Brown dog tick, *Rhipicephalus sanguineus sensu lato*, infestation of susceptible dog hosts is reduced by slow release of semiochemicals from a less susceptible host

Jaires Gomes de Oliveira Filho^{a#}, Lorena Lopes Ferreira^{a#}, André Lucio Franceschini Sarria^b, John A. Pickett^b, Michael A. Birkett^b, Gabriel Moura Mascarin^c, Adalberto A. Pérez de León^d, Lígia Miranda Ferreira Borges^{e*}

^a Escola de Veterinária e Zootecnia. Universidade Federal de Goiás. Campus Samambaia, Avenida Esperança, s/n, Campus Universitário, CEP: 74690-900, Goiânia, Goiás, Brazil.

E-mail addresses: jaires_filho@hotmail.com; loren4_lopes@hotmail.com

^b Biological Chemistry and Crop Protection Department, Rothamsted Research, West Common, Harpenden, Hertfordshire, AL5 2JQ, United Kingdom.

E-mail addresses: andre.sarria@rothamsted.ac.uk; mike.birkett@rothamsted.ac.uk; john.pickett@rothamsted.ac.uk

^c EMBRAPA Arroz e Feijão, Rodovia GO-462, Km 12, Zona Rural, Caixa Postal: 179, CEP: 75375-000 - Santo Antônio de Goiás, Goiás, Brazil.

E-mail address: gabriel.mascarin@embrapa.br

^d USDA, ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory and Veterinary Pest Genomics Center, 2700 Fredericksburg Road, Kerrville, TX 78028, USA

E-mail address: beto.perezdeleon@ars.usda.gov

^e Instituto de Patologia Tropical e Saúde Pública. Universidade Federal de Goiás, Rua 235 s/n, Setor Universitário, CEP: 74605050, Goiânia, Goiás, Brazil.

E-mail address: borges.ligia@gmail.com

*Corresponding author

These authors contributed equally to this work.

Abstract

Domestic dog breeds are hosts for the brown dog tick, *Rhipicephalus sanguineus sensu lato*, but infestation levels vary among breeds. Beagles are less susceptible to tick infestations than English cocker spaniels due to enhanced production of 2-hexanone and benzaldehyde that act as volatile tick repellents. We report the use of prototype slow-release formulations of these compounds to reduce the burden of *R. sanguineus* s. l. on English cocker spaniel dogs. Twelve dogs were randomly assigned to two groups with six dogs each. The treated group received collars with slow-release formulations of the compounds attached, while the control group received collars with clean formulations attached. Five environmental infestations were performed, with the number of ticks (at all stages) on the dogs being counted twice a day for 45 days. The counts on the number of tick stages found per dog were individually fitted to linear mixed effects models with repeated measures and normal distribution for errors. The mean tick infestation in the treated group was significantly lower than in the control group. For larvae and nymphs, a decrease in tick infestation was observed at the fifth count, and for adults, lower average counts were observed in all counts. The compounds did not interfere with the distribution of the ticks on the body of the dogs, as a similar percentage of ticks was found on the anterior half of the dogs (54.5% for the control group and 56.2% for the treated group). The biological and reproductive parameters of the ticks were not affected by the repellents. This study highlights for the first time the potential use of a novel allomone (repellent)-based formulation for reduction of tick infestation on susceptible dogs.

Key-words: non-host; allomone; 2-hexanone; benzaldehyde; formulation; SLM's.

1. Introduction

54

55 The brown dog tick, *Rhipicephalus sanguineus sensu lato*, has a cosmopolitan
 56 host distribution and, in addition to parasitizing domestic dogs, can parasitize birds,
 57 livestock and human beings (Borges and Silva, 1994; Louly et al., 2006; Rodríguez-Vivas et
 58 al., 2016; Szabó et al., 2012). *R. sanguineus* l. comprises a species complex (Nava et al.,
 59 2015; Szabó et al., 2005), and is of veterinary and public health importance due to its
 60 obligate blood feeding habit and role as a vector of pathogens such as *Babesia vogeli*,
 61 *Ehrlichia canis*, *Anaplasma platys*, *Rickettsia rickettsii* and *R. conorii* (Cardoso et al., 2010;
 62 Ereemeeva et al., 2011; Pacheco et al., 2011; Socolovich et al., 2009).

63 Tick infestation levels can vary within a single host species according to host
 64 breed, age, immunological state, and individual semiochemical production (Bunnett et al.,
 65 2011; Pickett et al., 2010; Weldon, 2010). Our hypothesis, relating to the ecological basis of
 66 ectoparasite repellency in hosts (Pickett et al., 2010), is that individual hosts, either closely
 67 related taxonomically or from a single taxon, differ in their interaction with ectoparasites
 68 due to differences in the production and emission of non-host chemical signaling.
 69 Furthermore, individual signal components emitted by each host can act synergistically or
 70 can exert antagonism to other ectoparasites (Nielsen et al., 2015; Weldon, 2010). For
 71 example, a blend of volatiles produced by the non-host waterbuck, *Kobus defassa*, was
 72 identified as being repellent to tsetse flies, *Glossina morsitans*, (Bett et al., 2015; Gikonyo
 73 et al., 2002; Gikonyo et al., 2003). However, little is known about host natural odors that
 74 are repellent for ticks.

75 In our previous work where it was shown that beagles were less infested with *R.*
 76 *sanguineus* l. than the English cocker spaniel, it was suggested that a host factor could
 77 determine differential tick load (Louly et al., 2009; Louly et al., 2010). Our subsequent work
 78 identified two volatile small lipophilic molecules (SLMs), 2-hexanone and
 79 benzaldehyde, which are produced in greater amounts in beagles, and which act as natural

80 repellents against *R. sanguineus* s.l. (Borges et al., 2015), and demonstrated constitutive
 81 release of these two SLMs in the odor of beagles (Oliveira Filho et al., 2016).

