1 Odour of domestic dogs infected with *Leishmania infantum* is attractive to
2 female but not male sand flies: evidence for parasite manipulation.

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22 **Short title** Odour of *Leishmania* infected dogs enhances female vector response

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26 **Key words:** *Leishmania infantum*; parasite manipulation; *Canis lupus familiaris*;

27 *Lutzomyia longipalpis*; volatile organic chemicals; volatile disease markers; entrainment;

28 bioassay;

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30 Abstract

31 Globally visceral leishmaniasis (VL) causes thousands of human deaths every year. In
32 South America, the etiologic agent, *Leishmania infantum*, is transmitted from an infected
33 canine reservoir to human hosts by the blood-feeding activity of the sand fly vector,
34 predominantly, *Lutzomyia longipalpis*. Previous evidence from model rodent systems have
35 suggested that the odour of infected hosts is altered by the parasite making them more
36 attractive to the vector leading to an increased biting rate and improved transmission
37 prospects for the pathogen. However, the effect of *Le. infantum* infection on the
38 attractiveness of naturally infected dogs which are integral to human infection, has not
39 been assessed.

40 Hair collected from infected and uninfected dogs residing in a VL endemic city in
41 Brazil. was entrained to collect the volatile chemical odours present in the headspace.
42 Female and male *Lu. longipalpis* sand flies were offered a choice of infected or uninfected
43 odour in a series of behavioural experiments. Control experiments established that female
44 and male *Lu. longipalpis* were equally attracted to uninfected dog odour, female *Lu.*
45 *longipalpis* were significantly more attracted to infected dog odour than uninfected dog
46 odour in all 15 experimental replicates (average 45.7 ± 0.87 females attracted to infected
47 odour; 23.9 ± 0.82 to uninfected odour; paired T-test, $P=0.000$). Male *Lu. longipalpis* did not
48 significantly prefer either infected or uninfected odour (average 36.1 ± 0.4 males to infected
49 odour; 35.7 ± 0.6 to uninfected odour; paired T-test, $P=0.722$). A significantly greater
50 proportion of females chose the infected dog odour compared to the males (paired T-test,
51 $P=0.000$).

52 The results show that dogs infected with *Le. infantum* are significantly more
53 attractive to blood-feeding female than male sand flies. This is strong evidence for parasite
54 manipulation of the host odour in a natural transmission system and indicates that infected

55 dogs may have a disproportionate significance in maintaining infection in canine and
56 human infection.

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58 **Author Summary**

59 Visceral leishmaniasis (VL) is a disease caused by the Protist parasite *Leishmania*
60 *infantum*. In Brazil and other South and Central American countries, the parasite is
61 transmitted by the blood-feeding activity of infected female *Lutzomyia longipalpis* sand
62 flies. The disease leads to thousands of human cases and deaths every year. Domestic dogs
63 are the reservoir of infection for humans therefore understanding the effect of infection on
64 dogs is important in developing an understanding of the epidemiology of the disease.
65 Although previous studies on rodent models of *Le. infantum* infection have shown that
66 infected Golden Hamsters are more attractive to *Lu. longipalpis* the attractiveness of
67 naturally infected dogs to the insect vector has not been previously been investigated. In
68 this study we showed that the odour of infected dogs is significantly more attractive to
69 female sand flies which can transmit the pathogen than to male sand flies which do not.
70 This clear-cut difference in attraction of female and males suggests that the females are
71 preferentially attracted by parasite infected hosts and may lead to enhanced infection and
72 transmission opportunities for the parasite.

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80 **Introduction**

81 In South America the protist *Leishmania (Le.) infantum* (Cunha & Chagas) (Kinetoplastida:
82 Trypanosomatidae) causes the disease visceral leishmaniasis (VL) in humans and dogs
83 (*Canis lupus familiaris*) [1]. The intracellular parasite, which is a pathogen of the immune
84 system, primarily affects the reticuloendothelial system, surviving and multiplying in
85 macrophage and dendritic cells [2]. In humans, VL is characterized by prolonged irregular
86 fever, splenomegaly, hepatomegaly, pancytopenia and weight loss and in the absence of
87 treatment the case fatality rate is 90% [3].

