sharps bin	Details of this risk assessment, scheme of work, correct handling of canines and safety procedures to be made clear to all team members prior to work commencing.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

eNose analysis of volatile chemicals from dogs naturally infected with *Leishmania infantum* in Brazil

Monica E. Staniek[1☯], Luigi Sedda[2☯], Tim D. Gibson[3], Cristian F. de Souza[4], Erika M. Costa[5], Rod J. Dillon[1], James G. C. Hamilton[1]*

1 Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancashire, United Kingdom, **2** Centre for Health Informatics Computation and Statistics, Lancaster Medical School, Faculty of Health and Medicine, Lancaster University, Lancashire, United Kingdom, **3** RoboScientific Ltd., Espace North, Littleport, Cambridgeshire, **4** Fiotec (Rio de Janeiro), Avenida Brasil, Manguinhos, Rio de Janeiro, Brazil, **5** Laboratório de Pesquisa em Leishmaniose, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil

☯ These authors contributed equally to this work.
* j.g.hamilton@lancaster.ac.uk

Citation: Staniek ME, Sedda L, Gibson TD, de Souza CF, Costa EM, Dillon RJ, et al. (2019) eNose analysis of volatile chemicals from dogs naturally infected with *Leishmania infantum* in Brazil. PLoS Negl Trop Dis 13(8): e0007599. https://doi.org/10.1371/journal.pntd.0007599

Editor: Guilherme L. Werneck, Universidade do Estado do Rio de Janeiro, BRAZIL

Received: April 16, 2019

Accepted: July 3, 2019

Published: August 6, 2019

Data Availability Statement: All data are included in the manuscript and supporting information, sections 3 and 4.

Funding: MS was the recipient of a PhD studentship from the Division of Biomedical and Life Sciences, Lancaster University. JGCH was the recipient of award 104250/B/14/Z from The Wellcome Trust (https://wellcome.ac.uk).The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Background

Visceral leishmaniasis (VL) in Brazil is a neglected, vector-borne, tropical parasitic disease that is responsible for several thousand human deaths every year. The transmission route involves sand flies becoming infected after feeding on infected reservoir host, mainly dogs, and then transmitting the *Leishmania infantum* parasites while feeding on humans. A major component of the VL control effort is the identification and euthanasia of infected dogs to remove them as a source of infection. A rapid, non-invasive, point-of-care device able to differentiate between the odours of infected and uninfected dogs may contribute towards the accurate diagnosis of canine VL.

Methodology/Principal findings

We analysed the headspace volatile chemicals from the hair of two groups of dogs collected in 2017 and 2018 using a bench-top eNose volatile organic chemical analyser. The dogs were categorised as infected or uninfected by PCR analysis of blood samples taken by venepuncture and the number of parasites per ml of blood was calculated for each dog by qPCR analysis. We demonstrated using a robust clustering analysis that the eNose data could be discriminated into infected and uninfected categories with specificity >94% and sensitivity >97%. The eNose device and data analysis were sufficiently sensitive to be able to identify infected dogs even when the *Leishmania* population in the circulating blood was very low.

Conclusions/Significance

The study illustrates the potential of the eNose to rapidly and accurately identify dogs infected with *Le. infantum*. Future improvements to eNose analyser sensor sensitivity, sampling methodology and portability suggest that this approach could significantly improve the

Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: TDG is a founding director and equity holder in Roboscientific Ltd. All the other authors declare that no competing interests exist.

diagnosis of VL infected dogs in Brazil with additional potential for effective diagnosis of VL in humans as well as for the diagnosis of other parasitic diseases.

Author summary

Visceral leishmaniasis (VL) is an insect transmitted, tropical parasitic disease and in Brazil it causes thousands of human deaths every year. Domestic dogs can also be infected, and they are a risk factor for people. The Brazilian Ministry of Health tries to control the disease in 3 ways; first by reducing the population of insects that can carry the disease, second by using therapeutic drugs to treat the disease in humans, and third by identifying and euthanising infected dogs. However, despite these efforts the burden of VL has doubled since 2010 and a significant contributing factor is the lack of a rapid and accurate pathway for diagnosing dogs. In this study we have shown that an eNose can differentiate between the smell of VL infected and uninfected dogs. The analysis was highly sensitive i.e. if the dog was infected, the eNose would detect it in > 97% of the cases and it was highly specific i.e. if the dog was uninfected eNose detect it in >94% of the cases. The outcome was not dependant on the numbers of parasites or the clinical status of the dog. The results suggest that eNose analysis could be used to identify VL infected dogs with improved the speed and accuracy compared to current methods.

Introduction

Visceral leishmaniasis (VL) is a neglected tropical disease caused by protist parasites belonging to the genus *Leishmania*. Globally over 350 million people are at risk of infection with an estimated 200–400 thousand cases annually and an estimated 10% fatality rate. Ninety percent of all reported VL cases occur in only six countries including Brazil[1, 2].

In Brazil, transmission of *Leishmania (Leishmania) infantum* (Kinetoplastida: Trypanosomatidae) occurs between domestic dogs *Canis familiaris* (Carnivora: Canidae) (the reservoir host) and from dogs to humans when an infected female sand fly vector *Lutzomyia longipalpis* (Diptera: Psychodidae) takes a blood meal.

Despite substantial efforts by the Brazilian Ministry of Health (MoH) the burden of VL in Brazil more than doubled between 1990 and 2016[3]. The increase is probably due to the spread of the vector into urban areas as a result of human migration into cities[4] and the expansion of the range of the vector into new areas because of environmental degradation[5–7]. Given the spread of the disease and increase in cases it is also likely that current VL control measures are inadequate[8].

The control of VL in Brazil has three main components. Insecticides are applied in houses and animal sheds to lower the vector population density and reduce vector-human contact. Secondly, diagnosis and treatment of human cases to prevent severe forms of the disease and death. Finally, the identification and euthanasia of seropositive canine cases to decrease the sources of infection for the vector[9, 10].

Modelling predicts that the dog-culling program in Brazil should be effective in areas of low, medium but not high *Leishmania* transmission[11]. However, the practice is controversial and despite the euthanasia of thousands of canines with suspected and confirmed infection each year the program has been unsuccessful[10, 12]. There are a number of possible

explanations for this situation A). Shortage of qualified professionals caused by financial constraints leading to delays in collections, performance of routine diagnostic tests and subsequent removal of seropositive dogs. B). Failure to identify and remove the high proportion of asymptomatic animals C). Refusal of dog owners to comply with surveillance measures. D). The high rate of dog replacement with young immunologically naïve dogs. E). Lack of an accurate point-of-care diagnostic test[13].

Identification of dogs infected with canine VL (CVL) follows a two stage serodiagnostic protocol recommended by the Brazilian MoH. Initial screening using the Dual-Path Platform (DPP CVL) immunochromatography diagnostic test is followed by a laboratory-based ELISA (EIE CVL) confirmatory test. Overall the 2-step protocol was reported to have a 73% sensitivity and 98% specificity however the relatively low sensitivity indicates the maintenance of false-negative dogs in endemic areas which represents a public health concern[14].

The DPP CVL test has also been assessed several times since it was introduced and most recently it has been shown that overall it has 86% sensitivity and 94% specificity[14] or 89% sensitivity and 70% specificity[15].

The concept of volatile organic compounds (VOCs) as diagnostic aids to signal a disease is well established and since antiquity, many physicians have used odours associated with disease to help diagnose their patients[16]. Modern analytical techniques such as single ion flow tube mass spectrometry (SIFT-MS) and chemi-resistive sensors have taken the concept to the point of widespread clinical application. Volatile markers from human breath can be used to identify a variety of disease states e.g. inflammatory bowel disease, chronic liver disease, diabetes, *Pseudomonas aeruginosa* infection and adenocarcinomas[17, 18]. A recent study has shown that the use of VOCs is sufficiently robust to discriminate between 14 cancerous and other disease states[19].

Parasite infections of humans and animals also alter the odour of the host animal. The odours of golden hamsters infected with *Le. infantum* are more attractive to female sand flies than the odours of uninfected hamsters[20, 21]. The odour obtained from the hair of dogs infected with *Le. infantum* in Brazil was found to be significantly different to the odour of uninfected dogs. These odour differences which were detected by coupled gas chromatography-mass spectrometry (GC/MS) and multivariate statistical analysis indicated the increased presence of a small number of primarily low molecular weight aldehydes (octanal, nonanal), alkanes (undecane, heptadecane) and 2-ethylhexyl-salicylate[22, 23]. More recently, odours were also implicated in children infected with the infectious gametocyte stage of the malaria parasite *Plasmodium falciparum were* found to be more attractive to the mosquito vector *Anopheles gambiae*[24]. This phenomenon occurred even when the gametocytemia was very low and was associated with changes in aldehyde concentration of the foot odours of the infected children[25, 26].

GC/MS analysis is a useful research tool but its use as a widely available diagnostic tool is unrealistic because of significant costs associated with the infrastructure and personnel costs. An alternative means of detecting the odour change associated with parasitaemia is required that would fulfil the majority of the World Health Organisation ASSURED criteria; affordable, sensitive, specific, user-friendly, rapid and robust, equipment free and deliverable to end-users [27] for all new point-of-care diagnostics tools. VOC analysers (eNoses) may fulfil WHO criteria, they can detect differences in the odours from sputum of tuberculosis (TB) infected and TB uninfected patients with sensitivity, specificity and accuracy of around 70%[28]. The aim of the present study was to determine if the odour of dogs naturally infected with *Le. infantum* could be detected with high sensitivity and specificity using a commercially available VOC analyser.

