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Pesticide exposure on sloths (*Bradypus variegatus* and *Choloepus hoffmanni*) in an agricultural landscape of Northeastern Costa Rica

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Abstract

Between 2005 and 2008, wild *Bradypus variegatus* and *Choloepus hoffmanni* inhabiting an agricultural landscape and captive animals from a rescue center in Northeastern Costa Rica were studied to assess exposure to pesticides. A total of 54 animals were sampled: 42 wild sloths captured at an agricultural landscape and 12 captive animals from a rescue center. Pesticides' active ingredients were determined in three sample matrices: hair, aqueous mixture (paws' wash) and cotton gauze (mouth clean) based on multi-residue gas chromatography methods. Recoveries tests ranged from 73 to 146% and relative standard deviations were less than 20% throughout all the recovery tests. Active ingredients detected in sloths samples were ametryn, chlorothalonil, chlorpyrifos, diazinon, difenoconazole, ethoprophos and thiabendazole. These active ingredients were used in intensive agricultural production for bananas, pineapples and other crops. Blood plasma cholinesterase activity (PChE) was determined by the Ellman method modified for micro plates. Enzyme activity determination was normalized to protein content in the samples according to Bradford method. Wild sloth PChE activity was similar for both species while sloths in captivity showed differences between species. Enzyme activity was significantly lower for two-toed sloths.This study showed that sloths were exposed to pesticides that caused acute and chronic effect in mammals and can also be a threat to other wildlife species. There is a need to better understand the potential effects of exposure to pesticides in sloths and other wild mammal populations, especially those threatened or endangered. More studies in this field must be carried out on the wildlife fauna inhabiting the agricultural landscape and its surroundings.

Key words

Blood, Cholinesterase, Exposure, Pesticides, Sloths

Introduction

Costa Rican economy is based on eco-tourism and agriculture. It is among the richest countries of the world in terms of biodiversity, with 25% of its territory protected, including national parks, wildlife refuges, protected areas, wetlands, biological reserves, forest reserves and diverse natural habits. In contrast, Costa Rica reported the highest imports of pesticides in the Mesoamerican region (Bravo *et al*., 2011); most of them known to have acute and chronic effects on human health and aquatic ecosystems (Castillo *et al.* 1997; Castillo *et al.* 2000; de la Cruz and Castillo 2002; de la Cruz *et al*. 2004; Castillo *et al.* 2006; Wesseling *et al*. 2006; Bravo *et al*. 2011). In 2006, it was calculated that 19.33 kg of active ingredient/ha was used in agriculture; which represents an approximate rate of 2.4 kg of active ingredients (ai) per inhabitant. Pesticide use in Costa Rica has increased from 2,648 tons of active ingredients in 1977 to 11,636 tons in 2006; which represents more than a fourfold increase over three decades (Ramirez *et al*. 2009; Bravo *et al*. 2011).

The trend for increased import and use of pesticides is due to intensive cultivation and an increase in crop areas. The area under pineapple cultivation alone has increased by 500% from 2000 to 2010. Recently, Costa Rica has become the world's largest exporter of pineapples.

Bravo *et al*. (2011) reported that historically fungicides (46%) have been the most common import, followed by herbicides (29%), insecticides-nematicides(16%) and fumigants (8%). Fungicides are used mainly on bananas. Herbicides and nematicides are used on bananas and pineapple (Bravo, 2007; Polidoro *et al.,* 2008). The cultivation of these crops involves frequent application of various pesticides in large quantities, exacerbated by the fact that production of bananas (49.29 kg a.i. ha^{-1} yr^{-1}) and pineapples (24.55 kg a.i ha^{-1} yr^{-1}) are the nation's largest and most important agricultural activities.

