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ORIGINAL PAPER

# Effects of the essential oils of *Lippia turbinata* and *Lippia polystachya* (Verbenaceae) on the temporal pattern of locomotion of the mosquito *Culex quinquefasciatus* (Diptera: Culicidae) larvae

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**Abstract** The essential oils (EO) of *Lippia turbinata* (TUR) and *Lippia polystachya* (POL) have shown lethal effects against mosquito larvae. The present work evaluated whether these EO at doses ranging from sublethal to lethal (20, 40 and 80 ppm) modify the temporal pattern of locomotion of *Culex quinquefasciatus* larvae. Larvae were individually placed in glass boxes, and their activity recorded at 0.3 s intervals during 40 min. Individuals treated with doses >40 ppm of either EO significantly decreased their ambulation speed and the percentage of total time ambulating compared to controls. TUR 80 ppm decreased their ambulation even sooner than POL 80 ppm, when compared to their respective controls. These findings are consistent with the neurotoxic effect against insects attributed to $\alpha$-Thujone, a main component of both EO. A detrended fluctuation fractal analysis evaluating the complexity and organisation of the temporal pattern of locomotion showed fractal patterns in all animals. Both sublethal and lethal doses of TUR and POL increased the complexity of ambulation. Interestingly, for POL 20 ppm, an increase in complexity was observed, while no changes in general activity were detected, suggesting that fractal analysis may be more sensitive to detect behavioural changes than general activity evaluation.

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## Introduction

Mosquitoes are insects of sanitary and economical importance both due to their irritant or allergenic bites and to the parasites they transmit (Snow et al. 2005). Vector control remains a primary element in the current global strategy for the control of vector-borne diseases (Zaim and Jambulingam 2004). The search for natural larvicides and efficient pesticide substances with low environmental toxicity is increasing (Chantraine et al. 1998; Kabir et al. 2003; Silva et al. 2004). Essential oils extracted from aromatic plants are good candidates, since they are, in some cases, highly bioactive, easily available in tropical countries and economically viable (Silva et al. 2008; Gleiser and Zygadlo 2008). Besides, they may be biodegraded to non-toxic compounds, minimising the accumulation of noxious residues in the environment (Macedo et al. 1997; Choochote et al. 2005). Essential oils of several plant species have shown bioactive effects on insects (Carvalho et al. 2003; Cavalcanti et al. 2004; Amer and Mehlhorn 2006; Gleiser and Zygadlo 2007; Gillij et al. 2008; Gleiser and Zygadlo 2008). In particular, *Lippia turbinata* Griseb and *Lippia polystachya* Griseb are lethal against mosquito larvae at doses ≥80 ppm after 1 h of exposure to the oils. Toxicity of both essential oils (EO) may be attributed to their main components $\alpha$-Thujone (representing 48% of the EO in

*L. turbinata* and 69% in *L. polystachya*) and Carvone (17.4 and 12%, respectively) (Gleiser and Zygadlo 2007). Acute doses of *L. polystachya* (synonym=*Aloysia polystachya* (Griseb.)) extract decreased total motility, locomotor activity and rearing behaviour and showed sedative, anxiolitic and antidepressant effects in rats (Mora et al. 2005). Individually, the compound α-Thujone has been found to have antinociceptive activity (Rice and Wilson 1976) and convulsionant activity in mice (Hold et al. 2000) and neurotoxicity in rats (Millet et al. 1981). In addition, it has been classified as a neurotoxic insecticide (Hold et al. 2001; Ratra and Casida 2001) and pesticide (Duke 2004). These effects could partly be explained by α-Thujone acting on $GABA_A$ receptors. α-Thujone is a competitive inhibitor of [$^3$H]EBOB binding (i.e. of the non-competitive blocker site of the GABA-gated chloride channel) and is a reversible modulator of the $GABA_A$ receptor (Hold et al. 2000). In addition, in human, the $GABA_A$ receptor α-Thujone, has a low potency overall, requiring 10,000 nM for 33% inhibition of Cloride uptake (Ratra and Casida 2001). Carvone has also shown effects on the nervous system, mainly an antinociceptive action associated to a decrease of the excitability of peripheral nerves, without depressing the central nervous system nor muscle relaxation (Ramos Gonçalves et al. 2008).

