



Innovative way to dispense pheromones for off-host control of *Rhipicephalus sanguineus* sensu lato ticks



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ABSTRACT

Vapour patches dispensing pheromones were evaluated as lures to increase the attractiveness of sticky tick traps for *Rhipicephalus sanguineus* sensu lato (s.l.). Sex pheromone (SP), assembly pheromone (AP) and a combination of SP + AP at optimal concentrations were impregnated in vapour patches. The responses of the different stages of *R. sanguineus* s.l. (i.e. larvae, nymphs and adults) to the pheromones were evaluated using a Petri dish bioassay. The impregnated vapour patches were retained as such for a period of two mo and their efficacy was reassessed. In a subsequent field trial, pheromone impregnated vapour patches were placed as lures in bamboo (*Bambusa vulgaris*) sticky traps designed for the control of ticks in dog kennels. *In vitro* AP impregnated vapour patches were effective in attracting the different life stages of *R. sanguineus* s.l. whereas SP was effective in attracting the unfed and fed male stages of *R. sanguineus* s.l. The field trial revealed that questing and engorged larvae, nymphs and females of *R. sanguineus* s.l. were attracted more towards AP impregnated vapour patches than SP and AP + SP impregnated vapour patches. Fed and unfed male ticks were lured effectively by SP alone. The combination of SP + AP revealed no potent additive or synergistic effect.

1. Introduction

Ticks are important vectors of infectious agents causing diseases in humans, companion animals and livestock (Parola and Raoult, 2001; Peter et al., 2005; de la Fuente et al., 2008). The endophilic and monotropic brown dog tick, *Rhipicephalus sanguineus* sensu lato (s.l.), is the most prevalent tick infesting dogs in Tamil Nadu, India (Koshy et al., 1983). It acts as a vector for transmission of *Ehrlichia canis*, *Babesia canis* and *Hepatozoon canis* (Baneth et al., 2001). The diseases caused by these pathogens can present as life threatening conditions in pet animals if not treated immediately. Being a three host tick, generally all life stages of *R. sanguineus* s.l. are seen inside dog kennels (Louly et al., 2007; Dantas-Torres and Otranto, 2011). Acaricides have been used effectively for on- and off-host control of ticks (Dhivya, 2013). However, years of use, misuse and over-use of the available acaricides has resulted in the evolution of resistance in ticks (Coles and Dryden, 2014). Moreover, the use of acaricides contributes to environmental pollution and health hazard to the pet owners.

Lure and kill strategies generally combine attractants with an

acaricide to form a slow-release formulation or device. The attractive compound lures the tick to some object that contains a contact acaricide. The delivery device provides a safer means of acaricide application both for the pest control operator and the environment since the amount of the pesticide applied is reduced with restricted bioavailability to the general landscape (Carr and Roe, 2016). Use of pheromones as lures has been considered in control of dog ticks (Dhivya, 2013). Pheromones form a major part of semiochemicals which are highly potent, intraspecifically biologically active substances released on the skin or outer surface of an individual and are perceived by other individuals of the same species (Gothe, 1987). There are many benefits to utilization of pheromones in integrated pest management (IPM) strategies. These molecules are naturally occurring and are generally environmentally friendly. Moreover, they act only on the specific species. Additionally, in IPM strategies, these compounds are used at concentrations close to those found in nature and the quantity of insecticide used is reduced (Heuskin et al., 2011). The cost of control is thus reduced compared to conventional control measures (Heuskin et al., 2011).

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Ticks have three categories of pheromones, namely assembly pheromone (AP), sex pheromone (SP) and attraction-aggregation-attachment pheromones (Hamilton, 1992). These pheromones have been identified in metastriate, ixodid and argasid ticks (Sonensine, 1991). AP are waste products of nitrogen metabolism in tick excreta (Dusbabek et al., 1998) as well as in tick exuvia (Sonensine, 2004). They result in a behavior termed 'arrestment', where in ticks cease their ambulatory activity and form clusters in their natural environment (Carde and Baker, 1984). This clustering is believed to enhance host-finding success and avoid stressful conditions (Sonensine, 1985). Sex pheromones are compounds or mixtures of compounds that guide different phases of the mate-finding process, culminating in insemination of the female partner (Sonensine, 2006). Three types of sex pheromones have been described: attractant sex pheromone (ASP), mounting sex pheromone (MSP) and genital sex pheromone (GSP) (Sonensine, 2006). Sex pheromones (SP) such as 2,6-dichlorophenol are highly volatile and unstable while guanine, adenine and xanthine, which are components of the AP, are less volatile compounds when compared to 2,6-DCP.

Degradation of the pheromone whether volatile or non volatile due to micro environmental factors can be minimized by encapsulation (Dhivya, 2013). The AP of ticks has been successfully encapsulated in natural polymers such as calcium alginate, chitosan and poly- ϵ -caprolactone for sustained release in control of dog ticks (Bhoopathy and Latha, 2016). Calcium alginate encapsulated AP was very effective in attracting the different stages of ticks when compared with chitosan and poly- ϵ -caprolactone. However, encapsulation within calcium alginate is labour intensive and requires repeated checks to ensure encapsulation is indeed done. Kulkarni and Vandana (2018) had used vapour patches to release essential oils in a sustained manner. Impregnation of vapour patches is relatively easy and hence, the present study attempted to evaluate the efficiency of vapour patches as a novel delivery device for SP, AP and a combination of SP + AP. Following an *in vitro* assessment, the efficiency of the pheromone impregnated vapour patches for off-host control of *R. sanguineus* s.l. ticks in kennels was assessed utilizing a bamboo sticky trap. The bamboo sticky trap was devised with the intension of prolonging the stickiness of the trap and thereby its effectiveness by protecting it from exposure to the dust and grime in the environment.