Methods

Study site

Governador Valadares (18˚51′S, 41˚56′W) (Minas Gerais State, Brazil), located in the valley of the Rio Doce 320 km northeast of Belo Horizonte is a city of approximately 280,000 people. The climate is temperate, characterised by dry winters and hot, wet summers[29]. Studies in Governador Valadares in 2013 found that an average of 30% of dogs from 16,529 samples taken from 35 urban and rural districts were seropositive for canine visceral leishmaniasis (CVL)[30]. From 2008 until 2017, 194 human VL cases were recorded in Governador Valadares with a fatality rate of 15.5%[31].

Ethics

Dog blood and hair samples were taken from dogs that were also microchipped with the informed consent of their owners. Ethical approval was obtained from the Comissão de Ética no Uso de Animais (CEUA), Instituto Oswaldo Cruz (licence L-027/2017) in Brazil and Lancaster University Animal Welfare and Ethics Review Board (AWERB) in the UK. The CEUA approval complies with the provisions of Brazilian Law 11794/08, which provides for the scientific use of animals, including the principles of Brazilian Society of Science in Laboratory Animals (SBCAL). The AWERB approval complies with the UK Home Office guidelines of the Animals in Science Regulation Unit (ASRU) and in compliance with the Animals (Scientific Procedures) Act (ASPA) 1986 (amended 2012) regulations and was consistent with UK Animal Welfare Act 2006.

Dog recruitment

A 2 year cohort study in the Altinópolos district of Governador Valadares was initiated in August 2017 by initial recruitment and sampling of 185 dogs. The area was chosen because of the high prevalence of CVL (average incidence 33.8%)[30] and the large population of household-owned dogs (ca. 2000) (Centro de Controle de Zoonoses (CCZ) survey) located there. The dogs were microchipped to aid their identification. Inclusion criteria: dogs aged $\geq$ 3 months, dogs without previous clinical assessment or laboratorial diagnosis for CVL. Exclusion criteria: pregnant/lactating bitches; aggressive dogs; stray dogs. In April 2018 149 dogs were sampled, this number included 133 dogs that were resampled from the 2017 cohort and an additional 16 from CCZ which had been collected in the same area and at the same time as our sample collections.

Between 5ml and 10ml of peripheral blood was collected in 10ml K2 EDTA-coated tubes (BD Vacutainer, UK) via cephalic or jugular venepuncture by a qualified vet in 2017 and by a CCZ qualified phlebotomist in 2018. Samples were placed in containers marked with the microchip bar code to aid subsequent tracking and identification. Blood samples were stored in a cool box with a freezer pack before being transferred to a fridge (4˚C) prior to processing.

Hair samples were obtained by cutting the dorsal hair close to the skin using surgical scissors that had been washed with hexane prior to the collection of each sample by members of the LU research team. A minimum of 2g of hair was collected from each dog. All hair samples were placed in individual foil bags (110mm x 185mm; Polypouch UK Ltd, Watford, England) heat sealed and stored at 4˚C prior to analysis.

All dogs were assessed for clinical signs of *Leishmania* infection by veterinarians and CVL control specialists at CCZ. The animals were classified according to the presence of clinical signs which were recorded for each dog. The main signs of CVL considered were onychogryphosis, ophthalmologic abnormalities, adenitis, cachexia, hepatosplenomegaly, alopecia,

crusted ulcers and lesions; dogs were classified as asymptomatic (the absence of clinical signs), oligosymptomatic (the presence of one to three clinical signs), or symptomatic (the presence of more than three clinical signs[32].

Molecular diagnosis of dogs

DNA extraction. Collected blood samples were centrifuged at 2500 x g for 10 minutes at room temperature and the top layer of buffy coat removed, placed in 1.5ml Eppendorf tubes and stored at -20˚C until DNA extraction. The DNA was extracted from 200μl of buffy coat samples using the QIAamp DNA Blood Mini Kit following the manufacturer's instructions. Cell lysis was mediated using protein kinase with a final elution volume of 50μl.

Qualitative detection of Leishmania DNA. Conventional PCR was initially used to ascertain which blood samples were positive for *Leishmania infantum*. Although the sensitivity of PCR is not 100% and is not considered to be as accurate as the direct parasitological assessment of lymph node or bone marrow aspirates, it is highly sensitive, rapid, requires minimal facilities, avoids potential dog-odour contamination issues and is less distressing for the dogs.

Following primer optimization, extracted DNA from canine blood obtained during August 2017 and April 2018 were tested using Primer pair MaryF (5'–CTT TTC TGG TCC TCC GGG TAG G– 3'), and MaryR (5'- CCA CCC GGC CCT ATT TTA CAC CAA– 3' [33]. The reactions were performed in a final volume of 25μl containing 0.5μl DNA template (100ng μl-1), 12.5μl Mastermix (dH_2O, Buffer 5x, MyTaq redmix polymerase, dNTP's) and 10μM of each primer. The PCR amplifications were performed in a TECHNE Prime Thermal Cycler (Cole-Palmer Ltd., Staffordshire, UK) using the following conditions: 95˚C for 5mins and 30 cycles of 95˚C for 30sec, 57˚C for 30sec and 72˚C for 60sec, followed by 72˚C for 10min.

The PCR products were analysed by gel electrophoresis using 2% agarose gels run at 90V for 1hr 30 minutes and visualized under UV light following the addition of 6.5μl of 10,000x SYBR Safe (Thermo Fisher Scientific, UK) to each gel. Samples were run 3 times and dogs were considered to be infected if 2 or 3 out of the 3 replicates were positive.

Canine beta globin house-keeping gene was used to monitor the performance of the amplification and check for DNA degradation as the samples were transported from Brazil to the UK. Amplification of the constitutive canine globin gene was performed using the primers: '5—CAA CTT CAT CCA CGT TCA CC– 3' and '5—ACA CAA CTG TGT TCA CTA GC– 3' [34]. Positive control was leishmania culture DNA at a 10^6 parasites ml^{-1} concentration. Negative control was obtained by performing DNA extraction on 200μl of water instead of buffy coat in Brazil under the same conditions as the blood.

Quantitative detection of Leishmania DNA. A real-time quantitative PCR (qPCR) for detection and quantification of *Le. infantum* DNA in positive dog samples from both sampling occasions (August 2017 and April 2018) was performed using MaryF/R primers.

The qPCR amplifications were performed on a Bio-Rad C1000 Thermal Cycler with each reaction consisting of a final volume of 13.0μl; 12.0μL of PCR mix plus 1μL of DNA (approximately 75–100 ng/μl per reaction). The qPCR mix was composed of 6.25 μL 2x QuantiNova SYBR Green PCR Master Mix, 0.5 μL of each primer (MaryF/R, corresponding to 10 mmol) and 4.75 μL of water[35].

The amplification was performed in triplicate[36] at 94˚C for 10 min, followed by 40 cycles at 94˚C for 30 sec, 60˚C for 20 sec and 72˚C for 20 sec. At the end of each run, a melt curve analysis was performed from 55˚C to 95˚C in order to identify the formation of non-specific products as well as primer dimers. A standard curve was established using extracted *Le. infantum* DNA; 1:10 serial dilutions, ranging from 10,000 to 0.01 parasites per ml and used to quantify the number of parasites in the dog blood samples.

VOC analysis

Initial VOC analysis was carried out on all (n = 11) of the infected dog hair samples and a subset of the uninfected dog hair samples (n = 44) collected in 2017. The choice of 44 uninfected dog hair samples (4 matched uninfected dog hair samples for each infected dog hair sample) was obtained by a power analysis to optimise the control size. The uninfected dogs were selected from groups of dogs matched by shared characteristics (age, sex and whether or not treatment for ectoparasites was received) with infected dogs (Table 1). Subsequently the VOC analysis was carried out on all the infected dog hair samples (n = 44, including 10 CCZ infected dogs) and all of the uninfected dog hair samples (n = 105, including 6 CCZ uninfected dogs) collected in 2018. The number of uninfected dog hair did not exceed the "4 uninfected for each infected dog" rule set above, and for this reason all the dogs were used.

A VOC analyser (Model 307, RoboScientific Ltd, Leeds, UK) with 11 functioning semi-conducting polymer sensors was used for the analysis. Each sensor has 2 outputs (positive and negative) giving a total of 22 responses. Two calibration points were automatically set by the sensor unit; the first was the baseline obtained when carbon-filtered air was passed over the sensor at a flow rate of 200ml min^{-1} which was automatically adjusted to zero on the Y-axis scale, and the second was a reference point obtained from sampling the head space of 5ml of a liquid water control in a plastic vial.

The chemical sensors were thin films of semi-conducting polymers deposited onto interdigitated gold structures on a silicon substrate. We used 12 different sensor types chosen from a group of polymers that included polyaniline, polythiophene and polypyrrole. Each sensor had semi-selectivity to a different group of volatile chemicals; aldehydes, alcohols, amines, organic acids and ketones etc. In this way a digital fingerprint of the VOC mixtures emanating from the samples was generated. Two similar sensor arrays were used in the study, the second array (used for the 2018 analysis) was a derivation of the first with 50% of the sensors being identical to the first array.

The interaction of the mixtures of VOCs in the samples with the semi-conducting polymer surfaces produced a change in electrical properties (e.g. voltage and resistance) over time. This change was measured, recorded and simultaneously displayed on the VOC analyser data logger screen for each sensor. Four parameters were used from each sensor response; the divergence from the baseline (maximum response), the integrated area under each response curve, absorbance and desorbance. Therefore, the total number of VOC measurements produced for each sample were 88 (11 sensors x 4 parameters and 2 outputs–positive or negative). The sampling profile was set at 2 seconds baseline, 7 seconds of absorption, a 1 second pause, 5 seconds desorption and 12 seconds flush to bring the sensors back to baseline.

Water (DD;10μl) was injected into each foil bag containing the dog hair samples with a Hamilton syringe and inflated with 140ml of laboratory air using a diaphragm pump. The samples were then incubated at 50˚C for 15 minutes in an oven, then allowed to cool to room temperature for 5 minutes prior to head space analysis.