Almost of all the chemicals applied for banana and pineapple production have acute, sub- chronic or chronic adverse effects on human and wildlife health (Wesseling *et al*., 2006; Bravo, 2007). The widespread use and environmental contamination from these pollutants suggest a high potential for exposure and bioaccumulation in wildlife inhabiting both cultivated and natural habitats surrounding these agricultural areas. However, in tropical countries, most research programs are limited to measured residues of pesticides in the environmental samples. Few studies have been conducted to determine wildlife exposure: most performed on aquatic organisms (Castillo *et al*., 1997; Castillo *et al.,* 2000; de la Cruz and Castillo, 2002; de la Cruz *et al*., 2004; Castillo *et al.,* 2006). Virtually no studies have evaluated the effects of exposure to pesticides such as organophosphate insecticide on terrestrial wildlife at the regional level (Cobos *et al*., 2006). There is a need for research using non-sacrifice methods to assess wildlife exposure and to evaluate chronic effects. This will increase our knowledge of the real impacts of agricultural practices on terrestrial wildlife. In this context, sloths (*Bradypus variegatus* and *Choloepus hoffmanni*) are suitable species for exposure studies. They are arboreal mammals living in and feeding on leaves from a large variety of trees used as living fences in agricultural areas or as shade for cocoa plantations (Vaughan *et al*., 2007). These mammals use trees for shelter, for support during rest time and to move from one tree to another everyday and a half or so. Compared to other mammals, sloths have a relative small home range (<5 ha) and a high population density (6-8.5 animal per ha); characteristics that make them a suitable species for contamination monitoring studies (Vaughan *et al*., 2007). Sloths and their food sources are potentially exposed to pesticides applied to agricultural crops located near their home range, especially those applied by aerial fumigation. The aim of this study was to evaluate pesticide exposure and blood plasma cholinesterase activity in a wild population of three-toed (*B. variegatus)* and two-toed sloths C*. hoffmanni*) inhabiting an agricultural landscape and captive animals on a nearby sloth rescue center. The results of this study would help in proper planning and implementation of conservation strategies for vulnerable species living in agricultural areas or their surroundings.

Materials and Methods

Study area: Field research was carried out during 2005-2006 and in 2008 in an agricultural landscape and a sloth rescue center; both areas are located in the Northeastern of Costa Rica. The agricultural landscape is composed of organic cocoa farming, intensive banana and pineapple cultivation, pastures with isolated trees, and riparian forests in the village of Pueblo Nuevo, Guácimo (10°20"N, 83º20'W). The rescue center, with an extension of 96 ha, is a protected area adjacent to large extensions of intensive banana plantations. Animals from this center were used as reference group.

Capture and handling of animals: A total of 54 animals were studied, twelve captive animals from the rescue center and forty-two wild animals from the agricultural landscape. Animals from the rescue center (six three-toed and six two-toed) were used as the reference group. The individuals included in the study were selected based on time spent in the center (≥ 2 yrs) and having had good health during the last two years. Wild animals (35 two-toed and 7 three-toed) from the agricultural landscape were captured and used as the exposure group. Their captures were centered in and around the FINMAC organic cocoa farm and they were located by intensive search. Once a sloth was located usually on a tree its GPS coordinates were collected (Magellan Sport Track GPS) and then the animal was hand-caught. All animals were individually transported from their capture site in a burlap sack to a small work station laboratory established at FINMAC. All animals were sedated with atipamezole (0.1 mg kg^{-1}) to minimize stress during manipulation. Sloths were weighed, sexed, measured and tagged. A pit tag was inserted sub-dermally between the shoulder blades (Trovan, Electronic Identification System, ID-100US). Only adult animals were sampled. Once awake each sloth was put in the cage it usually lived in (reference group) or released on to the same tree where it was captured, in the case of the wild group.

Sex determination: Sex determination in two-toed sloth was established by checking the presence of intra-abdominal penis and testes. The sex of adult three-toed sloths was determined by the presence of the dorsal, central, orange and black spot on males. Developmental stage status of sampled animals was estimated based on their body weight (Reid, 1997; Carrillo *et al*., 1999; Lara-Ruiz and Chiarello, 2005). For the two-toed sloth, an individual was considered an adult when it weighed over 2.7 kg and for the three-toed sloth when it weighed more than 2.3 kg.

