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Potential of the insect growth regulator, fluazuron, in the control of *Rhipicephalus sanguineus* nymphs (Latreille, 1806) (Acari: Ixodidae): Determination of the LD_{95} and LD_{50}

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ABSTRACT

Conventional pesticides have suffered two main drawbacks: (a) broad spectrum of action and (b) selection of target species resistant to the different active ingredients. Thus compounds that are less harmful to the environment and to human health, more specific and that do not induce resistance need to be developed. One alternative are insect growth regulators, such as fluazuron. The present study examined the efficacy of fluazuron (active ingredient of the acaricide Acatak®) and the sensitivity of *Rhipicephalus sanguineus* nymphs exposed to different doses of this chemical, and determined the lethal doses of fluazuron: 95% – LD_{95} and 50% – LD_{50}. Different doses of fluazuron were applied in duplicates on the dorsal region of hosts ("pour on"). Distilled water was used in the control group. On the first day after the treatment with fluazuron, hosts were artificially infested with *R. sanguineus* nymphs. After engorgement, nymphs were removed, placed on Petri dishes, identified, and maintained in BOD incubator for 15 days. Dead *R. sanguineus* nymphs after the treatment with 13 different doses of fluazuron were quantified and the LD_{95} was estimated to be 100 mg/kg and LD_{50}, 19.544 mg/kg (12.478–22.636), with a confidence interval of 95%. Nymphs of *R. sanguineus* were sensitive to fluazuron at various levels, indicating that this insect growth regulator (IGR) may be used to control this parasite in this stage of its biological cycle, reducing the significant damage it causes.

1. Introduction

The tick *Rhipicephalus sanguineus* is one of the main ectoparasites of medical and veterinary importance worldwide (Linardi and Nagem, 1973; Labruna and Pereira, 2001; Szabó et al., 2001; González et al., 2004; Soares et al., 2006), as it is the vector and reservoir of several pathogens and causes direct and indirect damage to their hosts, such as blood spoilage and skin lesions (Sonenshine, 1991).

Recently, *R. sanguineus* has become an important urban pest and a concern to public health agencies (Paz et al., 2008). As a result, the veterinary pharmaceutical industry has experienced a significant growth focused on the development of new acaricides (Labruna, 2004).

Synthetic acaricides have been the main method to control ticks, despite the high costs of products, facilities, qualified manpower, and concerns about the contamination of animal products by chemical residues, and the risks to human health and environmental contamination (Nolan, 1985; Pruett, 1999; Oliveira et al., 2008, 2009). Another concern is the selection of tick lineages resistant to the different active ingredients mainly due to the incorrect use of chemicals (Crampton et al., 1999). Therefore, the search for new acaricides with lower toxicity and less contaminants has been intensified.

A new category of compounds with the potential to control ticks are insect growth regulators (IGRs) (Coop et al., 2002). These chemicals act in the molting process by affecting the metabolism of chitin or the production of hormones involved, thus interfering in the growth and development of ectoparasites (Graf, 1993; Fournet et al., 1995; Pawar et al., 1995; Hoffmann and Lorenz, 1998; Taylor, 2001).

Since the synthesis of chitin in arthropods is a limiting factor for their success and the development of new compounds could have a

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very narrow specificity, high efficacy, and low toxicity to vertebrates and the environment, research on growth regulators has intensified even further and became more important in the current scenario (Bowman et al., 1997).

Among insect growth regulators are the compounds of the benzoylphenylurea chemical class, which includes diflubenzuron, lufenuron, flufenoxuron, triflumuron, and fluazuron (Correia, 2003).

Fluazuron (active ingredient of the acaricide Acatak®) was the first growth regulator to be registered for the control of ixodid ticks, but its use is restricted to a few species (Bull et al., 1996). In the case of *R. sanguineus*, few studies on the use of this chemical in tick control are currently available in the literature.

Therefore, the present study was aimed at assessing the susceptibility of *R. sanguineus* nymphs to different doses of fluazuron (Acatak®) and determining its lethal doses: 95% – LD_{95} and 50% – LD_{50}.

2. Materials and methods

2.1. Chemical compound

Fluazuron is a compound of the benzoylphenylurea chemical class, chemical name *N*-[[4-chloro-3-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]oxyphenyl]carbamoyl]-2,6-difluorobenzamide and CAS 86811-58-7. For experiments, the acaricide Acatak®, a commercial product produced by Novartis, was used.

