Leg loss in *Lutzomyia longipalpis* (Diptera: Psychodidae) due to pyrethroid exposure: Toxic effect or defense by autotomy?

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ABSTRACT

**Background & objectives:** Phlebotomine sandflies lose their legs after exposure to pyrethroids. In some insects leg loss helps to defend them from intoxication and predation, a phenomenon known as autotomy. A field observation has shown that sandflies that have lost some legs are still able to blood-feed. The aims of the study were to determine whether leg loss in sandflies, after exposure to deltamethrin, is due to autotomy and to establish the effect of the leg loss on blood-feeding.

**Methods:** Two experiments were carried out with *Lutzomyia longipalpis*: (i) Females were individually exposed to a sublethal time of deltamethrin and mortality and the number of leg loss were recorded; and (ii) Groups of females with complete legs or with 1–3 legs lost due to pyrethroid exposure were offered a blood meal and percentages of blood-fed and fully-fed females were recorded.

**Results:** Most females lost a median of 1 leg within 1–48 h post-exposure to deltamethrin. Mortality (after 24 h) was significantly higher for exposed females with lost legs (31.1%), compared to exposed females with complete legs (7.3%), and there were no differences in mortality between females with complete legs and the control (unexposed females). There were no differences between the three treatments in the percentages of blood-fed and fully-fed females.

**Interpretation & conclusion:** Leg loss in sandflies is a toxic effect of pyrethroids and there was no evidence of autotomy. The loss of up to three legs after exposure to pyrethroids does not affect blood-feeding behaviour in laboratory and probably also in wild conditions.

**Key words** Deltamethrin; leg loss; *Lutzomyia longipalpis*; pyrethroids; sandflies; toxicity

INTRODUCTION

Pyrethroid insecticides have been used frequently for the control of phlebotomine sandfly vectors of *Leishmania* spp., mainly through household spraying and insecticide-treated bednets preventing leishmaniasis indoor transmission. Studies on the effects of these insecticides have focused on evaluating mortality and knockdown effect on sandflies, with little attention paid to other intoxication symptoms such as the loss of legs. Leg loss has been reported in several Old World and New World sand flies species after tarsal contact with treated surfaces. Individuals of *Phlebotomus papatasi* lost their legs after exposure in laboratory to bednets treated with cyfluthrin and deltamethrin¹ or glass bottles treated with cypermethrin, permethrin, deltamethrin and lambda-cyhalothrin². *Lutzomyia longipalpis* lost their legs after exposure to filter papers impregnated with permethrin, deltamethrin and lambdacyhalothrin³ or glass bottles treated with the three latter pyrethroids and cypermethrin², ⁴ and in experimental chicken sheds treated with lambda-cyhalothrin⁵. Hence, it seems that leg loss in sandflies is due to a toxic effect of pyrethroids. On the other hand, leg loss is also common in mosquitoes where this phenomenon has been observed after exposure of several body parts to pyrethroids. *Anopheles arabiensis* and *Culex tarsalis* lost their legs after tarsal contact with deltamethrin and bifenthrin treated surfaces, respectively⁶–⁷. It has also been reported in *Aedes aegypti* after being sprayed with d-phenothrin, d-allethrin and tetramethrin in a wind tunnel⁸ or when they were treated topically with bioresmethrin⁹. For both sandflies and mosquitoes, it is suggested that leg loss occurs in an unspecified time before insect death¹, ⁷, ¹⁰. Nevertheless, leg loss has neither been quantified nor described in detail.

Based on laboratory testing, it is generally assumed that sandflies that have lost their legs due to pyrethroid exposure have little possibility of surviving, feeding on blood, or transmitting *Leishmania*. By contrast, it has been reported in laboratory, that females losing up to four legs (*Ph. papatasi*) or one or more legs (*Ph. papatasi* and *Lu. longipalpis*) for the same reason are still able to fly or...
blood-feed, respectively\textsuperscript{1–2}. In field conditions, a casual picture of a wild \textit{Lu. longiflocosa} female, feeding blood on a human host in a forest, revealed that the female had lost three of its legs from one side of its body (Fig. 1), supporting the authors observation, suggesting that partial leg loss, at most up to three, is not detrimental for sandflies and the females with this condition could survive and at least are able to feed under natural conditions. Besides the toxic effect, it is possible that the loss of legs could be an extreme defense response known as autotomy which in this case would be due to exposure to a toxic substance\textsuperscript{11}. There is no evidence of autotomy for haematophagous Diptera after exposure to pyrethroids. This phenomenon has been recorded in Lepidoptera for the crop pest diamondback moth \textit{Plutella xilostella} (Plutellidae), where it has been demonstrated that moths that lost their legs, after exposure to sublethal doses of the pyrethroid fenvalerate, had significantly lower mortality and recovered quicker from knockdown compared with moths that did not lose legs\textsuperscript{12}. Furthermore, it was shown that moths that lost legs did have a significant lower concentration of pyrethroid and metabolites compared with those moths that did not. These findings suggest that by autotomy, moths may be able to eliminate a portion of the pesticide along with their legs, preventing the lethal quantity reach to the rest part of their body. Autotomy could have important epidemiological consequences for sandflies. If leg loss in sandflies after pyrethroid exposure were due to autotomy, then it is expected that the proportion of leg loss survivor females in a population would eventually lead to an insecticide-resistant population over the time. Furthermore, these mutilated females would have the ability to bite, infect themselves with parasites, and transmit \textit{Leishmania}.