Sample collection: Hair was cut ~0.5 cm from the base of skin; composed samples (1 to 2 g) were taken from dorsal region (neck and legs) and ventral region (throat and arms) of each sloth and

were used for pesticides' analysis. Front paws were washed to the elbows with 75ml of Milli-Q water/isopropanol (1:1) for about 1min; the aqueous mixture was used for pesticide analysis. After sampling, paws were washed with tap water to remove residual solvent. Tongue, jaw and cheek were cleaned with sterile cotton gauze (8.5x5 cm, Klinion) and individual gauze was placed and stored in a separate sealed glass tube.

Blood samples were taken from saphenous or jugular vein with sterile needles and 1ml syringes previously rinsed with heparin (Baxter, USA). Blood samples were transferred to 5ml ependorff containers, previously heparinized and were centrifuged for 10 min at 3 000 rpm to obtain plasma. Plasma was used to determine cholinesterase activity.

All samples were stored and transported to the laboratories in dry ice. Paws-wash aqueous mixture was extracted within 48 hrs of collection. Hair, cotton gauze and plasma samples were frozen at -20 °C and analyzed as soon as possible.

Analysis of pesticide residue

Extraction and concentration: Samples were spiked with external standard ethion. Active ingredient recovery tests were performed for three matrices (hair, aqueous mixture and cotton gauze).

Three extraction methods were used: a) hair was extracted by ultra-sonic bath (acetone/cyclohexane 1:9, Branson 5210,USA); b) front paws by an aqueous mixture of a liquid-liquid method (dichloromethane) and c) mouth swabs extracted with microwave (acetone/hexane 1:1, CEM, MARS-X™, Canada). Each extract was evaporated to about 2 ml with a rotary evaporator (Büchi REIII, Switzerland), a water bath at 30°C (Büchi461), and recirculating bath (HAAKEG 000-5744) with water at <10°C. All extracts were concentrated with a stream of nitrogen with a purity of 99.99% and re-dissolved in acetone/cyclohexane 1:9. Hair and paws aqueous mixture wash extracts were filtered (0.2 µm GHP Acrodisc Pall, USA) with a 5 ml Hamilton syringe (#1005) before being decanted into injection vials. Extracts were stored at 4°C until analyzed by gas chromatography (GC). Sample blanks were strictly performed to detect any external source of contamination.

Multi-residues pesticides analysis: Extracts were analyzed by a multi-residue method with an Agilent 7890 Gas Chromatograph with 5975 C Mass Spectrometer (Agilent Technologies) with 1-2 ul splitless injection volume and high resolution capillary columns, HP5MS (30m x 0.250mm x 0.25mm) and DB-35ms (30m x 0.250mm x 0.25um) (T&W Scientific, USA)". All tests were performed at the Laboratorio de Análisis de Residuos de Plaguicidas (LAREP), Instituto Regional de Estudios en Sustancias Tóxicas (IRET), Universidad Nacional, Heredia, Costa Rica.

Blood plasma enzyme activity: Blood plasma obtained by centrifugation was used to determine cholinesterase activity (PChE). Enzyme activity was determine following Ellman method (Ellman *et al.,* 1961), adapted to micro-plates (Thermo Multi Skan Ascent, Thermo Electron Corporation). Absorbance was measured at 414nm and expressed as n mol min^{-1} mg^{-1} protein. Enzyme activity determination was normalized to protein content in the samples, according to the Bradford method (Bradford, 1976). All reagents were purchased from Sigma Chemical Company (USA) and Sigma-Aldrich Chemie Gmbh (Germany). These tests were conducted at the Laboratorio de Estudios Ecotoxicológicos (Ecotox), Instituto Regional de Estudios en Sustancias Tóxicas (IRET), Universidad Nacional, Heredia, Costa Rica.