88 Globally, approximately 50,000 to 90,000 new cases of VL occur each year with
89 more than 95% of them occurring in ten countries: Brazil, China, Ethiopia, India, Iraq,
90 Kenya, Nepal, Somalia, South Sudan and Sudan [4]. In the Americas, leishmaniasis is a
91 significant public health problem due to its' morbidity, mortality and broad geographical
92 distribution [5]. From 2001 to 2015, 52,176 cases of visceral leishmaniasis were registered in
93 the Americas, with 96.4% of these cases (50,268) reported from Brazil [5, 6]. Overall, the
94 burden of the disease (age-standardized DALY) in Brazil has almost doubled during the
95 period from 1990 to 2016 [7].

96 In Brazil *Leishmania infantum* infection is a zoonosis, with canines as the primary
97 reservoir hosts. Humans, which are considered to be a dead-end host for the parasite [8],
98 become infected when they are fed on predominantly by infected female sand flies of the
99 *Lutzomyia longipalpis* s.l. (Lutz & Neiva) (Diptera: Psychodidae) species complex [9]. In
100 dogs, VL is characterised by dermatitis, lymphadenomegaly, general muscular atrophy, and
101 renal disease [10].

102 Host seeking haematophagous insects orientate towards and identify potential host
103 animals by combinations of visual, thermal tactile and chemical (host odour) cues produced
104 by the host [11]. Together these stimuli, along with the individual response of female insects,

dictate the likelihood that a haematophagous insect will successfully obtain a blood meal [11-13]. For temporary ectoparasites (e.g. mosquitoes, triatomines, midges and sand flies) host odour is of primary importance in host location. Parasite manipulation of the host can enhance transmission opportunities in favour of the parasite. There is now significant evidence that parasite infection of the host animal alters both the vector host choice behaviour as well as vector biting rate and blood-meal size [14]. The choice to bite an infected or uninfected host is key for pathogen transmission and numerous empirical studies show that pathogens can modify the scent of infected hosts to attract vectors [15].

Mosquito vectors are more attracted to hosts, including humans, mice and birds, infected with malaria than to uninfected hosts [16-20]. Humans infected with *Plasmodium falciparum* are more attractive to *Anopheles gambiae s.l.* than uninfected humans. The increased attraction is most pronounced when the parasite is in the most transmissible (gametocyte) stage [21] and the increased host parasitaemia may partially, at least, be induced by mosquito biting activity which leads in turn to higher transmission rates [22]. The difference in attractiveness has been related to the presence of a parasite produced isoprenoid precursor, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) in host blood which has been shown to trigger an enhanced release of attractants which in turn increase the likelihood of an infected human host being bitten by the vector [23].

In contrast only a limited number of studies have been carried out to determine if *Leishmania* parasites manipulate host odour. In olfactometer choice experiments volatile odours collected from hamsters infected with *Le. infantum* (MHOM/BR/74/PP75) were found to be more attractive to female *Lu. longipalpis* sand flies than uninfected hamsters [24]. A subsequent laboratory study investigated the change in attractiveness of individual hamsters infected with *Leishmania infantum* and showed that the odour of a significant number of hamsters became significantly more attractive to the vector after the *Leishmania* parasites had

reached the metacyclic infection stage compared to the initial amastigote infection stage [25].
A coupled gas chromatography-mass spectrometry (GC/MS) and statistical analysis
(principal component analysis, PCA) compared the volatile organic chemical profile obtained
from the headspace of the hair of a small group of Brazilian dogs infected with *Le. infantum*
with an uninfected group of dogs and found a significant difference in the odour profiles of
the two groups [26, 27]. The key discriminatory volatile components identified by the PCA
were characterised by conventional analytical methods [27] and in a behavioural study were
found to be attractive albeit at very high concentrations ($\geq 50\%$ purity) predominantly to male
Lu. longipalpis [28].

None of these previous studies conclusively demonstrated that the parasite *Le.*
infantum enhanced the attractiveness of the odour of the natural canine reservoir host to the
sand fly vector; the experiments with hamsters were carried out with a non-natural
host/parasite system and the experiments with canine odour, although showing a difference in
odour profile between infected and uninfected dogs, did not test if the infected dog odour was
more or less attractive than the uninfected dog odour.

