RESEARCH ARTICLE

Acaricide resistance in the spinose ear tick, *Otobius megnini* (Acari: Argasidae) infesting racehorses in Sri Lanka

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Abstract: The spinose ear tick *Otobius megnini* is a causative agent of otoacariasis in horses and jockeys in the Nuwara Eliya racecourses. The present study aimed at investigating the status of acaricide resistance and underlying mechanisms in this tick species under laboratory conditions. Live ticks collected from the ear canal of horses were used to establish a laboratory colony. Larval bioassays were carried out with an organophosphate (malathion), pyrethroids (flumethrin and permethrin), and an organochlorine (DDT) using Larval Packet Tests (LPT). The LC₅₀ (24 h) values for malathion, flumethrin, permethrin and DDT were recorded as 0.11%, 0.0003%, 0.008% and 0.124%, respectively, and the respective values for LC₉₀ were 0.722%, 0.004%, 0.300% and 3.049%. Biochemical assays revealed that the mean specific activity levels of esterase, glutathione S-transferases (GSTs), and monooxygenase amounts were $0.064 \pm 0.008 \mu\text{mol min}^{-1} \text{mg}^{-1}$, $0.104 \pm 0.050 \mu\text{mol min}^{-1} \text{mg}^{-1}$, and 0.068 ± 0.020 equivalent units of cytochrome P450, respectively. The percentage remaining activity of the propoxur-inhibited Acetylcholinesterase (AChE) was $33.88 \pm 13.60\%$. Our data indicated that the *O. megnini* population of Nuwara Eliya, Sri Lanka was most susceptible to flumethrin and showed resistance to DDT. Development of resistance to malathion through increased activity of esterase and insensitive AChEs was evident. Since the activities of monooxygenases and GSTs were not enhanced, permethrin and DDT resistance may have developed through target site insensitivity.

Keywords: Biochemical assays, DDT, ectoparasiticide, flumethrin, malathion, permethrin.

INTRODUCTION

The spinose ear tick *Otobius megnini* (Acari: Argasidae) is a nidicolous soft tick whose immature stages (larvae and nymphs) parasitize domestic animals and occasionally humans causing otoacariasis. *Otobius megnini* has four developmental stages in its life cycle: egg, larva, nymph and adult (male/female) and completes its life cycle on a single host with larvae and nymphs parasitizing for several days to months (Nava *et al.*, 2009; Diyes & Rajakaruna, 2017). The unfed larvae are highly active, and it is the only life stage that participates in a host-seeking activity. Following attachment, larvae feed for 8–13 days before moulting to nymphs and these nymphs continue feeding for another 17–37 days. After that, fully engorged nymphs detach and moult to non-feeding adults on the ground. After successful mating, females lay eggs in a refuge. The life cycle of *O. megnini* stretches over 51 to 901 days (Diyes & Rajakaruna, 2017). The life cycle duration strongly depends on the prevailing temperature and relative humidity. The presence of the tick inside the ear canal causes severe pain and irritation and can lead to toxic conditions, eardrum perforation, muscle spasms, and otitis (Madigan *et al.*, 1995; Estrada-Peña & Jongejans, 1999). Additionally, disease-causing agents such as *Coxiella burnetii* (Desjardins *et al.*, 2018),

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Borrelia burgdorferi (Fivaz et al., 1991) and *Babesia* (Diyes et al., 2018) have been detected in blood meal analysis of *O. megnini*.

In Sri Lanka, this tick species appears to have a limited distribution. To date, there are no records on *O. megnini* infestations in any domesticated animals except the horses and jockeys in the Nuwara Eliya racecourses. However, the dynamic nature of the life cycle with high population stability has enabled *O. megnini* to become an invasive tick species in the Nuwara Eliya racecourse. Controlling *O. megnini* using acaricides is not very common due to its presence in the ear canal of the horse. Current control practices used to manage *O. megnini* infestations include regular cleaning of the ear canals of the horses and frequent renewal of the sawdust inside the stables. However, effective complete control of *O. megnini* infestations is hard. Consequently, alternative control strategies, including vaccines and, synthetic and botanical acaricides are essential.