Data analysis: The statistical analysis was conducted with R Development Core Team, Version 3.0.1, 2013.

Results and Discussion

Most of the wild sloths were caught in riparian forest; live fence rows at the borders of cacao farm and pasture land. The two-toed sloth was the most captured species; 35 animals from a total of 42 sloths, 17 males and 18 females. Only 7 three-toed sloths were captured, 3 female and 4 male. The higher frequency of sloth capture in riparian forest and living fences might be related to high diversity and density of tree species that serve as food in those sites and to the shelter provided for these animals by the cacao agro forest (Vaughan *et al*., 2007).

Two-toed species are larger and heavier than three-toed species (Reid, 1997; Carrillo *et al*., 1999). Captive and wild three-toed sloth average weight was 4.0±0.6 kg (means ±SD). Two-toed sloths in captivity (9.4±1.9 kg) were heavier than wild ones (5.6±0.9 kg). The difference in weight among the captive and wild sloths is related to their diet; the wild two-toed sloths feed on leaves and fruit, and sometimes lick and gnaw on tree trunks while in captivity they are fed with leaves, fruits and dog food as a source of proteins (Bermúdez, 2004; Ávila, 2007; Vaughan *et al*., 2007).

Pet foods contain proteins, carbohydrates, fat and trace elements, and may also contain toxic metals. Duran *et al.* (2010) reported that some metals like copper, iron and manganese are listed on most pet food labels, but there are other toxic metals like silver, cadmium, cobalt, chromium, lead, phosphorus and aluminum that do not need to be listed on the label. Toxic metals may cause damage to the animal's reproductive system, brain, heart, kidney, lungs, liver and nervous system.

Fig. 1 shows PChE activity in wild and captive sloths of both species, the triangle represents two-toed sloth and the dot three-toed sloth enzyme activity, and error bars indicate 95% confidence intervals, R Development Core Team (2013). In wild sloths, PChE activity was similar for both species while sloths in

captivity showed significant difference in PChE activity between species. Overall, captive three-toed sloths PChE activity was a factor of 0.58 lower than for wild sloths ($p>0.05$) (Table 1). Differences in enzyme activity between wild and captive sloths may be due to veterinarian treatments. Ivermectin is a broad-spectrum antiparasitic drug extensively used in veterinarian medicine that kills the parasites by interfering with their nervous system and muscular function (Campbell and Benz, 1984). Riquelme and Pérez, (1994) reported dog intoxications from ivermectin. The insecticide ivermectin is a commonly used parasitic infestation treatment for captive sloths.

Recovery tests for all the compounds analyzed varied among different matrices: hair (73-146%); aqueous mixture of paws (82-96%) and gauze samples of mouth (84-132%). The external standard recoveries ranged from 80 to 140%. The relative standard deviation was less than 20% throughout all recovery tests. In general, recoveries tests were between the range established by SANCO (2009). Out of 162 samples analyzed, active ingredients were detected in 42 samples. Pesticide residues' concentrations are shown in Table 2. Seven different active ingredients used in bananas, pineapples and other crops were detected in sloths samples. All seven active ingredients were detected in hair samples of wild sloths with the highest concentrations. On the other hand, diazinon, ethoprophos and difenoconazole were detected in paw samples and chlorothalonil in mouth samples.

The herbicide ametryn was detected in two sloths: one wild and one captive animal. This active ingredient is used in the production of bananas, pineapples and others crops. Although the rescue center is a protected area, adjacent to large areas of intensive banana plantations, the pesticide could be spread through air (Gillian *et al*., 2007; Gouin *et al*., 2008). The highest concentration of pesticides detected in wild sloths corresponded to difenoconazole (2.87µg g^{-1}) followed by diazinon (1.54µg g^{-1}) and thiabendazole (0.34µg g^{-1}) (Table 2). These active ingredients have also been reported in water samples, fish tissues and air samples collected close to study area (Castillo *et al*., 2000; Castillo *et al*., 2006; Gillian *et al*., 2007; Gouin *et al*., 2008).