82 Acaricidal treatments remain the most common practice to control *R.*
 83 *sanguineus* s.l. However, the indiscriminate use of products containing synthetic
 84 acaricides represents a strong selective pressure that results in the emergence of resistant
 85 tick populations to those active ingredients (Borges et al., 2007; Eiden et al., 2015; Miller et
 86 al., 2001). Considering the role of non-host chemical signaling in host selection by
 87 ectoparasites, it has been suggested that such signaling, produced by resistant hosts, may
 88 form the basis of ecologically-based repellents to control ectoparasite infestation (Pickett et
 89 al., 2010; Weldon, 2010), and may also reduce the risk of exposure to vector-borne
 90 pathogens. Following our earlier work on biting fly repellents from non-preferred hosts
 91 (Birkett et al., 2004; Logan et al., 2008; Logan et al., 2009), we hypothesized that tick
 92 numbers on a susceptible animal host could be reduced by application of natural
 93 repellents discovered in the odor of less preferred conspecifics. Here, we investigate the
 94 effect of a prototype system, delivering 2-hexanone and benzaldehyde via slow release
 95 from polyethylene sachets, upon *R. sanguineus* s.l. infestation on English cocker
 96 spaniels, following artificial exposure under environmentally controlled conditions.

97

98 **2. Material and Methods**

99

100 **2.1. Animals**

101 The use of animals (dogs and rabbits) in this study was approved by the
 102 Committee on Ethical Animal Use of the Federal University of Goiás (CEUA/UFG, protocol
 103 number 024/2014). The care and use of the animals during this study were undertaken
 104 according to bioethics and animal welfare guidelines required by CEUA/UFG. Eight male and
 105 four female English cocker spaniel dogs, with ages varying from 50 to 116 days old, were

106 obtained from different breeders. They were treated for intestinal worms (Drontal Puppy -
 107 Bayer), received all applicable vaccinations (Vanguard Plus and Defensor – Pfizer), and
 108 were not treated with acaricides for 30 days before the study was initiated. All the dogs
 109 had their pelage cut to the same length prior to the initiation of the study, in order to offer
 110 the same conditions for tick infestation and also to facilitate tick counts. Dogs were housed
 111 in a kennel at the Veterinary and Animal Husbandry School of the Federal University of
 112 Goiás, Brazil. The kennel has a total area of 26.6 m², with an internal area of 9.8 m² that
 113 was used to inspect the dogs and six individual stalls each one with 2.4 m² being a cover
 114 area with 1.28 m² and an open area with 1.12 m². The floor is cemented and the walls of
 115 the kennel are glazed for easy cleaning. A week prior to the beginning of the
 116 experiment, all areas of the kennel were sanitized with a flamer three times in a week.
 117 During the progress of the experiment, the animals were fed twice a day (Golden Filhotes-
 118 Premier®), following the amounts recommended by the manufacturer and given water *ad*
 119 *libitum*, while the floor was cleaned daily with water and neutral detergent. The health of
 120 the animals was monitored daily and clinical pathology exams were conducted
 121 when necessary. Dogs were randomly divided composing two groups (treated and control)
 122 with six animals each (four males and two females per group). The dogs were randomly
 123 divided into three per stalls in order to facilitate the handling of animals and cleaning
 124 facilities.

125

126 2.2. Ticks

127 Engorged females of *R. sanguineus* s.l. were harvested from naturally infested
 128 dogs for the establishment of a colony. The ticks were maintained in an acclimatized
 129 chamber (27±1°C and 80% R.H.) and fed on rabbits (*Oryctolagus cuniculus*) to obtain
 130 differing life stages for use in dog infestations (Loully et al., 2010). The ticks used in the
 131 experiments were aged between one and three weeks old. Rabbits were removed from

132 use after two consecutive infestations. During infestations, rabbits were examined daily
133 and none showed symptoms of damage due to tick parasitism.

134

135 **2.3. Preparation and use of prototype non-host semiochemical delivery system**

136 Samples of analytical grade 2-hexanone (Aldrich Chemical Co. Ltd., Dorset,
137 UK) and benzaldehyde (Sigma-Aldrich Co. Ltd., Steinheim, Germany) were used in this
138 study. Sheets of cellulose sponge (0.5cm thick, code 0032 6865J. Sainsbury plc),
139 previously soaked in chloroform overnight then washed in chloroform 3 times before drying
140 in a fume cupboard, were cut into pieces (~2.5 x 2 cm) and either treated with 400 µl of
141 benzaldehyde or 2-hexanone, or left untreated. Polyethylene sachets were prepared by heat
142 sealing the sponges inside polyethylene sheeting (~3 x 3 cm, Al Packaging Ltd., London
143 film type LFT size 50mm gauge and 62.5 mic lift). The mean release rate per day of the
144 compounds from the sachet formulations was determined over several weeks using
145 dynamic headspace collection (air entrainment) and GC analysis. Thus, three sachets
146 filled with either benzaldehyde or 2-hexanone were enclosed in a glass vessel (700mL).
147 Air was pumped through an activated charcoal filter into the vessel (1L/min) and was then
148 drawn (500 cc/min) into tubes containing the adsorbent Porapak Q (50 mg). After one
149 hour, volatiles collected on the Porapak Q were eluted with 750 µl of redistilled diethyl
150 ether and the samples were stored at -20°C until analysis. Extracts (1 µL) were analysed
151 on an Agilent 6890 N GC fitted with a 10 m x 0.32 mm i.d. HP-1 column. The oven
152 temperature was maintained at 30°C for 0.1 min, then programmed at 10°C min⁻¹ to 250°C
153 and held for 30 min. The quantity of compound captured per sample was expressed in
154 µg/day using the analytical method described by Oliveira-Filho et al. (2016). For 15 days,
155 the release rates were 30.44 ± 1.12 µg/day for benzaldehyde and 14.38 ± 1.26 µg/day for
156 2-hexanone, decreasing sharply to 11.18 µg/day and 1.29 µg/day, respectively, after the

157 15th day. The ratio of released 2-hexanone and benzaldehyde broadly matched the ratio of
 158 naturally release from beagles (Oliveira-Filho et al. (2016)), and so experiments were
 159 conducted with these prototype formulations. The sachets were stored at -20°C until
 160 required for tests. Sachets were attached to dogcollars using a stapler shooter containing
 161 light duty staples (5/16" 8mm – Stanley) and covered withgauze to prevent injury to the
 162 dogs' necks.A single2-hexanone sachet and a singlebenzaldehyde sachet was attached
 163 per dog collar. A collar with oneuntreated sachet was used in each of the untreated control
 164 group dogs.