Table 1. *Leishmania infantum* infection status of dogs sampled in Governador Valadares, Minas Gerais.

Month of sample collection	Source of dogs	Number of dogs sampled	Leishmania positive dogs (PCR)		
			symptomatic	oligosymptomatic	asymptomatic
Aug-17	Altinópolis	185	0	3	8
Apr-18	Altinópolis	133	3	7	24
Apr-18	CCZ GV	16	6	2	2

Dogs from Altinópolis, Governador Valadares sampled in August 2017 (n = 185) and April 2018 (n = 133). An additional 16 dogs, considered positive by CCZ, were sampled in April 2018. PCR experiments were performed in triplicate with both positive and negative dogs identified.

https://doi.org/10.1371/journal.pntd.0007599.t001

For the analysis each foil bag (containing the dog hair + water) was sampled by insertion of an 18-gauge needle connected to a PTFE tube through the sidewall of the bag with the tip placed into the head space of each bag. This was connected to the sample port of the VOC analyser and the head space sample was therefore passed over the 12 sensor surfaces. The original flow rate for the sampling was 200 ml min^{-1}. The headspace of each foil bag was sampled 4 times. The first sample was disregarded as potentially it could contain volatile carryover from the previous sample and thus, we retained the data from the next 3 samples for analysis. The individual dog hair samples in each experiment were tested randomly with each sample used once only.

Data analysis

To test the ability of the VOC analyser to differentiate between the odours of infected and uninfected dogs, we employed mclust[37], a model-based clustering and classification algorithm (R-CRAN statistical software[38]. This was applied to the known data classes (infected or uninfected dogs). The initial analysis indicated that the model was overfitted, therefore we identified the infected and uninfected dog sub-classes (unsupervised clustering) and the analysis was repeated [39]. The robustness of the classification was evaluated by out-of-sample cross validation (CV) while the within-group homogeneity of the overfitting models was evaluated by a novel algorithm developed by the authors and termed confounder cross validation (CCV). Finally, the importance of each variable produced by the VOC analyser in discriminating between the infected and uninfected sub-classes was assessed by variable permutation analysis. A more detailed explanation of the rationale for this analysis approach is provided in the S1 Material and a more extensive description of the algorithms is provided in[40].

The VOC analysis dataset contained data from:

a. Infected and uninfected dog hair collected in 2017.

b. Infected and uninfected dog odour collected in 2018 including samples collected from CCZ dogs.

Three replicate VOC analyser readings were obtained for each dog odour sample. These replicates were considered to be independent, i.e. the three VOC replicates for each dog were considered as coming from three different dogs (a common procedure for repeated data in clustering analyses).

The analysis aimed to identify any significant differences in the VOC analyser variables (used to obtain the means and covariances of the infected and uninfected classes and/or sub-classes) of infected and uninfected dogs so as to be able to accurately predict the infection state of newly sampled dogs. Initially, the data was evaluated to determine 1. if infected and uninfected dogs in both 2017 and 2018 could be statistically separated and 2. if the uninfected dogs in 2017 were statistically separate from uninfected dogs in 2018.

As described above, to take account of overfitting [41], we reclassified the infected and uninfected classes into sub-classes using the mclust function (mclust package). The optimal inferential method and number of subclasses for infected and uninfected classes was obtained by Bayesian information criterion (BIC) (S1 Material), bootstrapping and the likelihood ratio test (function mclustBootstrapLRT (mclust package).

Importance of variables: Cross-validation (CV) and confounder cross-validation (CCV) analyses. Once the best model (number of subclasses and model components) had been found, we tested for the importance of the variables in clustering by permutation analysis; while the predictive capacity of the model by using "leave-one-out" cross validation (CV); and finally, the capacity of the model to recognise sample confounders by developing a technique

named confounder cross-validation (CCV) (a test to evaluate the statistical homogeneity of the class). For the latter 10% of the data from the infected class were placed in the uninfected class and vice versa (leaving the remaining 90% in their correct class for training in both cases). These analyses were done by compiling algorithms that included some of the MCLUST components (Mclust, MclustDA, predict) and permutation functions. Additional information is provided in the S1 Material.

Results

Molecular diagnosis of dogs

Qualitative detection of Leishmania DNA. PCR revealed that 11/185 (6%) dogs were positive for *Le. infantum* infection in August 2017 and 34/133 (26%) in April 2018 (Table 1) representing a 20 percent increase in infection rate over the 8-month period between sampling points. The typical PCR results showed a band at 140bp of varying intensities representing a semi-quantitative indication of parasite presence in individual samples (Fig 1).

In 2017, 3 out of the 11 positive dogs presented as oligosymptomatic and 8 were asymptomatic. In 2018, 3 dogs were symptomatic, 7 were oligosymptomatic and 24 were asymptomatic (Table 1). Of the 174 uninfected 2017 dogs 42 were lost to follow-up in 2018. There were 7 oligosymptomatic dogs and 3 had become symptomatic in the remaining 133 dogs. In the 2017 cohort 55% of the infected dogs had 3 out of 3 positive PCR results and 45% had 2 out of 3 positive PCR results. In the 2018 field collected cohort 47% had 3/3 + PCR results and 53% had 2/3 + PCR results. In the 2018 CCZ collected cohort 80% had 3/3 + PCR results and 20% had 2/3 + PCR results. Of the 11 positive dogs found in 2017 only 1 (dog 105) was resampled in 2018. The fate of all the loss to follow-up dogs (n = 52) was recorded by CCZ and in common with other surveys [42] the dogs had mostly either; died (n = 20; 3 through non-illness related issues) or escaped, became lost or stolen (n = 4), their owners moved (n = 13) or refused further testing (n = 7).

The evaluation indicated that the most frequently occurring clinical signs were skin lesions including dermatitis (18% 2017; 27% 2018) and ulcerative lesions (0% 2017; 25% 2018), long nails (9% 2017; 25% 2018) and signs of conjunctivitis (18% 2017; 14% 2018).

PCR diagnosis of the 16 CCZ dogs, sampled in April 2018, that were assumed to be VL infected, indicated that 10 (63%) were positive and the remaining 6 cases were not infected.

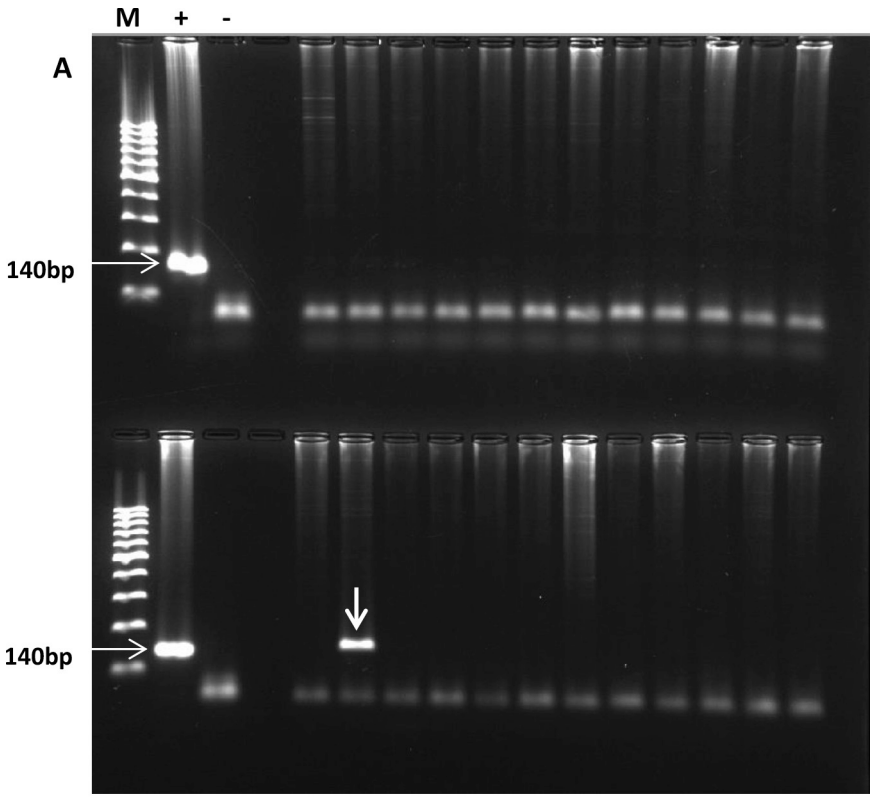
Quantitative detection of Leishmania DNA. The kDNA qPCR assay showed that parasite loads ranged from 0.4 to 103 parasites ml^{-1} in 2017 and from 1 to 850 parasite ml^{-1} in 2018 (both field and CCZ collected) (Fig 2).

This large variation in parasitic load over the study period can be observed through analysis of the median values which ranged from 5.06 parasites/ml (dog #116) in 2017 to 28.32 parasites/ml (dog #178) in 2018. Comparisons of parasitic load among the samples revealed that dog #126 in 2018 (853 parasites ml^{-1}) exhibited the highest degree of parasitism with dog #146 in 2017 (0.4 parasites ml^{-1}) exhibiting the lowest.

The average value of CT (ΔCT) obtained for dog #126 in 2018 (highest degree of parasitism) and for dog #146 in 2017 (lowest degree of parasitism) were the following: dog #126; 20.43 and dog #146; 30.45. A lower CT value correlates with a higher parasitic load per ml of blood.