Table 1 : Result of a linear model for PChE analyses for wild and captive three-toed and two-toed sloths, Northeastern Costa Rica during 2005-2006 and 2008

Coefficients	Estimate	SE	t value	Pr (>|t|)
Intercept	1.12	0.18	6.00	2.3e-07***
Captive B. v	-0.58	0.28	-2.04	0.05*
Wild Ch. h	-0.09	0.21	0.45	0.66
Site: species	0.89	0.37	2.41	0.02*

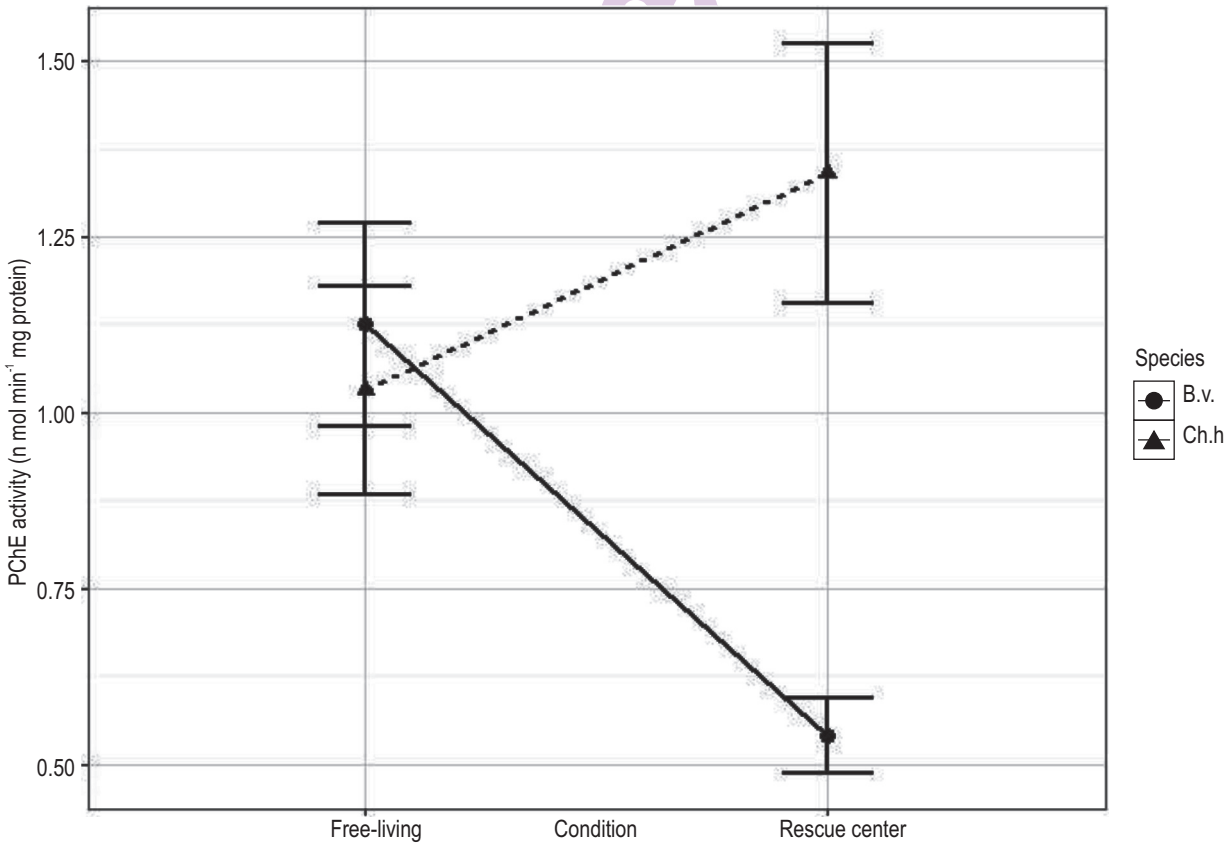

Fig.1 : Mean PChE activity (n mol min^{-1} mg^{-1} protein) in wild and captive two-toed (Ch. h) and three-toed (B. v) sloths, Northeastern Costa Rica in 2005-2006 and 2008