We therefore carried out a study on the odours collected from the hair of dogs in an
olfactometer, to determine if female or male *Lu. longipalpis* had a preference for either
infected or uninfected odour.

Results

Female sand fly response to infected and uninfected dog odour

In all 15 individual experimental replicates, female *Lu. longipalpis* were significantly
attracted to the infected dog odour compared to the uninfected odour (Fig 1A; S1 Table).

We combined the data from the blinded and unblinded experiments for each of the
infected and uninfected data sets as there was no significant difference between them

[infected dog odour; unblinded 45.6 ± 1.3 vs blinded 45.83 ± 1.0 (2-sample T-test, $P=0.869$); uninfected dog odour; unblinded 22.8 ± 1.1 vs blinded 25.5 ± 1.0 (2-sample T-test; $P=0.0874$)] and then compared the response to each (mean response to infected odour 45.7 ± 0.87 ; mean response to uninfected odour 23.9 ± 0.82). Female sand flies were significantly attracted to the infected odour (paired T-test, $P=0.000$).

The data were normally distributed (Anderson-Darling $P>0.1$ and Kolmogorov-Smirnov $P>0.1$).

Fig 1. Numbers of female (A) and male (B) *Lutzomyia longipalpis* attracted to volatiles collected from infected and uninfected dogs. Numbers of female (A) or male (B) *Lu. longipalpis* attracted to either the infected (grey bars) or uninfected (hatched bars) dog odour presented in the Y-tube choice olfactometer. The numbers attracted to each side are given beside each bar. The dog odour pairs are indicated on the left axis, the first dog in each pair is the infected animal. The superscripts indicate if the dog infection status was known to the experimenter or not; α for the female sand fly bioassay indicates that the status was known β indicates it was not; similarly, γ for the male sand fly assays indicates that it was known and δ indicates it was not. Statistical significance was analysed by binomial test comparing the actual number of sand flies that responded to infected odour compared to the number that responded to the uninfected odours (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, ns=not significant).

Male sand fly response to infected and uninfected dog odour

In all 15 of the individual experimental replicates, male *Lu. longipalpis* were not significantly more attracted to the infected dog odour entrainment extracts compared to the uninfected dog odour (Fig 1B; S2 Table).

We combined the data from the blinded and unblinded experiments for each of the infected and uninfected data sets as there was no significant difference between them [infected dog odour; unblinded 36.1 ± 0.5 vs blinded 36.0 ± 0.9 (2-sample T-test, $P=0.913$); uninfected dog odour; unblinded 35.8 ± 0.8 vs blinded 35.8 ± 0.9 (2-sample T-test; $P=0.890$)] and then compared response to each (mean response to infected odour 36.1 ± 0.4 ; mean response to uninfected odour 35.7 ± 0.6). Male sand flies were equally attracted to the infected and uninfected odours (paired T-test, $P=0.722$).

The data were normally distributed (Anderson-Darling $P>0.1$ and Kolmogorov-Smirnov $P>0.1$).

Comparison of female and male response to infected dog odour On average significantly more of the females that responded ($65.7 \pm 1.1\%$) were attracted to the infected dog odour than the males $50.3 \pm 0.6\%$ of the males (T-test; $P<0.000$).

Relationship between parasite load, discriminate value and the female and male sand fly response.

There was no significant relationship between the parasite load and the proportion of females (or males) that were attracted to the infected dog hair odour [Regression analysis: females $R^2(\text{adjusted}) = 0.00\%$; males $R^2(\text{adjusted}) = 0.00\%$]. There was a slight but non-significant relationship between the previously identified discriminate values [29] and the proportion of females (or males) that were attracted to the infected dog hair odour [Regression analysis: females: $R^2(\text{adjusted}) = 8.1\%$; males $R^2(\text{adjusted}) = 16.6\%$, $P=0.074$]. A correlation analysis suggested a negative relationship between the discrimination value of infected dog odour and male response (Pearson $r = -0.25$). Removal of the parasite load value of 853.44 parasites ml^{-1} , which is a potential outlier data point, had little effect on $R^2(\text{adjusted})$ for either the female or male response to parasite load however the relationship between discrimination factor

became slightly more important for the female sand fly response [R^2 (adjusted) = 8.9%
P=0.158] and for the males [R^2 (adjusted) = 9.4% P=0.152]. Correlation analysis suggested a
moderate relationship between the discriminate value and female response (Pearson r = 0.4)
and male response (Pearson r = -0.4).