165

166 **2.4.Tick infestation, evaluation, and sampling**

167 Five artificial environmental infestations were established each time by
 168 positioning breeding vials against the stall walls approximately 50 cm above the floor to
 169 release 2,000larvae, 100 nymphs, and 60 adults (30 males and 30 females) per stall. The
 170 first infestation was held 10 days before the attachment of the first collar, and afterwards
 171 four moreinfestations were carried out, every seven days.The collars were changed after
 172 two weeks, and were removed one week after the fifthinfestation and a count followed by
 173 an additional week. The dogs were rotated among the stalls on a weekly basis and in a
 174 clockwise fashion, which promoted standard experimental conditions to the animals.

175 Dogs were thoroughly inspected every day for larvae, nymphs and adults in the
 176 morning and afternoon, starting two days after the initial infestation and until seven days
 177 afterthe second set of repellent delivery collars were removed. A map of 24 body areas,
 178 including*R.sanguineuss*.l. sites, was used to standardize tick counts (Otranto et al., 2005).
 179 The final number of ticks per day was determined by the maximum number of ticks per
 180 area found in one day, and the counts representing the total of ticks found per dogs during
 181 a week, except at the first count that lasted 10 days.

During the counts, the dogs were brushed twice a day to collect all engorged stages of *R. sanguineus* s.l. Male ticks were not removed during the brushes. The ticks collected from the dogs were placed in a climate chamber (27±1°C and 80% R.H.) to assess the engorged female weight (EFW), egg mass weight (EMW), conversion of body weight on eggs (CBWE), conversion of body weight on larvae (CBWL), percentage of larval-hatchability (LH), percentage of larval ecdysis (LE), and percentage of nymphal ecdysis (NE). EFW was obtained after manual detachment of females while EMW was determined 15 days after the onset of egg laying. LH was evaluated by averaging the counts of two evaluators according to the estimate of the percentage of larvae that hatched from eggs (Bechara et al., 1994).

2.5. Statistical analysis

Count data on the number of larvae, nymphs and adults found per dog were individually fitted to linear mixed effects models with repeated measures and normal (Gaussian) distribution for errors. Normality assumptions were previously checked based on Shapiro-Wilk and Bartlett tests. In addition, goodness-of-fit of the models were assessed using residual plots. These models were implemented using the “lme4” package (Bates et al., 2015, <https://CRAN.R-project.org/package=lme4>) from the free statistical software R (R Core Team, 2014, <http://www.R-project.org/>). The linear predictors were represented by treatment (control vs. treated dogs wearing the repellent collar), infestations, and their interaction term. Since all dogs were repeatedly evaluated for all four weeks, dog was included as a random effect in models (i.e., to allow for non-independence of each time measurement from a given dog). Therefore, the model may be written as:

$$Y_{ijn} = \beta_0 + X_i + T_j + X_i^*T_j + \tau_n + \varepsilon_{ij}$$

207 Where Y_{ijn} is the value of the outcome variable (number of larvae, nymphs or
 208 adults per dog) for the i -th treatment of the j -th infestation at the n -th replicate (i.e., dog), β_0
 209 is the intercept, X_i is the treatment effect, T_j is the infestation time, $X_i * T_j$ is the interaction
 210 term, τ_n is the random effect for dogs, and ε_{ij} is the error for treatment i in time j . Then, type
 211 3 F -tests with Satterthwaite's approximation for degrees of freedom was employed to
 212 assess significance (P -values) of fixed effects using the "lmerTest" package from R
 213 (Kuznetsova, 2015, <http://CRAN.R-project.org/package=lmerTest>). When the interaction
 214 term was significant, multiple pairwise comparisons were performed based on differences
 215 of least squares means at $P < 0.05$.

216 The number of engorged larvae, nymphs and females recovered during the
 217 brushes was insufficient to evaluate the biological and reproductive parameters over the
 218 successive infestations. The Student t test at $P < 0.05$ was used to compare these
 219 parameters between treated and control groups adding the ticks recovered from all
 220 infestations, during the time the dogs were wearing the repellent collars.

221

222 3. Results

223 During the first infestation of *R. sanguineus* s.l. on dogs, conducted before the
 224 attachment of repellent-treated collars, the number of ticks (larvae, nymphs and adults) in
 225 the two groups was virtually similar, ranging from a rate of 30 ticks found on treated group
 226 and 27 ticks on the control group (larvae: $t = 0.2774$, $df = 5$, $P = 0.7926$; nymphs: $t = 0.0$,
 227 $df = 5$, $P = 1.0$; adults: $t = 0.3974$, $df = 5$, $P = 0.7075$). In the last count (6th week) realized
 228 without artificial infestation and in the absence of collars, a total (larvae, nymphs and
 229 adults) of 203 and 204 ticks in treated and control groups was observed, respectively. No
 230 larvae were found in either group, only five nymphs were found in the treated group vs. six
 231 in control group ($t = 0.3492$, $df = 5$, $P = 0.7412$), and the average number of adults was
 232 198 for untreated dogs vs. 198 for treated dogs ($t = 0.1661$, $df = 5$, $P = 0.8746$).

233 According to the *F*-test, treatment and infestation time (or sampling date) had a
 234 significant effect on the number of larvae, nymphs and adults of *R. sanguineus* s. l. found
 235 on dogs, as their interaction term was significant ($P= 0.0110$, $P= 0.0430$, $P= 0.0373$,
 236 respectively). This indicates that all life stages of this tick species considerably decreased
 237 across time in the presence of the repellent formulation (Fig 1). The compounds did not
 238 interfere with the distribution of the ticks on the body of the dogs, as a similar percentage
 239 of ticks was found on the anterior half of the dogs (54.5% for the control group and 56.2%
 240 for the treated group).

241 A more pronounced reduction in tick infestation due to the repellent effect was
 242 noted for larvae at the 5th count, with averages of 4.67 larvae in the treated group against
 243 10.67 larvae in the control group ($P< 0.0001$). Likewise, the counts of nymphs showed a
 244 significant difference in the same interval with average loads of 3.33 and 10.67 nymphs in
 245 the control and treated groups respectively ($P< 0.0001$). Averages for adult counts always
 246 showed significant reductions in infestation upon artificial infestations in the four
 247 consecutive weeks for animals wearing the repellent collar compared to those wearing
 248 untreated collars. For instance, in the 2nd infestation, the means of adult ticks found were
 249 12.67 and 21.33 in treated and control groups respectively ($P = 0.002$). In the 3rd
 250 infestation, the mean was 34.5 in treated group vs. 39.67 in control group ($P = 0.006$), in
 251 the 4th infestation the number was 41 in treated and 49.67 in control group ($P< 0.0001$),
 252 and in 5th infestation the mean of adults reached 39.67 and 48 in treated and control,
 253 respectively ($P< 0.0001$) (Fig 1).