2.2. R. sanguineus ticks (Latreille, 1806)

R. sanguineus nymphs were obtained from a colony maintained under controlled conditions (28 °C, 85% humidity, and 12-h photoperiod) in a BOD incubator, in a room of the Animal Facility of the Department of Biology – UNESP, Rio Claro campus/São Paulo, Brazil.

2.3. Hosts

Twenty-eight female New Zealand White rabbits, weighting between 3 and 3.5 kg, were used as hosts. Rabbits were obtained from the Animal Facility of UNESP – Botucatu Campus/São Paulo – Brazil and housed in the Animal Facility of UNESP – Rio Claro Campus/São Paulo – Brazil. Animals did not have prior contact with ticks or acaricides and were kept under controlled conditions. During the entire experiment, animals were maintained in cages and received water and rabbit food *ad libitum*.

This study was approved by the Committee of Ethics in Animal Research of UNESP/SP/Brazil, protocol # 09/2011.

2.4. Dosages of fluazuron

The initial dosage of fluazuron of the treatment group was defined based on the product label of the manufacturer of Acatak® (2.5 mg/kg and 1 mL/kg of fluazuron/body weight). The following dosages were prepared by diluting fluazuron (Acatak®) in distilled water and overdose (over 1 mL/kg). Dosages tested were as follows: 1.25, 2.5, 5, 10, 15, 20, 30, 40, 60, 80, 100, 125, and 150 mg/kg fluazuron/body weight.

All dosages of fluazuron were stored in labeled 100 mL volumetric flasks and beakers until use. Each treatment was conducted in duplicate.

2.5. Bioassays

For each dose of fluazuron (1.25, 2.5, 5, 10, 15, 20, 30, 40, 60, 80, 100, 125, and 150 mg/kg de fluazuron/body weight) and the control group, two female rabbits were used as hosts. Fluazuron was applied on the spine (''pour on'') of animals, and distilled water was used for control animals.

On the first day after the application of fluazuron and distilled water (control group), a feeding chamber was attached to a shaved dorsal area of the rabbit, following the methodology described by Bechara et al. (1995). Twenty-four hours after the chamber was attached, each rabbit was infested with *R. sanguineus* nymphs in a syringe. The chamber was then cover with a lid with holes to allow air circulation, but prevent escapes.

The first observation was carried out after 24 h (time needed for the ectoparasites to acclimate). The following observations occurred once a day until the total engorgement of nymphs. As soon as the engorgement process was completed, engorged nymphs (metanymphs) detached from the host and were collected and placed on identified Petri dishes. The same procedure was repeated for all treatments and for the control group.

Engorged nymphs were placed in BOD incubator and monitored daily for 15 days, as this is the maximum time needed for fluazuron to kill ectoparasites, according to the label of Acatak®.

After 15 days, dead engorged nymphs were accounted for each treatment. Ticks were considered dead when they did not respond, by moving their appendixes, when touched with a brush. Nymphs that molted were considered alive, as the drug did not inhibit the synthesis and/or deposition of chitin needed to form a new cuticle and thus did not prevent the development into the next instar (adult).

After the bioassay, the efficacy of the fluazuron was examined in this species.

2.6. Statistical analysis

The mortality of *R. sanguineus* nymphs fed on treated rabbits was compared with the Probit analysis with the program POLO-PC (LeOra SOFTWARE, 1987). The LD_{50}, the confidence interval 95% and the slope of the regression were calculated based on the relationship fluazuron dose/mortality of nymphs.

3. Results

The efficacy of the fluazuron on *R. sanguineus* nymphs was assessed based on 13 different doses of this compound tested in duplicates (Tables 1–4; see Graphical abstract).

The bioassay revealed that *R. sanguineus* nymphs are sensitive to all doses of fluazuron tested, although mortality was significantly different (Tables 1–4; see Graphical abstract).

Table 1
Number of dead *Rhipicephalus sanguineus* nymphs exposed to different doses of fluazuron – 1st day.

Fluazuron (doses) (mg/kg)	1°. Replicate		2°. Replicate		Sum	
	Alive	Dead	Alive	Dead	Alive	Dead
1.25	123	1	113	2	236	3
2.5	96	4	212	6	308	10
5	213	37	132	18	345	55
10	96	39	117	43	213	82
15	81	47	99	45	180	92
20	80	72	153	156	233	228
30	72	120	103	164	175	284
40	84	196	52	145	136	341
60	20	82	25	120	45	202
80	24	122	28	232	52	354
100	10	228	15	213	25	441
125	40	150	33	137	73	287
150	46	76	97	151	143	227

Table 2
Number of dead *Rhipicephalus sanguineus* nymphs exposed to different doses of fluazuron – 2nd day.

