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Original Article

In vivo effect of diflubenzuron, administered via mineral salt supplementation, against *Haematobia irritans* and *Rhipicephalus microplus* parasitizing cattle

Efeito *in vivo* do diflubenzuron, administrado via sal mineral, contra *Haematobia irritans* e *Rhipicephalus microplus* parasitando bovinos

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Abstract

This study involved two field trials with the aim of evaluating the efficacy of diflubenzuron, via mineral supplementation, against *Haematobia irritans* parasitizing cattle. Concomitantly with the main trial, a stall test was conducted to ascertain the effects of a different formulation with the same active ingredient against *Rhipicephalus microplus*, along with the action of diflubenzuron on the reproductive parameters of *R. microplus*females that had naturally detached from cattle. Against *H. irritans*, it was observed that the efficacy indexes fordiflubenzuron were low (≤ 31.3% or 44.6%) or null (0.0%) throughout the study. The anti-*R. microplus* efficacy of diflubenzuron, at weekly intervals, ranged from 0.0 to 13.7% over the entire experimental period. Null efficacy (0.0%) was registered for diflubenzuron in relation to the reproductive parameters of *R. microplus*females that had naturally detached from cattle. The different diflubenzuron formulations, administered via mineral salt supplementation, did not show satisfactory efficacy indexes against *H. irritans* and *R. microplus* parasitizing cattle, within the experimental design of the present study. In addition, this agent did not present any deleterious effects on the reproductive parameters of *R. microplus* females.

Keywords: Benzoylphenyl urea, cattle, chitin synthesis inhibitor, engorged females, reproductive parameters, *Rhipicephalus microplus*.

Resumo

O objetivo deste estudo foi avaliar a eficácia do diflubenzuron, administrado via suplementação mineral, contra *Haematobia irritans* parasitando bovinos, em dois testes à campo. Concomitantemente, foi realizado testes em estábulo para determinar os efeitos de uma formulação diferente, com o mesmo princípio ativo, contra *Rhipicephalus microplus*, bem como a ação do diflubenzuron nos parâmetros reprodutivos de fêmeas de *R. microplus* recolhidas após desprendimento natural do hospedeiro bovino. Contra *H. irritans*, foi observado que foram baixos (≤ 31,3% ou 44,6%) ou nulos (0,0%) os índices de eficácia do diflubenzuron. A eficácia anti-*R. microplus* do diflubenzuron, observada em intervalos semanais, variaram de 0,0% a 13,7% durante todo o período experimental. Com relação aos parâmetros reprodutivos das fêmeas de *R. microplus* recolhidas, foi observada eficácia nula (0,0%) para o diflubenzuron. Conclui-se que as diferentes formulações administradas via sal mineral no atual estudo, contra *H. irritans* e *R. microplus* parasitando bovinos, não apresentaram eficácia satisfatória. Este agente também não mostrou efeito deletério sobre os parâmetros reprodutivos de fêmeas de *R. microplus.*

Palavras-chave: Benzoylphenyl urea, bovino, inibidor de síntese de quitina, fêmeas ingurgitadas, parâmetros reprodutivos, *Rhipicephalus microplus*.

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Introduction

Despite the existence of many studies focusing on use of technologies against *Rhipicephalus microplus*, controlling this ectoparasite continues to be a major challenge in the different regions where it occurs. For many years, this tick species was controlled by using synthetic pyrethroids, organophosphates or even macrocyclic lactones. However, the increasing number of *R. microplus* populations that are resistant to active agents in these chemical groups (CORRÊA et al., 2015; CRUZ et al., 2015; MACIEL et al., 2016) has led to decreases in their efficacy. In their place, treatments with benzoylphenyl ureas are increasingly being used (CRUZ et al., 2015; GOMES et al., 2015).

Benzoylphenyl ureas belong to a chemical group in which active agents are selective growth regulators of insects. They also act as acaricides, through interference in chitin synthesis, thus making it impossible for larvae and nymphs to complete ecdysis. Consequently, these parasites lose hemolymph and death occurs through dehydration.

These chemicals present high specificity, low toxicity for mammals and long periods of efficacy at low concentrations against *R. microplus* (RETNAKARAM & WHIGHT, 1987; GRAF, 1993; BULL et al., 1996). Fluazuron, novaluron and diflubenzuron are the most prominent agents in this group. Regarding diflubenzuron, which is orally administered as a feed additive, studies have indicated that it demonstrates low toxicity towards vertebrates (KEGLEY et al., 2010), low deposition in muscle tissues of fish (WINKALER, 2008) and absence of residues in either meat or milk (TFOUNI et al., 2007). Thus, there is no requirement for withdrawal periods relating to consumption of products from animals that have been treated with this agent.

On the other hand, to the best of our knowledge, relatively few *in vivo* trials focusing on evaluation of diflubenzuron effects against *Haematobia irritans* and *Rhipicephalus microplus* have been published. The relatively scarce information available was the reason for conducting the experiments of the present study.

