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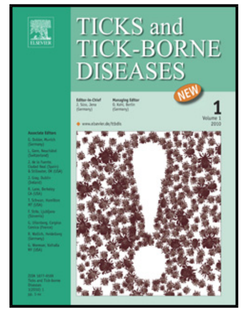


# Identification of a non-host semiochemical from miniature pinscher, *Canis lupus familiaris,* that repels *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae)

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**Identification of a non-host semiochemical from miniature pinscher, *Canis lupus familiaris,* that repels *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae)**

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**Abstract**

It is already known that the beagle breed of domestic dogs produces semiochemicals capable of repelling the brown dog tick, *Rhipicephalus sanguineus* sensu lato (s.l.). With a view to discovering new non-host semiochemicals as tick repellents, we compared the semiochemicals produced by a putative tick-resistant breed of dog, miniature pinscher, with known tick-resistant (beagle) and tick-susceptible (English cocker spaniel) breeds. Two non-host compounds produced by beagles, i.e. 2-

hexanone and benzaldehyde, were shown to be present in samples collected from all three breeds. Furthermore, two compounds, 6-methyl-5-hepten-2-one and 1,2,4-trimethylbenzene, were found in higher amounts in samples collected from miniature pinscher dogs. The mean amounts of benzaldehyde, 2-hexanone and 1,2,4-trimethylbenzene were similar for beagles and miniature pinschers ($P > 0.05$) and higher than the means observed for cocker spaniels ($P < 0.05$), whereas the mean amount of 6-methyl-5-hepten-2-one produced by miniature pinschers was significantly higher ($P < 0.05$) than for the other breeds of dogs. In Petri-dish assays with adult *R. sanguineus* s.l., 6-methyl-5-hepten-2-one was repellent for all observation periods evaluated for the two highest concentrations (0.100 and 0.200 $mg.cm^{-2}$, $P < 0.01$). The obtained results support our hypothesis that miniature pinschers are a tick-resistant dog breed and agree with previous observations of miniature pinschers being the breed least parasitized by ticks. Furthermore, the non-host semiochemical 6-methyl-5-hepten-2-one has potential to be developed for use as a repellent for the protection of susceptible dogs from *R. sanguineus* s.l. ticks.

**Keywords:** brown dog tick; miniature pinscher; 6-methyl-5-hepten-2-one; allomone.

## 1. Introduction

*Rhipicephalus sanguineus* sensu lato (s.l.) is a tick species complex associated with dogs around the world. Members of this complex have morphological, biological and genetic differences, are adapted to domestic environments and have been able to colonize both human and canine dwellings (Gray et al., 2013; Nava et al., 2015). They are vectors of several pathogens for dogs such as *Babesia vogeli* and *Ehrlichia canis*, and human beings like as *Rickettsia conorii* and *Rickettsia rickettsia*, besides they can causes direct injuries such us blood spoliation when in heavy infestations (Otranto et al., 2012; Raoult and Parola, 2007; Parola et al., 2009).

Previous studies on *R. sanguineus* s. l. behavior showed that there is a difference in susceptibility to parasitism between English cocker spaniel and beagle breeds of dog, with ticks feeding on beagles having their development impaired (Louly et al., 2009, 2010). More recently, Borges et al (2015) investigated the semiochemicals produced by resistant beagles and susceptible English cocker spaniels, which allowed them to identify two compounds, benzaldehyde and 2-hexanone, produced by beagles that were able to repel adult *R. sanguineus* s. l. in *in vitro* bioassays. A prototype system was developed using both allomones in a rate of release similar to that found naturally in beagles and was partially successful in controlling ticks in an artificially infested environment (Oliveira-Filho et al., 2016, 2017). The longevity, but not the level, of prototype efficacy was increased in a later study (Oliveira-Filho et al., 2018) by using higher doses of compounds compared to doses tested by Oliveira-Filho et al (2016).

Silva (2016) ranked dog breeds with respect to tick parasitism and hemoparasites transmitted and found that the English cocker spaniel had the highest prevalence of ticks (34.25%), while the miniature pinscher was the least parasitized (4.72%). Although ticks were not identified in this study, *R. sanguineus* s.l. has the highest prevalence on urban dogs in Brazil, more than any other tick species (Silva et al., 2017; Szabó et al., 2001, 2010). These results led us to hypothesize that the miniature pinscher breed could be resistant to parasitism by *R. sanguineus* s.l. similar to beagles (Louly et al., 2009, 2010), and that it may also produce semiochemicals which mediate tick repellency as was observed for beagles (Borges et al., 2015). Thus, the aim of the present study was to test the hypothesis that miniature pinschers produce compounds that repel *R. sanguineus* s.l. Odours of the two breeds of dogs that have already been studied, ie. beagle (resistant) and cocker (susceptible), in addition to miniature pinscher were collected and compared and *in vitro* behavioral tests were performed with compounds identified from miniature pinscher.