Fluazuron (doses) (mg/kg)	1°. Replicate		2°. Replicate		Sum	
	Alive	Dead	Alive	Dead	Alive	Dead
1.25	99	1	103	0	202	1
2.5	172	8	197	3	369	11
5	159	22	111	9	270	31
10	111	39	154	43	265	82
15	128	42	174	66	302	108
20	133	87	105	75	238	162
30	86	94	98	112	184	206
40	77	123	81	139	158	262
60	56	144	33	107	89	251
80	44	156	44	176	88	332
100	38	167	32	178	70	345
125	50	76	54	96	104	172
150	101	109	88	102	189	211

The percentage of dead nymphs (0.5–95%) directly increased with doses (1.25, 2.5, 5, 10, 15, 20, 30, 40, 60, 80, and 100 mg/kg). However, the progressive increase in mortality occurred until 100 mg/kg, which caused the death of 95% (DL_{95}) of the ticks exposed. In the groups treated with the two highest doses (125 and 150 mg/kg), the number of dead nymphs decreased to 80% and 62%, respectively (Tables 1–4; see Graphical abstract).

The first nymphs to engorge and detach from the treated host (5th day) were more affected by fluazuron than those that engorged later (7th day). This difference was observed since the treatment with fluazuron at 15 mg/kg. In some treatments, such as 100 mg/kg, the percentage of dead detached nymphs on the first day was 95%, against 81% on the second day (Tables 1–4).

After the treatment of hosts with lower doses of fluazuron (1.25, 2.5, and 5 mg/kg) and the end of the engorgement of ticks, no morphological or behavioral changes were observed in nymphs exposed during the 15 days of observation. However, nymphs from the treatments with higher doses of fluazuron (10–150 mg/kg) exhibited, morphological and behavioral changes, such as smaller size, elliptical shape, curved idiosoma, fragile and easily breakable integument, not well defined dorsal and ventral ridges, and lethargy. The nymphs that were able to molt into the next instar and become adults had a thin layer of the previous cuticle attached to the new cuticle. Dead nymphs were shriveled and darker.

In the control group, mortality was not statistically significant and no morphological changes or alterations in color and consistency of the tegument were observed during the bioassay (15 days).

The period of engorgement and molting of *R. sanguineus* nymphs of the control group and treated rabbits were also examined. The period of engorgement ranged between 5 and 6 days in the control group and 7 and 8 days in nymphs from fluazuron-treated rabbits. Despite the slightly longer period in the treated group, the size of engorged individuals was smaller compared to those detached from rabbits of the control group.

The molting period in the control group was 6–10 days while that of engorged ticks from treated rabbits was on average 13 days. In the group treated with lower doses of fluazuron, the molting period was shorter and similar to those of nymphs of the control group. The molting period gradually became longer as the fluazuron doses increased.

The mortality of *R. sanguineus* nymphs detached on the first day from fluazuron-treated hosts were compared with the Probit analysis and the LD_{50} and confidence interval (95%) were estimated

Table 3
Percentage of dead *Rhipicephalus sanguineus* nymphs exposed to different doses of the fluazuron – 1st day.

Fluazuron (doses) (mg/kg)	1°. Replicate		2°. Replicate		Average	
	Alive (%)	Dead (%)	Alive (%)	Dead (%)	Alive (%)	Dead (%)
1.25	99.2	0.8	98	2	98.7	1.3
2.5	96	4	97	3	96.8	3.2
5	85.2	14.8	88	12	86.25	13.75
10	71.12	28.88	73.13	26.87	72.2	27.8
15	63.28	36.72	68.75	31.25	66.18	33.82
20	52.63	47.37	49.51	50.49	50.55	49.45
30	37.5	62.5	38.57	61.43	38.12	61.8
40	30	70	26.4	73.6	28.51	71.49
60	19.6	80.4	17.25	82.75	18.22	81.78
80	16.44	83.56	10.77	89.23	12.8	87.2
100	4.2	95.8	6.57	93.43	5.35	94.5
125	21	79	19.41	80.59	20.28	79.72
150	37.7	62.3	39.11	60.89	38.65	61.35

Table 4
Percentage of dead *Rhipicephalus sanguineus* nymphs exposed to different doses of the fluazuron – 2nd day.