Thus, the aim of the present study was to analyze the efficacy of diflubenzuron, administered via mineral salt supplementation, against *H. irritans* parasitizing cattle, in two different trials. Concomitantly with the main trial, a stall test was performed, seeking to ascertain the effects of a different formulation of this same active agent against *R. microplus*, along with the action of diflubenzuron on the reproductive parameters of fully engorged females of this tick species after naturally detaching from cattle.

Materials and Methods

Efficacy against natural infestations of Haematobia irritans

The field studies were performed on two ranches, located in the municipalities of Formiga, state of Minas Gerais, in the southeastern region of Brazil, and Caçu, state of Goiás, in the central-western region of Brazil. These studies were conducted between December 2013 and April 2014 and between January and May 2015, respectively. On each ranch, 30 non-castrated male calves aged 18 to 20 months were selected from a herd of about 100 animals. The selection criterion was that these calves presented the highest horn fly infestations among the herd. This selection procedure was conducted on study days -2 and -1, based on *H. irritans* (horn fly) counts over the entire body surface of these animals.

Fly counts were simultaneously conducted by observers on each side of the animal (MACIEL et al., 2015), between 07:00 and 10:00 a.m. The same observer always performed counts on the same side of these animals on all post-treatment dates. Subsequently, they were ranked according to their mean numbers of flies and were placed in two groups of 15 animals each, followed by application of randomly allotted treatments. The cattle were divided into 15 blocks of two animals each, and within each block, the animals were randomly assigned to the treatment groups.

The animals started receiving diflubenzuron (Difly Mosca®, Champion Animal Health) on experimental day zero, and administration was performed in accordance with the manufacturer's recommendations. The product was mixed, using a mechanical mixer, with mineral salt at a rate of 1.0 g per kg of ready-to-use salt, and was then supplied to the animals. All of these materials came from the same production batch from the manufacturer. This proportion was determined assuming that the consumption of mineralized salt by these animals would be around 60 g/head/day. In this case, each animal consumed an average of approximately 120 mg of the diflubenzuron product (Difly Mosca®, Champion Animal Health) per day. This formulation was chosen because this is the only form of diflubenzuron commercialized on the Brazilian market against *R. microplus*. The mineral salt mixture containing diflubenzuron was supplied to the animals in covered troughs arranged in the paddocks. For control animals (untreated), the same mineralized salt, without addition of diflubenzuron, was provided. Every seven days, it was observed whether the salt had been ingested. A further 10 kg of saltwas also addedto the covered troughs by means of an automatic dispensing system, for each group (with or without diflubenzuron), [over the period of the trial? every seven days?].

Starting on experimental day zero, the cattle were kept in different paddocks (A and B), five kilometers apart, in order to prevent horn fly dispersal between the herds. The two paddocks presented the same topographic characteristics (no invading vegetation, nearby forests or places for animals to hide) and the same size and pasture species (*Urochloa decumbens*).

To evaluate the therapeutic efficacy indexes of each treatment scheme, the presence of *H. irritans* on the entire body surface of each animal was registered on days 3, 7, 14, 21, 28 and every seven days thereafter, until the 160th day after the start of treatment (DAST). Efficacy indexes for the treatments were calculated at each count using the following formula:

$$Efficacy = \frac{a-b}{a} \times 100 \quad (1)$$

In this equation, "**a**" represents the mean number of *H. irritans* specimens on animals in the untreated control group, while "**b**" represents the mean number of horn flies counted on animals in the treated group.

Efficacy against artificial R. microplus infestations

Location, animals and pre-treatment infestations

One experiment was conducted at the Animal Health Research Institute Ltd. (Instituto de Pesquisas em Saúde Animal Ltda., IPESA), which is located in the municipality of Formiga, state of Minas Gerais, southeastern Brazil.

Sixteen seven-month-old crossbred calves, weighing from 168 to 175 kg, which had not received any antiparasite treatment for at least 120 days before the experiment, were selected and identified with numbered ear tags. All the animals were held in individual raised pens (i.e. self-draining pens) that were appropriate for conducting "stall tests", starting on day 27 before treatment (D -27), for an acclimatization period. After this initial stage, each animal was subjected to infestation with approximately 5,000 *R.microplus* larvae (0.25 g of eggs), aged 14 to 28 days, on experimental days -24, -21, -19, -17, -14, -12, -10, -7, -5, -3 and -1, considering that day 0 was the treatment date (BRASIL, 1997). The animals were restrained in order to apply the infestation. The syringes containing the larvae were applied gently along the dorsal and/or lateral line of the animal, thus allowing the larvae to move and choose a fixation site. The animals were restrained for approximately 60 minutes. This strain of *R. microplus* had been obtained from the same location as that of the present study and had been maintained at IPESA since 2001, using cattle and a BOD incubator. The main compounds used against this strain of *R. microplus* have been macrocyclic lactones, fipronil and fluazuron; this strain has never been previously exposed to diflubenzuron.