2. Materials and methods

2.1. Collection of ticks

Engorged larvae, nymphs, females and fed males of *R. sanguineus* s.l. were collected from dogs housed in kennels at the Blue cross of India, a shelter for stray dogs located at Chennai, India. Ticks were removed from the dogs when they were brought to the Dermatology unit, Madras Veterinary College Teaching Hospital, Chennai with tick infestations without any harm or inconvenience to the dogs. The collected ticks were identified under a stereo microscope based on morphological characters (Patton and Cragg, 1984) (Fig. 1). The engorged female ticks were placed in dry plastic vials covered with cloth at room temperature (25°C). They were maintained for oviposition by placing them in a tray filled with moist sand to maintain the relative humidity of > 80% until oviposition was completed and the larvae hatched. The larvae so obtained were used as unfed larvae. Engorged larvae and nymphs collected from infested dogs were maintained as described above until they moulted into unfed nymphs and adults, respectively. *In vitro* bioassays were carried out using unfed and fed larvae, unfed and fed nymphs, unfed and engorged adult females and unfed and fed adult males.

2.2. Tick pheromones

Synthetic tick pheromones, namely SP (2,6-DCP) - D70201 and AP (Adenine - A8626, Guanine - G11950 and Xanthine - X0626), were

procured from Sigma-Aldrich, Germany (99% pure). The composition of AP was guanine-95 mg, adenine- 3.8 mg and xanthine- 3.8 mg dissolved in 4 ml of Millipore water. The AP solution (200 μ l) was used as an optimal concentration (Sonensine, 2004; Ranju, 2011; Bhoopathy et al., 2016 and Anish et al., 2017). The SP, namely 2,6-dichlorophenol - 8.23 mg, dissolved in 500 μ l of acetone (Louly et al., 2008; Ranju et al., 2012), was used at an optimal concentration in the present study. A combination of SP and AP was prepared by mixing the above two solutions and used as an optimal concentration 2.3. *Vapour patches*

Fabric based vapour patches of 150 g per square meter (gsm) with 350 mm diameter were procured from Crawford Wisdom, Delhi, India. The vapour patch was made from non-woven fabric designed to firmly retain the impregnated active ingredients so that there will be a sustained release. On one side of the patch, a self-adhesive material was present while the other side was covered with a fabric non-woven mesh into which the pheromones were impregnated. A layer of lamination is provided between the self-adhesive material and vapour patch so that the impregnated pheromone does not pass through the patch and cross-react with the adhesive material. The optimal concentration of 500 μ l of SP, 200 μ l of AP and a combination of 500 μ l of SP + 200 μ l of AP were directly impregnated into each vapour patch using micropipette. The patch was air dried for a period of 30 min. Plain vapour patches without any pheromone were used as negative controls. Acetone and Millipore water has not been used as control because it has been tried by previous researchers (Ranju, 2011; Dhivya, 2013 and Anish, 2015) with no impact on tick response. After complete drying, the impregnated patch was used for *in vitro* and field trials.

2.3. *In vitro* assessment – Petri dish bioassay

A method modified from Yoder and Stevens (2000) was employed in the current study. A glass Petri dish (Borosil, India) of 200 mm \times 30 mm diameter was used for the bioassay. Utmost care was taken in handling of ticks so that the first pair of legs as well as palps were not damaged. Ticks were handled with camel hair (No.9) brushes. Nitrile gloves were used to prevent the direct contamination of tick sensory organs by human skin volatiles. For each trial fresh active tick stages were used. Unfed stages were used within 24 hours (h) of moulting while fed stages were used the same day they dropped off in natural rhythm from the host. Vapour patches were used within 7 days (d) of being impregnated. All tests were conducted at room temperature and the results evaluated after 24 h. The number of ticks attracted to the pheromone-impregnated vapour patches affixed to one quadrant of the Petri dish was counted and recorded whereas the ticks in other three quadrant of the Petri dish was considered as not attracted to the pheromone impregnated vapour patches.

The SP, AP and SP + AP impregnated and control vapour patches were affixed to one quadrant of the different Petri dishes. Unfed larvae, nymphs and adult stages were placed in the opposite quadrant of the different Petri dishes affixed with pheromone impregnated vapour patches separately. A total of 500 unfed larvae and 500 engorged larvae, in batches of 100 larvae each, were exposed to each of the 3 treatments or the control while a total of 100 unfed and engorged nymphs, unfed and engorged females as well as unfed and fed male ticks were similarly exposed in batches of 20. The same vapour patches and the Petri dishes were used for all trials. The Petri dish was covered with another Petri dish and sealed with laboratory grade parafilm (Parafilm M, Germany) in order to prevent escape of ticks, and also to avoid response of the ticks to carbon dioxide emitted by the investigator which may lead to erroneous results.