Fluazuron (doses) (mg/kg)	1°. Replicate		2°. Replicate		Average	
	Alive (%)	Dead (%)	Alive (%)	Dead (%)	Alive (%)	Dead (%)
1.25	99	1	100	0	99.5	0.5
2.5	95.55	4.45	98.5	1.5	97.1	2.9
5	87.5	12.15	92.5	7.5	89.7	10.3
10	74	26	78.17	21.83	76.37	23.63
15	75.24	24.71	72.5	27.5	73.66	26.34
20	60.45	39.54	58.33	41.67	59.5	40.5
30	47.78	52.22	46.67	53.33	47.18	52.82
40	38.5	61.5	36.82	63.18	37.62	62.38
60	28	72	23.57	76.43	26.18	73.82
80	22	78	20	80	20.95	79.05
100	18.54	81.46	15.24	84.76	16.87	83.13
125	39.68	60.32	36	64	37.68	62.32
150	48	52	46.32	53.68	47.25	52.75

to be LD_{50} = 19.544 mg/kg (12.478–22.636) and slope ± standard deviation: 1.4025 ± 0.4023 (Table 5).

4. Discussion

There are currently two main drawbacks to control ectoparasites of medical and veterinary importance and economic interest with conventional pesticides: (a) broad spectrum of action and (b) development of resistance of target species o (Nolan, 1985; Pruett, 1999; Oliveira et al., 2008, 2009). Several studies have focused on the search for an efficient strategy to control ectoparasites (Saito et al., 2005). One alternative has been de use of insect growth regulators (IGRs).

Among these, fluazuron (active ingredient of the acaricide Acatak®), a chemical of systemic action, is the first growth regulator of mites to be registered for the control of ixodid ticks (Kemp et al., 1990; Bull et al., 1996). This chemical inhibits the synthesis and/or deposition of chitin in target organisms (preventing molting to the next instar) and is an alternative available in the market for the control of populations already resistant to acaricides. Fluazuron has been used in the control of *Rhipicephalus (Boophilus) microplus* ticks (Alves-Branco et al., 2002), but few studies are available on the control of *R. sanguineus* ticks.

Thus, the development and use of compounds that are less harmful to the environment, non-target organisms, and human health, and that are more specific and do not induce resistance are of great importance. Since *R. sanguineus* has become an important urban pest, and consequently of interest for the community in general, the present study examined the susceptibility of *R. sanguineus* nymphs exposed to different concentrations of fluazuron and estimated its lethal dose (95% (LD_{95}) and 50% (LD_{50})).

The results obtained in the present study revealed that *R. sanguineus* nymphs are sensitive to fluazuron. Mortality of nymphs was higher as the doses increased (up to 100 mg/kg). The LD_{95} was estimated to be 100 mg/kg. The efficacy of fluazuron is due to the inhibition of the synthesis and/or deposition of chitin, needed for the polymerization of a new cuticle during molting, resulting in an abnormal endocuticular deposition, affecting the elasticity and resistance of the cuticle, and consequently preventing the normal formation of a new cuticle and the completion of the molting process (Mikolajczyk et al., 1994; Oberlander and Silhacek, 1998; Palli and Retnakaran, 1999; Oberlander and Smagghe, 2001). According to Palli and Retnakaran (1999), the suppression of the synthesis and/or deposition of chitin occurs through the inhibition of the biochemical processes involved, such as inhibition of the enzyme chitin-synthetase or the transport of UDP-N-acetylglucosamine by biomembranes, blockage of the binding of chitin to cuticular proteins and of conversion of glucose into fructose-6-phosphate, and inhibition of DNA synthesis, among others. Similar results have been reported by Bull et al. (1996), for *R.* (*Boophilus*) *microplus* fed on treated cattle, in which fluazuron caused the death of many immature individuals (larvae and nymphs) by preventing the formation of a new cuticle and the molting of individuals to the next instars.

In rabbits treated with the highest doses of fluazuron (125 and 150 mg/kg), the percentage of dead nymphs was lower than those treated with 100 mg/kg. Side effects, however, were observed in rabbits, such as eye irritation, redness, watery eyes. These reactions might have occurred due to the large quantities of the chemical applied for relatively small hosts (3 kg) (overdose of Acatak® to obtain the doses tested). The doses were initially applied in the dorsal region animals, but spread to the sides, allowing the contact of the chemical with the rabbit's paws. While scratching themselves, the rabbits could have transferred the compound to others areas, possibly triggering an allergic reaction during the 36 h after the treatment, and affecting the efficacy of fluazuron.