Pre-treatment counts of engorged *R. microplus* females detached from cattle, and allocation of animals to treatment groups

On days -3, -2 and -1, fully engorged *R. microplus* females that naturally detached from each animal were counted. All counts were performed daily, in the mornings (between 08:00 and 09:00 a.m.).

Animals were allocated to treatment groups according to a randomized complete block design. Block formation was based on arithmetic mean counts offemale ticks that became detached from each animal prior to treatment (days -3, -2 and -1), and on individual pen location. In each experiment, the animals were divided into eight blocks of four animals each and, within each block, they were randomly placed in one of the treatment groups, as shown in Table 1. The blocks were assigned to sets of two nearby

Table 1. Efficacy of diflubenzuron added to mineral salt for controlling *Haematobia irritans* in crossbred cattle, in the municipality of Caçu, Goiás state, Brazil.

Day of the study	Number of animals per group in each data	Untreated		Treated		Efficacy	Teste U of Mann-Whitney	
		Average infestation/ Standard deviation[1]		Average infestation/ Standard deviation[1]			U	p-value
zero	15	88.0 ± 20.4	A	87.9 ± 20.4	A	_	111.5	0.9835
3	15	69.3 ± 16.9	A	73.9 ± 28.1	A	**0.0**	106.5	0.8195
7	15	63.0 ± 25.0	A	57.8 ± 20.7	A	**8.3**	105.5	0.7875
14	15	66.7 ± 49.5	A	49.3 ± 28.0	A	**26.1**	100.0	0.6187
21	15	58.8 ± 62.1	A	43.4 ± 15.5	A	**26.2**	98.5	0.5755
28	15	59.3 ± 58.1	A	40.4 ± 14.5	A	**31.9**	104.5	0.7557
35	15	61.1 ± 54.6	A	53.8 ± 28.6	A	**12.0**	111.5	0.9835
42	15	53.3 ± 23.9	A	49.1 ± 35.6	A	**7.9**	93.0	0.4306
49	15	58.8 ± 39.6	A	40.4 ± 35.6	A	**31.3**	74.0	0.1150
56	15	61.9 ± 30.1	A	66.6 ± 24.5	A	**0.0**	93.5	0.4429
63	15	54.7 ± 26.2	A	56.4 ± 54.0	A	**0.0**	83.0	0.2290
70	15	68.3 ± 56.7	A	48.1 ± 49.3	A	**29.5**	80.5	0.1914
77	15	71.3 ± 60.0	A	66.4 ± 40.3	A	**6.9**	105.5	0.7875
84	15	81.7 ± 56.8	A	60.1 ± 49.5	A	**26.4**	80.5	0.1914
91	15	71.5 ± 60.5	A	64.2 ± 71.5	A	**10.3**	99.5	0.6041
98	15	96.5 ± 105.5	A	82.3 ± 67.9	A	**14.7**	107.5	0.8519
105	15	79.1 ± 73.0	A	68.1 ± 71.9	A	**13.9**	93.0	0.4306
112	15	104.5 ± 70.3	A	87.2 ± 51.1	A	**16.6**	96.5	0.5203
119	15	102.7 ± 66.5	A	106.7 ± 103.3	A	**0.0**	108.0	0.8682
126	15	60.2 ± 36.1	A	69.9 ± 36.2	A	**0.0**	95.0	0.4807
133	15	42.6 ± 22.9	A	38.8 ± 24.5	A	**8.9**	100.0	0.6187
140	15	37.9 ± 20.4	A	36.9 ± 38.3	A	**2.6**	82.0	0.2134
147	15	24.0 ± 21.3	A	20.7 ± 20.8	A	**13.6**	100.5	0.6334
154	15	19.5 ± 11.2	A	16.1 ± 12.1	A	**17.1**	75.5	0.1300
160	15	19.9 ± 9.4	A	14.0 ± 8.4	A	**29.5**	70.5	0.0852

zero = Mean counts of the days -2 and -1; [1]Means values followed by the same letter on the same line for each parameter, do not differ by the test U Mann-Whitney ($p>0.05$).

pens, and the animals within each block were randomly allocated to individual pens within the set. Each animal constituted one experimental unit.

The cattle started receiving diflubenzuron (Difly S3®, Champion Animal Health) on experimental day zero, and administration was performed in accordance with the manufacturer's recommendations. Assuming that salt consumption would be around 60 to 80 g/day, 17 g of the tested compoundwere added per kilogram of salt (425 g per 25 kg bag). For consumption, the product was added to the daily feed, and thus the intake of daily recommended dosages of diflubenzuron by these animals was assured, since all of them consumed all the feed provided. The product used is the only diflubenzuron formulation commercially available for *R. microplus*on the Brazilian market.

Counts of engorged *R. microplus* females detached from each animal, and post-treatment infestations

Engorged female ticks that had become naturally detached from experimental cattle were counted daily, starting on day 1, up to the end of each trial (80th DAST). During the post-treatment period, all animals were subjected to infestation with approximately 5,000 viable unfed *R. microplus* larvae twice a week (every Tuesday and Thursday), up to the end of the study, as recommended by Holdsworth et al. (2006).