The application of synthetic acaricides such as carbamates, organophosphates, synthetic pyrethroids, formamidines, macrocyclic lactones and pyrazoles have played a pivotal role in controlling ticks worldwide (Lovis et al., 2011). However, the long-term use of these compounds has resulted in the development of acaricide resistance, thereby reducing the effectiveness (Graf et al., 2004). Therefore, constant monitoring of the level of susceptibility of pests to these chemicals is required to control the spread of any acquired resistance (Graf et al., 2004). However, to the best of our knowledge, there were no previously published reports on the susceptibility of *O. megnini*. Therefore, it is important to collect baseline data on the level of acaricide susceptibility of *O. megnini*.

In both insects and acarines, elevated enzymatic activity and target site insensitivity are the two major metabolic resistance mechanisms to insecticides (Karunaratne et al., 2018). The high enzymatic activity involves variations in insecticide metabolizing enzymes, i.e., esterases, glutathione-S-transferases (GSTs) and monooxygenases, which metabolize the acaricide before reaching the target site. However, target site insensitivity is the inability of mutated target sites to recognize the acaricide. Recently, Bandara and Karunaratne (2017) reported that DDT and malathion resistance in the cattle tick *Rhipicephalus microplus* from Sri Lanka was caused by both elevated esterase activity and insensitive target sites. Here, we determined the status of acaricide susceptibility of *O. megnini* larvae against four commonly used acaricides: malathion, flumethrin, permethrin, and DDT. The activity of acaricide metabolizing enzymes,

and the insensitivity of the organophosphate and carbamate target site to acetylcholinesterase were also investigated to understand the potential mechanism of resistance.

METHODOLOGY

Tick collection and rearing

Live specimens of *O. megnini* were collected from 14 stabled thoroughbred horses in a racecourse in Nuwara Eliya (GPS: 6.9628 N 80.7692 E). Immediately after collecting the ticks, they were transferred to a laboratory facility where they were weighed and separated according to life stage. Fully engorged nymphs weighing over 60 mg were used to establish the laboratory tick colony. Nymphs were not fed after the collection because female ticks do not require an additional blood-meal to produce eggs (Diyes & Rajakaruna, 2017). Tick rearing chambers were prepared according to Sonenshine (1993) and were maintained at $28 \pm 2^\circ \text{C}$ under 96% relative humidity (RH) until nymphs molted into adults. New horse-blood-fed nymphs were introduced biweekly to the tick-rearing chambers to maintain a high genetic diversity in the colony.

In total, 100 engorged females, weighing from 60 to 120 mg, were used in this study. Each female was transferred into a separate slot of a 30-well plastic grid plate. Multiple plates were used to house all the females. After that, males were introduced to each female. Plates containing mating pairs were placed in a glass chamber kept in darkness and maintained at $28 \pm 2^\circ \text{C}$ under 96% RH until oviposition. The eggs obtained from multiple females were mixed, subdivided into groups of 150 and then transferred into Eppendorf® tubes with perforated caps. Eggs, and later larvae, were maintained under the same conditions as those of females. Tubes were monitored daily for any fungal or mite infestations and were changed whenever contamination was observed. The tick colonies were maintained between September 2014 and March 2015. Larvae at the age of 14 to 21 days were used for all the bioassays.

Acaricide bioassays

A total of 8,408 larvae of *O. megnini* were subjected to acaricide bioassays. Acaricide bioassays were conducted using four technical grade acaricides, i.e., organochlorine (DDT), organophosphate (malathion) and two pyrethroids (permethrin and flumethrin) (Sigma Chemicals, UK) using the experimental protocol described by Larval Packet Test (LPT) (FAO, 2004). As there were no