2.4. Sustained release study

The impregnated vapour patches used in the previous trial were retained in individual plastic vials at room temperature for a period of 2 months (mo) for evaluating their efficacy for sustained release of

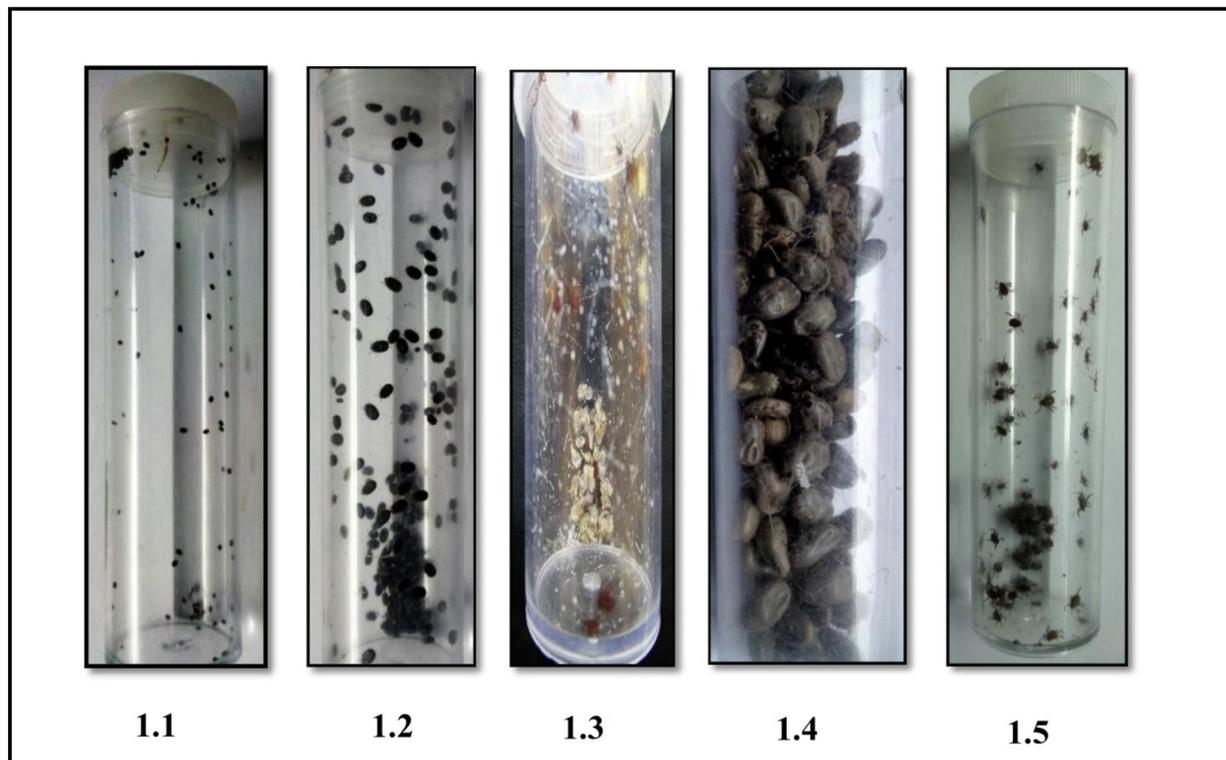


Fig. 1. Different stages of *Rhipicephalus sanguineus* s.l ticks used for Petri dish bioassay.
1.1 – Engorged larvae, 1.2 – Engorged nymphs, 1.3 – Unfed nymphs, 1.4 – Engorged females, 1.5 – Fed males.

pheromones. The Petri dish bioassays were then repeated as described above.

2.5. Field trials

2.5.1. Design of bamboo sticky tick trap device

A bamboo (*Bambusa vulgaris*) culm of 35 cm of length was cut at their internodes. It was then cut along the sulcus to divide it into two equal halves. Using metallic hinges, the two halves of the bamboo were fixed on one side. The other side was left open, to enable easy opening and closing of the trap for monitoring the trapped ticks. Two hooks were fixed near the hinge at the top for suspending the bamboo trap in the dog kennel. To allow tick entrance into the trap, two rows with six openings each of 1 cm diameter were made at equal distances using a drill on the sides. The trap so made was placed with the openings facing the wall (Fig. 2 – 2.1). Double sided adhesive tape (Camel, India) was affixed on the inner side of both halves of the bamboo culm, adjacent to the six openings, thus forming a sticky trap. In each trap, nine vapour patches impregnated with SP, AP, SP + AP or control were affixed to the inner surface of the bamboo with double sided sticky tape and used in field trials (Fig. 2 – 2.2).

2.5.2. Field evaluation of the bamboo sticky trap

Field trials were carried out at Blue Cross of India kennel, a private non-governmental organization. The kennel area measures 10 by 10 feet and the room is divided into 4 compartments by a 1.5 ft wall on either side. During the period of the experiment the kennel housed a total of 60–80 stray pups for a period ranging from 30 d to 6 m. The number of pups in each kennel compartment ranged from 4 to 8 animals. Water and food were available *ad libitum* to the pups. The population floats occurs due the quantity of dogs rescued or adopted. Since the kennel is a shelter for stray dogs and pups the level of infestation and re-infestation is very high in-spite of the regular use of deltamethrin sprays as well as flame guns to control ticks. The devices were hung on the wall about one meter from the ground so that it was

inaccessible to pups housed within (Fig. 3). A total of 12 bamboo sticky traps were used. The trial was repeated twice, in the interval of 7 days. Each trial consisted of three vapour patch traps impregnated with SP, AP or SP + AP, and three with plain vapour patches for control. The devices were hung in the kennel for a period of 10 days and after that the overall number of tick stages trapped was recorded.

2.6. Statistical analysis

Statistical analysis was done by using chi-square test using IBM®, SPSS® Version 20.0 for Windows® for comparison between the control and among the pheromone combinations whereas binary logistic regression was done to know the efficiency (odds ratio) of the pheromone to attract the different tick life stages. MANOVA was carried out to compare the groups (SP, AP and SP + AP) and tick categories (unfed/fed larvae; unfed/fed nymphs; unfed/engorged adult females; unfed/fed adult males) altogether. Results are presented as mean \pm S.E.