254 Regardless of the treatment, all tick life stages tended to increase with time, and
 255 population peaks were noted at the 4th or 5th week of evaluation (Fig 1). Overall, tick
 256 infestation loads in dogs wearing the collar containing the repellent formulation exhibited
 257 significantly lower numbers of larvae ($P=0.00049$), nymphs ($P= 0.00027$) and adults ($P<$
 258 0.0001), when compared to untreated dogs from the control group (Fig 2). The weight of

the female ticks, egg conversion, larval hatchability and ecdysis were statistically similar in treated and control groups (Table 1), suggesting that the repellent formulations had no effect on the biological parameters of this tick species.

262

263 4. Discussion

The results presented here support the hypothesis that non-host unsuitability can be conferred to a suitable host by treatment with non-host semiochemicals (Borges et al., 2015; Pickett et al., 2010; Weldon, 2010; Weldon, 2013). Our findings document, for the first time, *in vivo* activity of a natural repellent blend containing odor components of a resistant host, which could be regarded in general as a safer tool to be used to manage *R. sanguineus* s. l. infestations on dogs, and perhaps other susceptible host species. The doses of the two compounds tested are well below the median lethal dose (LD₅₀) for each compound. Furthermore, they are benign, are used as flavorings, perfume components and are present in food (ATSDR, 1995; FDA, 2013; Sigma-Aldrich, 2015). Experimental data showed that the release of 2-hexanone and benzaldehyde using the prototype delivery system prevented infestation levels in a dog breed known to be susceptible to parasitism by *R. sanguineus* s. l. The artificial environmental infestations were established to resemble more closely field conditions of dog exposure to *R. sanguineus* s. l. (Hansford et al. 2015; Rodriguez-Vivas et al. 2016).

Several non-host compounds deter parasitism of animals by hematophagous arthropods (Birkett et al., 2004; Borges et al., 2015; Douglas et al., 2004; Gikonyo et al., 2002; Gikonyo et al., 2003; Pageat, 2005). In several cases, however, their evaluation as the basis for innovative control technologies remains to be accomplished. Saini et al. (2013) tested a collar prototype with repellents identified on the non-host *Kobus defassa* by Gikonyo et al. (2003) in association with a synthetic repellent against *Glossinamorsitans*. A 90% reduction in the transmission of trypanosomiasis was observed

285 after the use of this prototype. Refinement of the blend constituents showed the potential to
 286 deploy the repellent-based technology to protect cattle from tsetse and the risk of
 287 exposure to *Trypanosoma* spp. (Bett et al., 2015). Pageat (2005) reported the presence of
 288 allomones bis(2-ethylhexyl) adipate and 2,2,4-trimethyl-1,3-pentanediol
 289 diisobutyrate from the secretion of uropygial gland of ducks that repel red poultry mites,
 290 *Dermanyssus gallinae*, and a product comprising these compounds is being manufactured
 291 and distributed in France for use in the control of *D. gallinae* on poultry farms. The use of
 292 a prototype system to deliver repellent compounds provides an opportunity to develop an
 293 optimal formulation, in terms of compound quantity and ratio and matrix for formulation
 294 stability, for efficient sustained release over several weeks, as has been attempted with
 295 other efforts to develop new formulations to control *R. sanguineus* s. l. infestations on dogs
 296 (Bhoopathy et al., 2014; Stanneck et al., 2012).

297 Our results indicated that the counts of larvae (0.47%) and nymphs (12.15%)
 298 are a fraction of the total released into the environment where the dogs were housed.
 299 Various sources of variability contributed to our inability to recover all the ticks released.
 300 Despite our methodical approach, it is possible some ticks were missed during visual and
 301 tactile inspection, and during brushing for ectoparasite enumeration, because of the small
 302 size of larvae and nymphs, immature ticks can also perish due to desiccation and predation
 303 (Apanaskevich and Oliver Jr., 2014; Troughton and Levin, 2007). Host grooming is a
 304 preponderant behavior that can result in the removal of up to 80% of the ticks, mainly
 305 larvae, because they can attach several times before feeding, which is accompanied by
 306 itching that triggers grooming (Hart, 2000; Mooring et al., 1996).

307 Higher counts for adults (180%) were observed parasitizing the dogs than those
 308 released in the environment. When the adults were counted, males and unengorged
 309 females were not removed from the dogs. Therefore, the higher number of adults found on
 310 dogs could be related to the fact that males of *R. sanguineus* s. l. can survive in the

environment for up to 568 days and seek the host repeatedly for feeding, or finding a mate thus moving between hosts and throughout the environment (Hooker et al., 1912; Little et al., 2007; Troughton and Levin, 2007). It is possible that male ticks may have been counted on the same dog in different areas or even on different dogs through time because our prototype formulation was repellent and not acaricidal.

Adult tick numbers on treated dogs were lower than on the control animals across all counts. However, this trend was observed for larvae and nymphs only in the fifth count. Synthetic compounds like DEET promote significant repellency against all *R. sanguineus* s.l. developmental stages, but the rate and duration of repellency tends to decrease with time when this compound was tested against adults (Kumar et al., 1992). The natural repellents 2-hexanone and benzaldehyde were observed to repel adult *R. sanguineus* s.l. *in vitro* as strongly as DEET (Borges et al., 2015). Further research is required to determine the relative repellency of 2-hexanone and benzaldehyde against *R. sanguineus* s.l. larvae and nymphs.

The recovery rate for engorged larvae, nymphs, and females was 0.17, 0.80 and 3.33%, respectively. Notwithstanding the number of engorged ticks that may have been missed during brushing, some ticks could have engorged and detached from the dogs before the animals were inspected to quantify the ectoparasite load. Engorged larvae detach from their hosts during the day and night, while engorged nymphs and females detach mainly during the night (Paz et al., 2008). Therefore, the majority of engorged ticks may have dropped off at night before the dogs were brushed during the day.

