Does Neem trigger the same response as Ivermectin? Dung beetle behaviour and physiology

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Potential negative effects of the synthetic veterinary pharmaceutical, Ivermectin, on non-target fauna have generated a search for less-toxic alternatives. Thus, Neem plant extract (*Azadirachta indica* A. Juss) has been used as a natural alternative to replace Ivermectin worldwide. However, little is known about the effects of this natural veterinary pharmaceutical's residues on the behaviour and physiology of adult dung beetles (Coleoptera: Scarabaeinae), which use livestock dung as a feeding and nesting resource. To understand such effects, we performed a non-choice experiment using *Dichotomius nisus* Oliver, 1798. We evaluated effects of Neem and Ivermectin residues on the ecological functions of dung burial and soil bioturbation performed by dung beetles. Additionally, we performed Soxhlet extraction of dung beetle body fat content to evaluate physiological stress in response to ingestion of Ivermectin or Neem. Our results showed that *D. nisus* do not alter their behaviour in the presence of Neem and Ivermectin residues in dung when contrasted with the control after 48 hours. However, individuals feeding on dung with Ivermectin residues for a period of twenty days had 5% more body fat content than those from control and Neem treatments. Our findings provide the first evidence that Neem can be a less toxic alternative to non-target fauna than Ivermectin.

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Abstract

Potential negative effects of the synthetic veterinary pharmaceutical, Ivermectin, on non-target fauna have generated a search for less-toxic alternatives. Thus, Neem plant extract (*Azadirachta indica* A. Juss) has been used as a natural alternative to replace Ivermectin worldwide. However, little is known about the effects of this natural veterinary pharmaceutical's residues on the behaviour and physiology of adult dung beetles (Coleoptera: Scarabaeinae), which use livestock dung as a feeding and nesting resource. To understand such effects, we performed a non-choice experiment using *Dichotomius nisus* Oliver, 1798. We evaluated effects of Neem and Ivermectin residues on the ecological functions of dung burial and soil bioturbation performed by dung beetles. Additionally, we performed Soxhlet extraction of dung beetle body fat content to evaluate physiological stress in response to ingestion of Ivermectin or Neem. Our results showed that *D. nisus* do not alter their behaviour in the presence of Neem and Ivermectin residues in dung when contrasted with the control after 48 hours. However, individuals feeding on dung with Ivermectin residues for a period of twenty days had 5% more body fat content than those from control and Neem treatments. Our findings provide the first evidence that Neem can be a less toxic alternative to non-target fauna than Ivermectin.

Key words: Pesticides Livestock, Ecological functions, Physiological stress, Fat storage

Introduction

Negative effects of pesticides used for pest control on cattle have become the subject of recent global discussion[1–4]. In the 1990's, the European Union started a program to assess the environmental risks caused by pesticides classified as veterinary pharmaceuticals or veterinary medicinals and aimed to identify ways to minimize their associated risks on non-target fauna [5,6].

This issue is of particular relevance to Brazil, which harbours more cattle than humans, with the biggest commercial livestock population in the world [7,8].

Ivermectin is a veterinary pharmaceutical used worldwide since the 1980's. It is a macrocyclic lactone that acts on signal transmission in neural and muscular cells, causing paralysis and death in nematode and arthropod parasites [9]. However, studies report resistance of target fauna [10,11] and lethal and sub-lethal effects on non-target fauna, such as dung beetles [3,5]. Neglect of the pesticide withdrawal time in cattle (i.e. the time between pesticide application and slaughter) increases the likelihood of Ivermectin residues being found in the milk and meat [12–15].

Neem plant extract (*Azadirachta indica* A. Juss, Meliaceae) has been proposed as a viable natural alternative to Ivermectin, due to its rapid degradation, low toxicity to mammals [16], and effectiveness in controlling nematodes and ticks [17,18]. The Neem's active substance, azadirachtin, a tetranortriterpenoid plant limonoid, is a potent insect feeding deterrent and, if consumed, causes growth disrupting effects [19], and reduced reproduction [20,21].