## 2. Material and methods

### 2.1. Tick colony

Engorged females of *R. sanguineus* s. l. were obtained from naturally infested dogs attended at the Hospital Veterinário of the Universidade Federal de Goiás (UFG), in Goiânia, Brazil. Ticks were not tested for any pathogens before incubation. The engorged females were incubated at 27±1°C and >80% relative humidity for egg posture. Larvae and nymphs were incubated at the same conditions described above, and naïve rabbits (*Oryctolagus cuniculus*) were used for feeding those stages, using a feeding apparatus that was glued onto the animal's shaved back (Louly et al., 2009). For the bioassays, unfed adults were used, aged between 7 and 21 days. This work was approved by the Ethics Committee on Animal Use of the UFG (CEUA-UFG) (Prot. nº 005/2017).

### 2.2. Semiochemical collection from dogs and chemical analysis

For semiochemical collection, 21 adult dogs were sampled: seven beagles (1 to 4 years old), six English cocker spaniels (2 to 3 years old) and eight miniature pinschers (1 to 9 years old). Each sample received a number in ascending order from "1" to the total of dogs of that breed. The dogs were mixed sex and were not bathed 15 days prior to odour collection. They were neither treated with topical acaricides for at least 30 days nor systemic acaricides (e.g. fluralaner, afoxolaner, sarolaner, etc.) for at least six months prior to odour collection.

Dog odours were collected using the technique described by Gikonyo et al. (2002) and modified by Oliveira-Filho et al. (2016). The collection was accomplished in the owners' house. For each collection, a rectangular capsule (10 cm × 9 cm × 8 cm) similar to an envelope was used, with one side made of clean aluminum foil and the other of wire-mesh stainless steel (100 µm), containing a filter paper sachet (9 cm × 9 cm × 7 cm) (Whatman no. 1 qualitative), with activated charcoal (350 mg, 40 µm; Fluka, Sigma–Aldrich) and octadecyl bonded silica (ODS)-$C_{18}$ (450 mg, particle size 40 µm; JT Baker-Hexis, Sigma–Aldrich) as adsorbents. One capsule was fastened onto the dorsum of each dog, with the steel face in contact with the skin, and left for 8 h. After this period, the sachet was taken to the laboratory and stored in a covered glass container until extraction. The adsorbents from the sachets containing trapped volatiles were placed into an empty glass vial (5 mL). Dichloromethane (DCM, 1 mL) was added to the vial and then the vial was sonicated (Ultrasonic cell disrupter - Unique) for 10 min. DCM extracts were transferred using glass pipettes to conical vials, and then evaporated to 100 µL under a gentle stream of nitrogen gas (Air Liquid Brazil Ltd). Concentrated extracts were sealed and stored at -20ºC until required for chemical analysis.

The concentrated extracts (4 µl) were analyzed by gas chromatography (GC) and coupled GC–mass spectrometry (GC–MS) as described by Borges et al. (2015). The identification of the compounds was made by comparing spectra with mass spectral databases and GC peak enhancement, using authentic samples of chemicals, as described below (Borges et al., 2015). To compare the spectra with mass spectral databases and GC peak enhancement, 2-hexanone, benzaldehyde, 6-methyl-5-hepten-2-one and 1,2,4-trimethylbenzene were purchased from Sigma-Aldrich. A calibration assay was done for determine the concentration of 2-hexanone, benzaldehyde, 6-methyl-5-hepten-2-one and 1,2,4-trimethylbenzene in the dogs extracts according with Oliveira Filho et al. (2016). Primary stock standard solutions of all compounds at a concentration of 700 µg $mL^{-1}$ were prepared using ether bi-distillate as solvent. Stock solutions were diluted serially to reach a concentration of 20.00 µg $mL^{-1}$, which was used to produce five different concentrations (20.00, 15.00, 8.00, 3.00, and 1.5 µg $mL^{-1}$). Each final concentration was tested in triplicate. One µl of each solution was injected and analyzed by GC-FID using the same method as detailed in the previous section. This method was adopted to account for technical variation in the calibration procedure.