The main goal of this study was to determine whether leg loss in females of the sandfly \textit{Lu. longipalpis} (Lutz & Neiva), after exposure to a sublethal dose of pyrethroid, is caused by autotomy and to identify the effect of this leg loss in blood-feeding behaviour.

**MATERIAL & METHODS**

**Preliminary test: Sublethal time needed to induce high leg loss and low mortality**

A preliminary test was carried out where three exposure times (0.5, 1, and 1.5 min) to deltamethrin, 55 mg/m\textsuperscript{2}, were evaluated in order to determine the appropriate sublethal time needed to induce the loss of legs (target of 1–3 leg loss) with the lowest mortality and knockdown effect in \textit{Lu. longipalpis} females. The selected sublethal exposure time was then used for the subsequent experiments. The study used unfed females with all of their legs, aged from 2–6 days, from a laboratory colony established at the National Health Institute in 1995 from individuals gathered in El Callejón (Cundinamarca, Colombia). For each exposure period, the individual females were exposed to a piece of bednet impregnated with deltamethrin, inside a modified device for sandflies designed initially for mosquitoes by Skovmand \textit{et al}\textsuperscript{13}. The recorded variables were mortality, knockdown effect and the number of loss legs.

The modified Skovmand device was made of transparent acrylic and consists of four parts: (i) A base for attaching the material to be tested, made up of two sheets with a central circular hole, with the material to be tested placed on the lower sheet (Fig. 2a\textsubscript{1}) and held in place by the upper sheet (Fig. 2a\textsubscript{2}); (ii) A sandfly exposure chamber made up of the space left by the hole in the upper sheet (Fig. 2b) and with a height of 2 mm defined by referring to the height of \textit{Lu. longipalpis} in a resting position (this value is only a fifth of the height of the chamber used by Skovmand); (iii) A movable lid, made up of a circular sheet which can be moved horizontally (Fig. 2c) with a central hole connected to a short tube for transferring sand flies (Fig. 2c\textsubscript{1}); and (iv) Horizontal and vertical movement restrictors for the movable lid (Figs. 2d\textsubscript{1} and 2d\textsubscript{2}). These are other additions to the Skovmand design. The material used in the test was a piece of a long lasting bednet treated with deltamethrin, 55 mg active ingredient (a.i.)/m\textsuperscript{2}, made of polyester with a maximum mesh size of 2 mm (Permanet 2.0). As the mesh size did not amount to a complete physical barrier for \textit{Lu. longipalpis} females, it was necessary to place an untreated piece of bednet below the piece of the long-lasting bednet. At the time of testing, the modified Skovmand device was placed
on a support in horizontal position. Then, a female *Lu. longipalpis* was introduced into the device and kept in contact with the piece of bednet during the exposure time to be evaluated. To stimulate contact between the female and the piece of bednet, a flashlight was placed below the device (Fig. 3). After exposure, each female was moved to an observation container with *ad libitum* supply of water and sugar solutions. Mortality was recorded immediately, at 1 h, and at 24 h post-exposure; the knockdown effect was assessed at 24 h; and the number of lost legs left by the female on the base of the observation container was also recorded. The condition of death or knockdown was determined based on the response of each female after being gently touched with a caliper. Females not showing any movement were considered dead, while those that responded showing some type of movement, but could not assume an upright position or fly normally were recorded as a knocked-down. Two types of mortality were recorded: (a) real mortality, based only on the counting of dead sandflies14, and (b) functional mortality, the sum of dead and knocked down sandflies15. The later was recorded because to date, no recuperation has been noted for *Lu. longipalpis* after knockdown caused by lethal doses of pyrethroids. The test was replicated 20 times.