A lack of effect on the biological and reproductive parameters of *R. sanguineus* s.l. was noted for 2-hexanone and benzaldehyde. However, *R. sanguineus* s.l. that fed on a resistant dog breed had their biological parameters impaired (Louly et al., 2009). *R. sanguineus* s.l. apparently senses repellent SLMs to distinguish between resistant and susceptible hosts, but also is deterred by contact compounds produced by resistant

337 animals (Louly et al., 2010). In comparison to chemosensation in congeneric ticks, it was
 338 hypothesized that *R. microplus* could perceive not only phagostimulants known to be
 339 present in host blood, but also anti-feeding substances that could exist in cattle serum
 340 associated with decreased susceptibility to tick infestation (Ferreira et al., 2015). Additional
 341 experiments are needed to determine if the deleterious effects observed in *R. sanguineus*
 342 s. l. upon feeding on resistant dogs are caused by compounds other than 2-hexanone and
 343 benzaldehyde, which could be present on host skin or circulating in the blood.

344 The specificity of SLM perception and susceptibility to their effects are linked to
 345 subtle ratios of the compounds produced by hosts and non-host species (Bruce et al.,
 346 2005; Logan et al., 2008; Weldon, 2010). The release rate adopted here roughly matched
 347 that observed by Oliveira Filho et al. (2016), where it was shown, using analytical chemistry
 348 experiments, that the mean ratio of 2-hexanone:benzaldehyde was ~ 1:2 in odor samples
 349 collected from beagles. Further work is needed to determine optimal doses and
 350 formulations for the two compounds, which will underpin our attempts to develop a
 351 commercial product.

352 Problems with the control of ticks such as *R. sanguineus* s. l. and the pathogens
 353 they transmit are increasing in complexity (Dantas-Torres et al., 2015; Esteve-Gassent et
 354 al., 2016). The One Health approach, which is the synthesis of strategies aimed
 355 at enhancing animal and human health that take into consideration our environment, has
 356 been proposed to address the problem of tick and tick-borne diseases as a way to facilitate
 357 the development of sustainable control solutions (Dantas-Torres et al., 2012; Pérez de León
 358 et al., 2010; Vayssier-Taussat et al., 2015). Work on natural products that repel arthropod
 359 vectors, including ticks, merits attention for development because they could be used as
 360 part of integrated pest management programs, and as tools to enable the rational use of
 361 acaricides (Guerrero et al., 2014; Mencke, 2013; Pérez de León et al., 2014).

Natural semiochemicals such as those tested here are ubiquitous and form part of the adaptive behavioral ecology of *R. sanguineus* s. l. (Nielsen et al., 2015; Pickett et al., 2010; Weldon, 2010). Further research will help understand the mode of repellency of 2-hexanone and benzaldehyde against *R. sanguineus* s. l. As has been noted with other species, knowledge gaps on the anatomy and molecular biology of chemosensation and chemoreception in ticks remain to be elucidated (Borges et al., 2016; Esteve-Gassent et al., 2016; Ferreira et al., 2015; Renthal et al., 2016). An enhanced understanding of how ticks detect and process chemical cues to find hosts or avoid unsuitable but potential hosts could offer further innovative technologies to mitigate the burden of these ectoparasites and vectors of pathogens that affect humans, domestic animals, and wildlife.

Acknowledgments.

We thank the National Council of Technological and Scientific Development (CNPq), Brazil, for its financial support (Grant no. #/2013-9) and for supply a Scholarship to LMFB (#/2015-0). Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the United Kingdom. USDA is an equal opportunity provider and employer.

References

- Apanaskevich, D.A., Oliver Jr, J.H., 2014. Life cycles and natural history of ticks. In: Sonenshine, D.E. and Roe, M. (Eds.), *Biology of ticks* Vol. 1. Oxford University Press, Oxford and New York, pp. 59-73.
- ATSDR - Agency for Toxic Substances and Disease Registry., 1995. 2-Hexanone CAS # 591-78-6. www.atsdr.cdc.gov/toxfaqs/tfacts44.pdf. Last access: January 2016.

- 386 Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. *lme4*: Linear mixed-effects models
 387 using Eigen and S4. R package version 1.1-9, [https://CRAN.R-](https://CRAN.R-project.org/package=lme4)
 388 [project.org/package=lme4](https://CRAN.R-project.org/package=lme4)>. Last access: January 2016.
- 389 Bechara, G.H., Szabó, M.P.J., Mukai, L.S., Rosa, P.C.S., 1994. Immunization of dogs,
 390 hamsters and guinea pigs against *Rhipicephalussanguineus* using crude unfed
 391 adult tick extracts. *Vet. Parasitol.* 52, 79–90.
- 392 Bett, M.K., Saini, R.K., Hassanali, A., 2015. Repellency of tsetse-refractory waterbuck
 393 (*Kobus defassa*) body odor to *Glossinapallidipes* (Diptera: Glossinidae):
 394 assessment of relative contribution of different classes and individual constituents.
 395 *Acta Trop.* 146, 17-24.
- 396 Bhoopathy, D., Latha, B.R., Uma, T.S., Sreekumar, C., Leela, V., 2014. A novel approach
 397 to control brown dog tick, *Rhipicephalussanguineus* using sustained release poly-ε-
 398 caprolactone-pheromone microspheres. *ActaParasitol.* 59, 153-157.
- 399 Birkett, M.A., Agelopoulos, N., Jensen, K.M.V., Jespersen, J.B., Pickett, J.A., Prijs,
 400 H.J., Thomas, G., Trapman, J.J., Wadhams, L.J., Woodcock, C.M., 2004. The role
 401 of volatile semiochemicals in mediating host location and selection by nuisance and
 402 disease-transmitting cattle flies. *Med. Vet .Entomol.* 18, 313–322.
- 403 Borges, L.M.F., Li, A.Y. Olafson, P.U., Renthal, R., Bauchan, G.R., Lohmeyer, K.H., Pérez
 404 de León, A.A., 2016. Neuronal projections from the Haller's organ and palp sensilla
 405 to the synganglion of *Amblyommaamericanum*. *Rev Brasil. Parasitol. Vet.* 25, 217-
 406 224.
- 407 Borges, L.M.F., Oliveira Filho, J.G., Ferreira, L.L., Louly, C.C.B., Pickett, J.A., Birkett,
 408 M.A., 2015. Identification of non-host semiochemicals for the brown dog tick,
 409 *Rhipicephalussanguineussensulato* (Acari: Ixodidae), from tick-resistant beagles,
 410 *Canis lupusfamiliaris*. *Ticks Tick Borne Dis.* 6, 676–682.