recommended dosages by FAO for soft ticks, the dosage recommended for hard ticks was used (Bandara & Karunaratne, 2017). Acaricide solutions with different concentrations were prepared using olive oil:acetone (1:2; analytical grade) as the solvent. All concentrations used ranged from 10^{-6} to 10%. These concentrations (v/v for malathion and w/v for DDT, flumethrin and permethrin) were determined using published dose-response data for ticks (Bandara & Karunaratne, 2017; Miller *et al.*, 1999) and by conducting preliminary studies. An aliquot of 0.7 mL from each dilution was evenly distributed on a Whatman No.1 filter paper (7.5 cm $\times$ 9.0 cm) (Miller *et al.*, 1999) and the control received the solvent alone. Acaricide impregnated papers were wrapped individually in aluminium foil and stored at 4° C until the larvae reached 14–21 days old (FAO, 2004). They were used within a month after preparation, and each paper was used in a maximum of two trials/replicates (WHO, 2016) as follows. An acaricide impregnated paper was folded along the middle line with the impregnated surface inside and two bulldog clips were fixed on two opening sides, making a packet (FAO, 2004). Tick larvae (150 larvae) were inserted into the packet with the aid of a fine brush through the remaining open side and a third bulldog clip was used to seal the opening end. The packets were kept in separate humidity chambers with a temperature of 27–28° C, and 85–95% RH. Mortality was recorded after 24 h after exposure to the treatment. Larvae that did not show a response to a needle touch were considered dead. A hand lens was used to confirm the response, whenever necessary. According to FAO (2004), if the mortalities of the controls were less than 5% of the mortalities of the treated, sample mortalities can be directly used for analyses without correction. However, if control mortalities were between 5–10%, the mortalities of the treated sample must be adjusted using Abbott's formula (FAO, 2004). Since there were no mortalities observed in the controls, the mortalities of the treated samples were processed normally. All the acaricide bioassays were performed in triplicate.

Statistical analysis

Mortality values were plotted against acaricide concentrations to obtain log concentration-probit regression lines using Sigma Plot software (version 10.0). For each tested acaricide, lethal concentrations that kill 50% (LC_{50}) and 90% (LC_{90}) of the packet population, confidence intervals (CI) of 95% and the slope of the regression line (through regression analysis) were determined. A Chi-square test was used to evaluate the goodness of fit of the linear regression

to log concentration-probit transformed data. Resistance discriminating dosages were not available for *O. megnini*. Therefore, we adopted a method similar to Bandara and Karunaratne (2017) to evaluate the current resistance/susceptibility status of *O. megnini* larvae. Here, the discriminating dosages available for *Rhipicephalus microplus* (Acari, Ixodidae) (FAO, 2004) were used to assess the susceptibility/resistance status of the *O. megnini* population: susceptible (98–100% mortality), possible resistant (90–97.9% mortality) and resistant (< 90% of mortality) (WHO, 2016). Discriminating dosages given for anopheline mosquitoes (WHO, 2016) were also used to evaluate the susceptibility/resistance status due to the absence of discriminating dosages for *O. megnini* or other ticks.

Biochemical assays

All the biochemical assays were carried out according to the procedures recommended by WHO (1998a) with slight modifications. Randomly selected *O. megnini* larvae were subjected to total protein, esterase, glutathione S-transferase (GST), monooxygenase and acetylcholinesterase assays in two replicates. Larvae were homogenized in groups of 25 individuals in 150 μ L ice-cold distilled water. An aliquot of 50 μ L was taken for the two replicates of AChE assay and the rest was centrifuged at 13,000 rpm for 2 min. The supernatant was used for esterase, GST, monooxygenase and protein assays (WHO, 1998a). This whole experiment was repeated 21 times. The average values from 21 homogenates (total number of larvae = 25 $\times$ 21) were used for the analysis. The results of the following biochemical assays are expressed as mean $\pm$ SD.

Acetylcholinesterase assay

Two 25 μ L aliquots of crude homogenate were added to two consecutive microtiter plate wells. The membrane-bound AChE in the mosquito homogenate was solubilized by adding 145 μ L of 1 % (v/v) Triton X-100 in 0.1 M phosphate buffer (pH 7.8). Ten microliters of the substrate solution dithobis-2 nitrobenzoic acid (DTNB) in phosphate buffer (pH 7.0) was added to each aliquot. To one replicate, 25 μ L of acetylthiocholine iodide (ASChI) and propoxur solution (10 mL of 0.01 M ASChI 20 μ L of 0.1 M propoxur in acetone) were added. To the other replicate, 25 μ L of 0.01 M ASChI alone was added. The plate was read at 405 nm for 5 min. Results were expressed as the percentage remaining activity in the inhibited fraction and was compared with the control (uninhibited) activity (WHO, 1998b).

Glutathione-S-transferase assay

To prepare the working solution, 0.5 mL of 63 mM I-chloro-2,4-dinitro benzene (CDNB) in methanol and, 9.5 mL of 10 mM reduced glutathione (GSH) in 100 mM phosphate buffer (pH 6.5) were mixed. Ten microliters of each insect homogenate was mixed with 200 μ L of freshly prepared GSH/CDNB working solution in a microtiter plate well. The enzyme reaction rate was measured at 340 nm at 12 s intervals for 5 min. An extinction coefficient of 5.76 mM⁻¹ (corrected for the path length of 0.6 cm) was used to convert the absorbance values to moles product. Thus, the GST activity per individual was reported as CDNB conjugated μ mol product min⁻¹mg⁻¹ protein (Karunaratne & Hemingway, 1996).