Assessment of reproductive parameters in pre-selected engorged female ticks

The following reproductive parameters of engorged female ticks were analyzed: female weight, egg mass weight, hatchability percentage, percentage of reduction in oviposition, percentage of reduction in hatchability, reproductive efficiency and control/efficacy percentage of formulations regarding reproductive parameters.

For this analysis, all engorged female ticks that became detached from animals were collected every morning, from day 1 up to the end of the experiment (day 80). For each group, 10 engorged female ticks were randomly selected each day, weighed, fixed in Petri dishes using scotch tape and moved to a BOD incubator at 27 °C and approximately 85% relative humidity, to stimulate oviposition.

After 20 days of laying eggs in the BOD incubator, the engorged female ticks were discarded, and the weights of their egg masses were recorded for each group and each post-treatment day. Subsequently, the egg mass of each group and each post-treatment day was transferred to an adapted 3 ml syringe and was returned to the BOD incubator (27 °C and approximately 85% relative humidity), to stimulate larval hatching.

After another 20 days, when larval hatching had ended, the hatchability rate was calculated in accordance with methodology described by Gonzales et al. (1993). The hatchability percentage from each sample group (derived from the daily sample of engorged female ticks) was visually estimated using a stereo microscope with an eyepiece grid, by comparing the proportion of larvae in relation to unhatched eggs for each group and each post-treatment day (DRUMMOND et al., 1973; GONZALES et al., 1993; GEORGE & DAVEY, 2004; HOLDSWORTH et al., 2006).

Reduction in the number of engorged females counted

Acaricide efficacy indexes from each formulation were calculated using arithmetic means from counts of engorged female ticks that had become detached from animals in each group. The data thus collected were grouped into seven-day intervals. The formula for this calculation, as recommended by Roulston & Wharton (1967), is described below:

$$Efficacy\ percentage = \left[1 - \frac{Ta\ x\ Cb}{Tb\ x\ Ca}\right] x\ 100 \qquad (2)$$

In this equation, "**Ta**" represents the average number of engorged female ticks counted on treated animals post-medication; "**Tb**" is the average number of engorged female ticks counted on treated animals during the three days prior to treatment; "**Ca**" is the average number of engorged female ticks counted on control animals after the experiment began; and "**Cb**" is the average number of engorged female ticks counted on control animals during the three days that preceded treatment.

Reproductive parameters of pre-selected engorged female ticks

To assess the impact of treatments on reduction of oviposition and hatching, the following equations were used, as described by Drummond et al. (1973):

Percentage oviposition reduction = [(average egg mass weight of control group – average egg mass weight of treated group) / average egg mass weight of control group] X 100;

Percentage hatching reduction = [(average hatchability of control group – average hatchability of treated group) / average hatchability of control group] X 100.

To estimate the extent of reproduction and percentage control or efficacy, the following equations, described by Drummond et al. (1973), were applied:

Estimate of reproduction (ER) = (egg weight / female weight) X hatching % X 20,000[1];

Control or efficacy percentage = [(ER of control group – ER of treated group) / ER of control group] X 100.

All experiments were blinded; thus, completely reliable data were obtained. Furthermore, all animals received the envisaged doses of the active agent.

Data analysis

The data obtained from the counts of *R. microplus* and *H. irritans* did not meet the requisites of normality and homogeneity of variances. The Mann-Whitney U test (at a significance level of 5%) was used to compare the treatments, within each experimental date, using the npar1way Wilcoxon SAS procedure (SAS Institute, 2008).

[1] Constant corresponding to an estimate of the number of larvae contained in 1 g of eggs.

The numerical observations regarding the reproductive parameters (engorged female weight, egg mass and hatchability) of pre-selected engorged female ticks in both experimental groups met the requisites of normality, homogeneity of variances and residue analysis. The data were analyzed by analysis of variance (ANOVA) using the SAS GLM procedure and the means of the treatments were compared by means of Tukey's test (Tukeys's Stundentized Range – HSD) at a significance level of 5%.

Results

No side effects from the treatments were observed among the cattle medicated with different formulations containing diflubenzuron.

Haematobia irritans

The results regarding average fly counts and efficacy indexes obtained from diflubenzuron treatments in the trial in central-western Brazil are shown in Table 1. It can be seen that the animals kept as untreated controls were constantly challenged by this parasite up to the 126th DAST. In subsequent counts, on the 133rd, 140th, 147th, 154th and 160th DAST, *H. irritans* infestations in the control group diminished considerably. This interfered with efficacy results on those experimental days. Nonetheless, it could be seen that efficacy indexes for this active agent against horn flies parasitizing cattle were either very low or null throughout the experiment.

This formulation reached a maximum efficacy index of 31.9% on the 28th DAST. Between the 3rd and 126th DAST, the efficacy values ranged from 0.0% to around 30% (Table 1). The average horn fly counts obtained among control animals (untreated) did not differ significantly ($p > 0.05$) from the average counts of *H. irritans* on cattle treated with diflubenzuron via mineral salt, on all days of observation post-treatment (Table 1).