Control

Both female and male sand flies were significantly attracted to dog odour. A significantly
greater proportion of female sand flies (66.5 %) were attracted to the uninfected dog volatiles
compared to hexane (Binomial test; P=0.000) and of the male sand flies that responded,
62.9% were attracted to uninfected dog odour compared to the hexane control (Binomial test;
P=0.000) (Table 1).

Table 1 Response of female and male *Lutzomyia longipalpis* to odour of uninfected
dogs and hexane only in Y-tube olfactometer choice experiments.

female sand fly response					
expt	dog i.d.	uninfected dog odour	hexane	no-response	P
1	138	48	21	11	***
2	128	45	29	6	*
3	027	46	20	14	***
cumulative		139	70	31	****
male sand fly response					
expt	dog i.d.	uninfected dog odour	hexane	no-response	P
4	138	43	27	10	*
5	128	47	22	11	***
6	027	42	29	9	*
cumulative		132	78	30	****

Female/male sand fly response = control experiments measuring either the response of the
female or male sand flies in the Y-tube olfactometer. expt = experimental replicate; dog i.d. =
dog identification number; uninfected dog odour = number of sand flies attracted to the dog
odour sample; hexane = number of sand flies attracted to hexane only; no-response = number
of sand flies that did not respond. Statistical significance was analysed by binomial test

comparison of number of sand flies responding to the dog odour vs hexane (* $P \leq 0.05$,
*** $P \leq 0.001$, **** $P \leq 0.0001$).

Discussion

Domestic dogs, *Canis lupus familiaris*, are the primary reservoir host of the Protist parasite *Le. infantum* which is transmitted by the blood-feeding activity of female *Lu. longipalpis* sand flies in Brazil. This study has shown that the odour of dogs naturally infected with *Le. infantum* was significantly more attractive to female *Lu. longipalpis* than uninfected dog odour. In addition, the odour of infected and uninfected dogs was equally attractive to male *Lu. longipalpis*.

In each of the 15 experimental replicates where female *Lu. longipalpis* were offered a choice of infected dog odour or uninfected dog odour, the females chose the infected dog odour. Overall, 685 (mean $\pm$ sem: 45.7 $\pm$ 0.9) female sand flies chose the infected odour compared to 358 (23.9 $\pm$ 0.8) that chose the uninfected dog odour. By comparison, in all 15 experimental replicates the male *Lu. longipalpis* did not differentiate between infected and uninfected dog odour. Overall, 541 (36.1 $\pm$ 0.4) males chose the infected odour and 536 (35.7 $\pm$ 0.6) chose the uninfected odour. In both sets of experiments female and male *Lu. longipalpis* were highly attracted to dog odour with only 13 and 10% respectively failing to respond to the dog odour in the olfactometer.

Previous studies using model infection systems have indicated that the odour of host animals infected with *Le. infantum* is more attractive to the sand fly *Lu. longipalpis*. A study with Golden Hamsters showed that the odour of hamsters infected with *Le. infantum* was more attractive than odour from uninfected hamsters [24] and a subsequent study showed that the odour of 6 out of 13 Golden hamsters infected with *Le. infantum* became significantly more attractive to the majority of *Lu. longipalpis* females after a prolonged period of

infection [25]. Both of these studies used Golden Hamsters which would not be infected with *Le. infantum* or exposed to blood-feeding by *Lu. longipalpis* in the wild. In that study *Le. infantum* infected Golden Hamsters were not attractive to males. This is the first occasion, that we are aware of, when *Lu. longipalpis* females and males have been offered the choice of entrained headspace volatile odours from infected and uninfected dogs. The results are significant because in South and Central American, and Mediterranean countries, dogs are the reservoir of infection and are essential to the maintenance of the zoonotic transmission cycle that leads to human infection [30]. The results of this study differ in 2 important ways from those obtained from previous work using infected Golden Hamster odour [25]. First, only 6 out of 13 of the infected hamster's odours were attractive to females after infection whereas in this study all the randomly chosen infected dog odours were attractive and second, only approximately 50% of the male *Lu. longipalpis* responded to the infected or uninfected hamster odour whereas in this study 90% of males were responsive. These results highlight the potential problems of using model rather than real systems to explore pathogenic parasite host vector interactions.