3. Results

3.1. Petri dish bioassay

The response of different life stages of *R. sanguineus* s.l. ticks to freshly impregnated and 2 mo old vapour patches in Petri dish bioassays, along with statistical analysis, is summarized in Tables 1 and 2. MANOVA analysis is summarized in Table 3.

3.2. Response of unfed larvae

When unfed larvae (n = 500) were exposed to vapour patches freshly impregnated with SP, AP, or SP + AP, it resulted in 63.6%, 85.2% and 63.6% of attraction, respectively, after 24 h compared with 9.2% attraction for plain control vapour patches. Unfed larvae exhibited intense questing behavior, initially moving towards the SP impregnated vapour patches. After contact, they assumed a typical feeding



2.1



2.2

Fig. 2. Bamboo sticky trap.

2.1 Basic design of the bamboo trap showing the holes and hinges. 2.2 Bamboo sticky trap with double sided sticky tape and vapour patches.



Fig. 3. Field trials at the Blue cross kennel using bamboo sticky trap device (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

posture with raised posterior and lowered palps. No similar characteristic behavioral response was recorded in the control. When exposed to AP, clustering behavior was seen. Binary logistic regression revealed AP ($B = 56.81$) was highly significant ($p < 0.01$) in attracting unfed larvae as compared with SP ($B = 17.24$), SP + AP ($B = 17.2$) and control ($B = 0.10$).

When exposed to 2 mo old impregnated vapour patches with SP, AP, SP + AP and control resulted in 54%, 85.4%, 59.6% and 9% attraction, respectively. Binary logistic regression revealed that AP attracted 1.7 times ($B = 59.14$) more unfed larvae as against SP ($B = 11.8$) and SP + AP ($B = 14.9$) which was highly significant when compared with control ($B = 0.09$).

3.3. Response of engorged larvae

A total 48.7% engorged larvae ($n = 500$) were attracted to different pheromone impregnated vapour patches and control during the study period. Being, 48.7% attracted 36.4%, 88.6%, 62.8% and 7% were attracted to SP, AP, SP + AP and control vapour patches, respectively. The response was highly significant ($p < 0.01$) when compared with control and pheromones used. Binary logistic regression revealed that AP attracted 2.01 times ($B = 103.25$) more engorged larvae followed by SP + AP ($B = 22.4$) as against SP ($B = 7.60$) which was highly significant when compared with control ($B = 0.07$).

Exposure of the engorged larvae ($n = 500$) to SP, AP, SP + AP

impregnated two mo old vapour patches and control revealed that 30.8%, 80.6%, 44.8% and 8% of ticks were allured, respectively. Binary logistic regression revealed that AP attracted 1.67 times ($B = 47.77$) more engorged larvae followed by SP + AP ($B = 9.33$) as against SP ($B = 5.11$) which was highly significant when compared with control ($B = 0.08$).

3.4. Response of unfed nymphs

Amongst the pheromone complexes highly significant ($p < 0.01$) difference in alluring unfed nymphs was observed in SP with AP whereas non-significant ($p > 0.05$) results was observed when SP (55%), AP (77%) compared with SP + AP (64%) complex. Thus, during the study period, 52.75% nymphs were attracted to the pheromone impregnated vapour patches. The control patches attracted 15% of unfed nymphs. Statistically, AP alone lured 1.27 ($B = 18.97$) times more unfed nymph as against SP + AP ($B = 10.07$) and SP ($B = 6.92$) when compared with control ($B = 0.17$).

When unfed nymphs ($n = 100$) were exposed to SP, AP, SP + AP impregnated, two mo old vapour patches, it resulted in 49%, 65%, 51% of attraction, respectively, while only 11% attraction was observed to control vapour patch. Unfed nymphs were lured 1.17 times more to AP ($B = 15.02$) as against SP + AP ($B = 8.42$) and SP ($B = 7.77$) in two mo old impregnated vapour patches when compared with control ($B = 0.12$).

3.5. Response of engorged nymphs

During the study the % nymphs attracted to SP, AP, SP + AP and control was 33%, 90%, 52% and 12% respectively. The effect of luring the engorged nymph was highest in AP followed by SP + AP. Amongst the pheromones SP was ranked last in luring the tick stage. Binary logistic regression revealed that AP impregnated patches attracted 1.81 ($B = 66$) times more nymphs than control ($B = 0.13$) while SP + AP attracted 0.89 ($B = 7.94$) times more nymphs than SP ($B = 3.61$).

The response of the engorged nymphs ($n = 100$) in two mo old impregnated vapour patches in Petridish bioassay was that a total of 39.25% ticks were attracted to different pheromone impregnated two mo old and control vapour patch during the study period. Out of the attracted engorged nymph 20%, 76%, 45% and 16% ticks were

Table 1
Response of *R. sanguineus* stages to fresh pheromone impregnated vapour patches- Petri dish bioassay.