Data analysis

The best model for the analysis of all infected and uninfected dog classes was EEE apart from the uninfected 2017 dogs which was VVI [40]. The EEE model assumes ellipsoidal covariances and equal shape, volume and orientation for all the classes. The VVI model assumes diagonal covariances with orientation parallel to the coordinate axes with variable shape and volume for all the classes [43]. Between 14 models x 1 to 9 classes were assessed (i.e. 126 mixture models)


M + -
A
140bp
140bp

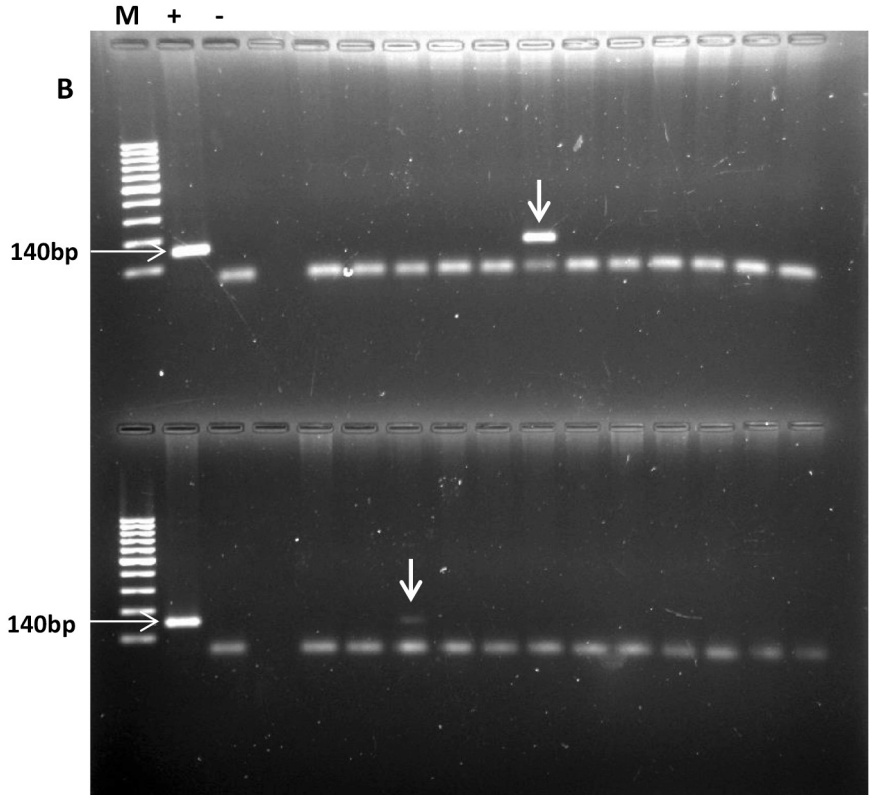

M + -
B
140bp
140bp

Fig 1. Preliminary detection of *Leishmania infantum* in dog blood samples. SYBR safe-stained 2% agarose gels showing the results of electrophoresis of 24 random PCR products from A; August 2017 dogs and B; April 2018 dogs, used to identify the presence of *Leishmania* in dog blood. Positive dogs are indicated by a 140bp band visible in line with the PCR product for the *Leishmania* positive control. M, molecular weight marker (100bp DNA ladder); "+" = positive control; "-" = negative control.

https://doi.org/10.1371/journal.pntd.0007599.g001

[44] (S1 Table (2017 data) and S2 Table (2018 data)). Clustering analysis of 2017 dogs identified 1 class for uninfected dogs and 3 classes for infected dogs and for the 2018 dogs 2 classes for uninfected dogs and 6 classes for infected dogs were identified.

Confusion matrices of the separation obtained from the training set of uninfected vs infected dogs in 2017 and uninfected vs infected dogs in 2018 without sub-classes are given in Table 2A and 2C respectively and with sub-classes in Table 2B and 2D respectively below.

These data show that in both years the infected dog odours were significantly different from the uninfected dog odours. In 2017 uninfected dogs were discriminated with 96% specificity and 97% sensitivity, that was improved to 100% for both metrics when the data was divided in sub-classes. The overall training error was reduced (from 2.8% to 0%) when the 2017 data was divided in sub-classes.

In 2018 uninfected and infected dogs were discriminated with 89% specificity and 100% sensitivity and that was improved to 94% specificity and 97% sensitivity when the data was divided in sub-classes. The overall training error was reduced (from 7.6% to 4.2%) when the 2018 data was divided in sub-classes.

Cross-validation (CV) and confounder cross-validation (CCV) analysis. When considering only the infected and uninfected classes, the CV analysis returned a reduced sensitivity of 50% and specificity of 84% for 2017 dogs, and 48% sensitivity and 96% specificity for 2018 dogs (Table 3A and 3B first line and first two columns) indicating a reduced capacity to estimate true positives compared to the training set (as reported in Table 2) due to overfitting. The CCV analysis suggested high heterogeneity of the infected and uninfected classes since the model is unable to identify the false positive and false negatives in the training groups. However, when the analyses were repeated on the EDDA models with sub-classes, both cross validation (CV) and confounder cross validation (CCV) calculations of sensitivity and specificity improved substantially (Table 3A and 3B second line). Thus, by identifying sub-classes for infected and uninfected dogs it was possible to obtain a better delineation of the multivariate space with improved predictivity capacity (CV analysis) and recognition of false positive and false negative in the two main macro-classes (infected and uninfected) of the training sets (increased homogeneity into sub-classes; CCV analysis).

The eNose variables important in the clustering are shown in S3 Table (2017 data) and S4 Table (2018 data). A 0.99 P-value indicates that in 99% of the permutations the number of optimal clusters changed, indicating a strong influence of the variable in the final clustering.

Discussion

The results presented in this study show that by combining VOC (eNose) data with robust clustering analysis we can identify dogs infected with *Le. infantum* by analysis of their odour with very high sensitivity and specificity, regardless of parasite load or the presentation of clinical symptoms. We observed this outcome in two data sets from dog hair samples collected in 2017 (99% [0.95,0.99] specificity and 90% [0.75,0.96] sensitivity) and in 2018 (89% [0.85,0.92] specificity and 100% 0.97,1] sensitivity). When the small size of both data sets (2017, 55 dog hair samples: 2018, 149 dog hair samples) and consequent potential for overfitting was accounted for by improving the mixture of the models, both sensitivity and specificity

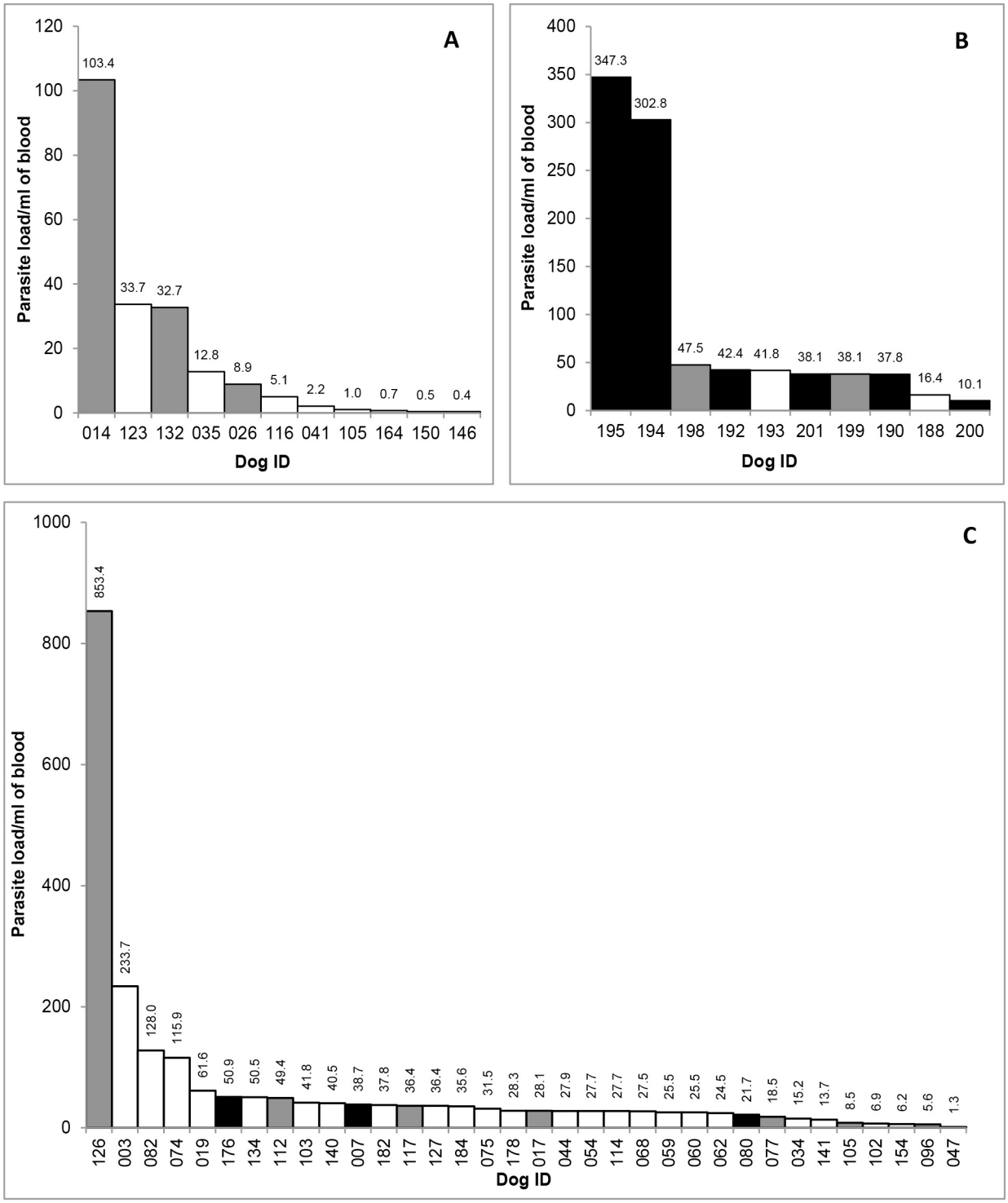


Fig 2. Quantitative estimation of *Leishmania infantum* in blood samples from infected dogs. kDNA qPCR assay showing the quantification of all positive samples from A; August 2017, B; CCZ (April 2018) and C; in the field (April 2018). All positive samples previously determined by conventional PCR were qualitatively analysed by qPCR to determine parasite loads of each positive dog. Range of parasite load; 0.4 parasites mL^{1} to 853 parasites mL^{-1}. White bars; asymptomatic dogs, grey bars; oligosymptomatic dogs and black bars; symptomatic dogs. Parasite load is per mL of blood. For clarity parasite load is given for all dogs in 2A and 2B but is excluded from dogs with similar loads in 2C. Dog identification number is given on the X-axis.

https://doi.org/10.1371/journal.pntd.0007599.g002

Table 2. Confusion matrices for Gaussian mixture model EDDA classification.