The efficacy of fluazuron, starting with doses of 15 mg/kg, was influenced by the time of engorgement of nymphs in hosts, as the effects of the chemical increased inversely to the period of engorgement. Therefore the fastest the engorgement, the higher the mortality of nymphs. As the first nymphs to detach consumed higher amounts of the chemical, the doses of fluazuron (in the first day of collection) caused a higher number of deaths.

In nymphs fed on rabbits treated with lower doses of fluazuron (1.25, 2.5, and 5 mg/kg), no side effects were observed throughout the 15 days of observation. However, in ticks exposed to higher doses of fluazuron (10 and 150 mg/kg), several morphological and behavioral alterations were observed: smaller size, elliptical shape, curved idiosoma, fragile integument, not well defined dorsal and ventral ridges, and lethargy. Similar results were reported by Da Glória (1988), in studies conducted with (*Boophilus*) *microplus* exposed to fluazuron.

According to Kemp et al. (1990), these morphological changes may be associated with the patency of salivary ducts when the synthesis of chitin is affected, causing a hemolymph imbalance, and compromising the entire individual. The absence of dorsal and ventral ridges in treated nymphs might be due to the abnormal formation of apodemes (cuticle invaginations that act as pillars for the insertion and support of muscles) (Kemp et al., 1990), as the synthesis and/or deposition of chitin needed to form the cuticle is affected by fluazuron.

The difficulty of ticks to move might be correlated to the absence of sustaining muscles, as well as the lack of balance due to the elliptical shape, and during molting, the thin skin that remain adhered to the new cuticle (Citroni et al., 1999). These alterations are results of the inhibition of the synthesis and/or deposition of chitin caused by fluazuron.

In nymphs from the control group, no morphological and/or behavioral changes were observed during the entire observation period (15 days).

The engorgement and molting period was 5–6 days and 6–10 days, respectively, for the control group, similar to the results reported by Bechara et al. (1995) in *R. sanguineus* under laboratory conditions, and in nymphs from fluazuron-treated rabbits, 7–8 days and 13 days (surviving nymphs), respectively. These differences between the control group and treatments were not statistically significant, although nymphs from treatments were smaller. The molting period became longer as the doses of fluazuron increased, indicating that the chemical affected this process, as observed by Splinder et al. (1990) and Chen (1987) in other ticks exposed to fluazuron. According to these authors, this was due to the inhibition of synthesis and/or deposition of chitin, preventing the periodic molting in exposed individuals.

The efficacy of fluazuron in *R. sanguineus* on the first day of detachment from the host were compared with the Probit analysis with the software POLO-PC (LeOra SOFTWARE, 1987). The slope ± standard deviation was 1.4025 ± 0.4023. The lethal dose 50% (DL_{50}) of fluazuron and the confidence intervals (upper and lower limits) at 95% of probability, was *g* (0.95): DL_{50} = 19.544 mg/kg (12.478–22.636). Compared with the efficacy of other pesticides, fluazuron (100 mg/kg) caused the death of 95% of *R. sanguineus* nymphs fed on treated hosts. These findings are similar

Table 5
Results of the Probit analysis based on the mortality of *R. sanguineus* nymphs exposed to the fluazuron.

LD_{50}	19.544 limits: 12.478–22.636
Regression equation	$y = 500.6617.10^{-2}$–$0.4346.10^{-2}$ log*x*
Slope	1.4025 ± 0.4023
Estimation of the confidence interval *g* (0.95)	0.207

to the results obtained with the association of 10% imidacloprid and 50% permethrin (96.52%), but higher than those obtained with the association of 10% fipronil and 12% methoprene (72.40%), both applied on the dorsal region of dogs (Otranto et al., 2005). This indicates the high potential of this compound in the control of nymphs of this species.

Therefore, our findings demonstrated the susceptibility of *R. sanguineus* nymphs to various concentrations of fluazuron, indicating its potential in the control of this tick by inhibiting the synthesis and/or deposition of chitin in the target organisms, and preventing the molting of the ectoparasite into the next stage. Since this compound is more specific, it does not induce resistance in target organisms, decreasing the risks of environmental contamination and non-target organisms, mainly vertebrates, as they are not dependent on chitinous structures.

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