Stages of ticks	n	Sex pheromone (SP)		Assembly pheromone (AP)		SP + AP		Control	
		Attracted	Not attracted	χ ²	Attracted	Not attracted	χ ²	Attracted	Not attracted
Unfed larvae	500	318 (63.6 ± 0.4)	182 (36.4 ± 0.4)	a**,b ^{NS} ,c**	426 (85.2 ± 0.5)	74 (14.8 ± 0.5)	d**,e**	318 (63.6 ± 1.0)	182 (36.4 ± 1.0)
Engorged larvae	500	182 (36.4 ± 0.7)	318 (63.6 ± 0.7)	a**,b**,c**	443 (88.6 ± 0.7)	57 (11.4 ± 0.7)	d*,e**	314 (62.8 ± 3.0)	186 (37.2 ± 1.3)
Unfed nymphs	100	55 (7.8 ± 1.0)	45 (6.4 ± 0.7)	a**,b ^{NS} ,c**	77 (11 ± 1.7)	23 (3.2 ± 0.2)	d ^{NS} ,e**	64 (9.1 ± 1.8)	36 (5.1 ± 0.3)
Engorged nymphs	100	33 (4.7 ± 0.9)	67 (9.5 ± 0.8)	a**,b**,c**	90 (12.8 ± 1.6)	10 (1.4 ± 0.2)	d**,e**	52 (7.4 ± 1.3)	48 (6.8 ± 0.4)
Unfed females	100	6 (0.8 ± 0.2)	94 (13.4 ± 1.5)	a**,b ^{NS} ,c**	91 (13 ± 1.6)	9 (1.2 ± 0.1)	d**,e**	44 (6.2 ± 1.6)	56 (8 ± 0.3)
Engorged females	100	13 (1.8 ± 0.4)	87 (12.4 ± 1.6)	a**,b**,c ^{NS}	80 (11.4 ± 1.9)	20 (2.8 ± 0.3)	d**,e**	47 (6.7 ± 1.1)	53 (7.5 ± 1.0)
Unfed males	100	17 (2.4 ± 0.4)	83 (11.8 ± 0.9)	a**,b ^{NS} ,c ^{NS}	79 (11.2 ± 1.6)	21 (3 ± 0.4)	d**,e**	57 (8.1 ± 1.2)	43 (6.1 ± 0.5)
Fed males	100	93 (13.2 ± 1.7)	7 (1.7 ± 0.2)	a ^{NS} ,b**,c**	86 (12.2 ± 1.5)	14 (2.7 ± 0.2)	d**,e**	51 (7.2 ± 1.2)	49 (7.7 ± 0.4)

a = SP compared with AP, b = SP compared with SP + AP, c = SP compared with control, d = AP compared with SP + AP, e = AP compared with control, f = SP + AP compared with control.
^{NS} Non-significant (p > 0.05), * Significant (p < 0.05), ** Highly significant (p < 0.01), Figures in the parenthesis indicate mean ± SE.

Table 2
Response of *R. sanguineus* stages to two month old impregnated vapour patches- Petri dish bioassay.

Stages of ticks	n	Sex pheromone (SP)		Assembly pheromone (AP)		SP + AP		Control	
		Attracted	Not attracted	χ ²	Attracted	Not attracted	χ ²	Attracted	Not attracted
Unfed larvae	500	270 (54 ± 2.3)	230 (46 ± 2.3)	a**,b ^{NS} ,c**	427 (85.4 ± 1.4)	73 (14.6 ± 1.4)	d**,e**	298 (59.6 ± 1.9)	202 (40.4 ± 1.9)
Engorged larvae	500	154 (30.8 ± 1.1)	346 (69.2 ± 5.0)	a**,b**,c**	403 (80.6 ± 0.9)	97 (32.3 ± 0.9)	d**,e**	224 (44.8 ± 1.2)	276 (92 ± 1.2)
Unfed nymphs	100	49 (7 ± 1.2)	51 (12.7 ± 0.6)	a ^{NS} ,b ^{NS} ,c**	65 (9.2 ± 1.2)	35 (8.7 ± 0.6)	d*,e**	51 (7.2 ± 1.2)	49 (12.2 ± 0.5)
Engorged nymphs	100	20 (2.8 ± 0.6)	80 (11.4 ± 1.1)	a**,b**,c ^{NS}	76 (10.8 ± 1.7)	24 (3.4 ± 0.4)	d**,e**	45 (6.4 ± 0.9)	55 (7.8 ± 1.0)
Unfed females	100	10 (1.2 ± 0.1)	90 (11.2 ± 1.4)	a ^{NS} ,b ^{NS} ,c ^{NS}	81 (10.1 ± 1.1)	19 (2.5 ± 0.3)	d**,e**	35 (4.3 ± 0.9)	65 (8.1 ± 0.5)
Engorged females	100	11 (1.5 ± 0.4)	89 (12.7 ± 0.8)	a ^{NS} ,b ^{NS} ,c ^{NS}	62 (8.8 ± 2.0)	38 (5.4 ± 0.8)	d**,e**	36 (5.1 ± 1.0)	64 (9.1 ± 0.3)
Unfed males	100	20 (2.5 ± 0.7)	80 (10 ± 0.6)	a**,b ^{NS} ,c*	71 (8.8 ± 1.2)	29 (3.6 ± 0.3)	d**,e**	44 (5.5 ± 1.4)	56 (7 ± 0.4)
Fed males	100	72 (10.2 ± 1.2)	28 (4 ± 0.4)	a ^{NS} ,b**,c**	80 (11.4 ± 1.3)	20 (3 ± 0.3)	d**,e**	47 (6.7 ± 1.1)	53 (7.5 ± 0.4)

a = SP compared with AP, b = SP compared with SP + AP, c = SP compared with control, d = AP compared with SP + AP, e = AP compared with control, f = SP + AP compared with control.
^{NS} Non-significant (p > 0.05), * Significant (p < 0.05), ** Highly significant (p < 0.01), Figure in the parenthesis indicate mean ± SE.

Table 3
Overall comparison of Petridish bioassay using MANOVA.

Pheromone	Different Ticks stages attracted	Percentage of Different Ticks stages attracted	Different Ticks stages Not attracted	Percentage of Different Ticks stages Not attracted
SP	1318	41.2%	1882	58.8%
AP	2632	82.2%	568	17.8%
SP + AP	1716	53.6%	1484	46.4%
Control	296	9.2%	2904	90.8%

attracted to SP, AP, SP + AP and control. The AP impregnated vapour patch attracted 1.20 (B = 16) times more nymphs than control (B = 0.19) while SP + AP attracted 0.62 (B = 4.2) times more nymphs than SP (B = 1.32).