For statistical analysis of the mean quantities of each substance found in different breeds of dogs, the BioEstat software version 5.0 was used. For normally distributed data the means were compared using analysis of variance (ANOVA) and Tukey test, whereas for non-normally distributed data, the means were compared using Kruskal-Wallis analysis and Student-Newman-Keuls test ($p$<0.05).

### 2.3. Petri-dish bioassay

The compounds produced in higher amounts by miniature pinschers, ie. 6-methyl-5-hepten-2-one and 1,2,4-trimethylbenzene, and a 1:1 mixture of the two compounds, were tested in an *in vitro* Petri-dish bioassay at three different concentrations ie. 0.050, 0.100 and 0.200 mg $cm^{-2}$ (1.8%, 3.6% and 7.2%, respectively). These concentrations were also used by Borges et al (2015) and were selected because commercially available formulations with the repellent N,N-diethyl-m-toluamide have this compound at the highest concentration ie. 7.2%. The compounds were diluted in hexane.

The experiments were performed in an acclimatized room under both controlled temperature (27±1ºC) and relative humidity (70±10%), in complete darkness (Bissinger et al. 2009; Borges et al. 2015; Ferreira et al., 2017). A glass petri-dish of 10 cm diameter, containing two semi-circles (31.8 cm² of area) filter paper (Whatman cat nº 1001) covering the bottom of the plate was used. One semicircle received 87 µl of one of the above-mentioned compounds in each concentration mentioned. The other semicircle received the same volume of the solvent (control side). The treated filter papers were dried for 10 min inside an exhaust hood and then used in the assays. Six adult ticks (three males and three females) were placed in the Petri-dish along the line formed by the junction of the treated and untreated papers. Two control tests were performed: solvent (hexane) versus clean paper (non-treated paper) and clean paper versus clean paper. The positions of the ticks were evaluated at 5, 10 and 30 min after the beginning of each experiment and the tests were replicated 10 times (total of 60 ticks per treatment). The percentage of repellence of each compound and concentration was calculated based on the number of ticks that remained in the control side of the Petri-dish. A Chi-square test was performed for comparison of the tick choices, taking the significance level of $p < 0.05$.

## 3. Results

Chemical analysis of collected dog odour extracts showed that the mean amounts of benzaldehyde, 2-hexanone and 1,2,4-trimethylbenzene released per hour were similar for beagles and miniature pinschers and higher than the mean amounts observed for cocker spaniels. On the other hand, extracts from miniature pinschers contained greater mean amounts of 6-methyl-5-hepten-2-one released per hour (222.61 ± 26.56 ng/h), which was statistically significantly different when compound to beagles (104.94 ± 20.13 ng/h) and cocker spaniels (33.53 ± 3.90 ng/h). The mean amount ratio between benzaldehyde and 2-hexanone for beagles was 2.58 ± 1.36, for cocker spaniels 0.14 ± 0.35 and for miniature pinschers 1.75 ± 0.30 (Fig. 1, Table 1).

In both control Petri-dish bioassays, ticks were homogeneously distributed (data not shown). The compound 6-methyl-5-hepten-2-one was repellent at all concentrations and times evaluated, with the exception of the lowest concentration at 10 and 30 min. The mean repellency of 6-methyl-5-hepten-2-one varied from 65% at the lowest concentration of 0.050 $mg.cm^{-2}$ at 5 min, to 71.7% at the highest concentration of 0.200 $mg.cm^{-2}$ in the same period of observation. On the other hand, 1,2,4-trimethylbenzene was only repellent at the highest concentration in the 5-min observation (65%). When both compounds were tested together in a 1:1 mixture, there was no improvement in the efficacy of repellency, with activity only observed at the concentration of 0.100 mg $cm^{-2}$ varying from 66.1% to 76.3% (Table 2).

## 4. Discussion

In this paper we show that miniature pinscher, a putative tick-resistant breed of dog, produces the non-host semiochemicals benzaldehyde and 2-hexanone in a similar manner to beagles, ie. the known tick-resistant breed as shown by Borges et al. (2015). We also show that greater amounts of another non-host semiochemical for this tick, 6-methyl-5-hepten-2-one, are also produced by miniature pinschers. The present study corroborates previous results obtained by Borges et al (2015), in that cocker spaniels produce lower amounts of benzaldehyde and 2-hexanone compared to beagles, and the ratio of these two compounds in beagles is similar to that reported by Oliveira-Filho et al (2016).