A. 2017: 1 uninfected and 1 infected dog class.				
observed\predicted	uninfected	infected	specificity	sensitivity
uninfected	111	1	0.99 (0.95,0.99)	
infected	3	28		0.90 (0.75,0.96)
training error:	0.028			
B. 2017. VVI model: 1 uninfected and 3 infected dog classes.				
uninfected	112	0	1 (0.96,1)	
infected	0	31		1 (0.88,1)
training error:	0			
C. 2018: 1 uninfected and 1 infected dog class.				
uninfected	280	34	0.89 (0.85,0.92)	
infected	0	132		1.00 (0.97,1)
training error	0.076			
D. 2018. EEE model: 2 uninfected and 6 infected dog classes.				
uninfected	298	16	0.94 (0.91,0.96)	
infected	3	129		0.97 (0.93,0.99
training error	0.042			

Training error is the average error, i.e. the ratio between correctly predicted class members and the total number of records e.g. in 1A above (111+28)/(111+28+1+3). Specificity and sensitivity 95% confidence intervals (based on binomial probabilities) are reported in brackets.

https://doi.org/10.1371/journal.pntd.0007599.t002

increased (2017; 100% [0.96,1] specificity, 100% [0.88,1] sensitivity: 2018; 94% [0.91,0.96] specificity, 97% [0.93,0.99] sensitivity).

The robustness of the models was further tested by cross-validation and an novel approach which we have termed confounder cross validation analyses. The model prediction was poor when we used 2 classes (infected and uninfected). However, when we accounted for the

Table 3. Comparison of sensitivity and specificity after CV and CCV analysis.

A	2017 dog samples			
Model	CV sensitivity	CV specificity	CCV sensitivity	CCV specificity
2 classes	0.50 (0.40,0.59)	0.84 (0.75,0.89)	0.11 (0.09,0.13)	0.60 (0.56,0.62)
4 classes	0.75 (0.65,0.82)	0.80 (0.71,0.86)	0.60 (0.56,0.62)	0.70 (0.67,0.72)
2 classes = 1 uninfected + 1 infected class			4 classes = 1 uninfected + 3 infected classes	
B	2018 dog samples			
2 classes	0.48 (0.38,0.57)	0.96 (0.90,0.98)	0.18 (0.15,0.20)	0.67 (0.64,0.69)
8 classes	0.93 (0.86,0.96)	0.92 (0.85,0.95)	0.74 (0.71,0.76)	0.84 (0.81,0.86)
2 classes = 1 uninfected + 1 infected			8 classes = 2 uninfected + 6 infected	

Specificity and sensitivity 95% confidence intervals (based on binomial probabilities) are reported in brackets. CV- cross validation; CCV- confounder cross validation.

https://doi.org/10.1371/journal.pntd.0007599.t003

heterogeneity within each of these classes and subdivided them into either 4 subclasses (2017 data) or 8 subclasses (2018 data) the sensitivity and specificity and their confidence intervals improved substantially. In both cases the models accurately placed dog odours in the correct infected or uninfected class with a high degree of specificity and sensitivity (93% sensitivity and 92% specificity).

The results suggested that the VOC analyser response was not related to the parasite load in the dog peripheral blood. As the analysis gave sensitivity and specificity responses substantially better than 90%, the effect of parasite load on the VOC analyser response is unlikely to have been significant as the majority of infected animals, regardless of parasite load were detected. However, determining the limits of detection will be important in the future.

Previous work has suggested that symptomatic dogs with a greater parasite load produced greater quantities of volatiles than infected asymptomatic dogs[23]. However, asymptomatic dogs can contribute to disease transmission and VL control strategies should target infectious dogs rather than infected dogs *per se* and in particular the super-spreaders in the population [21, 45]. In this study we identified *Leishmania* DNA in circulating blood obtained by cephalic and jugular venepuncture, however the relationship between numbers of circulating parasites in peripheral blood and the infection status of the dog is unclear. In future studies, the skin parasite load, which appears to be more closely related to infectiousness[45] could be correlated with the odour profile.

In our study we used molecular techniques, PCR and qPCR, to diagnose and quantify *Le. infantum* infection in dogs. Although the gold standard diagnosis is considered to be the direct parasitological assessment of lymph node or bone marrow aspirates, in this study we chose to take blood and hair samples from the dogs at their homes. This methodology reduced the possibility of cross-contamination between infected and uninfected dog odour which might have occurred if the dogs had been kept together e.g. at the CCZ facility. This less invasive sampling protocol also reduced stress on the dogs, did not require large facilities (e.g. for sedation required to obtain bone marrow aspirates), reduced the risk of infection to the animal and was more likely to receive owner consent and compliance.

A recent study[46] evaluated the accuracy of serological tests, immunochromatographic (Dual Path Platform: DPP) and enzyme-linked immunosorbent (ELISA EIE), for CVL in relation to the detection of Leishmania DNA through real-time PCR) in samples from symptomatic and asymptomatic dogs. The PCR analysis demonstrated greater homogeneity between symptomatic and asymptomatic groups of infected dogs compared with DPP and ELISA. Solcà et al. showed that The diagnosis of CVL through the amplification of kinetoplast DNA presented the highest rates of sensitivity and specificity in comparison with parasitological and serological methods[47]. These authors concluded that molecular methods are required to confirm the infection. Even though serological tests are routinely employed for diagnosing CVL, they have limitations in sensitivity, especially in asymptomatic dogs, and therefore may underestimate *Leishmania* infection rates[48]. Despite the high specificity, the serological tests present low capacity to detect Leishmania infection in relation to molecular tests [49]. The authors of that study concluded that their study "demonstrated that real-time PCR identified the presence of Leishmania DNA in asymptomatic dogs that had a negative result in serological tests recommended by the official Brazilian protocol for CVL. In addition in a recent study [50], 34 out of 36 (96%) *Leishmania* isolates from dogs sampled in GV were found to be *Le. infantum*, the other 2 isolates were from the *Leishmania mexicana* complex, *Le.* (*Le.*) *amazonensis Le.* (*Le.*) *mexicana*). Therefore, for the purposes of the current study our molecular diagnosis was likely to be representative of the true infection status of the dogs.

The prevalence of CVL recorded in our 2017 sample (6%) is low compared to the prevalence recorded in our 2018 sample (25.6%). It is possible that the extensive monitoring carried

out by Governador Valadares health authorities in the Altinópolis district of GV, where the study was carried out, immediately prior to our sample collection in 2017, had an impact on CVL prevalence.

However, these values are within the range of prevalence seen previously in studies carried out in the State of Minas Gerais generally e.g. a prevalence of 8.1% was observed in Belo Horizonte in dogs surveyed between 2008–2010[42] and 13.6% in Divinópolis in 2011[51]. In GV specifically, a study carried out in Altinópolis, between 2008–2011 found 33.8% of dogs surveyed to be infected[30] whereas a survey of dogs carried out in 2014–2015 found 22% of dogs to be positive by serology[52].

It has been suggested that change in odour of dogs infected with *Le. infantum* might be related to the immune response[23]. Changes in odour profile have been observed in other disease states where changes in relatively low molecular weight compounds were expressed as distinct and immediate changes arising from pathophysiological processes occurring and altering the body's metabolism[19]. However, although the very low parasite loads in some dogs might suggest recent infection, parasite load in the peripheral blood is not indicative[53] and as this study did not determine if the dogs had seroconverted it therefore remains unclear if the odour changes are related to the host immune response or not.

Our results also suggest that there was no relationship between clinical state of infection (symptomatic, oligosymptomatic and asymptomatic) and detector response. The analyser could accurately detect asymptomatic dogs with low parasite levels as well as symptomatic dogs with high parasite loads.

It has been proposed that manipulation of the hosts chemical communication system could enhance the transmission of the parasite to the insect vector and potentially have a significant effect on the epidemiology of the disease[20, 54, 55]. Our study examined volatile odours present on the dog hair only, it did not consider the effect of other volatiles, semi-volatiles and non-volatiles from other sources e.g. breath compounds, specialized scent gland secretions, sweat, urine or faeces[56]. The source of the odours that were detected by the VOC analyser is not clear, they could have arisen from the skin, as a result of the metabolic activity of skin microbiota[57], the immune response or potentially directly from the *Le. infantum* parasites.

Our study did not examine the effect of other infections and the ability of the VOC analyser to differentiate between dogs infected with *Le. infantum* and other *Leishmania spp*. or other infections was not determined. In Governador Valadares dogs infected with *Le. amazonensis* have been found[50] and the sand fly vector *Lu. longipalpis* infected with multiple *Leishmania spp*. have been also been found[58] indicating that the epidemiological features require further work.