**Experiment 1: Relationship between leg loss due to a sublethal exposure time to deltamethrin, and female mortality and knockdown after 24 h post-exposure**

This experiment was carried out to determine the relationship between leg loss, mortality and knockdown effect on females of *Lu. longipalpis* after deltamethrin exposure to a sublethal time of 1 min. This exposure time was selected because during the preliminary test this was the period which presented simultaneously, a high proportion of females with leg loss and low functional mortality. Females were individually exposed to the sublethal time in the modified Skovmand device as described in the preliminary test. Two randomly-assigned treatments were compared: (i) a piece of long-lasting bednet treated with deltamethrin, 55 mg a.i./m² (Permanet 2.0); and (ii) a piece of untreated polyester bednet with a maximum mesh size of 0.5 mm (control). After exposure, females were kept individually in an observation container for 24 h. Immediately, after the test and at 24 h post-exposure, mortality, both real and functional (as explained in the preliminary test), knockdown, and the number of leg loss were recorded. The test was replicated 158 times.

**Experiment 2: Effect of leg loss, due to sublethal pre-exposure to deltamethrin, on blood-feeding behavior**

The experiment was aimed to determine if leg loss (1–3 legs), after pyrethroid exposure, could modify blood-feeding behavior of female *Lu. longipalpis*. In addition, this experiment provided information about female mortality up to 48 h post-exposure time and the position of the legs loss on the thorax. The females used were those which survived after 24 h in the experiment 1. Three groups of females were compared, each comprising 22
individuals on average: (i) females that did not lose their legs after 1 min exposure to deltamethrin, 55 mg a.i. /m²; (ii) females that lost 1–3 legs after exposure to the same pyrethroid, dose and exposure time; and (iii) females that did not lose their legs after being exposed during 1 min to a piece of bedding that has not been treated with the pyrethroid (control). Each group of females was provided with blood meal in a transparent acrylic tunnel (120 × 30 × 30 cm) made up of two identical sections: (i) a feeding chamber where a previously anesthetized golden hamster, *Mesocricetus auratus*, was placed; and (ii) a release chamber where a group of females to be tested were introduced. The anesthetized hamster was offered in the chamber for 90 min (two hamsters each for a period of 45 min). The tests were performed in the daytime (0900–1500 hrs), and to simulate darkness during feeding, the tunnel was covered with a thick synthetic covering. When the blood-feeding time was over, the females were transferred to a container and observed over 24 h. The females surviving this time period were killed by freezing. Percentages of females feeding on blood (any amount of blood) and blood-feeding success (percentage of fully-fed females) were recorded. Also, immediately after the test (i.e. 24 h after insecticide exposure) and 24 h later (i.e. 48 h after insecticide exposure) real mortality was recorded. Only alive females which survived immediately after the test were taken into account to calculate 48 h mortality. Finally, for each female included in the test, the numbers of legs lost according to the position on the thorax segments (prothorax, mesothorax, and metathorax) and on the sides of the body (right or left) were recorded up to 48 h after exposure. One test was conducted per day, with random assignment of treatments. The test was replicated five times. The temperature during the tests fluctuated between 24 and 26°C and the relative humidity between 55 and 65%.

**Statistical analysis**

Most of the evaluated variables are presented as percentages of the total number of females, each with their corresponding 95% confidence interval (CI). However, for the preliminary test, only percentages are shown. The statistical analysis was carried out mainly with chi-square ($\chi^2$) test and Fisher’s exact test when the expected number was < five. In experiment 1, the statistical comparisons of the number of leg loss between alive and dead females were made with the Mann-Whitney test for independent samples; and the possible association between mortality and the number of leg loss, recorded under three categories (0, 1–3 and 4–6 legs), was assessed using chi-square test for trend. In experiment 2, the comparison of real mortality among all three treatments of leg loss condition and exposure time was initially analysed with the Likelihood ratio chi-square test ($L\chi^2$), followed by pairwise comparisons for appropriately collapsed tables (if the initial test was statistically significant)\(^{16}\). Additionally, comparisons between number of legs loss according to thoracic segments were analyzed with a $\chi^2$ for one variable, comparing observed percentages of females with leg loss over specific thoracic segments (four categories: prothorax, mesothorax, metathorax and combination of segments) or sides of their bodies (three categories: left, right and both sides) with equal expected percentages according to each category. Analyses were conducted using Stata 12 and EpilInfo 7 software.

**Ethics**

The use of laboratory animal in this study was approved by the Comité de Ética en Investigación, Instituto Nacional de Salud de Colombia (Approved by agreement No.5 Jun 25, 2009).