Table 2 : Active ingredients detected in the three sample matrices of wild and captive sloths, North eastern Costa Rica during 2005-2006 and 2008

Matrix	Pesticide	dl	ql	Wild sloths		Captive sloths	
				Three-toed	Two-toed	Three-toed	Two-toed
				Maximum concentration of pesticide detected			
Paws aqueous	diazinon	0.005	0.03	traces	0.10	nd	nd
mixture	difenoconazole	0.3	1.0	0.90	0.71	nd	nd
(ug ml^{-1})	ethoprophos	0.02	0.08	traces	nd	nd	nd
Mouth gauze	chlorothalonil	0.03	0.09	nd	traces	nd	nd
	ametryn	nd	nd	0.02	nd	nd	0.06
Hair (ug g^{-1})	chlorothalonil	0.03	0.09	nd	0.07	nd	nd
	chlorpyrifos	0.01	0.03	0.01	0.02	nd	nd
	diazinon	0.005	0.03	1.03	1.54	nd	nd
	difenoconazole	0.30	1.0	2.87	1.03	nd	nd
	ethoprophos	0.02	0.08	0.05	0.07	nd	nd
	thiabendazole	nd	nd	nd	0.34	nd	nd

Out of 162 samples analyzed 42 of them were detected with active ingredients; dl: detection limit; ql: quantification limit; nd: not detected (below the limit of detection); Traces: between limit of quantification and detection limit

The organophosphate pesticides (OP) chlorpyrifos, ethoprophos and diazinon were among 22 active ingredients imported to Costa Rica during the period 1977 to 2006 (Ramírez *et al.,* 2009). Chlorpyrifos is used throughout the year in banana plantations in impregnated plastic bags to protect the fruit from insect pests. Although, the maximum concentration detected was below the acute toxicity reported for rats, it is important to bear in mind the risk of chronic exposure to this compound and the fact that it can be transported via air over long distances (Gillian *et al*., 2007).

The insecticide/nematicide ethoprophos is used mainly in banana production and applied to the soil 1 to 2 times a year and with an average of 9 kg a.i. ha^{-1} application. The insecticide diazinon is mostly applied in pineapple cultivation, at a ratio of 0.18 kg a.i. ha^{-1}application, 6-13 times a year. This organophosphate was detected in the sloths samples in high concentration. Samples that showed positive test to this pesticide correspond to animals caught at edges of the cocoa farm and in the riparian forest, close to banana and pineapple plantations. Even if sloths' hair functions as a protective barrier that prevents contact of toxic substance with the sloths' skin (Smith *et al*., 2007), high application throughout the year might cause chronic effects. On the other hand, substances such as diazinon could enter the body by inhalation or by intake of contaminated food and could accumulate and detected in the hair (Tutudaki *et al*., 2003).

Our results did not reveal a clear effect of these active ingredients on PChE activity, but **c**hemical compounds detected in the samples indicated that sloths are exposed to pesticides. Although the effects are still unclear, these results are an important contribution to the proper planning and implementation of conservation strategies for species living in or near agricultural areas, since chronic exposure to low concentrations of organophosphate pesticides can cause adverse effect in the development of animals, such as reduction in bone formation and reduced sperm quality (Tutudaki *et al*., 2003).

There is a need to better understand the potential effects of exposure to pesticides in sloths and other wild mammal populations, especially those threatened or endangered. More studies in this field must be carried out on the wildlife fauna inhabiting the agricultural landscape and its surroundings.

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