Dung beetles are a relevant group to cattle management, as they perform the important ecological function of dung burial, which helps to control flies and nematodes of veterinary importance [22]. However, this behaviour, as well as dung beetle body fat content, may be negatively affected by veterinary pharmaceuticals, such as Ivermectin and Neem. For example, Ivermectin residues can affect attraction to cattle dung and be lethal or sub-lethal for dung beetles in different developmental stages [3]. In addition, Ivermectin causes disorders in sensory and locomotor systems in dung beetles [2], reduces body size of offspring, and changes the sex ratio [23]. Dung beetles under stress accumulate fat, which is related to population reduction of some species. Thus, analyses of body fat content have been used as a successful tool to assess physiologic stress responses to environmental conditions in tropical dung beetles [24].

For this experimental study, we selected *Dichotomius nisus*, which is a large tunneler dung beetle that is abundant in both exotic and native pastures and is considered the most important species performing the ecological function of dung burial in Brazil [25]. We performed a non-choice experiment to evaluate the behavioural responses of exposure to Ivermectin and Neem residues in cattle dung. To evaluate physiological stress, we also measured changes to fat storage in response to feeding on dung with Ivermectin and Neem residues.

Methods

Dung beetle collection

We captured live dung beetles in exotic pastures in March 2014 in the municipality of Carrancas, South Minas Gerais state, Brazil, (21°28'24"S, 44°39'05"W), using 50 randomly-distributed pitfall traps placed 10 meters apart and baited with human dung (50 g). The bait was placed in a plastic cup suspended above the pitfall trap. Traps consisted of a plastic container (20 cm deep, 13 cm in diameter) buried in the ground and filled up to a quarter with a soil and litter mixture, to provide a substrate for beetles and prevent their death. The beetles were taken to the Laboratory of Invertebrate Ecology and Conservation at the Universidade Federal de Lavras (UFLA), and maintained at 29 ± 2°C, 70 ± 10% RH and a 12:12 LD photoperiod.

Dung collection

We used cattle dung produced according to three distinct regimes of parasite management adopted on a privately-owned farm: 1) with Ivermectin, 2) with Neem and 3) untreated control. We collected all dung samples without interfering with the daily routine of the farm as agreed with the landowners Francisco Altamiro Reis and Fátima Lúcia Tito Reis. Therefore, no ethics committee licence was required. We choose to use dung provided by a farm to simulate field conditions to Ivermectin and Neem residues on cattle dung. Ten Girolando cattle, a mix of *Bos* *indicus* (Gir cattle) and *Bos taurus* (Holstein cattle), weighing an average of 500 kg, were exposed to subcutaneous injections of 1% Ivermectin solution. Another ten were fed with a mixture of 1:100 kg of Neem pie (seeds and leaves of Neem plant mixed and crushed) and mineralized salt. As a control, a further ten were not exposed to any veterinary products. We collected the dung samples with Ivermectin residues after five days of Ivermectin application. The dung samples with Neem residues were collected two weeks after Neem administration started, as this was the activation period for pest control indicated on the product label. All collected dung was taken to the laboratory and maintained in sealed containers at −5º C. The dung was defrosted one day before being used in the experiments.

Experimental design

A non-choice experiment, with eighteen replicates per treatment, was carried out to evaluate the behavioural and ecological responses of *D. nisus* exposure to Neem and Ivermectin residues in dung, in regard to dung burial and soil bioturbation. For each treatment type (Neem, Ivermectin, or control), the replicate consisted of a plastic bucket filled with 6 kg of soil and five unsexed individuals of *D. nisus* provided with 500 g of bovine dung type corresponding to treatment type. After 48 hours, we weighed both the remaining dung and the soil removed due to soil excavation. To control for the effect of natural dung desiccation in dung burial analysis, we installed four replicates per treatment without beetles. We used the ratio of initial to final weight after 48 h as a humidity loss percentage parameter to correct the values associated with dung beetle activity.

Fat extraction

After the ecological functions analysis, we continued to evaluate ten replicates per treatment with the same five individuals for another twenty days, to assess the effects of feeding on dung with Neem or Ivermectin residues on body fat content. We replaced the 500 g of dung every five days. After the feeding period, the dung beetles were collected, frozen and taken to the Oilseeds Plants Laboratory (G-Oil – UFLA) where we performed a continuous fat extraction process, using Soxhlet type extractors and hexane (C_6H_{14})as the solvent. All beetles were weighed individually before fat extraction. The samples remained for at least 4 hours in the extractor apparatus at a temperature of over 69°C (hexane boiling point). Samples were dried in a drying oven at 60°C for one day and were then weighed again. To determine the body fat content, we use the following formula: (1- (initial weight– weight of the fat extracted)/ initial weight)).