In the experiment conducted in the southeastern region of Brazil (municipality of Formiga, state of Minas Gerais), diflubenzuron administered via mineral salt presented no efficacy (0.0%) against *H. irritans* parasitizing cattle up to the 77th DAST (Table 2). Between the 84th and 147th DAST, diflubenzuron showed efficacy values ranging from 32.5% to 44.6%, while immediately afterwards (154th and 160th DAST), the efficacy indexes decreased to 11.4% and 9.2%, respectively. Statistical analysis on the results from fly counts showed that the average numbers of *H. irritans* on animals that received diflubenzuron via mineral salt were significantly lower ($p \leq 0.05$) than the numbers obtained from the untreated control group between the 84th and 140th DAST. On other experimental days, the average numbers of horn flies did not differ significantly ($p > 0.05$) between untreated animals (control) and animals that received diflubenzuron (Table 2).

Table 2. Efficacy of diflubenzuron added to mineral salt for controlling *Haematobia irritans* in crossbred cattle, in the municipality of Formiga, Minas Gerais state, Brazil.

Day of the study	Number of animals per group in each data	Untreated		Treated		Efficacy	Teste U of Mann-Whitney	
		Average infestation/ Standard deviation[1]		Average infestation/ Standard deviation[1]			U	p-value
zero	15	71.4 ± 18.9	A	71.3 ± 18.9	A	_	111.5	0.9835
3	15	69.3 ± 16.9	A	73.9 ± 28.1	A	**0.0**	106.5	0.8195
7	15	81.3 ± 55.1	A	91.1 ± 54.9	A	**0.0**	94	0.4553
14	15	81.1 ± 53.6	A	92.0 ± 63.8	A	**0.0**	92	0.4068
21	15	83.5 ± 49.5	A	90.1 ± 54.9	A	**0.0**	92	0.4068
28	15	85.1 ± 57.6	A	106.1 ± 48.7	A	**0.0**	79.5	0.1776
35	15	100.5 ± 59.7	A	105.3 ± 61.1	A	**0.0**	107.5	0.8519
42	15	86.7 ± 42.9	A	92.6 ± 69.2	A	**0.0**	111	0.9669
49	15	105.5 ± 53.2	A	114.3 ± 63.6	A	**0.0**	99	0.5897
56	15	95.9 ± 43.4	A	105.5 ± 56.2	A	**0.0**	96	0.5069
63	15	82.6 ± 55.2	A	95.1 ± 48.3	A	**0.0**	96.5	0.5203
70	15	93.3 ± 40.1	A	96.7 ± 60.1	A	**0.0**	109	0.9010
77	15	97.3 ± 53.9	A	102.5 ± 49.9	A	**0.0**	102	0.6783
84	15	128.4 ± 54.9	A	86.7 ± 53.4	B	**32.5**	65.5	0.0438
91	15	90.3 ± 61.7	A	55.7 ± 37.9	A	**38.3**	74.5	0.1198
98	15	103.9 ± 53.4	A	61.1 ± 29.6	A	**41.2**	62	0.0881
105	15	99.9 ± 70.1	A	55.3 ± 26.4	B	**44.6**	68.5	0.0412
112	15	108.5 ± 53.3	A	65.1 ± 40.7	B	**40.0**	57.5	0.0238
119	15	106.0 ± 37.8	A	65.2 ± 28.7	B	**38.5**	40.5	0.0030
126	15	105.1 ± 50.7	A	60.1 ± 29.5	B	**42.8**	55	0.0181
133	15	89.1 ± 40.2	A	58.3 ± 26.2	B	**34.5**	57.5	0.0238
140	15	109.8 ± 34.8	A	69.5 ± 40.5	B	**36.7**	53.5	0.0152
147	15	93.7 ± 46.9	A	60.0 ± 25.9	A	**36.0**	60.5	0.0527
154	15	91.5 ± 38.2	A	81.0 ± 43.4	A	**11.4**	93	0.4306
160	15	98.2 ± 45.0	A	89.1 ± 59.1	A	**9.2**	89.5	0.3507

zero = Mean counts of the days -2 and -1. [1]Means values followed by the same letter on the same line for each parameter, do not differ by the test U Mann-Whitney ($p>0.05$).

Rhipicephalus microplus

The anti-*R. microplus* efficacy of a different formulation containing diflubenzuron, which was calculated based on seven-day tick count intervals, was below 14% (arithmetic means) during the entire experiment. A maximum efficacy of 13.7% was reached by this formulation between the 15th and 21st DAST. Between the 22nd and 28th DAST, values of 12.6% were registered. The efficacy decreased to values lower than 10% after the 28th DAST (Table 3). The average numbers of *R. microplus* on control animals (untreated) did not differ statistically ($p > 0.05$) from average numbers of ticks on animals that were subjected to treatment, with diflubenzuron via mineral salt, at any time during the trial (Table 3). Based on concern for animal welfare and good clinical practice, allied to low efficacies demonstrated by diflubenzuron throughout the experiment, all the cattle received specific treatment against *R. microplus* on the 80th DAST and experimental tick counting was discontinued.