- 411 Borges, L.M.F., Silva, C.R.F., 1994. Ixodídeos parasitos de bovinos e equinos da
412 microrregião de Goiânia, Goiás. Rev. Parasit. Trop.23, 69-74.
- 413 Borges, L.M.F., Soares, S.F., Fonseca, I.N., Chaves, V.V., Louly, C.C.B., 2007.
414 Resistência acaricida em larvas de *Rhipicephalussanguineus* (Acari: Ixodidae)de
415 Goiânia-GO, Brasil. Rev. Pat. Trop. 36, 87–95.
- 416 Bruce, T.J.A., Wadhams L.J., Woodcock, C.M., 2005. Insect host location: a volatile
417 situation. Trends Plant. Sci. 10, 269-274.
- 418 Bunnell, T., Hanisch, K., Hardege, J.D., Breithaupt, T., 2011. The fecal odor of sick
419 hedgehogs (*Erinaceuseuropaeus*) mediates olfactory attraction of the tick
420 *Ixodeshexagonus*. J. Chem. Ecol. 37, 340–347.
- 421 Cardoso, L., Tuna, J., Vieira, L., Yisaschar-Mekuzas, Y., Baneth, G., 2010. Molecular
422 detection of *Anaplasmaplatys* and *Ehrlichia* in dogs from the North of
423 Portugal.Vet. J. 183, 232–233.
- 424 Dantas-Torres F., 2015. Climate change, biodiversity, ticks and tick-borne diseases: the
425 butterfly effect. Int. J. Parasitol. Parasites Wildl. 4, 452-461.
- 426 Dantas-Torres, F., Chomel, B.B, Otranto, D., 2012.Ticks and tick-borne diseases: a One
427 Health perspective. Trends in Parasitol.28, 437-346.
- 428 Douglas, H.D., Co, J.E., Jones, T.H., Conner, W.E., 2004. Interspecific differences in
429 *Aethia spp.* auklet odorants and evidence for chemical defense against
430 ectoparasites.J.Chem. Ecol. 30, 1921-1935.
- 431 Eiden, A.L., Kaufman, P.E., Ol, F.M., Allan, S.A., Miller, R.J., 2015. Detection of
432 permethrin resistance and fipronil tolerance in *Rhipicephalussanguineus* (acari:
433 ixodidae) in the United States. J. Med. Entomol. 52, 429-36.
- 434 Eremeeva, M.E., Zambrano, M.L., Anaya, L., Beati, L., Karpathy, S.E., Santos-Silva, M.M.,
435 Salceda, B., Macbeth, D., Olguin, H., Dasch, G. A., Aranda C.A., 2011. *Rickettsia*
436 *rickettsii* in *Rhipicephalus* Ticks, Mexicali, Mexico.J. Med. Entomol.48, 418-421.

- 437 Esteve-Gassent, M.D., Castro-Arellano, I., Feria-Arroyo, T.P., Patino, R., Li, A.Y., Medina,
 438 R.F., Pérez de León, A.A., Rodríguez-Vivas, R.I., 2016. Translating ecology,
 439 physiology, biochemistry, and population genetics research to meet the challenge of
 440 tick and tick-borne diseases in North America. Arch. Insect Biochem. Physiol. 92,
 441 38-64.
- 442 FDA - Food and Drug Administration., 2013. Everything added to food in the United
 443 States. www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=eafusListing. Last
 444 access: January 2016.
- 445 Ferreira, L.L., Soares, S.F., Oliveira Filho, J.G., Oliveira, T.T., Pérez de León, A.A.,
 446 Borges, L.M.F., 2015. Role of *Rhipicephalus microplus* cheliceral receptors in
 447 gustation and host differentiation. Ticks Tick Borne Dis. 6, 228–233.
- 448 Gikonyo, N.K., Hassanali, A., Njagi, P.G.N., Gitu, P.M., Midiwo, J.O., 2002. Odor
 449 composition of preferred (Buffalo and Ox) and nonpreferred (Waterbuck) hosts of
 450 some savanna tsetse flies. J. Chem. Ecol. 28, 969–981.
- 451 Gikonyo, N.K., Hassanali, A., Njagi, P.G.N., Saini, R.K., 2003. Response of
 452 *Glossina morsitans morsitans* to blends of electroantennographically active
 453 compounds in the odor of its preferred (buffalo and ox) and nonpreferred
 454 (waterbucks) hosts. J. Chem. Ecol. 29, 2331–2345.
- 455 Guerrero, F.D., Pérez de León, A.A., Rodríguez-Vivas, R.I., Jonson, N., Miller, R.J.,
 456 Andreotti, R., 2014. Acaricide research and development, resistance and resistance
 457 monitoring. In: Sonenshine, D.E. and Roe, M. (Eds.), Biology of ticks Vol. 2. Oxford
 458 University Press, Oxford and New York, pp. 353-381.
- 459 Hansford, K.M., Pietzsch, M., Cull, B., Medlock, J.M., 2015. Brown dog tick infestation of a
 460 home in England. Vet. Rec. 176, 129-130.
- 461 Hart, B.L., 2000. Role of grooming in biological control of ticks. Ann. N. Y. Acad. Sci. 916,
 462 565-569.