The application of VOC analyser technology is potentially a significant step towards the application of volatile odour analysis in diagnosis of parasitic disease. It raises the possibility that in the future a modified VOC device could provide a rapid, accurate, non-invasive point-of-care diagnostic tool for the specific diagnosis of leishmaniasis in dogs and humans. In our study we found that a small proportion of the sensor variables (2 out of 88 in 2017 and 3 out of 88 in 2018) contributed significantly to the outcome. Therefore, there is considerable scope for enhancing the sensitivity and specificity of the device through modifications to the sensor chemistry as well as incorporating further improvements to the field collection and analysis of odour. As well as further developments in robustness, portability and simplicity of the device all of which would improve the reliability and utility in the field.

A reliable, rapid, accurate, non-invasive additional point-of-care test that identifies *Leishmania* infection using a different set of disease markers in addition to the DPP CVL test could potentially eliminate the need for the in-laboratory ELISA confirmatory test that currently fails to rapidly diagnose and remove infected dogs from the population. The ability of a VOC

analyser, that conforms to the WHO ASSURED criteria, to recognise infection in asymptomatic dogs and dogs with low levels of infection would be of great benefit to VL control programs.

The possible integration of a rapid, non-invasive and point-of-care test would be much better accepted by dog owners and could be an important epidemiological tool. In addition to being useful for the selection of infected animals for euthanasia, it could also be useful in the implementation, monitoring and evaluation of leishmania control activities such as insecticide impregnated dog collars that are currently being implemented and sex pheromone-based *Lu. longipalpis* control programs that are currently being evaluated[59, 60].

Further work to compare the sensitivity and specificity of a VOC test combined with DPP CVL diagnostics against DPP CVL combined with ELISA is required. The development of a non-invasive POC diagnostic tool based on host odour opens up a myriad range of opportunities to diagnose *Leishmania* infections in humans and other diseases such as malaria, trypanosomiasis and Chaga's disease.

Supporting information

S1 Table. Outcome of the mixture model analysis showing the top three models for the 2017 uninfected and infected dog data.
(DOCX)

S2 Table. Outcome of the mixture model analysis showing the top three models for the 2018 uninfected and infected dog data.
(DOCX)

S3 Table. Relative importance of different sensor variables in the contribution to the clustering observed in 2017 data.
(DOCX)

S4 Table. Relative importance of different sensor variables in the contribution to the clustering observed in 2018 data.
(DOCX)

S1 Material. Rationale for the data analysis.
(DOCX)

Acknowledgments

We are grateful to CCZ in GV for their permission and practical support to help carry out the work. Ricardo Alves Miranda phlebotomist (CCZ, Governador Valadares, MG) for collecting the dog blood samples in 2018. Ms M. Bell Lancaster University for providing technical support. Prof P. Bates (Division of Biomedical and Life Sciences, Lancaster University) who supplied *Leishmania infantum* promastigotes (strain M4192). We are profoundly grateful to the dog owners of Altinópolos, GV for allowing us to carry out this study on their cherished companion animals.

Author Contributions

Conceptualization: James G. C. Hamilton.

Formal analysis: Luigi Sedda, Tim D. Gibson, Rod J. Dillon, James G. C. Hamilton.

Funding acquisition: James G. C. Hamilton.

Investigation: Monica E. Staniek, Luigi Sedda, Cristian F. de Souza, Erika M. Costa, Rod J. Dillon.

Methodology: Monica E. Staniek, Luigi Sedda, Tim D. Gibson, Cristian F. de Souza, Erika M. Costa, Rod J. Dillon, James G. C. Hamilton.

Project administration: James G. C. Hamilton.

Resources: Tim D. Gibson.

Supervision: Rod J. Dillon, James G. C. Hamilton.

Writing – original draft: Monica E. Staniek, Luigi Sedda, Tim D. Gibson, Rod J. Dillon, James G. C. Hamilton.

Writing – review & editing: Monica E. Staniek, Luigi Sedda, Tim D. Gibson, Cristian F. de Souza, Erika M. Costa, Rod J. Dillon, James G. C. Hamilton.

References

1. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PloS one. 2012; 7(5):e35671–e. https://doi.org/10.1371/journal.pone.0035671 PMID: 22693548.
2. Organisation PAHOWH. Leishmaniasis: Epidemiological report in the Americas. 2019 Contract No.: 7.
3. Martins-Melo FR, Carneiro M, Ramos AN Jr., Heukelbach J, Ribeiro ALP, Werneck GL. The burden of Neglected Tropical Diseases in Brazil, 1990–2016: A subnational analysis from the Global Burden of Disease Study 2016. PLoS Negl Trop Dis. 2018; 12(6):e0006559. Epub 2018/06/05. https://doi.org/10.1371/journal.pntd.0006559 PMID: 29864133; PubMed Central PMCID: PMC6013251.
4. Werneck GL. Geographic spread of visceral leishmaniasis in Brazil. Cad Saude Publica. 2010; 26 (4):644–5. Epub 2010/06/01. PMID: 20512199.
5. Brazil RPB-R, U.; Brazil, B.G., editor Impact of climatic changes and habitat degradation on Phlebotominae (Diptera: Psychodidae) distribution and leishmaniases dispersion in Brazil. 7th International Symposium on Phlebotomine Sand flies 2011 25–30 April 2011; Turkey.
6. Salomon OD, Feliciangeli MD, Quintana MG, Afonso MM, Rangel EF. Lutzomyia longipalpis urbanisation and control. Mem Inst Oswaldo Cruz. 2015; 110(7):831–46. Epub 2015/10/31. https://doi.org/10.1590/0074-02760150207 PMID: 26517497; PubMed Central PMCID: PMC4660613.
7. Casanova C, Colla-Jacques FE, Hamilton JG, Brazil RP, Shaw JJ. Distribution of Lutzomyia longipalpis chemotype populations in Sao Paulo state, Brazil. PLoS Negl Trop Dis. 2015; 9(3):e0003620. Epub 2015/03/18. https://doi.org/10.1371/journal.pntd.0003620 PMID: 25781320; PubMed Central PMCID: PMC4363371.
8. Quinnell RJ, Courtenay O. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology. 2009; 136(14):1915–34. https://doi.org/10.1017/S0031182009991156 PubMed PMID: WOS:000273515200006. PMID: 19835643
9. Ministério da Saúde B. Manual de vigilância e controle da leishmaniose visceral In: Epidemiológica SdVeSDdV, editor. 1 ed. Brasília: Ministério da Saúde.; 2014. p. 120.
10. Albuquerque e Silva RdA A.J.; Quint B.B.; Raffoul G.E.S.; Werneck G.L.; Rangel E.F.; Romero G.A.S. Effectiveness of dog collars impregnated with 4% deltamethrin in controlling visceral leishmaniasis in Lutzomyia longipalpis (Diptera: Psychodidade: Phlebotominae) populations. Mem Inst Oswaldo Cruz. 2018; 113:9. https://doi.org/10.1590/0074-02760170083
11. Costa DN, Codeco CT, Silva MA, Werneck GL. Culling dogs in scenarios of imperfect control: realistic impact on the prevalence of canine visceral leishmaniasis. PLoS Negl Trop Dis. 2013; 7(8):e2355. Epub 2013/08/21. https://doi.org/10.1371/journal.pntd.0002355 PMID: 23951375; PubMed Central PMCID: PMC3738479.
12. Dantas-Torres F, Solano-Gallego L, Baneth G, Ribeiro VM, de Paiva-Cavalcanti M, Otranto D. Canine leishmaniosis in the Old and New Worlds: unveiled similarities and differences. Trends Parasitol. 2012; 28(12):531–8. Epub 2012/09/22. https://doi.org/10.1016/j.pt.2012.08.007 PMID: 22995719.
13. Zuben AP, Donalisio MR. Difficulties in implementing the guidelines of the Brazilian Visceral Leishmaniasis Control Program in large cities. Cad Saude Publica. 2016; 32(6). Epub 2016/06/23. https://doi.org/10.1590/0102-311X00087415 PMID: 27333135.

14. Fraga DB, Pacheco LV, Borja LS, Tuy PG, Bastos LA, Solca Mda S, et al. The Rapid Test Based on Leishmania infantum Chimeric rK28 Protein Improves the Diagnosis of Canine Visceral Leishmaniasis by Reducing the Detection of False-Positive Dogs. PLoS Negl Trop Dis. 2016; 10(1):e0004333. Epub 2016/01/06. https://doi.org/10.1371/journal.pntd.0004333 PMID: 26731098; PubMed Central PMCID: PMC4701173.

15. Figueiredo FB, Vasconcelos TCB, Madeira MF, Menezes RC, Maia-Elkhoury ANS, Marcelino AP, et al. Validation of the Dual-path Platform chromatographic immunoassay (DPP(R) CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. Mem Inst Oswaldo Cruz. 2018; 113(11):e180260. Epub 2018/11/01. https://doi.org/10.1590/0074-02760180260 PMID: 30379198; PubMed Central PMCID: PMC6216333.

16. Liddell K. Smell as a diagnostic marker. Postgrad Med J. 1976; 52(605):136–8. Epub 1976/03/01. https://doi.org/10.1136/pgmj.52.605.136 PMID: 1264934; PubMed Central PMCID: PMC2496390.

17. Kumar S, Huang J, Abbassi-Ghadi N, Mackenzie HA, Veselkov KA, Hoare JM, et al. Mass Spectrometric Analysis of Exhaled Breath for the Identification of Volatile Organic Compound Biomarkers in Esophageal and Gastric Adenocarcinoma. Ann Surg. 2015; 262(6):981–90. Epub 2015/01/13. https://doi.org/10.1097/SLA.0000000000001101 PMID: 25575255.

18. Smith D, Spanel P. Status of selected ion flow tube MS: accomplishments and challenges in breath analysis and other areas. Bioanalysis. 2016; 8(11):1183–201. Epub 2016/05/24. https://doi.org/10.4155/bio-2016-0038 PMID: 27212131.