In a series of analytical chemistry studies, the odour of dogs infected with *Le. infantum* was shown to have a different odour profile to uninfected dogs [27]. Subsequently, 4 compounds identified as potential biomarkers of infection were tested in an olfactometer at either 50% or 100% concentration. In that study, the males were found to be more responsive than females in a wind tunnel olfactometer [28]. The response of both male and female *Lu. longipalpis* was also substantially lower than the response seen in this study. It is not possible to be sure if the observed differences are because of differences in olfactometer design, small sample size or are related to the range of different compounds and concentrations presented to the sand flies and which differed substantially between the two studies.

Although both male and female *Lu. longipalpis* were attracted to uninfected dog odour only the female sand flies showed an enhanced attraction to infected dog odour. The differences between the female and male sand fly response to infected dog odour observed in this study may also be important to transmission of the parasite. *Lutzomyia longipalpis* mating behaviour is believed to be driven by male lekking behaviour in which male *Lu. longipalpis* first locate the host and establish mating aggregations on or near potential host animals [31]. Males compete with each other for territory, produce a sex/aggregation pheromone and then females are attracted by a combination of the host odour and pheromone [32-34]. The females arrive at the lekking site, choose a mate, take a blood-meal and depart [35]. In this model, males first select the host animal and the females arrive later [32, 33, 36]. The current study suggests a more nuanced situation where females can choose host animals independently of their need for associated male pheromone and it is this behaviour that can be manipulated by the parasite. The majority of sand fly species do not lek and therefore it might be possible for female *Lu. longipalpis* to also locate a host and take a blood meal without being attracted to a lek site. By not attracting male *Lu. longipalpis* to infected dogs, male aggregations would be more likely to take place on uninfected dogs (as they would be the majority of the canine population). Thus, infected female sand flies might be more likely to feed on uninfected dogs with established leks and thereby satisfying the proposed need [14] of infected vectors to feed on uninfected hosts to maintain disease transmission. To test this hypothesis in the future it will be important to determine the relative attractiveness of sex pheromone plus uninfected dog odour versus infected dog odour alone, as well as the attractiveness of infected and uninfected dog odours to infected female sand flies.

Our results showed that the parasite load in the infected dogs was not significantly related to attractiveness for female *Lu. longipalpis*. It is likely that this may be because our analysis of parasite load did not differentiate between the stage of the parasite development

and thus infectivity of the animal [37]. Both these factors have been shown in other studies to be important factors in the manipulation of host attractiveness, as parasite manipulation would be most pronounced when the parasite was at a stage suitable for infection of the vector [8, 38, 39].

There was a slight positive correlation between odours that were more attractive to females and the eNose discrimination response (obtained from a previous study [29]) and a slightly greater negative correlation in the male response. This suggests that the sand flies and eNose detectors are responding to similar cues.

In the future it will be important to determine if this observed phenomenon also occurs with live infected/infectious dogs and wild *Lu. longipalpis* in the peridomestic environment where a number of other factors (e.g. odour of competing alternative animal hosts, the presence of sex/aggregation pheromone and plant odours) might contribute to, divert or dilute the response of the female sand flies to infected dog odour. In addition, synthetic sex/aggregation pheromone, formulated in controlled release lures [40] is a strong attractant that maintains the aggregations of both male and female *Lu. longipalpis* [41] while also drawing them away from chicken sheds (Gonzalez et al., 2020 In Press, Gonçalves et al., 2020 Submitted), it will be important to determine if the synthetic pheromone lure can disrupt the attractiveness of infected dogs.