Most work studying the effects of essential oils on mosquito larvae focus on lethal doses and time to achieve lethal effects, but mode of action are in general not fully elucidated (Shaaya and Rafaela 2007). The study of how larvicides affect locomotor behaviour and, in particular, its temporal pattern, may aid in understanding their physiological effects on the insects. Besides, changes in mosquito behaviour as a result of exposure to sublethal doses of a larvicide may influence their vulnerability to other factors affecting their survival.

According to Brackenbury (2001), locomotion is not an end per se but a means to achieve a goal, normally food acquisition, finding a mate, finding shelter against adverse environmental conditions or predators or for oviposition. Diving behaviour of mosquito larvae and pupae has been proposed as an anti-predator response, a mechanism to avoid mechanical shock from rain drops, a way to avoid flushing by water currents and necessary for bottom-feeding (Romoser and Lucas 1999; Tuno et al. 2004, 2007). In addition, diving behaviour of larvae (Tuno et al. 2007) and pupae (Romoser and Lucas 1999) has been observed to vary between and within species and, in some cases, depends on the developmental stage (Strickman 1989; Tuno et al. 2007). Moreover, depth, duration and/or quantity of dives may be influenced by factors such as amount of light, food availability (Workman and Walton 2003) and water turbidity (Tuno et al. 2004) and depth (Tuno et al. 2007).

In recent years, fractal analysis, such as detrended fluctuation analysis (DFA), has emerged as an effective tool to measure the temporal organisational complexity of a particular behaviour (Ho et al. 1997; Peng et al. 2000). Because this analysis considers the complete sequence of a given behaviour, they provide additional and complementary information to the standard (conventional) behavioural analyses that often summarises a specific behavioural activity performed without considering the structure of the activity sequence (Kembro et al. 2008a). This analysis may be useful to detect subtle changes in behavioural patterns due to sublethal doses of the toxic compound that could go undetected by conventional behavioural analyses, such as total distance ambulated or number and duration of dives.

The present work evaluated whether the EO of *L. turbinata* and *L. polystachya* at doses ranging from sublethal to lethal (20, 40 and 80 ppm) modify the temporal pattern of locomotion of *Culex quinquefasciatus* larvae using both conventional behavioural analyses and fractal analysis.

## Materials and methods

### Essential oils

The essential oils of *L. polystachya* (POL) and of *L. turbinata* (TUR) were extracted by hydro-distillation for 2 h (Clevenger type apparatus, with a separated extraction chamber). The resulting EO were dried over anhydrous-sodium sulphate to extract the oil and analysed with gas–liquid chromatography and mass spectrometry (Perkin Elmer Q-700 equipment). A polar column of 30 m×0.25 mm×0.25 μm (CBwax) and an apolar column of 30 m×0.25 mm×0.25 μm (DB-5) were successively used. Both columns were held for 3 min at 60°C and then programmed at 4°C/min to 240°C. The injector was held at 250°C. Helium was used as the carrier gas at a 0.9 ml/min caudal. The mass spectrum was obtained at a 70-eV ionisation voltage. The identification of components was done with the use of the NIST (version 3.0) database, bibliography (Adams 1969) and authentic standards. The concentration of each EO component was calculated from the integration area of the chromatographer (Gleiser and Zygadlo 2007).

### Procedure

Fourth-instar *C. quinquefasciatus* larvae reared in the laboratory (following Gerber et al. 1994) were exposed to test concentrations of 20, 40 and 80 ppm of *L. polystachya* or *L. turbinata* essential oil in dechlorinated tap water (POL 20, POL 40, POL 80, TUR 20, TUR 40 and TUR 80 ppm, respectively), with ethanol (1%) as control (the essential oils were first dissolved in ethanol (99.0%) because they are insoluble in water; Gleiser and Zygadlo 2007).

Seven larvae (one from each treatment) were individually and simultaneously tested in identical experimental glass boxes (4×4×16 cm) with one transparent and three white vertical walls. The seven boxes were lined approximately 3 cm apart, so that each larva could not visualise the larvae in the next neighbouring box. The assay was recorded during 40 min with a video camera installed directly in front of the boxes. During the observation period, the experimenter remained out of the room to minimise disturbances.