19. Nakhleh MK, Amal H, Jeries R, Broza YY, Aboud M, Gharra A, et al. Diagnosis and Classification of 17 Diseases from 1404 Subjects via Pattern Analysis of Exhaled Molecules. ACS Nano. 2017; 11(1):112–25. https://doi.org/10.1021/acsnano.6b04930 PMID: 28000444

20. O'Shea B, Rebollar-Tellez E, Ward RD, Hamilton JGC, El Naiem D, Polwart A. Enhanced sandfly attraction to Leishmania-infected hosts. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2002; 96(2):117–8. https://doi.org/10.1016/s0035-9203(02)90273-7 PMID: 12055795

21. Nevatte TM, Ward RD, Sedda L, Hamilton JGC. After infection with Leishmania infantum, Golden Hamsters (Mesocricetus auratus) become more attractive to female sand flies (Lutzomyia longipalpis). Sci Rep. 2017; 7(1):6104. Epub 2017/07/25. https://doi.org/10.1038/s41598-017-06313-w PMID: 28733676; PubMed Central PMCID: PMC5522394.

22. de Oliveira LS, Rodrigues FdM, de Oliveira FS, Mesquita PRR, Leal DC, Alcântara AC, et al. Headspace solid phase microextraction/gas chromatography–mass spectrometry combined to chemometric analysis for volatile organic compounds determination in canine hair: A new tool to detect dog contamination by visceral leishmaniasis. Journal of Chromatography B. 2008; 875(2):392–8. https://doi.org/10.1016/j.jchromb.2008.09.028.

23. Magalhaes-Junior JT, Mesquita PR, Oliveira WF, Oliveira FS, Franke CR, Rodrigues Fde M, et al. Identification of biomarkers in the hair of dogs: new diagnostic possibilities in the study and control of visceral leishmaniasis. Anal Bioanal Chem. 2014; 406(26):6691–700. Epub 2014/08/31. https://doi.org/10.1007/s00216-014-8103-2 PMID: 25171830.

24. Lacroix R, Mukabana WR, Gouagna LC, Koella JC. Malaria infection increases attractiveness of humans to mosquitoes. PLoS Biol. 2005; 3(9):e298. Epub 2005/08/04. https://doi.org/10.1371/journal.pbio.0030298 PMID: 16076240; PubMed Central PMCID: PMC1182690.

25. Busula AO, Bousema T, Mweresa CK, Masiga D, Logan JG, Sauerwein RW, et al. Gametocytemia and Attractiveness of Plasmodium falciparum-Infected Kenyan Children to Anopheles gambiae Mosquitoes. J Infect Dis. 2017; 216(3):291–5. Epub 2017/09/02. https://doi.org/10.1093/infdis/jix214 PMID: 28859429.

26. Robinson A, Busula AO, Voets MA, Beshir KB, Caulfield JC, Powers SJ, et al. Plasmodium-associated changes in human odor attract mosquitoes. Proc Natl Acad Sci U S A. 2018; 115(18):E4209–E18. Epub 2018/04/19. https://doi.org/10.1073/pnas.1721610115 PMID: 29666273; PubMed Central PMCID: PMC5939094.

27. Peeling RW, Holmes KK, Mabey D, Ronald A. Rapid tests for sexually transmitted infections (STIs): the way forward. Sex Transm Infect. 2006; 82 Suppl 5:v1–6. Epub 2006/12/08. https://doi.org/10.1136/sti.2006.024265 PMID: 17151023; PubMed Central PMCID: PMC2563912.

28. Kolk A, Hoelscher M, Maboko L, Jung J, Kuijper S, Cauchi M, et al. Electronic-nose technology using sputum samples in diagnosis of patients with tuberculosis. J Clin Microbiol. 2010; 48(11):4235–8. Epub 2010/08/20. https://doi.org/10.1128/JCM.00569-10 PMID: 20720034; PubMed Central PMCID: PMC3020808.

29. Alvares CA, Stape J.L., Sentelhas P.C., de Moraes G., Leonardo J., Sparovek G. Köppen's climate classification map for Brazil. Meteorologische Zeitschrift. 2013; 22:17.

30. Barata RA, Peixoto JC, Tanure A, Gomes ME, Apolinario EC, Bodevan EC, et al. Epidemiology of visceral leishmaniasis in a reemerging focus of intense transmission in Minas Gerais State, Brazil. Biomed

Res Int. 2013; 2013:405083. Epub 2013/09/04. https://doi.org/10.1155/2013/405083 PMID: 24000322; PubMed Central PMCID: PMC3755404.

31. Saúde BMd. Sistema de Informação de Agravos de Notificação—Sinan Net. 2019 [cited 2019 July 17]. Available from: http://tabnet.datasus.gov.br/cgi/deftohtm.exe?sinannet/cnv/leishvmg.def.
32. Mancianti F, Gramiccia M, Gradoni L, Pieri S. Studies on canine leishmaniasis control. 1. Evolution of infection of different clinical forms of canine leishmaniasis following antimonial treatment. Trans R Soc Trop Med Hyg. 1988; 82(4):566–7. Epub 1988/01/01. https://doi.org/10.1016/0035-9203(88)90510-x PMID: 3076714.
33. Mary C, Faraut F, Lascombe L, Dumon H. Quantification of Leishmania infantum DNA by a real-time PCR assay with high sensitivity. J Clin Microbiol. 2004; 42(11):5249–55. Epub 2004/11/06. https://doi.org/10.1128/JCM.42.11.5249-5255.2004 PMID: 15528722; PubMed Central PMCID: PMC525214.
34. Greer CE, Peterson SL, Kiviat NB, Manos MM. PCR amplification from paraffin-embedded tissues. Effects of fixative and fixation time. Am J Clin Pathol. 1991; 95(2):117–24. Epub 1991/02/01. https://doi.org/10.1093/ajcp/95.2.117 PMID: 1846996.
35. Costa Lima Junior MS, Zorzenon DCR, Dorval MEC, Pontes ERJC. Sensitivity of PCR and real-time PCR for the diagnosis of human visceral leishmaniasis using peripheral blood. Asian Pacific Journal of Tropical Disease. 2013; 3:6.
36. Ceccarelli M, Galluzzi L, Migliazzo A, Magnani M. Detection and characterization of Leishmania (Leishmania) and Leishmania (Viannia) by SYBR green-based real-time PCR and high resolution melt analysis targeting kinetoplast minicircle DNA. PLoS One. 2014; 9(2):e88845. Epub 2014/02/20. https://doi.org/10.1371/journal.pone.0088845 PMID: 24551178; PubMed Central PMCID: PMC3923818.
37. Fraley C, Raftery AE. MCLUST: Software for model-based cluster analysis. J Classif. 1999; 16(2):297–306. PubMed PMID: WOS:000083771300007.
38. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2018.
39. Karystinos GN, Pados DA. On overfitting, generalization, and randomly expanded training sets. IEEE Trans Neural Netw. 2000; 11(5):1050–7. Epub 2008/02/06. https://doi.org/10.1109/72.870038 PMID: 18249833.
40. Scrucca L, Fop M, Murphy TB, Raftery AE. mclust 5: Clustering, Classification and Density Estimation Using Gaussian Finite Mixture Models. R J. 2016; 8(1):289–317. PubMed PMID: WOS:000385276100022. PMID: 27818791
41. Bettenburg N, Nagappan M, Hassan AE. Towards improving statistical modeling of software engineering data: think locally, act globally! Empir Softw Eng. 2015; 20(2):294–335. https://doi.org/10.1007/s10664-013-9292-6 PubMed PMID: WOS:000353824000002.
42. Coura-Vital W, Ker HG, Roatt BM, Aguiar-Soares RD, Leal GG, Moreira N, et al. Evaluation of change in canine diagnosis protocol adopted by the visceral leishmaniasis control program in Brazil and a new proposal for diagnosis. PLoS One. 2014; 9(3):e91009. Epub 2014/03/13. https://doi.org/10.1371/journal.pone.0091009 PMID: 24608904; PubMed Central PMCID: PMC3946667.
43. Fraley C, Raftery AE. Model-based methods of classification: Using the mclust software in chemometrics. J Stat Softw. 2007; 18(6). PubMed PMID: WOS:000244067800001.
44. Andrews JL, McNicholas PD. Model-based clustering, classification, and discriminant analysis via mixtures of multivariate t-distributions. Stat Comput. 2012; 22(5):1021–9. PubMed PMID: WOS:000305963700003.
45. Courtenay O, Carson C, Calvo-Bado L, Garcez LM, Quinnell RJ. Heterogeneities in Leishmania infantum infection: using skin parasite burdens to identify highly infectious dogs. PLoS Negl Trop Dis. 2014; 8(1):e2583. Epub 2014/01/15. https://doi.org/10.1371/journal.pntd.0002583 PMID: 24416460; PubMed Central PMCID: PMC3886905.
46. de Carvalho FLN, Riboldi EdO, Bello GL, Ramos RR, Barcellos RB, Gehlen M, et al. Canine visceral leishmaniasis diagnosis: a comparative performance of serological and molecular tests in symptomatic and asymptomatic dogs. Epidemiology and Infection. 2018; 146(5):571–6. Epub 2018/02/26. https://doi.org/10.1017/S0950268818000225 PMID: 29477155
47. Solca MdS, Bastos LA, Guedes CES, Bordoni M, Borja LS, Larangeira DF, et al. Evaluating the accuracy of molecular diagnostic testing for canine visceral leishmaniasis using latent class analysis. PloS one. 2014; 9(7):e103635. PubMed PMID: Medline:25076494. https://doi.org/10.1371/journal.pone.0103635 PMID: 25076494
48. Grimaldi G Jr., Teva A, Ferreira AL, dos Santos CB, Pinto Id-S, de-Azevedo CT, et al. Evaluation of a novel chromatographic immunoassay based on Dual-Path Platform technology (DPP CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2012; 106(1):54–9. PubMed PMID: Medline:22137538. https://doi.org/10.1016/j.trstmh.2011.10.001 PMID: 22137538

49. Lopes EG, Seva AP, Ferreira F, Nunes CM, Keid LB, Hiramoto RM, et al. Serological and molecular diagnostic tests for canine visceral leishmaniasis in Brazilian endemic area: one out of five seronegative dogs are infected. Epidemiology and infection. 2017; 145(12):2436–44. PubMed PMID: Medline:28726597. https://doi.org/10.1017/S0950268817001443 PMID: 28726597

50. Valdivia HO, Almeida LV, Roatt BM, Reis-Cunha JL, Pereira AA, Gontijo C, et al. Comparative genomics of canine-isolated Leishmania (Leishmania) amazonensis from an endemic focus of visceral leishmaniasis in Governador Valadares, southeastern Brazil. Sci Rep. 2017; 7:40804. Epub 2017/01/17. https://doi.org/10.1038/srep40804 PMID: 28091623; PubMed Central PMCID: PMC5238499.