It has been suggested that human odour-baited traps combined with insecticides could improve capture and kill of mosquito vectors [42-44] or cattle or pig odour could be useful for control of tsetse flies [45]. Our work suggests that infected dog odour may also provide opportunities to develop new vector control methods. The observed sex specific increase in attraction to infected dogs demonstrates a potential new approach for the development of a novel tool for use in vector control. Further studies are required to evaluate and implement dog odour baited traps for sand fly control [46]. The use of infected dog odour as a lure could

be a widespread methodology used throughout Brazil. Combining such traps with current and proposed insecticide-based methodologies could lead to a significant reduction in the number of female sandflies [40, 47].

Materials and Methods

Field study site, dog recruitment and sample collection

The study was carried out on the entrained odour of hair collected from infected and uninfected dogs recruited in April 2018 in Governador Valadares (GV), a municipality of approximately 280,000 people in Minas Gerais State, Brazil (18°51'12"S W; 41°56'42"W, altitude 170m, Fig. 1) 320 km northeast of Belo Horizonte, the state capital.

GV is situated in the Rio Doce basin within the Atlantic Forest region and local topography consists of valleys and hills. The climate is Aw (tropical sub-warm and sub-dry) according to the Köppen–Geiger classification [48]. The city has an average temperature of 24.2°C (annual range 15.2 - 33°C) and the average annual rainfall of 1109 mm is concentrated between October and March [49].

Fig. 1. The municipality of Governador Valadares (Minas Gerais State Brazil) and its location in Brazil. The hair samples were from dogs recruited in the Altinópolis district of the city, bordered with a red line. Map in was constructed in the QGIS 3.10, using OpenStreetMap (Map data copyrighted OpenStreetMap contributors and available from <https://www.openstreetmap.org>) as base map.

The area is a focus of intense VL transmission and is also endemic for cutaneous leishmaniasis where the sand fly vector, *Lu. longipalpis* is abundant [50, 51]. In GV, 212 cases of human VL were reported between 2008-2015, the cumulative VL incidence was 7

cases per 100,000 [52] with case fatality rate estimates of 8.9%-16.3% [53, 54]. In 2017, just before this study, case fatality rate was 6.4 per 100,000 [55]. Canine infection reported for the 2008-2012 period was 29% (8,622/29,724) in GV [56].

Recruitment and sampling of 133 dogs was carried out in the Altinópolis district of Governador Valadares which was chosen because of the high prevalence of canine VL (cVL) (average incidence reported in 2013 was 33.8%) [50] and the presence of a large population of household-owned dogs (n = approx. 2000) (Centro de Controle de Zoonoses (CCZ) survey). The recruitment of domestic dogs and collection of individual hair and blood samples was undertaken with the informed consent of the dog's owners [29]. Briefly, dogs were chosen at random by walking through the area and visually identifying potential recruits and then seeking owner consent. Inclusion criteria were; dogs aged ≥ 3 months, without previous clinical assessment or laboratory diagnosis for cVL. Exclusion criteria were; pregnant/lactating bitches, aggressive or stray dogs. Canine dorsal hair and blood samples were obtained as previously described [29].

Diagnosis of dogs and parasite load calculation

The diagnosis of each dog was performed previously as reported [29]. Briefly, DNA was extracted from the buffy coat of each individual dog and a PCR was performed in triplicate using the primer pair Mary F and Mary R [57]. Diagnosis was based on the outcomes of three PCR analyses. Parasite load was calculated by qPCR also using the primer pair Mary F and Mary R and a standard curve was established using extracted *Le. infantum* DNA; 1:10 serial dilutions, ranging from 0.01 to 10,000 parasites per ml and used to quantify the number of parasites in the dog blood samples [29].

Entrainment of volatile organic compounds

Volatile organic chemicals (VOCs) from all the dog hair samples were entrained as described previously [58]. Briefly, charcoal-filtered air (15ml sec⁻¹) was passed through the inlet port of a quick-fit Dreschel head into a 50ml round-bottom quick-fit glass flask which contained the dog hair (1gram). The air from the effluent port of the Dreschel head passed into a 3.5-inch long Orbo-402 Tenax-TA trap (Sigma-Aldrich Company Ltd, Dorset, UK) via a 10cm length of Teflon tubing. Hair samples were entrained for 2.5h and the collected VOCs were recovered by elution of the Orbo tube with *n*-hexane (SupraSolv grade; Merck, Germany) (2ml). A fresh Orbo tube was used for each entrainment dog hair sample. Each eluted sample was concentrated to a final volume (ca. 500µl) under nitrogen and then sealed in a clean glass Pasteur pipette and stored (-20°C) until further analysis.