Concerning deleterious effects that might have been caused by diflubenzuron to the reproductive parameters of *R. microplus* females, this compound generally presented low or null efficacy. It showed a maximum percentage reduction of oviposition of 3.0% between the 71st and 77th DAST. On other days, this compound had no effect on this parameter (0.0%). Regarding percentage larval hatchability, reduction rates caused by diflubenzuron remained ≤ 7.5% throughout the trial. These results may have been reflected in the efficiency indexes of this active agent regarding the reproductive parameters of *R. microplus* females, with null efficacy (0.0%) observed for diflubenzuron during the entire experimental period (Table 4).

Results from the statistical analysis concerning the reproductive parameters of fully engorged females that had naturally detached from cattle reinforced inferences that had previously been described. Specifically, regarding tick weights, females obtained from the control group weighed less ($p \leq 0.05$) than did the females that became detached from animals that received diflubenzuron on some dates (15th to 21st and 29th to 35th DAST). Between the 36th and 42nd and between the 71st and 77th DAST, females selected from cattle that received diflubenzuron were significantly lighter ($p \leq 0.05$) than females obtained from untreated animals. The weight of egg masses from females detached from treated cattle was statistically greater ($p \leq 0.05$) than the weight of egg masses from *R. microplus* females that were obtained from the control group between the 8th and 35th DAST, and between the 43rd and 56th DAST. Regarding percentagelarval hatching, there was no significant difference ($p > 0.05$) in the values obtained from the two groups, except between the 22nd and 28th DAST, when the percentage of larvae that hatched from the treated (diflubenzuron) group was higher ($p \leq 0.05$) than the percentage of larvae that hatched from the control group (Table 4).

Discussion

In vitro, the effects of diflubenzuron on immature stages of *H. irritans* had already been demonstrated by Silva & Mendes (2002) and Dell'Porto et al. (2012). Silva & Mendes (2002) showed that third-stage larvae were more susceptible to diflubenzuron than were first and second-stage larvae. Moreover, this active agent achieved 100% lethality, under laboratory conditions, at concentrations of 300, 100 and 50 ppb. Dell'Porto et al. (2012) found that flies kept as controls presented 86% emergence (based on egg numbers), while a group cultivated in feces from animals that had been treated with diflubenzuron presented a 1% rate.

Miller et al. (1986), Cilek & Knapp (1991) and Tomberlin et al. (2007) studied the efficacy of diflubenzuron directly on hosts.

Table 3. Efficacy of diflubenzuron added to mineral salt for controlling *Rhipicephalus microplus* in crossbred cattle, in the municipality of Formiga, Minas Gerais state, Brazil.

Day of the study	Number of animals per group in each data	Mean value of engorged females detached of cattle /Experimental groups				Efficacy (%)	Teste U of Mann-Whitney	
		Untreated Average[1] infestation/Standard deviation		Treated Average infestation/Standard deviation			U	p-value
zero	8	47.1 ± 16.1	A	51.7 ± 16.3	A	-	15	0.6889
1 to 7	8	65.0 ± 12.5	A	67.0 ± 26.0	A	**6.0**	16	0.8102
8 to 14	8	70.3 ± 13.7	A	72.7 ± 15.2	A	**5.8**	14	0.5752
15 to 21	8	79.9 ± 23.4	A	75.6 ± 27.7	A	**13.7**	15	0.6889
22 to 28	8	80.5 ± 21.2	A	77.2 ± 34.1	A	**12.6**	13	0.4712
29 to 35	8	80.6 ± 7.7	A	80.6 ± 36.4	A	**8.8**	14	0.5752
36 to 42	8	82.5 ± 14.4	A	85.0 ± 41.0	A	**6.1**	15	0.6889
43 to 49	8	85.1 ± 20.4	A	86.1 ± 27.0	A	**7.7**	18	0.9362
50 to 56	8	76.6 ± 22.6	A	86.9 ± 25.5	A	**0.0**	15	0.6889
57 to 63	8	84.3 ± 30.5	A	91.9 ± 28.5	A	**0.6**	15	0.6889
64 to 70	8	90.9 ± 33.1	A	94.0 ± 28.5	A	**5.7**	17	0.9362
71 to 77	8	95.0 ± 27.9	A	93.9 ± 26.8	A	**9.9**	18	0.9362
78 to 80	8	94.3 ± 31.2	A	95.2 ± 25.5	A	**8.0**	18	0.9362

[1]Means values followed by the same letter on the same line for each parameter, do not differ by the test U Mann-Whitney ($p \geq 0.05$).

Table 4. Effect of diflubenzuron, administered orally to cattle, on reproductive parameters of engorged *Rhipicephalus microplus* females , in the municipality of Formiga, Minas Gerais state, Brazil.