51. Penaforte KM, Belo VS, Teixeira-Neto RG, Ribeiro RA, de Oliveira RB, Schettini DA, et al. Leishmania infection in a population of dogs: an epidemiological investigation relating to visceral leishmaniasis control. Rev Bras Parasitol Vet. 2013; 22(4):592–6. Epub 2014/01/30. https://doi.org/10.1590/S1984-29612013000400022 PMID: 24473887.

52. Leal GGdA, Carneiro M, Pinheiro AdC, Marques LA Ker HG, Reis AB, et al. Risk profile for Leishmania infection in dogs coming from an area of visceral leishmaniasis reemergence. Preventive Veterinary Medicine. 2018; 150:1–7. https://doi.org/10.1016/j.prevetmed.2017.11.022 PMID: 29406075

53. Doehl JSP, Bright Z, Dey S, Davies H, Magson J, Brown N, et al. Skin parasite landscape determines host infectiousness in visceral leishmaniasis. Nature Communications. 2017; 8. https://doi.org/10.1038/s41467-017-00103-8 PubMed PMID: WOS:000404778800001. PMID: 28680146

54. Hamilton JGC, Hurd H. Parasite manipulation of vector behaviour. In: Lewis EE, Campbell J.F., Sukhdeo M.V.K., editor. The Behavioual Ecology of Parasites: CAB International; 2002. p. 384.

55. Cator L. Editorial: Host Attractiveness and Malaria Transmission to Mosquitoes. J Infect Dis. 2017; 216 (3):289–90. Epub 2017/06/22. https://doi.org/10.1093/infdis/jix216 PMID: 28633282.

56. Drea CM, Boulet M, Delbarco-Trillo J, Greene LK, Sacha CR, Goodwin TE, et al. The "secret" in secretions: methodological considerations in deciphering primate olfactory communication. Am J Primatol. 2013; 75(7):621–42. Epub 2013/03/26. https://doi.org/10.1002/ajp.22143 PMID: 23526595.

57. Ezenwa VO, Williams AE. Microbes and animal olfactory communication: Where do we go from here? BioEssays. 2014; 36(9):847–54. https://doi.org/10.1002/bies.201400016 PMID: 24986361

58. Cardoso MS, Bento GA, de Almeida LV, de Castro JC, Reis-Cunha JL, Barbosa VA, et al. Detection of multiple circulating Leishmania species in Lutzomyia longipalpis in the city of Governador Valadares, southeastern Brazil. PLoS One. 2019; 14(2):e0211831. Epub 2019/02/06. https://doi.org/10.1371/journal.pone.0211831 PMID: 30721272; PubMed Central PMCID: PMC6363391.

59. Gonzalez MA, Bell MJ, Bernhardt SA, Brazil RP, Dilger E, Courtenay O, et al. Susceptibility of wild-caught Lutzomyia longipalpis (Diptera: Psychodidae) sand flies to insecticide after an extended period of exposure in western Sao Paulo, Brazil. Parasit Vectors. 2019; 12(1):110. Epub 2019/03/16. https://doi.org/10.1186/s13071-019-3364-4 PMID: 30871639; PubMed Central PMCID: PMC6419423.

60. Courtenay O, Dilger E, Calvo-Bado LA, Kravar-Garde L, Carter V, J. BM, et al. Sand fly synthetic sex-aggregation pheromone co-located with insecticide reduces canine Leishmania infantum infection incidence: a stratified cluster randomised trial. BioRxiv. 2019. https://doi.org/10.1101/712174.

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

Instituto Oswaldo Cruz
Comissão de Ética no Uso de Animais - CEUA/ IOC

Rio de Janeiro, 23 de maio de 2017.

Prezado Dr. Reginaldo Peçanha Brazil,

No dia 23 de maio de 2017 representantes da CEUA-IOC se reuniram e deliberaram sobre o projeto intitulado "O papel do odor de cães infectados por *Leishmania infantum* na atração de *Lutzomyia longipalpis*, e sua utilização como base para diagnóstico precoce da leishmaniose visceral, Governador Valadares, Minas Gerais, Brasil", proposto por V.S.ª. Com base nos documentos apresentados, a comissão se posicionou favorável à aprovação da licença após as correções sugeridas abaixo. Favor executar as correções utilizando sempre o formulário atual disponível no site do IOC (http://www.fiocruz.br/ioc/cgi/cgilua.exe/sys/start.htm?sid=386) e a ferramenta "marcador de alterações" do seu processador de texto, enviando seu novo protocolo revisado para o email ceua.ioc@ioc.fiocruz.br.

Favor verificar erros de digitação/português ao longo de todo o texto do formulário.

Item 1. Título: Corrigir "*Lutzomyia Longipalpis*" para "*Lutzomyia longipalpis*".

Item 7. Resumo: (1) Mencionar que, em caso de detecção de animais infectados ou que se infectarem durante a pesquisa, as autoridades municipais de saúde serão notificadas; (2) Substituir "veia radial" por "veia cefálica"; (3) Respeitar o limite de caracteres do texto.

Item 8. Justificativa: (1) Explicar a expressão "(...) a medicina utiliza-se do cheiro (ou mesmo gosto) de urina para diagnosticar doenças"; (2) Respeitar o limite de caracteres do texto.

Item 9. Modelo animal: A idade mencionada "<3 meses" difere da mencionada no resumo ">3 meses".

Item 10.1. Detalhamento de cada procedimento:

Procedimento 1: (1) No título deste procedimento, substituir o termo "assepsia" por "antissepsia"; (2) No texto, substituir "veia radial" por "veia cefálica"; (3) Como no resumo, mencionar "veia jugular" como alternativa para a coleta de sangue.

Procedimento 2: (1) Substituir o termo "cabelo" por "pelo"; (2) Substituir "luvas de laboratório" por "luvas de procedimentos".

Procedimento 3: (1) Favor mencionar que os microchips serão implantados subcutaneamente. (2) Favor substituir "ISSO" por "ISO".

Item 10.2. Desenho experimental: (1) O procedimento 3, "Implante de microchip nos cães", ocorrerá somente uma vez. A leitura do microchip implantado é que ocorrerá três vezes. (2) Mencionar que a coleta será feita de animais de ambos os gêneros, conforme previamente mencionado na observação do item 9.

TCLE: (1) Revisar o Português; (2) No título, corrigir "*Longipalpis*" para "*longipalpis*"; (3) Numerar as páginas (Pág. 1 de 4, Pág. 2 de 4, etc.); (4) Substituir "veia radial" por "veia cefálica"; (5) Mencionar que, em caso de detecção de animais infectados ou que se infectarem durante a pesquisa, as autoridades municipais de saúde serão notificadas; (6) Explicar a expressão "(...) a medicina utiliza-se do cheiro (ou mesmo gosto) de urina para diagnosticar doenças".

Atenciosamente,

Comissão de Ética no Uso de Animais
Instituto Oswaldo Cruz - CEUA/ IOC

Instituto Oswaldo Cruz

Comissão de Ética no Uso de Animais - CEUA/ IOC

LICENÇA L-027/2017

Certificamos que o protocolo (CEUA/IOC-018/2017), intitulado "O Papel do odor de cães infectados por *Leishmania infantum* na atração de *Lutzomyia longipalpis*, e sua utilização como base para diagnóstico precoce da leishmaniose visceral, Governador Valadares, Minas Gerais, Brasil.", sob a responsabilidade de **REGINALDO PEÇANHA BRAZIL** atende ao disposto na Lei 11794/08, que dispõe sobre o uso científico no uso de animais**,** inclusive, aos princípios da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL). A referida licença não exime a observância das Leis e demais exigências legais na vasta legislação nacional.

Esta licença tem validade até 30/06/2020 e inclui o uso total de:

Animal	Espécie ou linhagem	Quant (total)	♂	♀	Idade	Peso	Origem (*)
(X) Outros	*Canis familiaris*	300			>3 meses		Serão utilizados cães que se encontram em residências do município de Governador Valadares.

Observação: Esta licença não substitui outras licenças necessárias, como Certificado de Qualidade em Biossegurança para animais geneticamente modificados, certificado do IBAMA para captura de animais silvestres ou outros.

Rio de Janeiro, 28 de junho de 2017.

Flávio Alves Lara

Coordenador da CEUA/Instituto Oswaldo Cruz

Fundação Oswaldo Cruz

FIOCRUZ-Fundação Oswaldo Cruz/IOC-Instituto Oswaldo Cruz
Av. Brasil, 4365 - Manguinhos - Rio de Janeiro - RJ - Brasil
CEP: 21040-360 Tel: (21) 2562-1056