The experiment was carried out in two consecutive days, and a total number of 63 larvae were tested. Records were made between 8:00 A.M. and 5:00 PM. For data analysis, the observation period considered was 2,400 s. The locomotor activity of the larva was determined from the video recordings using ANY-maze behavioural tracking software. The position of the animal was recorded at 1/3-s time interval. A value of 1 represented the animal moving during a time interval (and the distance ambulated was also recorded), or alternatively a 0, representing immobility, was assigned if the animal did not move during a time interval. Thus, a time series of the locomotor activity during the 40-min test period (i.e. 7,200 time intervals) was constructed for each larva. In addition, three zones with equal dimensions were defined within a box: surface, centre and bottom. The zone that the larva was situated in during each time interval was also registered. Four animals could not be included in the analysis because of failures during video tracking.

The following variables were measured for each larva:

- *Total distance ambulated (cm):* the total (cumulative) distance ambulated by the animal during the test period
- *Average ambulation speed (cm/s):* distance ambulated divided by the time spent ambulating
- *Percentage of total time ambulating:* $t_{\%} = \frac{\Sigma t_i}{N}.100$, where $t_i$ is the time interval (s) in which the animal is ambulating and $N$ is the total number of data points of the test (s)
- *Diving duration (s):* time since leaving the surface zone, reaching the bottom zone and returning to the surface zone
- *Number of dives:* number of times the animal leaves the surface zone, reaches the bottom zone and then returns to the surface zone
- *Percentage of animals that dived:* percentage of animals that dived at least once during the assay period
- *Ambulation event:* interval of time (>0.6 s) in which the animal moves continuously
- *Immobility event:* interval of time (> 0.6 s) in which the animal remains immobile
- *Number of ambulatory/immobility events*
- *Average duration of an ambulatory event:* sum of the duration of each individual ambulation event divided by the number of ambulatory events
- *Distance moved per ambulatory event (cm):* sum of the distance moved in each individual ambulatory event divided by the number of ambulatory events
- *Percentage of time ambulating at 5 min intervals*
- *Proportion of time within each zone:* Proportion of the total test time that the animal was found within the surface, centre and bottom zone

## Data analysis

*Detrended fluctuation analysis* The locomotor activity time series of the larvae were submitted to a fractal analysis, as described in detail elsewhere (Kembro et al. 2008a,b). This method has been introduced by Peng et al. (1994) to analyse the temporal organisation of behavioural time series. We applied DFA method to the complete locomotion time series.

Briefly, in DFA, a self-similarity parameter ($\alpha$ value) is calculated that is related to the autocorrelation structure of the time series. If $\alpha$=0.5, then the series is uncorrelated (random) or has short-range correlations (i.e. the correlations decay exponentially), whereas the situation of $0.5<\alpha<1$ indicates long-range autocorrelation (correlations decaying as a power-law) exist, meaning that ongoing behaviour is influenced by what has occurred in the past (Kantelhard et al. 2001). $\alpha$ value provides a measure of the “roughness” of the original time series: the larger the value of $\alpha$, the smoother the time series (Peng et al. 2000). Furthermore, in DFA, the exponent $\alpha$ is inversely related to a typical fractal dimension, so in this case, the value increases with increasing regularity (or decreasing complexity) in the time series (Rutherford et al. 2003).

DFA was calculated with different detrending orders, and we selected the lowest detrending order (DFA2) that was able to eliminate trends in all data series. We used this order for comparison of the locomotor pattern of larvae treated with the essential oils. In order to accurately use DFA, a given time series must not present periodic oscillations (Kembro et al. 2008b). Thus, the presences of periodic oscillations were also evaluated using the power spectrum analysis tool from SigmaPlot (SPSS 2001), and no periodic oscillations were observed in our data.

*Statistical analysis* An analysis of variance (ANOVA) was used to evaluate the effect of the essential oil treatment on total distance ambulated, average ambulation speed, percentage of total time ambulating, dive duration, number of dives, number of immobility events, number of ambulatory events, distance moved per ambulatory event and average duration of an ambulatory event. All parameters except average ambulation speed were transformed to ranks to fit the ANOVA assumptions (Shirley 1987). Fisher’s least significant difference tests were used for post hoc comparisons of group means. To estimate dive duration, only animals that dived (i.e. that reached the bottom) at least once were considered in this parameter estimation. A two-sample proportion test was used to compare the proportion of animals that dive between groups (Statistix 2000).