Carboxylesterase assay

A stock solution of 100 mM *p*-nitrophenyl acetate (*p*NPA) was prepared in acetone. The working solution of *p*NPA (1 mM) was prepared freshly for each assay by adding 50 μ L of 100 mM *p*NPA stock solution into 5 mL of 50 mM phosphate buffer (pH 7.4). Ten microliters of each homogenate were mixed with 200 μ L of the 1 mM *p*NPA working solution in a microliter plate well. The reaction rate was read immediately at 12s intervals at 405 nm for 2 min at 21 °C. An extinction coefficient of 6.53 mM⁻¹ (correlated for the path length of 0.6 cm) was used to convert the absorbance values to moles product. The *p*NPA activity per individual was reported as μ mol product min⁻¹ mg⁻¹ protein.

Cytochrome P450 monooxygenase assay

Twenty microliters of each insect homogenate were mixed with 80 μ L of potassium phosphate buffer (pH 7.2) + 200 μ L of 6.3 mM tetramethylbenzidine (TMBZ) working solution [0.01 g TMBZ dissolved in 5 mL methanol and then in 15 mL of sodium acetate buffer (pH 5.0) + 25 μ L of 3% H₂O₂ solution] in a microtiter plate well. After 2 h of incubation at room temperature, the plate was read at 630 nm as an endpoint assay. This assay does not measure the monooxygenase activity but titrates the amount of heme in the mosquito homogenate. Since heme is present in the active site of the monooxygenases, the amount of heme corresponds to the amount of monooxygenases present. Using a standard curve of cytochrome C, an estimate of the amount of monooxygenases present was obtained and expressed as equivalent units of cytochrome P450.

Protein assay

To obtain specific activities of enzymes, protein concentrations of the tick homogenates were determined by BIO-RAD protein determination kit, using bovine serum albumin as the standard protein. Ten microliters of homogenate were mixed with 300 μ L of BIO-RAD working solution (prepared according to the instructions of the manufacturer), and the absorbance was read at 630 as an endpoint assay after a 5-minute incubation at room temperature.

RESULTS AND DISCUSSION

Dose-response mortalities against DDT, malathion, flumethrin and permethrin are summarized in Table 1 and the log concentration-probit mortalities are shown in Figures 1–4. No larval mortalities were detected in untreated controls. LC₅₀ and LC₉₀ (24 hours exposure) values along with 95% confident intervals (CI) are listed in Table 2. Ninety-five percent CI values were narrow at LC₅₀ and LC₉₀ levels in all graphs except for malathion and DDT at the LC₉₀ level, where they were much wider. Except for permethrin, χ^2 values for the

Table 1: Percentage mortalities of *Otobius megnini* for malathion, flumethrin, permethrin and DDT as determined by Larval Packet Test (LPT) bioassays (FAO, 2004)

Acaricide concentration (%)	Mean (%) mortality			
	Malathion	Flumethrin	Permethrin	DDT
0.00001	*	7.29	*	*
0.0001	12.28	7.89	7.65	1.77
0.0005	27.91	*	16.22	*
0.00075	*	*	17.53	*
0.001	18.31	81.50	22.81	3.56
0.005	22.68	*	47.92	*
0.01	62.92	98.74	53.43	4.70
0.05	*	99.62	*	*
0.1	63.86	99.62	100	11.30
0.5	50.00	100	*	*
1	97.78	*	*	59.77
2	*	*	*	93.97
5	*	*	*	99.46
7	*	*	*	100

* Not tested

goodness-of-fit estimations were high and significant (Table 2). The regression coefficient was highest for permethrin and similar for the other three acaricides (Table 2). Since discriminating dosages of acaricides are not available for *O. megnini* in the literature, the resistance of *O. megnini* was measured using the established discriminating dosages for the Australian and Yeerongpilly strains of *Rhipicephalus microplus* (FAO, 2004) and anopheline mosquitoes (WHO, 2016). Although a direct determination of resistance cannot be made using discriminating dosages specified for other species, a comparison was attempted in order to obtain a rough estimation (Figures 1 to 4 and Table 2). According to anopheline discriminating dosages, the *O. megnini* population had possible resistant genes for malathion (2.93% survivals), permethrin (5.38%) and DDT (8.0%). With Australian *R. microplus* discriminating dosages, *O. megnini* population was susceptible to flumethrin