Bioassay

Sand flies. The sand flies used in the bioassay were from a colony maintained at Lancaster University that was originally established from individuals collected in Jacobina, Brazil (40°31' W, 11°11'S). Flies were reared according to the method of Lawyer *et al* [59] and adults maintained in Nylon Barraud cages (18 x 18 x 18 cm) at 27°C (range 25-29°C), 95% RH with a 12:12 h (L:D) photoperiod. Female sand flies were fed sheep blood (TCS Biosciences Ltd, Buckingham, UK) via a chicken skin membrane before egg laying [60-62]. To obtain virgin female and male *Lu. longipalpis* for use in the bioassays newly eclosed adults were separated within 6 h of emergence (before rotation of external male genitalia) and fed on saturated sucrose solution only. They were then held in a Barraud cage, kept inside a plastic bag to maintain humidity at 27°C with access to sugar (*ad libidum*) in the insectary for 5 to 7 days until used in the bioassay.

Bioassays were performed as previously described [58]. One hour prior to the start of the experiment the sand fly holding cage was moved into the bioassay room where the plastic

bag was removed and the sand flies allowed to acclimatise to the room conditions (temp; mean 27°C, range 25-29°C; humidity; mean 75%, range 68-78%). All bioassays were performed at the same time of day to minimise any potential effect of circadian rhythms on flight activity [63].

Y-tube olfactometer. The Y-tube olfactometer and associated Teflon tubing were as previously described [34, 58]. This apparatus allowed us to enter the entrained dog odour solutions into the air flowing through the Teflon tubing connected to the olfactometer arm. The solution was injected onto a rolled up filter paper disk (Grade 1; 20mm diam., Whatman International Ltd., Maidstone, United Kingdom) via a small hole drilled in the wall of the tubing. A flow of clean air (ZeroGrade; BOC address) through the apparatus was controlled by a two-stage cylinder regulator valve (BOC Series 8500 Air Regulator BOC Lancaster, UK) and a rotameter (Sigma-Aldrich Company Ltd, UK), adjusted to 5ml sec⁻¹ and periodically confirmed using a bubble meter. All connections between Teflon tubing components were made airtight by sealing with Teflon tape (Sigma-Aldrich Company Ltd, UK). Glass wool was inserted into the end of each Y-tube olfactometer arm to prevent flies from escaping into the apparatus.

All Teflon tubing and glassware was cleaned 24h prior use. Glassware was washed with 10% Teepol solution, distilled water and then acetone before being baked overnight at 225°C. Teflon tubing was rinsed with hexane (Pesticide Grade, Fisher Scientific UK Ltd, Loughborough, UK) and allowed to air dry overnight.

To perform the bioassay, the Y-tube was placed horizontally on a solid bench, 1µl of an infected dog odour extract was injected onto the filter paper in one side of the apparatus and 1µl of an uninfected dog odour extract for comparison was injected into the other side of the apparatus. The holes in the wall of the Teflon tubes were sealed with PTFE ® tape

To test the response of sand-flies, each fly was removed from the holding cage and released individually at the open end of the stem of the Y-tube olfactometer. A timer was started and after three minutes the position of the sand fly within the Y-tube was recorded; either the test or control arm or, if it remained in the stem, a “no choice” was recorded. After 10 replicates, the rolled-up filter paper was removed and replaced, and to reduce any potential positional bias in the apparatus, the position of the test and control arms were swapped round by rotating the olfactometer through 180° in the horizontal plane. In total 80 female or 80 male sand flies were used per experimental replicate (i.e. each pair of infected and uninfected dog odour combinations were tested with 80 sand flies).

Sample selection. From the pool of available (n=133) dog hair odour samples, we selected the extracts collected from the hair of 15 infected and 15 uninfected dogs (n=15). Selection of the infected and uninfected dog hair odour extracts was based on PCR and qPCR analysis results. The selected infected dog hair extracts were from dogs with a parasite load that ranged from 1.3 ml⁻¹ to 853.4 parasites ml⁻¹ of blood. The uninfected dog hair extracts were from dogs with no detectable parasite DNA.