- 463 Hooker, W.A., Bishopp, F.C., Wood, H.P., 1912. The life history and bionomics of some
464 North American ticks. USDA Bur. Ent. Bul. 106, 239.
- 465 Kumar, S., Prakash, S., Kaushik, M.P., Rao, K.M., 1992. Comparative activity of here
466 repellents against the ticks *Rhipicephalussanguineus* and *Argas persicus*. Med. Vet.
467 Entomol. 6, 47-50.
- 468 Kuznetsova, A., Brockhoff, P.B., Christensen, R.H.B., 2015. lmerTest: Tests in Linear
469 Mixed Effects Models. R package version 2.0-29. [http://CRAN.R-](http://CRAN.R-project.org/package=lmerTest)
470 [project.org/package=lmerTest](http://CRAN.R-project.org/package=lmerTest). Last access: January 2016.
- 471 Little, S.E., Hostetler, J., Kocan, K.M., 2007. Movement of *Rhipicephalussanguineus* adults
472 between co-housed dogs during active feeding. Vet. Parasitol. 150, 139–145.
- 473 Logan, J.G., Birkett, M.A., Clark, S.J., Powers, S., Seal, N.J., Wadhams, L.J., 2008.
474 Identification of human-derived volatile chemicals that interfere with attraction of
475 *Aedes aegypti* mosquitoes. J. Chem. Ecol. 34, 308–322.
- 476 Logan, J.G., Seal, N.J., Cook, J.I., Stanczyk, N.M., Birkett, M.A., Clark S.J., Gezan, S.A.,
477 Wadhams, L.J., Pickett, J.A., Mordue (Luntz), J.M., 2009. Identification of human-
478 derived volatile chemicals that interfere with attraction of the scottish biting midge
479 and their potential use as repellents. J. Med. Entomol. 46, 208-219.
- 480 Louly, C.C.B., Fonseca, I.N., Oliveira, V.F., Borges, L.M.F., 2006. Ocorrência de
481 *Rhipicephalussanguineus* em trabalhadores de clínicas veterinárias e canis
482 domicílio de Goiânia, Go. Cienc. Anim. Bras. 7, 103–106.
- 483 Louly C.C.B., Soares, S.F., Silveira, D.N., Guimarães, M.S., Borges, L.M.F., 2010.
484 Differences in the behavior of *Rhipicephalussanguineus* tested against resistant and
485 susceptible dogs. Exp. Appl. Acarol. 51, 353-362.
- 486 Louly, C.C.B., Soares, S.F., Silveira, D.N., Neto O.J., Silva, A.C., Borges, L.M.F., 2009.
487 Differences in the susceptibility of two breeds of dogs, English cocker spaniel and
488 beagle, to *Rhipicephalussanguineus* (Acari: Ixodidae). Int. J. Acarol. 35, 25-32.

- 489 Mencke, N., 2013. Future challenges for parasitology: vector control and 'One health' in
 490 Europe: the veterinary medicinal view on CVBDs such as tick borreliosis,
 491 rickettsiosis and canine leishmaniosis. *Vet. Parasitol.* 195, 256-271.
- 492 Miller, J.R., George, J.E., Guerrero, F., Carpenter, L., Welch, J.B., 2001. Characterization
 493 of acaricide resistance in *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae)
 494 collected from the Corozal Army Veterinary Quarantine Center, Panama. *J. Med.*
 495 *Entomol.* 38, 293-302.
- 496 Mooring, M.S., McKenzie, A.A., Art, B.L.H., 1996. Role of sex and breeding status in
 497 grooming and total tick load of impala. *Behav. Ecol. Sociobiol.* 39, 259–266.
- 498 Nava, S., Estrada-Peña, A., Petney, T., Beati, L., Labruna, M.B., Szabó, M.P.J., Venzal,
 499 J.M., Mastropolo, M., Mangold, A.J., Guglielmone, A.A., 2015. The taxonomic status
 500 of *Rhipicephalus sanguineus* (Latreille, 1806). *Vet. Parasitol.* 208, 2–8.
- 501 Nielsen, B.L., Rampin, O., Meunier, N., Bombail, V., 2015. Behavioral responses to odors
 502 from other species: introducing a complementary model of allelochemicals involving
 503 vertebrates. *Front. Neurosci.* 9, 1-11.
- 504 Oliveira Filho, J.G., Sarria, A.L.F., Ferreira, L.L., Caulfield, J.C., Powers, S.J., Pickett, J.A.,
 505 Pérez de León, A.A., Birkett, M.A., Borges, L.M.F., 2016 Quantification of brown
 506 dog tick repellents, 2-hexanone and benzaldehyde, and release from tick-resistant
 507 beagles, *Canis lupus familiaris*. *J. Chromatogr. B.* 1022, 1-6.
- 508 Otranto, D., Lia, R.P., Cantacessi, C., Galli, G., Paradies, P., Mallia, E., Capelli, G., 2005.
 509 Efficacy of a combination of imidacloprid 10% permethrin 50% versus fipronil 10%
 510 (S)-methoprene 12%, against ticks in naturally infected dogs. *Vet. Parasitol.* 130,
 511 293–304.
- 512 Pacheco, R.C.; Moraes-Filho, J.; Guedes, E., Silveira, I., Richtzenhain, L. J., Leite, R.C.,
 513 Labruna, M.B., 2011. Rickettsial infections of dogs, horses and ticks in Juiz de Fora,

- 514 southeastern Brazil, and isolation of *Rickettsia rickettsii* from
 515 *Rhipicephalus sanguineus* ticks. Med. Vet. Entomol. 25, 148–155.
- 516 Pageat, P., 2005. Allomone repulsive compositions for controlling
 517 arachnids. PCT/EP2003/007143.
- 518 Paz, G.F., Labruna, M.B., Leite, R.C., 2008. Drop off rhythm
 519 of *Rhipicephalus sanguineus* (Acari: Ixodidae) of artificially infested dogs. Rev. Bras.
 520 Parasitol. Vet. 17, 139-144.
- 521 Pérez de León, A.A., Strickman, D.A., Knowles, D.P., Fish, D., Thacker, E., de la Fuente,
 522 J., Krause, P.J., Wikel, S.K., Miller, R.S., Wagner, G.G., Almazán, C., Hillman, R.,
 523 Messenger, M.T., Ugstad, P.O., Duhaime, R.A., Teel, P.D., Ortega-Santos, A.,
 524 Hewitt, D.G., Bowers, E.J., Bent, S.J., Cochran, M.H., McElwain, T.F., Scoles, G.A.,
 525 Suarez, C.E., Davey, R., Howell Freeman, J.M., Lohmeyer, K., Li, A.Y., Guerrero,
 526 F.D., Kammlah, D.M., Phillips, P., Pound, J.M., Group for Emerging Babesioses and
 527 One Health Research and Development in the U.S., 2010. One Health approach to
 528 identify research needs in bovine and human babesioses: workshop report.
 529 Parasit. Vectors. 3, 36.
- 530 Pérez de León, A.A., Teel, P.D., Li, A., Ponnusamy, L., Roe, R.M., 2014. Advancing
 531 integrated tick management to mitigate burden of tick-borne diseases. Outlooks
 532 Pest Management. 25, 382-389.
- 533 Pickett, J.A., Birkett, M.A., Dewhirst, S.Y., Logan, J.G., Omolo, M.O., Torto, B., Pelletier,
 534 J., Syed, Z., Leal, W.S., 2010. Chemical ecology of animal and human pathogen
 535 vectors in a changing global climate. J. Chem. Ecol. 36, 113-121.
- 536 R Core Team (2014). R: A language and environment for statistical computing. R
 537 Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>.
 538 (accessed 06.01.2016).