(0.05% survivals) but resistant to DDT (13.77% survivals). However, *O. megnini* population was resistant to flumethrin when the discriminating dosage of the Ceremonially strain of *R. microplus* was considered (10.79% survivals) (Table 2). It is important to develop separate discriminating dosages for *O. megnini* using a laboratory-maintained susceptible strain to obtain accurate results. S-shaped mortality curve in Figure 5 indicates the heterogenic response (i.e. both susceptible and resistant) of the *O. megnini* population to DDT (FAO, 2004). The mean specific activity levels of esterases and GSTs, and the amount of monooxygenases obtained from the biochemical assays were $0.064 \pm 0.008 \mu\text{mol min}^{-1} \text{mg}^{-1}$, $0.104 \pm 0.05 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and 0.068 ± 0.02 equivalent units of cytochrome P450, respectively. The percentage remaining activity of the target site AChE, after propoxur inhibition was 33.88 ± 13.6 .

Table 2: LC₅₀ and LC₉₀ values for malathion, flumethrin, permethrin, and DDT as determined by Larval Packet Test (LPT) acaricide bioassays (FAO, 2004) for the Sri Lankan population of *Otobius megnini*

Acaricide	Slope	SE	LC ₅₀ (%) (95% CI)	LC ₉₀ (%) (95% CI)	χ^2 (df)	R ²	DD (%)	R (%)	Resistance status**
Malathion (n=1741)	8.87	13.30	0.011 (0.048–0.002)	0.722 (60.284–0.113)	28.11* (7)	0.84	5 ^a	2.93	Possible resistant
Flumethrin (n=2567)	7.56	10.14	0.0003 (0.001–0.0001)	0.004 (0.015–0.001)	18.19* (5)	0.73	0.01 ^{bl}	0.05	Susceptible
							0.0036 ^{b2}	10.79	Resistant
Permethrin (n=2087)	10.88	2.60	0.008 (0.0132–0.0049)	0.300 (1.2112–0.111)	1.18 (5)	0.95	0.75 ^a	5.38	Possible resistant
DDT (n=2013)	9.42	19.40	0.124 (2.852–0.013)	3.049 (1703.73–0.291)	51.53* (6)	0.77	4 ^a	8.00	Possible resistant
							2 ^{bl}	13.77	Resistant

N = total number of individuals used; SE = standard error; LC = lethal concentration; CI = confident intervals; χ^2 = goodness of fit (Log concentration-probit transformed data); R² = regression coefficient; DD = discriminating dosage; R = resistance percentage; * = $p < 0.05$

^a = discriminating dosage for anopheline mosquitoes (WHO, 2016)

^b = discriminating dosage for ^{bl}Australian and ^{b2}Yeerongpilly strains of *Rhipicephalus microplus* (FAO, 2004).

** Resistance status was determined as susceptible (98–100 % mortality), possible resistant (90–97.9 % mortality) and resistant (< 90 % mortality) (WHO, 2016)

This study reports the first evidence of the development of acaricide resistance in spinose ear ticks, which infest the horses in the world. Resistance status was assessed using the larval packet test described by Stone and Haydock (1962) and later adopted and recommended by the Food and Agriculture Organization (FAO, 2004). This is a low cost straightforward method, which has been widely used to assess the resistance levels in hard and soft ticks, including *R. microplus*, *R. sanguineus*,

Hyalomma anatolicum and *Ornithodoros savignyi* (Miller *et al.*, 2001; Ahmed *et al.*, 2007; Lovis *et al.*, 2011; Shyma *et al.*, 2012; Bandara & Karunaratne, 2017). Ahmed *et al.* (2007) executed the same larval packet test for the soft tick *O. savignyi* but did not provide a discriminative dosage. Recently, Surbhi *et al.* (2018) used an adult immersion test for resistance trials in *O. savignyi* but results from this technique were difficult to compare with LPT results.

Figure 1: Log concentration-probit mortality of *Otobius megnini* for malathion as determined by the Larval Packet Test (LPT). The RDD A line indicates the resistance discriminating dosage (5%) for anopheline mosquitos (WHO, 2016).