When studying the prevalence of ticks and tick-borne diseases in Rio de Janeiro state, Brazil, Silva (2016) pointed out that the miniature pinscher breed was the least parasitized out of nine dog breeds. However, tick species were not identified in this study; *R. sanguineus* s.l. has the highest prevalence on urban dogs in Brazil, more than any other tick species (Silva et al., 2017, Szabó et al., 2001, 2010). The compound 6-methyl-5-hepten-2-one was found in greater amounts in extracts collected from miniature pinschers when compared to beagles and cocker spaniels. When tested in Petri dish assays, this compound was consistently repellent against *R. sanguineus* s. l. and stood out when compared to benzaldehyde and 2-hexanone (Borges et al., 2015). 6-Methyl-5-hepten-2-one has already been identified from waterbuck (*Kobus defassa*) and zebra (*Eguus quagga*) and shown to play a role as a repellent against *Glossina* species (Diptera) (Gikonyo et al., 2002, 2003, Olaide et al., 2019). Therefore, based on our results with 6-methyl-5-hepten-2-one, and the presence of benzaldehyde and 2-hexanone in miniature pinschers in similar amounts and ratios to that observed for tick-resistant beagles observed here and by Borges et al. (2015), we postulate that miniature pinscher is a breed that is resistant to *R. sanguineus* s.l. Based on the above it is possible also to propose that miniature pinschers are even more resistant to *R. sanguineus* s.l. than beagles, considering that they produce the highest amount of 6-methyl-5-hepten-2-one. However, studies to compare the performance of the tick on both breeds as well on a susceptible breed such as English cocker spaniel are required in order to confirm those suppositions.

Only one out of six cocker spaniels was shown to emit benzaldehyde in its odour. This dog shared a dwelling with a miniature pinscher that emitted 58.25 ng/h of benzaldehyde (Table S1). It is possible to assume that this cocker spaniel acquired this volatile organic compound through contact with the miniature pinscher. However, studies to confirm this are necessary. It is known that some birds and mammals exhibit anointing and fumigation behaviour, rolling on or wiping themselves with fruits, leaves, or other plant parts or incorporating aromatic leaves or bark in their nests (Weldon et al., 2011). This behavior can deter nuisance arthropods. We do not believe that cocker spaniels purposefully avoid parasitism through such behaviour. However, it would be interesting to evaluate if dogs which are susceptible to ticks, such as cocker spaniels (Louly et al., 2009, 2010) can be less parasitized by *R. sanguineus* s.l. when sharing a dwelling with less susceptible breeds.

Studies developed by Oliveira Filho et al. (2017, 2018) observed the potential of the allomones produced by beagles, 2-hexanone and benzaldehyde, to control artificial infestations by *R. sanguineus* s.l. on dogs.  Both studies concluded that although these repellents represent a new and innovative technology to control *R. sanguineus* s.l., high efficacy in reducing tick burden is desirable. Currently, we are evaluating these repellents on dogs, along with fungi associated with tick pheromones, as part of a push-pull strategy to control *R. sanguineus* s.l. In this case, ticks repelled from the dogs would be attracted by arrestment pheromones (Gowrishankar et al., 2019) to traps containing entomapathogenic fungi that would kill them (Alves et al., 2017). The results obtained in the present study can also help to improve the efficacy of the repellent-based strategy by incorporating 6-methyl-5-hepten-2-one in the repellent blend.  Studies to evaluate the repellent activity *in vitro* and also on animals are necessary in order to demonstrate this.

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## Credit author statement

**Viviane Zeringota**: Investigation, Formal Analysis, Writing - Original draft preparation. **Ronaldo Alves**: Investigation**. André Sarria: Investigation, Formal Analysis,** Writing- Original draft preparation. **Ariele Henrique: Investigation. Michael Birkett:** Resources, Supervision, Writing- Reviewing and Editing. **Lígia Borges** Resources, Conceptualization, Supervision, Writing- Review and Editing

**Conflict of interest** On behalf of all authors, the corresponding author states that there is no conflict of interest.

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**Fig. 1.** Mean amount (± SEM) of semiochemicals collected per hour (ng/h) from beagle (n = 7), English cocker spaniel (n = 6) and miniature pinscher (n = 8) breeds of dog. Means for the same semiochemicals with different letters differ significantly ($P < 0.05$).