A repeated measures ANOVA examined the effect of essential oil treatment on the proportion of time within each zone (surface, center and bottom) and the percentage of time ambulated at 5-min intervals. The proportion of time in each zone were transformed to square root, and the percentage of time ambulated at 5-min intervals were transformed to ranks to fit better the ANOVA assumptions. Fisher's test was also used for post hoc comparisons.

A probability level of ≤0.05 was considered to represent significant differences.

## Results

A one-way ANOVA of the ambulation of mosquito larvae exposed to essential oils showed a significant effect of essential oil treatment regarding total distance ambulated (Fig. 1a; $F_{6,52}$=2.34; $p$=0.04), average ambulation speed (Fig. 1b; $F_{6,52}$=4.45; $p$=0.01), percentage of total time ambulating (Fig. 1c; $F_{6,52}$=2.74; $p$=0.02), number of dives (Fig. 1e; $F_{6,52}$=3.46; $p$=0.006), number of ambulatory events (Fig. 2b; $F_{6,52}$=2.30; $p$=0.05), distance moved per ambulatory event (Fig. 2c; $F_{6,52}$=4.31; $p$=0.001) and average duration of an ambulatory event (Fig. 2d; $F_{6,52}$= 3.96; $p$=0.002). A trend towards significant differences between treatments was observed in dive duration (Fig. 1d; $F_{6,19}$=2.12; $p$=0.10), and no significant differences were detected in the number of immobility events (Fig. 2a; $F_{6,52}$=