Figure 2: Log concentration-probit mortality of *Otobius megnini* for flumethrin as determined by the Larval Packet Test (LPT). The RDD B1 and RDD B2 lines indicate the resistance discriminating doseages for Australian (0.0036%) and Yeerongpilly strains (0.01%) of *Rhipicephalus microplus*, respectively (FAO, 2004).

The discriminative dosage is generally assessed by doubling the mean $LC_{99.9}$ for a particular acaricide derived from a susceptible population (WHO, 2016). Susceptible strains of ticks are usually maintained under optimal conditions within laboratories for generations. Since this

is the first study carried out in Sri Lanka for *O. megnini*, a reliable susceptible-haplotype could not be found during the investigation. Since data for a susceptible *O. megnini* population were lacking, discriminating dosages available for hard tick *R. microplus* (FAO, 2004) were

used to assess the acaricide susceptibility of *O. megnini*. The presence of resistance was seen only against DDT. Two Sri Lankan populations of *R. microplus* have shown LC_{50} values of 0.04% and 0.03% for malathion, 0.002% and 0.001% for permethrin, 3.68% and 0.21%

for DDT, respectively (Bandara & Karunaratne, 2017). In comparison to Bandara and Karunaratne (2017), the present study showed that the tested *O. megnini* population has a similar LC_{50} value for malathion, a higher value for permethrin and a lower value for DDT.

Figure 3: Log concentration-probit mortality of *Otobius megnini* for permethrin as determined by the Larval Packet Test (LPT). The RDD A line indicates the resistance discriminating dosage (0.75%) for anopheline mosquitos (WHO, 2016).

Figure 4: Log concentration-probit mortality of *Otobius megnini* for DDT as determined by the Larval Packet Test (LPT). The RDD A and RDDB lines indicate the resistant discriminating dosages for anopheline mosquitos (4%) and *Rhipicephalus microplus* (2%), respectively (FAO, 2004; WHO, 2016).

However, mortality outcomes must be analysed carefully and interpreted cautiously when comparing data across different species as there are several species-specific

interfering factors, including rate of acaricide/ insecticide absorption and physiology of the organism, which can contribute to the mortality rate.

Figure 5: Line and scatter plot for the mortality response of *O. megnini* to DDT as determined by the Larval Packet Test (LPT). The RDD A and RDDB lines indicate the resistant discriminating dosages for anopheline mosquitos (4%) (WHO, 2013) and *R. microplus* (2%) (FAO, 2004), respectively. The two parallel broken lines represent hypothetical homogeneously susceptible (HHS) and hypothetical homogeneously resistant (HHR) populations.

Log concentration-probit mortality of DDT for the *O. megnini* population did not fit into a linear regression line indicating heterogeneous response for DDT (WHO, 2016; Karunaratne *et al.*, 2018). The high slope in the regression line with low LC₅₀ and LC₉₀ values indicated that the population was homozygous susceptible for flumethrin. However, there are no previous studies for *O. megnini* for comparison.

In Sri Lanka, DDT was extensively used in agriculture and to control malaria vectors for more than three decades (Kondradsen *et al.*, 2000) until it was replaced by malathion in the mid-1970s due to the development of resistance in *Anopheles* mosquitos (Kondradsen *et al.*, 2000). The use of organophosphates was legally limited to the health sector and carbamate was limited to the agricultural sector as a strategy to prevent resistance development. In the 1990s, pyrethroids were introduced to both agriculture and health sectors to control insect pests/vectors. Presently, pyrethroids are widely utilized in tick control programs in the country (personal communication

with veterinarians). The results of our study showed the presence of resistance for DDT of *O. megnini* despite the absence of DDT usage for more than four decades. It has been reported that intensive use of DDT in antimalarial and agricultural programs within the country from 1946 to 1977 has caused irreversible DDT resistance in almost all arthropod species including mosquitoes, bedbugs, ticks and agricultural pests (Damayanthi & Karunaratne, 2005; Karunaratne *et al.*, 2007, 2013; Perera *et al.*, 2008; Bandara & Karunaratne, 2017). This may be due to the selection of the same molecular mechanism initially developed to provide DDT resistance to *O. megnini* using insecticides. Alternatively, the evolved resistance mechanisms for other molecules may also be capable of causing DDT resistance in *O. megnini* (Bandara & Karunaratne, 2017).