Day post treatment	Total number of engorged female		Engorged female weight (g)		Egg mass weight (g)		Hatching percentage (%)		Reduction (%)		Reproductive efficiency		Efficacy
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Treated	Treated	Untreated	Treated	Treated
1 to 7	10	10	2.93 ± 0.222 A*	2.97 ± 0.180 A	0.93 ± 0.360 A	1.02 ± 0.278 A	89.8 ± 6.2 A	92.9 ± 4.6 A	0.0	0.0	571486.42	635130.36	0.0
8 to 14	10	10	2.98 ± 0.241 A	3.08 ± 0.287 A	0.74 ± 0.363 B	1.11 ± 0.265 A	86.0 ± 12.2 A	88.6 ± 3.9 A	0.0	0.0	428839.32	636576.09	0.0
15 to 21	10	10	2.84 ± 0.189 B	3.29 ± 0.301 A	0.72 ± 0.287 B	1.18 ± 0.179 A	92.0 ± 4.8 A	85.6 ± 10.2 A	0.0	7.0	465865.32	612902.56	0.0
22 to 28	10	10	3.22 ± 0.239 A	3.37 ± 0.501 A	0.86 ± 0.405 B	1.19 ± 0.314 A	72.6 ± 35.2 B	87.9 ± 8.5 A	0.0	0.0	385795.58	619073.72	0.0
29 to 35	10	10	3.12 ± 0.337 B	3.42 ± 0.076 A	1.15 ± 0.205 B	1.45 ± 0.261 A	94.1 ± 3.5 A	88.1 ± 8.6 A	0.0	6.4	691570.94	746869.67	0.0
36 to 42	10	10	3.57 ± 0.208 A	3.16 ± 0.538 B	1.19 ± 0.243 A	1.27 ± 0.327 A	92.6 ± 4.2 A	90.6 ± 5.2 A	0.0	2.1	616279.20	728940.86	0.0
43 to 49	10	10	3.49 ± 0.448 A	3.60 ± 0.304 A	1.10 ± 0.415 B	1.63 ± 0.200 A	87.3 ± 7.4 A	89.2 ± 5.3 A	0.0	0.0	547561.67	809959.16	0.0
50 to 56	10	10	2.96 ± 0.127 A	2.91 ± 0.322 A	0.84 ± 0.358 B	1.20 ± 0.313 A	91.1 ± 3.1 A	92.7 ± 2.1 A	0.0	0.0	515581.17	766674.28	0.0
57 to 63	10	10	3.06 ± 0.184 A	3.07 ± 0.402 A	1.14 ± 0.239 A	1.39 ± 0.169 A	89.2 ± 10.2 A	91.4 ± 4.4 A	0.0	0.0	663328.11	828089.06	0.0
64 to 70	10	10	2.88 ± 0.145 A	2.95 ± 0.331 A	0.98 ± 0.216 A	1.15 ± 0.174 A	86.1 ± 4.8 A	80.7 ± 11.5 A	0.0	6.3	586061.55	628911.46	0.0
71 to 77	10	10	2.94 ± 0.208 A	2.56 ± 0.153 B	1.26 ± 0.277 A	1.22 ± 0.157 A	91.9 ± 1.7 A	85.3 ± 9.5 A	3.0	7.2	789218.46	815681.25	0.0
78 to 80	10	10	2.82 ± 0.067 A	2.61 ± 0.090 A	1.08 ± 0.045 A	1.13 ± 0.063 A	93.8 ± 0.8 A	86.7 ± 8.1 A	0.0	7.5	721282.05	752653.32	0.0

*Means values followed by the same letter on the same line for each parameter, do not differ significantly at a 95% confidence interval.

Diflubenzuron was administered as a bolus for cattle in the studies of Miller et al. (1986) and Cilek & Knapp (1991), in the United States and Canada. These authors collected manure samples and then incubated them at 27 °C and maintained them for pupation and emergence of adults. Miller et al. (1986) reported that a commercial bolus formulation (10% diflubenzuron) used in manure samples from treated animalswas able to prevent development of horn fly larvae (*Haematobia irritans*) (14 weeks of protection), face fly larvae (*Musca autumnalis*) (17 weeks of inhibition), along with immature stages of stable flies (*Stomoxys calcitrans*) and house flies (*Musca domestica*).

A different trial tested diflubenzuron bolus in two cattle herds that were carrying horn flies that were resistant to permethrin and stirophos. Testes treatments reduced resistant populations of adult horn fly population by more than 80% during the first two and a half months (CILEK & KNAPP, 1991). Tomberlin et al. (2007) evaluated the flies' capacity to reach the adult stage through recording the percentages of deformed pupae that developed on manure samples from three pastures treated with diflubenzuron (at a dosage of 59 per 0.4 hectares). The capacity of*H. irritans*to reach adult stages was lower in the treated samples than in the control manure samples, on 11 of the 15 sampling dates. Accordingly, a significantly greater percentage of deformed pupae was recorded among the samples from the treated sites ≤ 17 days post-treatment.