To determine if any attraction observed in the Y-tube experiments might be related to the discrimination value previously obtained from a VOC analyser (eNose) study [29] the 15 infected and 15 uninfected dog hair odour samples were also selected according to their discrimination values. Therefore, each infected and uninfected dog hair sample group had 5 high, 5 medium, 5 low discriminated individuals, and a cross comparison performed. The pairings are summarised in S1 Table.

Study design

To carry out the study, the response of female (or male sand flies) to each infected dog hair odour sample was compared with their response to an uninfected dog hair sample in the Y-tube olfactometer. High, medium and low discriminated infected samples were cross compared with either a high, medium or low discriminated uninfected sample. The same 15 pairs of dog hair odour were used in both the female and male bioassays, although in a different order (S1 Table and S2 Table).

To avoid experimenter bias, the 15 infected and uninfected dog hair pairs were divided into 2 sub-groups. The infection status was known to the experimenter (unblinded) in one subgroup of nine dogs but unknown (blinded) in the other subgroup of 6 dogs.

All dog odour extracts were diluted by a factor of 1:10 to achieve optimal sand fly response [64]. Control bioassays were performed to determine female and male response to uninfected dog hair odour extract compared to a hexane control.

Data Analysis

Binomial tests [65] were used to determine whether a greater number of female or male sand flies were attracted to infected dog odour extract than uninfected dog odour extract than would be expected by chance (50/50) for each experimental replicate. A binomial test was also used to compare response of female and male *Lu. longipalpis* to uninfected dog odour.

Response of female or male sand flies to infected or uninfected dog odour in the unblinded and blinded experiments was compared using two-tailed T-tests.

Response of female and male sand flies to the infected dog odour or uninfected dog odour was compared using two-tailed paired T-tests.

Response of female sand flies to infected dog odour extract was compared to response of male sand flies to infected dog odour extract using a two-tailed T-test.

Normality tests (Anderson Darling and Kolmogorov-Smirnov) were used to confirm that data were normally distributed and control data were analysed by binomial test.

To determine if there was a relationship between parasite load and the female and male sand fly response, we carried out regression analyses on the proportion of females (and males) that were attracted to the infected dog hair odour and the parasite load of the dog. A correlation analysis was used to determine if there was a relationship between the discrimination status of odour extracts obtained in the VOC analyser study and the proportion of female or male sand flies that responded to the extracts.

All statistical analyses were performed using MINITAB 19 (Minitab, Inc. 2020).

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.

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Author roles

Conceptualization	JGCH
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Supporting information captions

S1 Table. **Female *Lutzomyia longipalpis* response to entrained odour of infected and uninfected dogs in unblinded and blinded Y-tube olfactometer experiments.** Summary of

blinded and unblinded Y-tube bioassay experiments for female *Lu. longipalpis*. The odour extracts from infected dogs (in bold) and uninfected dogs are shown. Infected = the number of male sand flies attracted to the arm of the Y-tube containing infected odour; uninfected = the number of male sand flies attracted to the arm of the Y-tube containing uninfected odour; no-response = number of sand flies that did not respond in the Y-tube experiment. a = unblinded experimental replicates, b = blinded experimental replicates. Statistical significance was analysed by binomial test comparison of number of sand flies responding to test and control odours (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

S2 Table. Male *Lutzomyia longipalpis* response to entrained odour of infected and uninfected dogs in the unblinded and blinded Y-tube olfactometer experiments.

Summary of blinded and unblinded Y-tube bioassay experiments for male *Lu. longipalpis*. The odour extracts from infected dogs (in bold) and uninfected dogs are shown. Infected = the number of male sand flies attracted to the arm of the Y-tube containing infected odour; uninfected = the number of male sand flies attracted to the arm of the Y-tube containing uninfected odour; no-response = number of sand flies that did not respond in the Y-tube experiment. a = unblinded experimental replicates, b = blinded experimental replicates. Statistical significance was analysed by binomial test comparison of number of sand flies responding to test and control odours (*ns* = not significant). The experimental outcomes are presented in the same order as for the female bioassays (Table 1) for ease of comparison.