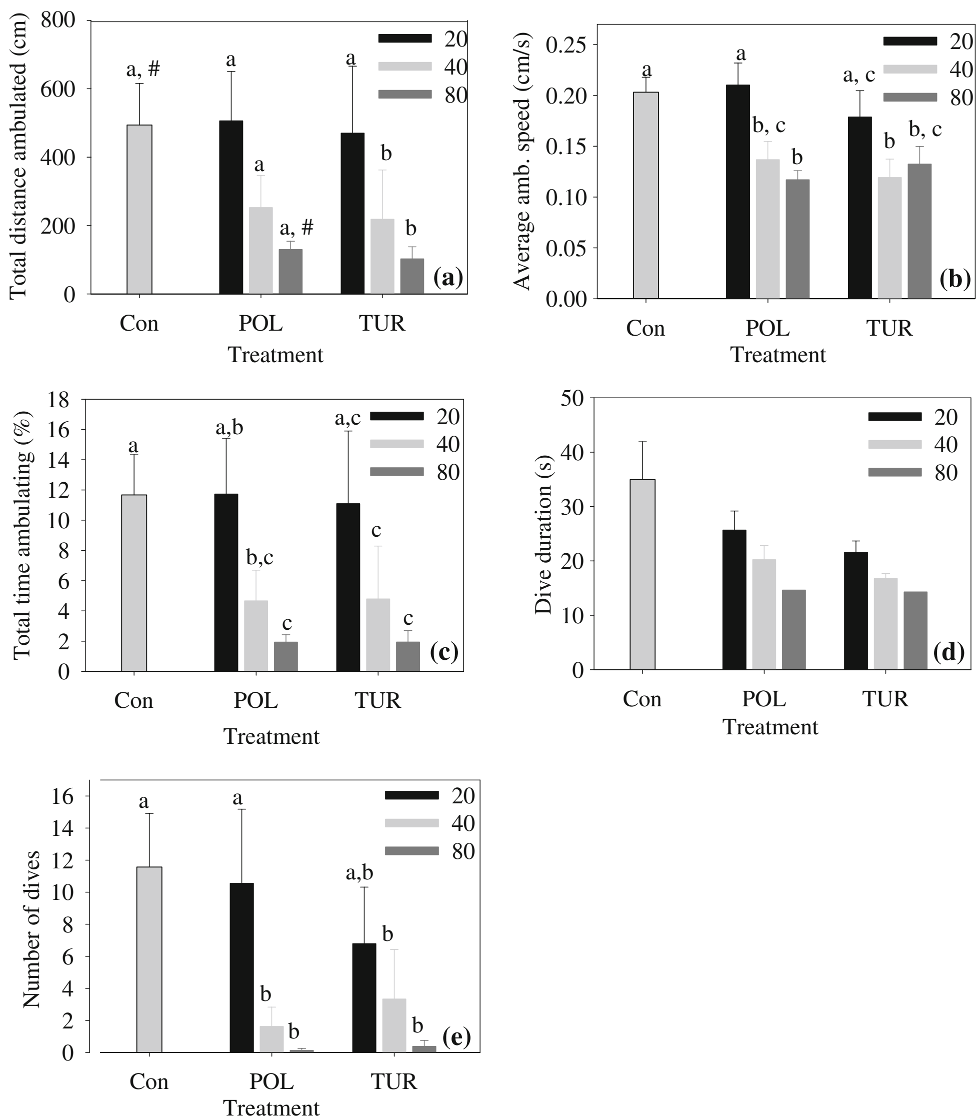


**Fig. 1** Parameters of activity of *Culex quinquefasciatus* from the control (*Con*) group and from the larvae treated with essential oil of *Lippia polystachya* or *Lippia turbinata* at concentrations of 20, 40 and 80 ppm (POL 20, 40 and 80, and TUR 20, 40 and 80, respectively). **a** Total distance ambulated, **b** average ambulation speed, **c** total time ambulating, **d** dive duration and **e** number of dives. Treatments without a common letter differ significantly with a $p<0.05$; $^{\#}p=0.07$

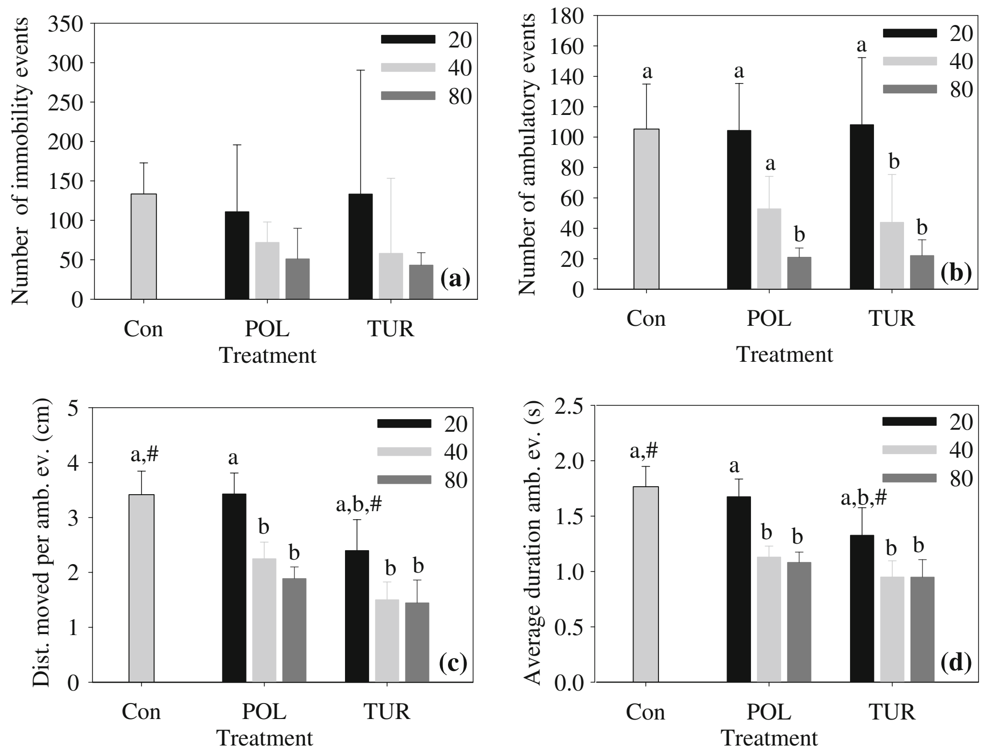


**Fig. 2** Ambulatory and immobility events (time >0.6 s in which *Culex quinquefasciatus* larva either continuously moves or continuously remains immobile, respectively) in the control (*Con*) group and in larvae treated with essential oil of *Lippia polystachya* or *Lippia turbinata* at concentrations of 20, 40 and 80 ppm (POL 20, 40 and 80, and TUR 20, 40 and 80, respectively). **a** Number of immobility events, **b** number of ambulatory events, **c** average distance moved per ambulatory event and **d** average duration of ambulatory events. Treatments without a common letter differ significantly with a $p<0.05$; $^{\#}p=0.08$

1.56; $p=0.18$). Table 1 shows that a lower proportion of larvae treated with POL 80, TUR 40 and TUR 80 ppm dive in comparison to the control group.