Metabolic resistance based on GSTs is the major mechanism of DDT resistance (Hemingway *et al.*, 2004; Karunaratne *et al.*, 2018). Susceptible populations of the mosquitoes *Anopheles gambiae* and *Culex*

quinquefasciatus had GST specific activities of 0.42 ± 0.06 and $0.34 \pm 0.007 \mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively, which was higher than the observed specific activity of *O. megnini* ($0.104 \pm 0.007 \mu\text{mol min}^{-1} \text{mg}^{-1}$) tested in the present study. Bandara and Karunaratne (2017) reported 0.38 and $0.13 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for Sri Lankan *R. microplus*. Therefore, it appears that GSTs are not responsible for the observed high DDT tolerance of *O. megnini*. The presence of mutations in the DDT target site, which is the voltage-gated sodium channel (VGSC) on the nerve membranes, may provide this resistance by making the target site insensitive to DDT. Although the pyrethroids share this target site, mutations of VGSC gene which provide DDT resistance does not necessarily provide resistance to pyrethroids (Hemingway *et al.*, 2004).

Increased esterase activity is a well-known mechanism for protecting organophosphates and carbamates as they are rich with ester bonds (Karunaratne *et al.*, 2018). The *O. megnini* population showed an esterase activity of $0.06 \pm 0.01 \mu\text{mol min}^{-1} \text{mg}^{-1}$, which is higher than that of a susceptible population ($0.02 \pm 0.007 \mu\text{mol min}^{-1} \text{mg}^{-1}$) but lower than that of a resistant population ($0.92 \pm 0.08 \mu\text{mol min}^{-1} \text{mg}^{-1}$) of the mosquito *Culex quinquefasciatus* (Karunaratne & Hemingway, 1996). Two *R. microplus* tick populations with esterase-based resistance had 0.09 ± 0.02 and $0.08 \pm 0.04 \mu\text{mol min}^{-1} \text{mg}^{-1}$ esterase activities (Bandara & Karunaratne, 2017). Esterases of *O. megnini* population may therefore play a role in the development of resistance against organophosphates similar to the malathion. The results of AChE assay can be used to distinguish susceptible homozygous (< 30% remaining activity), heterozygous (30–70% remaining activity), and resistant homozygous (> 70% remaining activity) populations for altered or insensitive AChE mechanisms (WHO, 1998b). Accordingly, the *O. megnini* population has slightly more than 30% remaining activity indicating that the resistant alleles have been introduced in the population, expressing altered AChE mechanism, which in turn act in OP resistance.

Monooxygenases are an essential and diverse family of heme-containing enzymes which unitize as a resistance mechanism against pyrethroids (Karunaratne *et al.*, 2018). A discriminating amount of 0.35 equivalent units of cytochrome P450 was introduced to enhance monooxygenase activity. The introduced amount of cytochrome P450 was based on the monooxygenase amounts given for susceptible *An. culicifacies* and *An. subpictus* by Perera *et al.* (2008). The Sri Lankan cattle tick *R. microplus* had 0.14 and 0.34 equivalent units

of cytochrome P450 as monooxygenase amounts from two populations (Bandara & Karunaratne, 2017). Since the studied *O. megnini* population had a lower amount of monooxygenase activity (0.068 ± 0.02 equivalent units of cytochrome P450), it can be inferred that this mechanism is absent in *O. megnini* population and does not support pyrethroid resistance.

The incidence of acaricide resistance is highest in one-host ticks with short life cycles such as *R. microplus* compared to multi-host ticks (Wharton & Roulston 1970; Shyma *et al.*, 2013). This is because one-host ticks spend a more significant period of their life cycle on the same host under constant exposure to chemical acaricide/insecticides (Kiffner *et al.*, 2011; Leger *et al.*, 2013). Although most soft ticks have multi-host feeding patterns with shorter parasitic periods, *O. megnini* has a one-host life cycle with a high degree of host and feeding site predilection (Sonenshine, 1993).

CONCLUSION

Otobius megnini collected from racehorses from Nuwara Eliya, Sri Lanka, are susceptible to flumethrin and resistant to DDT. The development of organophosphate resistance through enhanced activity of esterases and altered target site AChE were evident. Future research is needed to generate more information on the persistence of acaricide resistance in *O. megnini*.

Conflict of interest

The authors declare that they have no competing interests

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