Statistical analyses

We used Generalized Linear Models (GLM) with normal distribution to analyze whether there were differences in the ecological functions of dung burial and soil bioturbation among treatments. We used both dung burial and soil bioturbation as response variables, and treatment as the explanatory variable (3 levels: Neem, Ivermectin, Control). To evaluate the difference in body fat content, we performed generalized linear mixed models (GLMM) with binomial distribution. We used percentage of body fat as the response variable, treatments as the explanatory variable (3 levels), and replicate as a random effect. All analyses were performed using the *lme4* package v1 in R v3.2.0 (R Core Team 2015).

Results

We did not observe any between-treatment differences in dung burial ($F = 0.167$, d.f.=1 $p = 0.84$) or soil bioturbation ($F= 0.692$, d.f.=1 $p = 0.50$) performed by *D. nisus*. Mean dung burial and standard deviation (SD) were 190.53 ± 78.50 g for the control, 184.29 ± 89.43 g for the Ivermectin treatment, and 175.71 ± 63.26 g for the Neem treatment. Mean soil bioturbation and

SD were 295.50 ± 77.55 g for the control, 327.22 ± 85.64 g for Ivermectin, and 315.31 ± 73.21 g for Neem (Figure 1).

We found a significant difference in the body fat content of *D. nisus* dung beetles between treatments (χ^2=16.712, d.f=2, p < 0.001; Figure 2). The mean body fat content (as a percentage of body weight) and SD were 55.34 ± 13.49% for control, 60.25 ± 13.25% for Ivermectin, and 56.69 ± 8.6% for Neem. *Dichotomius nisus* individuals exposed to Ivermectin residuals had on average 5% more body fat content than individuals feeding on dung with Neem or residue-free control.

Discussion

Our results show that Ivermectin and Neem residues did not affect the behaviour of dung burial and soil bioturbation in *D. nisus*. However, dung beetles feeding on dung contaminated with Ivermectin showed a 5% increase in body fat content. Our findings provide the first evidence that Neem can be a less toxic alternative to non-target fauna than Ivermectin. In addition, they add to the list of physiological disorders shown by dung beetles after consuming dung with Ivermectin residues.

It is assumed that adult dung beetles are less susceptible to Ivermectin residues than developmental stages [26]. Still, *Dichotomius bos* (Blanchard, 1843) showed reduced dung burial behaviour in a non-choice test when exposed to dung with Ivermectin residues [27]. Damage caused by Ivermectin goes beyond ingestion, since mere contact with the drug can cause a reduction in mobility in mature insects [28]. Ivermectin inhibits olfactory and locomotion abilities in adult dung beetle *Scarabaeus cicatricosus* (Lucas, 1846) [2] and decreases muscle mass in *Euoniticellus intermedius* (Reiche, 1850) [23]. *Dichotomius nisus* olfactory and locomotory systems might not be affected by residues of Ivermectin and Neem present in the dung pads.

Further studies evaluating the olfactory system of *D. nisus* using an electroantennography and muscle mass assessment may provide more information about the behavioural responses of this species.

The 5% increase in body fat content observed in *D. nisus* exposed to Ivermectin may be related to the detoxification mechanisms conducted by the insect fat body. Insects resistant to pesticides exhibit high activity of detoxification enzymes that result in toxin immobilization in the fat body [29,30]. Indeed, Ivermectin is a highly lipophilic compound [31]. Detoxification mechanisms may be a response strategy that promotes survival of dung beetles feeding on contaminated dung. However, in many insects fat accumulation is associated with aging and reproduction reduction, and may even lead to population decline [24,32]. Further studies pertaining to physiology of the *D. nisus* fat body and activity of detoxification enzymes may help to better understand the responses to Ivermectin.

Conclusion

Neem may offer an alternative pesticide treatment for farmers due to its lack of negative effects on non target fauna. Identification of such alternatives has become particularly important since the Brazilian government banned macrocyclic lactones with concentrations over 1% in 2014, because high levels of Ivermectin residues in cattle meat were above the safety limit for human consumption. Nonetheless, further field tests evaluating the responses of dung beetle communities to the use of Neem would provide a better assessment of the safety of its use. Overall, our work shows that tests at multiple levels encompassing ecology, behaviour and physiology of non-target fauna such as dung beetles may offer a broader assessment of the risks associated with the use of synthetic or natural veterinary pharmaceuticals.