The effect of essential oil treatment on the temporal distribution of larval ambulation was assessed (Fig. 3). A significant main effect was observed for treatment ($F_{6,52}$= 2.75; $p=0.02$), time of exposure ($F_{7,364}$=19.20; $p<0.0001$) and a tendency ($F_{42,364}$=1.38; $p=0.07$) to treatment×time interaction was observed. Animals treated with POL 80 ppm ambulated a shorter proportion of time during the 0–5-min interval than the rest of the treatment groups ($p<0.05$). Larvae treated with POL and TUR 40 and 80 ppm spent less time ambulating after 10 min than the control group.

**Table 1** Effect of treatment with essential oils of *Lippia polystachya* and of *Lippia turbinata* at concentrations of 20, 40 and 80 ppm (POL 20, 40 and 80; TUR 20, 40 and 80, respectively) on the proportion of *Culex quinquefasciatus* mosquito larva that dive at least once

| Treatment | Animals that dive (%) | *Z* | *p* value | *n* |
|---|---|---|---|---|
| Control | 86 | | | 7 |
| POL 20 | 78 | 0.40 | 0.34 | 9 |
| POL 40 | 50 | 1.46 | 0.07 | 8 |
| POL 80 | 11 | 2.98 | 0.01 | 9 |
| TUR 20 | 56 | 1.24 | 0.10 | 9 |
| TUR 40 | 22 | 2.52 | 0.01 | 9 |
| TUR 80 | 12.5 | 2.84 | 0.01 | 8 |

*p value* probability value estimated with a proportion test resulting from the comparison of the proportion of animals diving from each treatment group compared to the control group; *n* samples size

The effect of essential oil treatment on the proportion of total time that a larva was found in each zone (Fig. 4) was analysed. A significant main effect of zone factor ($F_{2,104}$= 43.14; $p<0.0001$) and a significant interaction between treatment and zone ($F_{12,104}$=2.11; $p=0.02$) were observed. All treatments stayed a longer proportion of time in the surface compared to the central zone ($p<0.05$), and except TUR 40 and TUR 80, all stayed longer in the surface than in the bottom ($p<0.05$). POL 40, TUR 40 and TUR 80 stayed longer in the bottom compared to the central zone ($p<0.05$).

The temporal organisation of the locomotion of mosquito larvae in water or water treated with an essential oil was evaluated with DFA2. A lineal relation was observed between log fluctuation and log window size ($R^2>0.99$), for window size smaller than 60 s, in all but two of the larvae assessed. Significant differences were found in the $\alpha$ values between the essential-oil-treated groups ($F_{6,49}$=3.10; $p$= 0.01). Higher values of $\alpha$ (closer to 1) were seen in control and POL 20 ppm groups compared to POL 40, POL 80, TUR 20 and TUR 40 ppm ($p<0.04$). Furthermore, the $\alpha$ values of the control group were significantly higher ($p$= 0.03) and those of POL 20 ppm tended to be higher ($p$= 0.07) than the values found for TUR 80 ppm group (Table 2). In all, results show that POL and TUR increase the complexity of the ambulatory pattern of the larvae.