3.6. Response of unfed females

Unfed females (n = 100) of *R. sanguineus* s.l. were highly attracted to AP (91%) followed by SP + AP (44%) and SP (6%). Statistical analysis with binary logistic regression revealed highly significant difference in AP (B = 116.27) against SP + AP (B = 9.03) followed by SP (B = 0.73) with control (B = 0.08).

Two mo old impregnated SP, AP, SP + AP vapour patches and control resulted in 10%, 81%, 35%, 5% attraction, respectively. Statistical analysis with binary logistic regression revealed highly significant difference in AP (B = 81) followed by SP + AP (B = 10.23) and SP (B = 2.11) as against control (B = 0.05).

3.7. Response of engorged females

The active engorged female ticks (n = 100) when exposed to different pheromone impregnated fresh vapour patches resulted in 13%, 80%, 47% and 11% attraction to SP, AP, SP + AP and control patch, respectively, after 24 h. Binary logistic regression revealed that AP (B = 32.36) was highly significant (p < 0.01) in attracting engorged female when compared with SP + AP (B = 5.62), SP (B = 1.20) and control beads (B = 0.12).

The active engorged female ticks (n = 100) when exposed to SP, AP, SP + AP impregnated two mo old vapour patches and control resulted in 11%, 62%, 36% and 14% attraction, respectively. Binary logistic regression revealed that AP (B = 10.02) was highly significant (p < 0.01) in attracting engorged females when compared with SP + AP (B = 3.45), SP (B = 0.75) and control beads (B = 0.16).

3.8. Response of unfed males

Hundred unfed males on exposure to different pheromones resulted in 17%, 79%, 57% attraction to the SP, AP, SP + AP complex, respectively, whereas 8% attraction was observed in control vapour patch. Binary logistic regression revealed that AP attracted 1.63 times (B = 43.26) more unfed males as against SP (B = 2.35) and SP + AP (B = 15.24) which was highly significant when compared with control (B = 0.08).

The percentage of attraction by the unfed males (n = 100) to SP, AP, SP + AP and control vapour patches were 20%, 71%, 44% and 10%, respectively. Binary logistic regression revealed that AP attracted 1.34 times (B = 22.03) more unfed males as against SP (B = 2.25) and SP + AP (B = 7.07) which was highly significant when compared with control (B = 0.11).

3.9. Response of fed males

Fed males (n = 100) were strongly attracted to SP (93%) followed by AP (86%) and SP + AP (51%). Statistically, the response of the fed male ticks with control (15%) and different pheromone complexes was highly significant (p < 0.01). The response of SP (B = 66) in luring fed

males was highly significant as against AP (B = 38.36), SP + AP (B = 7.66) and control (B = 0.11) by binary logistic regression.

Fed males (n = 100) were highly attracted to AP (80%) followed by SP (72%) and SP + AP (47%) in two mo old impregnated vapour patches, respectively. The response of SP (B = 36) in luring fed male was highly significant as against AP (B = 23.14), SP + AP (B = 7.98) and control (B = 0.11) by binary logistic regression.

3.10. Comparison between fresh and two mo old impregnated vapour patches

The attractiveness for questing and engorged ticks was similar for freshly impregnated and 2 mo old vapour patches regardless of treatment (SP, AP or SP + AP). However, AP was very effective in luring all the stages of ticks when compared with SP + AP and SP.

Comparison between fresh and two mo old SP impregnated vapour patches in attracting different stages of *R. sanguineus* s.l. ticks revealed highly significant (p < 0.01) difference for unfed larvae and fed male ticks whereas only significant (p < 0.05) difference was observed in engorged nymph as against non-significant (p > 0.05) attraction in engorged larvae, unfed nymph, unfed female, engorged female and unfed male.

The AP vapour patches resulted in non-significant (p > 0.05) difference amongst unfed larvae, unfed nymph, unfed male and fed male ticks followed by significant (p > 0.05) difference in unfed female as against highly significant (p < 0.01) difference in engorged larvae, engorged nymph and engorged female.

The response of engorged larvae to SP + AP entrapped fresh and 60 d old vapour patch was statistically highly significant (p < 0.01) whereas non-significant (p > 0.05) difference in attraction was observed in all other tick stages.

3.11. Bamboo sticky trap device with vapour patch based lure

Overall, the bamboo sticky traps with SP, AP, or SP + AP impregnated vapour patches or control patches yielded a total 20,393 *R. sanguineus* s.l. ticks during a period of 20 d. This included 10161 (49.8%) engorged and unfed larvae, 3097 (15.18%) unfed nymphs, 5502 (26.97%) engorged nymphs, 370 (1.81%) engorged females, 673 (3.3%) fed males and 590 (2.89%) unfed adults respectively. The results of field trials were summarized in Table 4.

The sticky traps baited with the AP impregnated vapour patches (Fig. 4.1) yielded (27.36%) engorged and unfed larvae, 1859 (9.115%) unfed nymphs, 2211 (10.84%) engorged nymphs, 168 (0.82%) engorged females, 237 (1.16%) fed males and 487 (2.3%) unfed adults.

The percentage of different life stages of ticks lured to the bamboo sticky trap baited with SP + AP impregnated vapour patches (Fig. 4.2) was 2562 (12.5%) larvae, 815 (3.99%) unfed nymph, 1602 (7.8%) engorged nymphs, 116 (0.5%) engorged females, 165 (0.8%) fed males and 68 (0.33%) unfed adults.