- 539 Renthall, R., Maghnani, L., Bernal, S., Qu, Y., Griffith, W., Lohmeyer, K., Guerrero, F.,
 540 Borges, L.M.F., Pérez de León, A.A., 2016. The chemosensory appendage
 541 proteome of *Amblyomma americanum* (Acari: Ixodidae) reveals putative odorant-
 542 binding and other chemoreception-related proteins. *Insect Sci.*
- 543 Rodríguez-Vivas, R.I., Apanaskevich, D.A., Ojeda-Chi, M.M., Trinidad-Martínez I., Ryes-
 544 Noveloc, Esteve-Gassent M.D., Pérez de León A.A., 2016. Ticks collected from
 545 humans, domestic animals, and wildlife in Yucatan, Mexico. *Vet. Parasitol.* 215,
 546 106–113.
- 547 Saini, R., Andoke, J., Muasa, P., Mbuvi, D., Marete, T., Ngiela, J., Mbahin, N., Affogon, H.,
 548 2013. Cows in “waterbuck” clothing for protection against tsetse flies and enhanced
 549 productivity. In: Il'ichev, A.L., Cardé, R.T.D., Williams, G., Zalucki, M.P. (Eds.),
 550 Conference Program and Abstracts Handbook of International Chemical Ecology
 551 Conference. 19–23 August, 2013, Melbourne, p. 59.
- 552 Sigma Aldrich., 2015. Safety Data Sheet of 2-hexanone and benzaldehyde.
 553 <http://www.sigmaaldrich.com/>. (accessed 13.01.16).
- 554 Socolovicsh, C., Bitam, I., Raoult, B., Parola, P., 2009. Transmission of *Rickettsia conorii*
 555 *conorii* in naturally infected *Rhipicephalus sanguineus*. *Clin. Microbiol. Infect.* 15,
 556 319–321.
- 557 Stanneck, D., Kruehwagen, E.M., Fourie, J.J., Horak, I.G., Davis, W., Krieger, K.J., 2012.
 558 Efficacy of an imidacloprid/flumethrin collar against fleas, ticks, mites and lice on
 559 dogs. *Parasit. Vectors.* 5, 102.
- 560 Szabó, M.P.J., Mangold, A.J., Joao, C.F., Bechara, G.H., Guglielmone, A.A., 2005.
 561 Biological and DNA evidence of two dissimilar populations of the
 562 *Rhipicephalus sanguineus* tick group (Acari: Ixodidae) in South America. *Vet.*
 563 *Parasitol.* 130, 131–140.

- 564 Szabó, M.P.J., Rossi, G.F., Cabral, D.D., Martins, M.M., Amorim, G.M.P., Tsuruta, S.A.,
565 2012. Experimental evaluation of birds as disseminators of the
566 cosmopolitan tick *Rhipicephalus sanguineus* (Acari: Ixodidae). Exp. Parasitol. 132,
567 389–393.
- 568 Troughton, D.R., Levin, M.L., 2007. Life cycles of seven ixodid tick species (Acari:
569 Ixodidae) under standardized laboratory conditions. J. Med. Entomol. 44, 732-740.
- 570 Vayssier-Taussat, M., Cosson, J.F., Degeilh, B., Eloit, M., Fontanet, A., Moutailler, S.,
571 Raoult, D., Sellal, E., Ungeheuer M.N., Zylbermann, P., 2015. How a
572 multidisciplinary 'One Health' approach can combat the tick-borne pathogen threat
573 in Europe. Future Microbiol. 10, 809–818.
- 574 Weldon, P.J., 2010. Nuisance arthropods, nonhost odors, and vertebrate chemical
575 aposematism. Nat. Wiss. 97, 443–448.
- 576 Weldon, P.J., 2013. Chemical aposematism. Chemoecol. 23, 201-202.
- 577
- 578
- 579

580 **Figure legends.**

581 Figure 1. Mean ($\pm$ SD, standard deviation) number of ticks (larvae, nymphs, and adults)
 582 retrieved from untreated (control) and treated (repellent collar) dogs across infestation
 583 periods. Vertical arrows indicate when dogs received the repellent collar. At 4th infestation
 584 interval, the repellent collar was replaced by a new one and in the 5th the repellent collar
 585 was removed. The counts during a week, except the first count during 10 days. Significant
 586 difference between control and treated dogs is indicated by $P < 0.05$ (*) or $P < 0.01$ (**).

587

588 Figure 2. Overall mean ($\pm$ SD, standard deviation) number of ticks (larvae, nymphs, and
 589 adults) retrieved from untreated (control) and treated (repellent collar) dogs. Significant
 590 difference between control and treated dogs is indicated by $P < 0.05$ (*) or $P < 0.01$ (**).

591

592

593 Table 1 - Mean ($\pm$ SD) of biological parameters of *Rhipicephalus sanguineus sensu lato* ticks
 594 obtained along successive infestations on dogs treated or non- treated with 2-hexanone
 595 and benzaldehyde.

596

597

598

599

600

601

602

603

604

605

606

607

608

609

610

611

Table 1

Parameters	Groups	
	Treated	Control
EFW (g)	93.00 ± 30.00 ^a (n=10)	81.00 ± 34.00 ^a (n=10)
EMW (g)	50.00 ± 25.00 ^a (n=10)	40.00 ±21.00 ^a (n=10)
LH (%)	83.55 ±31.64 ^a	83.33 ±31.72 ^a
CBWE (%)	51.99 ±16.41 ^a	52.61 ±20.72 ^a
CBWL (%)	42.87 ±22.39 ^a	49.45 ±19.96 ^a
LE (%)	64.08 ±21.27 ^a (n=23)	79.16 ±24.57 ^a (n=39)
NE (%)	77.77 ±40.36 ^a (n=9)	75.17.±41.83 ^a (n=7)

All parameters evaluated were statistically similar between treated and control groups by *t*-Student test (P > 0.05). EFW: engorged female weight, EMW: egg mass weight, LH: larval-hatchability rate, CBWE: conversion body weight on eggs, CBWL: conversion body weight on larvae, LE: percentage of larval ecdysis, NE: percentage of nymphal ecdysis.