**RESULTS**

**Preliminary test: Sublethal time needed to cause high proportion of females with leg loss and low mortality**

Considering the time for leg loss, immediately after exposure to the deltamethrin, no any leg loss was observed in the *Lu. longipalpis* females. Leg loss started within the first hour of exposition to deltamethrin, in the two treatments, i.e. 0.5 min and 1 min of exposure periods, in which one female lost one leg. After 24 h post-exposure, leg loss was evident in all the treatments, and percentages of leg loss (in which females lost at least one leg) fluctuated from 45% in the treatment of 0.5 min exposure, to 80% in the treatment of 1.5 min exposure, with an apparent proportional increase as the exposure time passed (Table 1). In terms of number of lost legs per female, the results (for all treatments) showed that the majority of *Lu. longipalpis* females (85 to 100%) which shed their legs, lost between 1 to 3 legs. Mortality rates and knockdown effect after 24 h post-exposure showed low values, but also with an apparent positive relationship with the exposure time (Table 1). The 1 min sublethal exposure time was the only treatment that caused simultaneously a high (70%) proportion of females with leg loss and low (15%) functional mortality.

**Experiment 1: Leg loss due to exposure to deltamethrin, and female mortality and knockdown after 24 h post-exposure**

When females were evaluated immediately after 1
min exposure to a treated piece of bednet containing deltamethrin 55 mg a.i./m², little to no effect was observed in the exposed individuals. Females in the sublethal exposure time and control treatments neither experienced leg loss nor real mortality. Only one female was recorded under knockdown effect from the sublethal exposure time treatment. At 24 h post-exposure, 65.2% (103/158) of females in the sublethal treatment had experienced loss of legs, meanwhile none of the 158 control females suffered such phenomenon. To this point, the mortality rate of females exposed to sublethal treatment corresponded to 16.5% (26/158) compared with only 4.4% (7/158) in the control treatment.

With respect to the relationship between mortality and the condition of having lost legs at 24 h post-exposure to a sublethal time, it was found that each female lost on average 1 leg (lower quartile = 0, upper quartile = 2) after 24 h post-exposure to the sublethal time. Dead females lost significantly higher number of legs, 4 legs (lower quartile = 1, upper quartile = 6), compared with alive females, 1 leg (lower quartile = 0, upper quartile = 1) (Mann-Whitney test, \( z = 5.59, p < 0.001 \)). The comparison between the number of leg loss (three categories: 0, 1–3, 4–6 leg loss) by each female exposed to the sublethal time and mortality, showed that mortality increased with increase in leg loss (Fig. 4), suggesting an apparent positive relationship, which was confirmed statistically for both real (\( \chi^2 \) for trend = 23.18, \( df = 1, p < 0.001 \)) and functional mortality (\( \chi^2 \) for trend = 37.92, \( df = 1, p < 0.001 \)).

The knockdown effect (24 h), was very low, 6.3% (10/158), in females exposed to the sublethal time. Taking into account this, it was not analysed.

**Experiment 2: Leg loss and blood-feeding**

Mortality was low immediately after the experiment in all treatments, i.e. in control 5% (6/119), in females

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**Table 1. Effect of three sublethal exposure times to a treated bednet (deltamethrin 55 mg/m²) on leg loss, mortality and knockdown effect in Lutzomyia longipalpis females (n = 20) after 24 h individual exposure in the modified Skovmand device.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Exposure time (min)</th>
<th>% (N°)</th>
<th>% (N°)</th>
<th>% (N°)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>1</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Females with leg</td>
<td>45 (9)</td>
<td>70 (14)</td>
<td>80 (16)</td>
<td></td>
</tr>
<tr>
<td>loss</td>
<td>Real mortality</td>
<td>5 (1)</td>
<td>5 (1)</td>
<td>15 (3)</td>
</tr>
<tr>
<td>Knockdown</td>
<td>5 (1)</td>
<td>10 (2)</td>
<td>20 (4)</td>
<td></td>
</tr>
<tr>
<td>Functional</td>
<td>10 (2)</td>
<td>15 (3)</td>
<td>35 (7)</td>
<td></td>
</tr>
<tr>
<td>mortality</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**Table 2. The effect of individually exposure to 1 min sublethal time to a piece of bednet treated with deltamethrin (55 mg/m²), using the modified Skovmand device, on leg loss of Lutzomyia longipalpis females, according to mortality and knockdown, 24 h post-exposure.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control without leg loss (n = 158)</th>
<th>Exposure to sublethal time</th>
<th>Without leg loss (n = 55)</th>
<th>With leg loss (n = 103)</th>
<th>Total (n = 158)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% (95% CI)</td>
<td>N°</td>
<td>% (95% CI)</td>
<td>N°</td>
<td>% (95% CI)</td>
</tr>
<tr>
<td>Real mortality</td>
<td>4.4 (1.8–8.9)</td>
<td>7</td>
<td>5.5 (1.1–15.1)</td>
<td>3</td>
<td>22.3 (14.7–31.6)</td>
</tr>
<tr>
<td>Knockdown</td>
<td>0 (0–2.3)</td>
<td>0</td>
<td>1.8 (0.04–9.7)</td>
<td>1</td>
<td>8.7 (4.1–15.9)</td>
</tr>
<tr>
<td>Functional</td>
<td>4.4 (1.8–8.9)</td>
<td>7</td>
<td>7.3 (2–17.6)</td>
<td>4</td>
<td>31.1 (22.3–40.9)</td>
</tr>
<tr>
<td>mortality</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**Notes:**
- Total number of females;
- 95% confidence interval, except for knockdown in the control treatment where it was 97.5%;
- Number of females by treatment and variable.
that did not lose their legs 24 h after exposure to the sublethal time 3.7% (4/107), and in females that had lost 1–3 legs 24 h after exposure to the sublethal time 10% (11/110). As most of this mortality was probably caused by physical damage due to handling of females during the experiment, the presented data excluded females found dead immediately after the experiment, except for the description of the site of the thorax where the legs were lost. It is important to point out that the majority of dead females, except one, did not take a blood meal.