The bamboo sticky trap baited with SP impregnated vapour patch (Fig. 4.3) resulted in mortality of 1819 (8.9%) larvae, 416 (2.03%) unfed nymphs, 1576 (7.7%) engorged nymphs, 76 (0.37%) engorged females, 263 (1.28%) fed males and 25 (0.12%) unfed adults.

In case of control (Fig. 4.4), very few ticks were lured to the trap and found dead. The % mortality of larvae (199) was found to be 0.97 while

Table 4
Results of field trials using bamboo sticky trap impregnated with vapour patches.

Different stages of ticks	No. of different stages of ticks trapped in bamboo sticky trap with				Total
	AP impregnated vapour patches	SP + AP impregnated vapour patches	SP impregnated vapour patches	Control	
Larvae	5581	2562	1819	199	10161
Unfed nymphs	1859	815	416	7	3097
Engorged nymphs	2211	1602	1576	113	5502
Engorged females	168	116	76	10	370
Fed males	237	165	263	8	673
Unfed adults	487	68	25	10	590
Total	10543	5328	4175	347	20393

7 (0.03%) unfed nymphs, 113 (0.55%) engorged nymph, 10 (0.049%) engorged female, 8 (0.03%) fed male and 10 (0.04%) unfed adult were lured and trapped.

It was also noticed that many of the ticks attracted to the pheromone impregnated vapour patches were found stuck below the patches. Although most of the lured ticks were found dead on the sticky tape some engorged ticks were alive and were observed ovipositing on the sticky trap. Hatching of few of the eggs also had commenced and the newly hatched larvae were also found trapped on the sticky surface.

4. Discussion

The findings of this study are very interesting and provide encouraging data on the potential application of pheromone-based lures for the environmental control of the brown dog tick, especially in suitable environments such as kennels. Researchers have showed that different devices contain AP, SP and combination of SP + AP was successful in trap stages of *R. sanguineus* s.l. in the lab and field. The study evaluated the response of different life stages (unfed and fed) of *R. sanguineus* s.l. to SP, AP and a combination of SP + AP as well as the efficacy of vapour patches in sustained release of pheromones in both *in vitro* and field trials.

Researchers have used vapour patches, a novel transdermal drug delivery system for the first time to impregnate the tick pheromones for sustained release because SP is highly volatile. Such vapour patches has been used as mosquito repellants by Kulkarni and Vandana, 2018 to release essential oils in a sustained manner. SP impregnated in Whatmann no.1 filter paper for controlled release was not effective (Ranju et al., 2012) whereas vapour patches has been successful in sustained release of SP. AP encapsulated in calcium alginate, chitosan and poly-ε-caprolactone as a delivery device in conjunction with deltamethrin as well as in gold nanoparticles to ensure sustained release for successful control of dog ticks in kennels (Bhoopathy and Latha, 2016; Dhivya, 2013; Anish et al., 2017). Encapsulation of pheromones is tedious process whereas impregnation is simple and cost effective. Pheromones may react with minerals and metals during encapsulation process, which may lead to degradation. The current study employed the impregnation of pheromones for controlled release in off-host control of dog ticks.

Pheromones namely AP, SP and combination of SP + AP following successful impregnation in vapour patches were initially assessed for their efficacy for luring the different stages of *R. sanguineus* by *in vitro* bioassay. Laboratory bioassay, generally used for testing attractants namely Petri dish bioassay was utilized in the current study (Otiño et al., 1985). A modification of Petri dish bioassay was implemented for evaluation of the vapour patches as a delivery device namely use of large Petri dish to accommodate the large vapour patch and to ensure sufficient distance between the test material and tick stages.

Response of all the stages of ticks to SP impregnated vapour patch showed behavioral modifications such as feeding, probing, resting and questing postures which were not evident in the control. The behavioral modifications observed in the current study were similar to the study

conducted by Ranju et al., 2012. Only fed or feeding males can detect and respond to SP (Sonenshine, 2006). However, in the current study all stages of ticks exhibited behavioral responses. This may be because 2,6-DCP is not exclusively a sex pheromone and it might have additional role as attractant stimulant (Norval et al., 1991). Sonenshine (1991) and Carnohan et al. (2017) also stated that reaction of *R. sanguineus* ticks to 2,6-DCP was not specific to it but due to generalized responses to phenols in the context of host finding. When exposed to AP encapsulated microparticles clustering behavior was noticed in all stages of ticks. Clustering of both immature and mature ticks helps in decreasing water loss, in reduction in risk of predation and physical injury (Sonenshine, 2006). The theory of power in numbers reduces the risk to individuals (Carr and Roe, 2016). Although ticks exhibited behavioral changes to AP and SP separately they did not exhibit any characteristic behavioral response on exposure to a combination of SP + AP impregnated vapour patches. This may be due to the potential inhibition of the individual pheromonal effect which needs to be further proved with appropriate experiments.

Significant numbers of unfed larvae, engorged larvae, unfed nymphs, engorged nymphs, unfed females and engorged females of *R. sanguineus* s.l. were attracted to vapour patches impregnated with AP, followed by SP + AP and SP in the *in vitro* assay. It was observed that AP alone elicited higher percentage of attraction in all stages of ticks in the *in vitro* assay. The percentage of attraction of engorged tick stages was similar to that seen in the study of Dhivya (2013) and Anish (2016) who had employed Petri dish bioassay using AP in combination with deltamethrin and AP encapsulated in gold nanoparticles, respectively. The current study, in addition, also evaluated the effect of AP on unfed stages of larvae, nymph, female and male *R. sanguineus* s.l. and found it was quite effective in attracting the unfed stages of ticks. The percentage of attraction varied from a highest of 85% in unfed larvae to a lowest of 77% in unfed nymphs.