Regarding the field efficacy of diflubenzuron against *R. microplus*, a single trial exists. This was conducted in the municipality of Campo Grande, the capital of the state of Mato Grosso do Sul, in the central-western region of Brazil. Itused beef cattle that were maintained under pasture conditions and were experimentally infested with this ectoparasite (ANDREOTTI et al., 2015). In that study, a reduction in parasite burden of approximately 50% was observed 15 days after the treatment with diflubenzuron had started, in comparison with cattle that were kept as untreated controls. These authors also observed that diflubenzuron had effects on *R. microplus* only at larval stages. In the present study, using a stall test on artificially infested cattle, diflubenzuron demonstrated minimal efficacy against *R. microplus*, with indexes ≤ 15% throughout the experiment.

It is important to emphasize that the relatively low efficacy observed in the *R. microplus* studies should not be directly correlated with resistance to the insecticide tested. Although diflubenzuron resistance has been previously reported in the Australian blow fly *Lucilia cuprina* (KOTZE & SALES, 2001; LEVOT & SALES, 2002), resistance of *R. microplus* to this insecticide has not been reported so far. It is also important to highlight that pour-on fluazuron (a compound in the same family as diflubenzuron) has been shown to have elevated therapeutic and residual efficacy (≥ 90%) on farms where the *R. microplus* strain was the same as the one used for stall tests in the present study (CRUZ et al., 2014; GOMES et al., 2015; MACIEL et al., 2016; LOPES et al., 2017).

The main hypothesis to explain the failure of diflubenzuron in controlling *H. irritans* and *R. microplus* that wasobserved in the present study would be the insecticide administration route. In the studies by Miller et al. (1986) and Cilek & Knapp (1991), in the United States and Canada, diflubenzuron was administered via a bolus andhad a significant effect against insect larvae. On the other hand, the formulations used in the present study were administered via mineral salt supplementation, in accordance with the manufacturer's recommendations, and there are no known bioavailability studies on this molecule in animals exposed to diflubenzuron via mineral salt.

The route of administration for benzoylphenylureas is a significant factor in relation to the effect of a particular active component (GOMES et al., 2015). Against *R. microplus*, the efficacy indexes for pour-on fluazuron are higher than 90% (CRUZ et al., 2014; GOMES et al., 2015; MACIEL et al., 2016; LOPES et al., 2017), which contrasts with efficacy rates of less than 50%for this same molecule administered through the subcutaneous route (GOMES et al., 2015), against the same strain of *R. microplus*. The same may have occurred for diflubenzuron administered via mineral salt, though further studies should be conducted to substantiate this hypothesis.

Another issue that can interfere with the efficacy of a formulation relates to the availability and quantity of the product that can be administered to the animals (when these are supplied to the herd in covered troughs arranged in paddocks). There is also the impact of possible social interactions in this factor, since some cattle present dominance behavior and this may influence the amount of product ingested, in relation to other cattle that are classified as "less dominant". However, it is important to note that, in our study evaluating the efficacy of diflubenzuron against *R. microplus*, the cattle were housed in individual pens, so that the mineral salt containing the active substance was individually provided to the animals.

To the best of our knowledge, there is a lack of studies conducted using diflubenzuron with the aim of evaluating the effects of this molecule on the reproductive parameters of fully engorged *R. microplus* females that naturally detach from cattle. This makes it impossible to discuss or formulate comparative analyses on the data obtained. Nevertheless, based on the results obtained from the present study, diflubenzuron did not lead to any deleterious effects on the reproductive parameters of *R. microplus*, among females that had become detached from cattle during an experiment that was conducted over 80 consecutive days of evaluation.

However, studies on the effects of other insect growth regulators on the cattle tick have shownvarying results. Martins et al. (1995) used different concentrations of fluazuron (1 mg/kg and 2 mg/kg) and found that there was no oviposition among engorged females on days 7-11 after treatment, or that it occurred at a very low percentage (<15%). Mendonça (2010) evaluated a formulation of 3.0 mg/kg fluazuron + 0.5 mg/kg abamectin on a different strain of *R. microplus*. They observed that this compound had deleterious effects on the reproductive parameters of fully engorged *R. microplus* females, with 100% efficacy on several post-treatment days. Cruz et al. (2014) observed oviposition among pre-selected females that had become detached, in groups treated with two formulations (2.5 mg/kg fluazuron and 3.0 mg/kg fluazuron + 0.5 mg/kg abamectin) over the entire experimental period.

Further *in vivo* studies with different formulations containing diflubenzuron are needed, in order to understand and elucidate the effects of this active agent against *Haematobia irritans* and *Rhipicephalus microplus* parasitizing cattle. The different diflubenzuron formulations administered via mineral salt within the experimental design of the present study, against *H. irritans* and *R. microplus* parasitizing cattle, did not show satisfactory efficacy. Nor did this agent show any deleterious effects on the reproductive parameters of *R. microplus* females that had naturally detached from cattle.

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