In regard to the feeding behaviour, nevertheless, the percentages of blood-fed females were higher for the treatments exposed to the sublethal time; 55.3% for females that did not lose their legs and 55.6% for females that lost 1–3 legs, compared with the 43.4% for the control, these differences were not significant ($\chi^2 = 4.24, df = 2, p = 0.120$). This result was similar for blood-feeding success. The percentages of fully-fed females were higher for the treatments exposed to the sublethal time; 66.7% for females that did not lose their legs and 65.5% for females that lost 1–3 legs, compared with the 55.1% for the control, again with no significant differences ($\chi^2 = 1.75, df = 2, p = 0.416$) (Table 3).

It was remarkable that leg loss appeared at 24 h post-feeding (i.e. 48 h post-exposure to the sublethal time) in the two treatments (females exposed to sublethal time that had not lost their legs and the control) where no female had lost their legs at the beginning of the experiment. Nevertheless, the percentage of females that lost their legs, in the treatment exposed to the sublethal time without leg loss at the beginning of the experiment was significantly higher i.e. 34% (35/103), compared with the 5.3% (6/113) of the control ($\chi^2 = 28.80, df = 1, p < 0.001$).

Real mortality 48 h after post-exposure to the sublethal time was higher, 42.4% (42/99), in the treatment of females that lost 1–3 legs post-exposure time, followed by the treatment of exposed females that had not lost their legs at the beginning of the experiment, 26.2% (27/103), and the control 18.6% (21/113), this difference were significant ($L\chi^2 = 14.93, df = 2, p = 0.001$). The mortality in the treatment of females that lost 1–3 legs post-exposure time was significantly higher compared with the combine mortality of the other two treatments ($L\chi^2 = 13.12, df = 1, p < 0.001$) and there was not statistical difference in mortality between the treatment of exposed females that had not lost their legs at the beginning of the experiment and the control, also with no leg loss ($L\chi^2 = 1.82, df = 1, p = 0.178$).

Position of lost legs and their site of detachment on the thoracic segments were also investigated at the end of the experiment, in the two treatments exposed to the sublethal time. From 144 females that lost their legs, the legs were always detached in the trochanter-femur joint. Each female lost on average (median) 1 leg (lower quartile = 1, upper quartile = 2). According to the position on the thoracic segments, it was found that the highest percentages of females lost their legs from the prothorax (fore legs), 30.6%; and from any combination of two thoracic segments, 29.9%; followed by 20.1% from mesothorax (middle legs), 15.3% from metathorax (hind legs), and 4.2% from all three thoracic segments (Table 4). These

![Fig. 4: Relationship between mortality and number of leg loss by *Lutzomyia longipalpis* females, 24 h post-exposure to 1 min sublethal time to a piece of bednet treated with deltamethrin (55 mg/m²), using the modified Skovmand device. aNumber of females by category of number of leg loss, except for the control treatment where all females (Nº = 158) were grouped into category of 0 legs loss. Error bars corresponds to the 95% confidence intervals.](image-url)

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**Table 3. Effect of leg loss of *Lutzomyia longipalpis* females, after 24 h sublethal exposure time to deltamethrin (55 mg/m²), on hamster blood-feeding.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control without leg loss</th>
<th>Without leg loss after sublethal exposure</th>
<th>1–3 legs loss after sublethal exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%  (95% CI)⁴</td>
<td>% (95% CI)</td>
<td>% (95% CI)</td>
</tr>
<tr>
<td>Blood-fed females</td>
<td>43.4 (34.1–53)</td>
<td>55.3 (45.2–65.1)</td>
<td>55.6 (45.2–65.5)</td>
</tr>
<tr>
<td>Feeding success ³</td>
<td>55.1 (41.1–68.3)</td>
<td>66.7 (34.1–88.5)</td>
<td>65.5 (32.9–88.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 113</td>
<td>n = 103</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 49</td>
<td>n = 57</td>
</tr>
</tbody>
</table>

⁴95% confidence interval; ³Total number of females; ³Fully-fed females; ⁴Total number of blood-fed females.
the loss of legs in sandflies after an exposure to pyrethroid treatment did. Despite some authors having observed females losing some legs, none of the females in the experiment lost legs at 24 h post-exposure to deltamethrin, whereas 65.2% of the females in experiment 1, where a sublethal time of 1 min to deltamethrin was used, had lost legs. Further research is needed to confirm this observation.