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Figure 1(on next page)

Ecological functions performed by *D. nisus* exposed to distinct veterinary pharmaceutical (Control, Neem and Ivermectin).

(A) Amount of dung burial performed by *D. nisus* individuals exposed to distinct veterinary pharmaceutical (Control, Neem and Ivermectin). Same letters above bars indicate no significant difference (p = 0.84). (B) Amount of soil bioturbation performed by *D. nisus* individuals exposed to distinct veterinary pharmaceutical (Control, Neem and Ivermectin). Same letters above bars indicate no significant difference (p = 0.50).

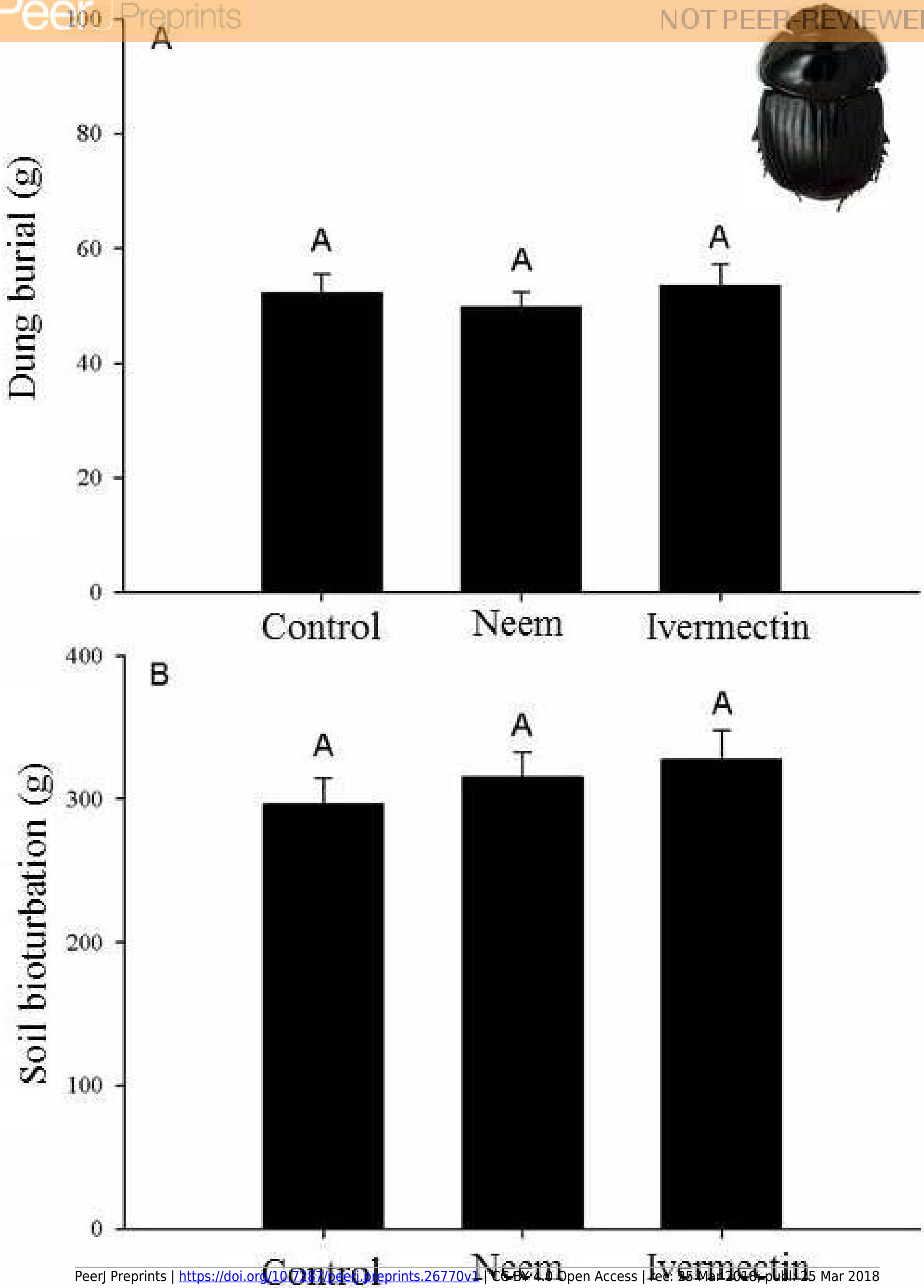

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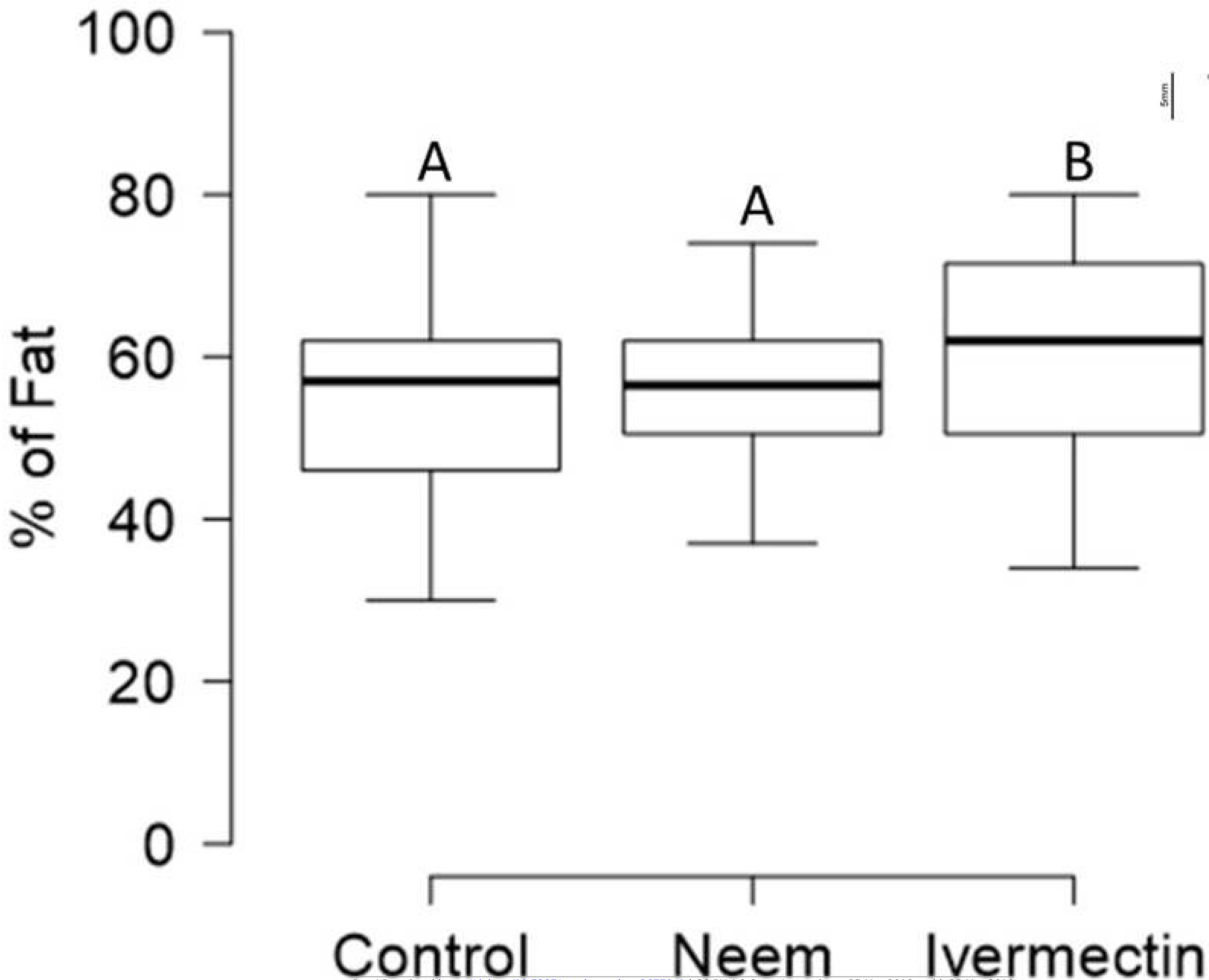
Figure 2 (on next page)

Increase of body fat content of *D. nisus* individuals exposed to Ivermectin compared to Neem and Control.

Percentage body fat content of *D. nisus* individuals exposed to distinct veterinary pharmaceutical (Control, Neem and Ivermectin). Distinct letters above bars indicate a significant difference ($p < 0.001$).


100
80
60
40
20
0
% of Fat
A
A
B
Control
Neem
Ivermectin