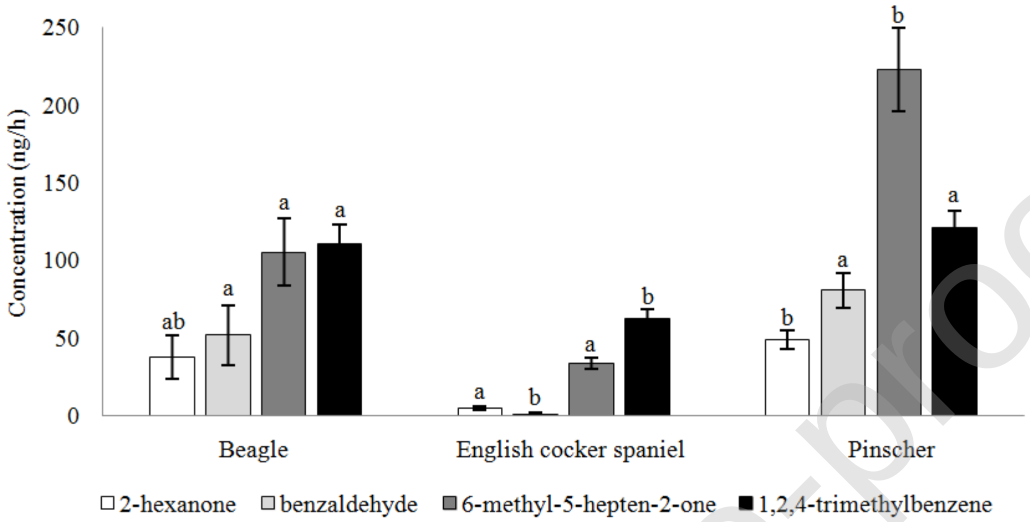


**Table 1** Mean amount and SEM of semiochemicals collected per hour (ng/h) from beagles (n = 7), English cocker spaniels (n = 6) and miniature pinschers (n = 8), and the ratio of benzaldehyde to 2-hexanone in odour samples.

| Breed | 2-hexanone | benzaldehyde | 6-methyl-5-hepten-2one | 1,2,4-trimethylbenzene | Ratio benzaldehyde:2-hexanone |
|---|---|---|---|---|---|
| Beagle | $37.69^{ab}$ (13.00) | $51.68^{a}$ (17.70) | $104.94^{a}$ (20.13) | $110.60^{a}$ (11.05) | 2.58 (1.36) |
| English cocker spaniel | $4.90^{a}$ (1.06) | $0.94^{b}$ (0.94) | $33.53^{a}$ (3.90) | $62.39^{b}$ (6.34) | 0.14 (0.35) |
| Miniature pinscher | $48.42^{b}$(6.09) | $80.64^{a}$ (11.25) | $222.61^{b}$ (26.56) | $120.72^{a}$ (11.24) | 1.75 (0.30) |

Means followed by different letters in the same column differ significantly ($P <$ 0.05).

**Table 2** Response of adult *Rhipicephalus sanguineus* s.l. to 1,2,4-trimethylbenzene, 6-methyl-5-hepten-2-one and a 1:1 mixture of 1,2,4-trimethylbenzene and 6-methyl-5-hepten-2-one in a Petri-dish bioassay. Data are represented as the mean (± SEM) % of ticks found on the untreated side of a Petri-dish 5, 10 and 30 minutes after experiments began. A stimulus was considered repellent if the % of ticks on the untreated side of the Petri-dish was statistically higher than the % on the stimulus side. Data were analyzed using a Chi-squared test. * $P < 0.05$; ** $P < 0.01$.

| Concentrations (mg $cm^{-2}$) | Time (min) | 1,2,4-trimethylbenzene | 6-methyl-5-hepten-2-one | 1,2,4-trimethylbenzene:6-methyl-5-hepten-2-one (1:1) |
|---|---|---|---|---|
| 0.050 | 5 | 48.3 ± 2.8 | 65.0* ± 3.6 | 35.0 ± 2.1 |
| | 10 | 43.3 ± 3.1 | 53.3 ± 3.2 | 41.0 ± 2.2 |
| | 30 | 53.3 ± 2.4 | 50.0 ± 2.3 | 39.0 ± 1.4 |
| 0.100 | 5 | 44.3 ± 2.2 | 68.0** ± 2.9 | 66.3* ± 2.4 |
| | 10 | 51.7 ± 3.8 | 70.0** ± 2.6 | 74.6** ± 3.1 |
| | 30 | 52. ± 2.0 | 70.0** ± 2.6 | 76.3** ± 2.1 |
| 0.200 | 5 | 65.0* ± 2.9 | 71.7** ± 3.5 | 60.0 ± 2.5 |
| | 10 | 58.3 ± 2.1 | 70.0** ± 1.3 | 56.7 ± 2.7 |
| | 30 | 60.0 ± 2.5 | 66.7** ± 2.7 | 60.0 ± 2.5 |