The leg was always detached from the joint between the trochanter and femur. It appears that there is a tendency for the leg to detach from the joint, but further research is needed to confirm this observation.

The present study, however, suggests that the time of leg loss can extend up to 48 h post-exposure. The delay in the time of leg loss does not match with the hypothesis of a possible autotomic response to the presence of a toxic substance, since in that case the female, as a defence mechanism, should quickly detach the contaminated legs, getting rid of the toxic substance. Conversely, in the moth Plutella xylostella autotomy has been shown after exposure to fenvalerate (250 ng/cm²) for 1 min, and it has been detected that 35% of the individuals lost at least one leg within the first 30 min post-exposure. As far as we know, there is only one previous publication regarding the time of leg loss in sandflies as a result of pyrethroid exposure. The study assessed the susceptibility of sandflies to insecticides and it was found that, for four pyrethroids, the leg loss of Lu. longipalpis and Ph. papatasi occurs both during the exposure time (60 min) and after it, however the exact time of leg loss was not specified. The phenomenon of leg loss during the exposure time may be explained by the longer exposure time used in comparison with the present study. In anopheline and culicine mosquitoes, leg loss caused by exposure to pyrethroids has been reported from 2 min post-treatment, after topical application of the pyrethroid, bioresmethrin, to individual insects. The quickest leg loss in the latter study is explained by the difference in the method of pyrethroid application.

In the present study, legs detachment occurred in the joint between the trochanter and femur, as reported earlier in most insects. It seems that this location is independent from the cause of the loss. For example, legs detachment at the trochanterofemoral joint may occur due to an intoxication with pyrethroids in the mosquitoes Aedes, Anopheles, Culex and Culiseta; autotomy in a defensive response to the exposure to insecticides in moths of the genera Plutella, Choristoneura, Pandemis differences were statistically significant ($\chi^2 = 10.12$, $df = 3$, $p = 0.018$), excluding the category of all three thoracic segments which presented low numbers. Furthermore, when the females who lost their legs simultaneously from the prothorax and any of the other two thoracic segments were summed, the percentage of leg loss from the prothorax increased to 55.6% (80/144). Finally, there was no any difference in leg loss with respect to the side of the body ($\chi^2 = 1.17$, $df = 2$, $p = 0.558$) (Table 4).

**DISCUSSION**

**Description of the leg loss due to a sublethal exposure to deltamethrin**

In connection with the leg loss phenomenon, the combined results of the three experiments (including the preliminary test), indicated that loss of legs in most female sandflies occurred during the a period of 1 to 24 h post-exposure to a sublethal time of 1 min to deltamethrin. However, time of leg loss can extend up to 48 h post-exposure. Each female lost on average (median) one leg. The leg was always detached from the joint between the trochanter and femur. It appeared that there is a tendency to lose the prothoracic legs; nevertheless this observation needs further research.

The phenomenon of losing legs by the exposure to pyrethroid was confirmed with the experiment 1, where after 24 h post-exposure to deltamethrin, 65.2% of the females lost some legs, whilst none of the females in the control treatment did. Despite some authors having observed the loss of legs in sandflies after an exposure to pyrethroid treated surfaces, this phenomenon had not been fully described before.

Even though in the current study, the period of time during which the leg loss occurred was not accurately determined, the results of the preliminary test indicate that for most of the females, the leg loss occurs from one to 24 h after 1 min exposure to deltamethrin. The reason for this was that before 1 h post-exposure, just one of the females had lost some of its legs, whilst within 24 h after the test, 70% of the females had already lost at least one of their legs. Furthermore, 48 h post-exposure to the pyrethroid (experiment 2), loss of some legs was observed in 34% of females belonging to the treatment exposed to the sublethal time which had not lost any of the legs at the beginning of the experiment. This indicates that the leg loss phenomenon can occur at least up to 48 h post-exposure. The delay in the time of leg loss does not match with the hypothesis of a possible autotomic response to the presence of a toxic substance, since in that case the female, as a defence mechanism, should quickly detach the contaminated legs, getting rid of the toxic substance.

Other observations were made during the preliminary test. For example, females used in experiment 1 on the effect of leg lost on blood-feeding, which have lost their legs at 48 h post-exposure to a sublethal time of deltamethrin (sum of the two treatments exposed to the sublethal time) were not able to feed.