In *in vitro* bioassay, unfed and engorged stages of ticks when exposed to SP impregnated in vapour patches were attracted to a limited extent. However, fed male was the stage most attracted to SP in *in vitro* assay. Sonenshine (2004) stated that only fed or feeding male can recognize SP while Haggart and Davis (1981) felt that unfed males do not respond in spite of being able to detect the pheromone. Ranju et al. (2012) observed 64% adult male and 71% unfed larvae were attracted to 0.1 M SP impregnated in filter paper. In the current study, a larger number of adult male ticks were attracted to SP possibly because impregnation in vapour patches ensured sustained release whereas rapid evaporation of acetone probably occurred in impregnated filter paper. It is also possible that males secrete small amounts of 2,6-DCP to function as an aggregation pheromone thereby increasing the feeding success in both immature and mature adult ticks (Carr and Roe, 2016).

The combination of SP + AP attracted less number of ticks in Petri dish bioassay when compared to AP and SP alone, so no synergistic effect between SP and AP. Even the fed males attracted to SP, failed to get attracted in sufficient numbers to the combination (SP + AP). This reveals that although synergistic effect was anticipated the actual effect was quite the opposite. Bioinformatics study using auto-docking



1. Trap with Assembly Pheromone impregnated vapour patch revealing the presence of different stages of dead ticks and eggs



2. Trap with SP+AP impregnated vapour patch revealing the presence of different stages of dead ticks and eggs



3. Trap with SP impregnated vapour patch revealing the presence of different stages of dead ticks and eggs



4. Trap with control vapour patch revealing the presence of few dead ticks

Fig. 4. Results of field trials in bamboo sticky trap with pheromone impregnated vapour patches.

revealed that the compound 2,6- DCP when added to AP reacts with phenol present in SP to form an unstable compound. Similar reaction could have occurred during the *in vitro* trial which may have had an impact on decreasing the synergistic or potentiating or additive effect of the pheromones.

For field evaluation bamboo traps were designed because it was easily available, economical and fitted perfectly with the tag of being eco-friendly. The device showed advantages was made with a natural and low cost material, ensured the stickiness of the double sided tape since it remains closed in the environment, presented flexibility for placement and was able to attract the different tick life stages.

In the present study, field trials revealed that both questing and engorged larvae, nymphs and adults of *R. sanguineus* s.l. were attracted more towards vapour patches impregnated with AP. The presence of AP indicates that the region is more conducive for the survival of ticks, that it is a safe place for engorged females to oviposit with minimal desiccation, while for engorged nymphs and larvae it indicates a sheltered location to moult and then subsequently seek a host (Sonensine, 1991; Sonensine, 2006).

Vapour patches impregnated with SP used as bait in bamboo sticky trap also lured and killed all different stages of unfed and engorged *R. sanguineus* s.l. ticks. Amongst the engorged stages, engorged nymphs

were more attracted followed by fed males and engorged females. Yoder and Stevens (2000) reported that 2,6-DCP was attractive to unfed nymph and larvae of *D. variabilis* ticks. However, the males of *Dermacentor nitens* do not detect the females and mate in an environment saturated with 2,6-DCP (Borges et al., 2007). The range of detection of 2,6-DCP is limited and usually only 3 or 4 cm (Sonenshine, 2006). Vapour patches with combination of SP + AP impregnation attracted more ticks when compared with that of SP alone. The results of the field trials were contradictory to what was observed with the Petri dish bioassay. Although bioassays are the only means available to assess the effect of any attractant on the acarines the results of these trials may not and cannot reflect the reality faced in the field condition. Changes in temperature from one day to the next or even during a typical day also affect vaporization of chemical compounds and tick activity (Carr and Roe, 2016).

The ticks died in the bamboo trap because they were glued to the adhesive tape in such a way that the stickiness prevented the breath of ticks as well as the desiccation and exhaustion that occurs during a struggle to free themselves from the adhesive. The tick stages use spiracular respiration except larvae which has cutaneous respiration by diffusion across the integument (Sonenshine, 1991).

The bamboo sticky trap with impregnation of AP into vapour patches is considered apt for use in integrated pest management of ticks. Each bamboo lure can be exploited maximum for a period of 10 days and remain effective in the field up to 2 months. However, it depends on the severity of tick infestation, the number of pups housed in and saturation of the sticky tape. These traps along with routine tick control practices such as regular use of flame gun, plastering of cracks and crevices, etc. would be ideal for effective tick control. Results show, indeed, that the assembly pheromone (AP) impregnated vapour patches hold great promise for such an application. Nonetheless, the results here presented pave the way for further improvements, with special regards to the delivery systems, scalability and potential industrialisation of such control tools. Moreover, potential further combinations, with acaricide ingredients as well as other tick-specific pheromones and chemical and physical attractants, should also be explored at a fundamental stage of research.

5. Conclusion

Vapour patches were used for the first time to impregnate the different pheromones of *R. sanguineus* s.l. AP was found to be very effective in luring all the unfed and engorged stages of ticks. A novel, eco-friendly and economical bamboo sticky tick trap was innovated for use in dog kennels. Both unfed and fed larvae, nymphs and adults were equally attracted to the lure. Since, the vapour patches were effective even after two mos in dispensing the pheromone it can be used as a potent delivery device. The sticky trap device uses pheromone without any acaricide. So, the device is most suitable as a green technology for use in integrated pest management.

Declaration of Competing Interest

There is no conflict of interest in this research study.

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