**Table 4. Position of leg lost on the thorax of Lutzomyia longipalpis females used in experiment on the effect of leg lost on blood-feeding, which have lost their legs at 48 h post-exposure to a sublethal time of deltamethrin (sum of the two treatments exposed to the sublethal time)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Females that lost legs (144)</th>
<th>%</th>
<th>(95% CI)</th>
<th>Nº</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thoracic segment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prothorax</td>
<td>30.6</td>
<td>(23.2–38.8)</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Mesothorax</td>
<td>20.1</td>
<td>(13.9–27.6)</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Metathorax</td>
<td>15.3</td>
<td>(9.8–22.1)</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Mixed of two segmentsd</td>
<td>29.9</td>
<td>(22.5–38.0)</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>All three segments</td>
<td>4.2</td>
<td>(1.5–8.8)</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Side of the body</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>34.7</td>
<td>(27.0–43.1)</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>36.1</td>
<td>(28.3–44.5)</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Both sides</td>
<td>29.2</td>
<td>(21.9–37.3)</td>
<td>42</td>
<td></td>
</tr>
</tbody>
</table>

*Total number of females; †95% confidence interval; ‡Number of females by category of variable; ‡Mixed of prothorax and mesothorax, prothorax and metathorax or mesothorax and metathorax.
and Cydia\textsuperscript{17, 19}; or (c) autotomy in response to the attack of predators or molting complications in stick insects of the order Phasmdida\textsuperscript{20}. Although the details of the legs detachment are unknown, the events that trigger this phenomenon are well known. Pyrethroids are neurotoxins which affect mainly the sodium channels in the cell membrane of the neuronal cells. Deltamethrin cause membrane depolarization accompanied by suppression of the action potential\textsuperscript{21}. Intoxication symptoms presented in insects are ataxia and lack of coordination with intense hyperactivity periods and convulsions, what is probably responsible for the leg loss, followed by prostration, paralysis and eventually death\textsuperscript{22}.

A greater percentage, \textit{i.e.} 30.6\% of Lu. longipalpis females lost the legs of the prothorax part. An explanation for this differential leg loss might be an unequal surface contact of the three pairs of legs. Consequently, the legs with greater contact with the treated surface might absorbed a higher amount of insecticide, causing detachment more easily. This hypothesis has support on a study in \textit{An. stephensi}, where the exposure to surfaces treated with a sublethal dose of deltamethrin cause a significantly greater loss of the metathoracic legs, which are the legs more in contact with resting surfaces\textsuperscript{23}. For sand flies this hypothesis seems unlikely. Unfed females of \textit{Lu. longipalpis} rest in a position so that their metathoracic legs, similar to mosquitoes, have more contact with the resting surface, whilst the forelegs have less contact with the resting surfaces.

Finally, the results of this study indicated that leg loss in phlebotomine sand flies is possible not only as a result to toxic exposure (\textit{e.g.} pyrethroids), but also due to accidents in the natural environment and by handling in laboratory. The photograph taken by the authors (Fig. 1) is the first documented evidence of this phenomenon in wild sandflies. It is unlikely that the photographed female had lost its legs due to the contact with insecticide, because when the photograph was taken there were no household interventions with insecticides and the inhabitants do not use domestic insecticides. The leg loss in this case might occur due to the attack of predators or contact with sticky surfaces. Concerning the leg loss by handling, evidence of this comes from the 5.3\% of females which lost their legs in the control group in experiment 2 after been handled during the exposure to the pyrethroid and the blood-feeding.

\textbf{Leg loss and female mortality}

In experiment 1, in the treatment with females exposed to sublethal time, both mortalities (real and functional) were significantly higher in the group of females that lost legs, 22.3 and 31.1\%, respectively, compared with the group of females that did not lose any leg, 5.5 and 7.3\%, indicating that leg loss is associated with mortality. This result was confirmed by the real mortality recorded 48 h post-exposure to sublethal time in experiment 2. In this experiment a significant higher mortality, 42.4\%, was found in the treatment with females which were exposed to pyrethroid and lost up to three legs, compared with the mortality, 26.2\%, in the treatment of females which were exposed and did not lose legs at the beginning of the experiment. The reason for the highest mortality in the females that lost legs may be intraspecific differences in susceptibility to the pyrethroid. Alternatively, this group of females might have been exposed to a higher toxic dose because they kept longer contact with the pyrethroid treated material. The latter explanation seems unlikely, because the exposure to pyrethroid using the modified Skovmand device ensured that, during the all exposure time, each female were in contact with the treated material.

On the other hand, a lower toxic effect, or no apparent effect, was evident in the group of females which were exposed to pyrethroid and did not lose legs in both experiments 1 and 2. In experiment 1, there were not significant differences in real and functional mortality 24 h post-exposure between the before mentioned female group and the control. The same result was given in experiment 2 for real mortality at 48 h post-exposure.

The apparent positive association between the number of legs loss and real and functional mortalities, in experiment 1, suggests that the number of legs loss may be used, in exposures to sublethal doses, as an indicator of the degree of toxicity of the pyrethroids. Nevertheless, these results should be taken with caution, because the explored association was carried out using categories of numbers and not the number of lost legs. This association has not been previously reported in blood sucking Diptera.

On the other hand, it is remarkable, in experiment 1, the high percentage of survivorship in the group of females that lost 1–3 legs, 85\% (inferred of 15\% of functional mortality), with the ability to stay straight or to fly after 24 h post-exposure time, and the very low survivorship, over 20\%, in the group of females that lost 4–6 legs (Fig. 4). This confirmed the observations in field (Fig. 1) and in laboratory, by other authors\textsuperscript{1}, which suggest that sandflies females which lose some of their legs could survive without many troubles. By contrast, problems with leg loss have been visualized by Denlinger \textit{et al.}\textsuperscript{2}, who...
observed that leg loss could be a potential physical challenge for female sandflies at least during blood-feeding as they saw that females with this condition often lose their balance and need to relocate to blood-feed.

Toxic effect vs autotomy

The results of the current study indicate that leg loss in females of *Lu. longipalpis* after being exposed to a sublethal dose of pyrethroids is due to a toxic effect and not because of an autotomy phenomenon, which may defend the females from the pyrethroid. The reasons for this are: (1) in females exposed to pyrethroid, the mortality was significantly higher in those females that lost their legs compared to females that did not lose legs; in other words, the loss of legs did not protect females against the lethal effect of the pyrethroid; and (2) leg loss for most females was relatively delayed, at some time between 1 and 24 h post-exposure. To be effective against a toxic substance leg loss should occur quickly, within minutes.

Leg loss and blood-feeding

There was no any statistical difference between treatments (females exposed to pyrethroid that lost 1–3 legs, females that did not lose legs and control) in the percentage of blood-fed females, just as in females with blood-feeding success (fully-fed females), indicating that, the blood-feeding is not affected by the loss of some legs due to the exposure to pyrethroids, under laboratory conditions. The observations agree with the comment of Denlinger *et al*\(^2\), that under laboratory conditions, females of *Lu. longipalpis* and *Ph. papatasi* exposed to pyrethroids that have lost at least one leg were still capable of blood-feeding on mice. Similar observations have been made in mosquitoes. Females of *Ae. aegypti* with four missing legs due to a sublethal exposure to biorestemethrin were able to find and blood-feed on a restrained guinea pig\(^9\) notwithstanding their decreased flight activity. Females of *An. gambiae*, that were not exposed to pyrethroids, but that had a simulation of leg loss (one or two legs of their metathorax) before the blood meal, laid the same amount of eggs than control females\(^24\). This suggests that leg loss did not affect feeding behavior of sand flies and mosquitoes significantly. The observation of a wild female *Lu. longiflucosa*, that did not had three legs on its left side and still was able of blood-feeding on a human host (Fig. 1) shows that, under field conditions, female sandflies with this condition are not seriously affected for their host seeking and feeding behaviour. Therefore, we propose that female sand flies, under field conditions, that had lost up to three legs for different reasons may survive and are capable of host seeking, blood-feeding and transmit *Leishmania* parasites.

CONCLUSION

The obtained results showed that leg loss caused by the exposure to a sublethal time of deltamethrin did not reduce female mortality; however this condition was associated with higher mortality. This observation along-with the relatively slow process of leg loss with time, revealed that there is no evidence of autotomy. The loss of legs in *Lu. longipalpis* is just one of the toxic effects caused by the exposure to pyrethroids. The apparent positive association between the numbers of leg loss, after exposure to a sublethal dose of deltamethrin, in female *Lu. longipalpis*, and the mortality need to be confirmed in future studies. Taking into account the short period (1 min) of pyrethroid exposure that caused the loss of legs in the majority (65%) of females at 24 h post-exposure, it is suggested that leg loss is an indicator of contact between sand flies and pyrethroids. Besides, if a reference percentage of legs loss caused by the exposure to pyrethroids would be established (in a susceptible sand fly population), a reduction in leg loss in a sandfly population under selection pressure with pyrethroids could be used as an indicator that the population is moving towards resistance development as already suggested for mosquitoes. The study showed that leg loss (up to three legs) independent of their cause, does not visibly affect feeding behaviour neither in the laboratory, nor in field. The possible survival of wild blood-fed females that lost their legs, after exposure to sublethal doses of pyrethroids, should be investigated as they might contribute in the development of resistant populations.

Conflict of interest

The authors declare that they don’t have any conflict of interest.

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