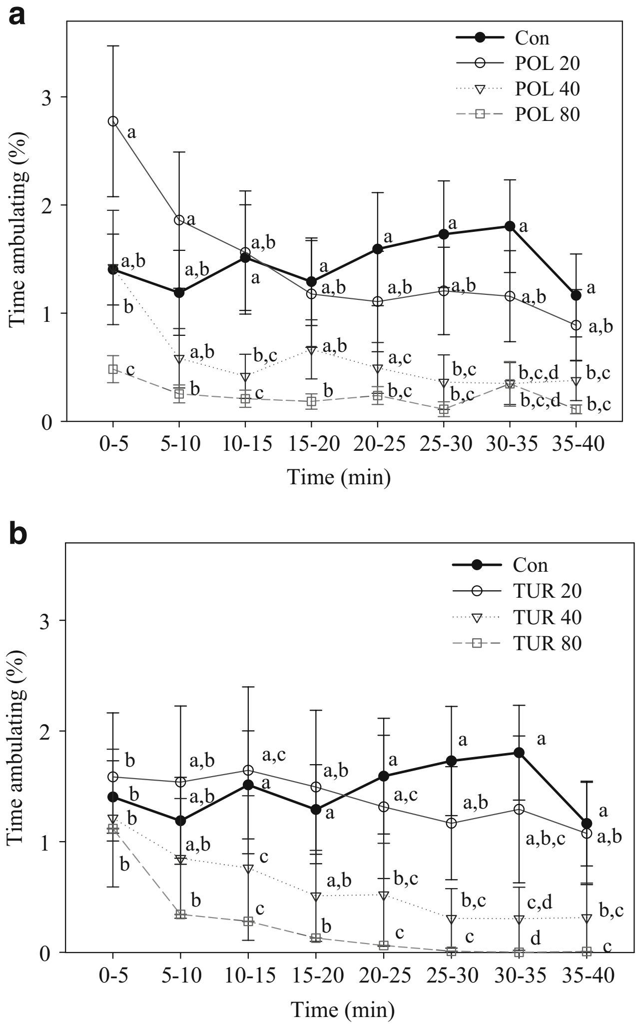


**Fig. 3** Percentage of time ambulated in five min intervals. Control (*Con*); **a** essential oil of *Lippia polystachya* at concentrations of 20, 40 and 80 ppm (POL 20, 40 and 80, respectively); **b** essential oil of *Lippia turbinata* at concentrations of 20, 40 and 80 ppm (TUR 20, 40 and 80, respectively). Within the 5-min interval, treatments without a common letter differ significantly with a $p<0.05$

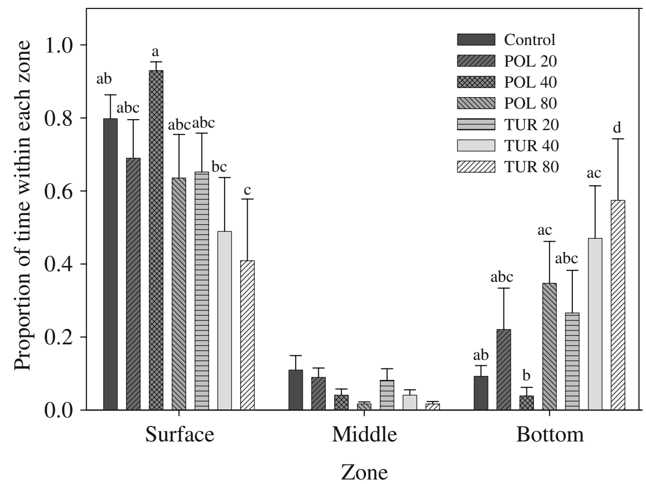


**Fig. 4** Proportion of total assay time within each zone (surface, center and bottom) of *Culex quinquefasciatus* mosquitoes from the control group (*Con*) and treated with essential oil of *Lippia polystachya* at concentrations of 20, 40 and 80 ppm (POL 20, 40 and 80, respectively), and essential oil of *Lippia turbinata* at concentrations of 20, 40 and 80 ppm (TUR 20, 40 and 80, respectively). In a zone, treatments without a common letter differ significantly with a $p<0.05$. $^{\#}p=0.08$; $^{*}p=0.07$; $^{+}p=0.09$. Significant differences are observed ($p<0.05$) between the proportion of time spent in the surface zone in comparison to the bottom zone in control, POL 20, 40 and 80 and TUR 20 groups

## Discussion

Larvae of the *Culex* genus have been categorised as collector and filter feeding organisms that feed in water columns (Dahl et al. 1988; Merritt et al. 1992). *Culex pipiens* larvae rest under the water surface connected by their respiratory siphon or glide propelling themselves by their mouth brushes, and when disturbed, they make a quick dive by vigorously flexing their body from side to side (Brackenbury 2001). Then, they may return to the surface by flotation because they are less dense than water. In the present study, where animals were not disturbed, had no food source and conditions throughout the test were constant, we observed a diving behaviour comparable to that described for most *C. quinquefasciatus* larvae. Dive duration (voluntarily diving to the bottom and returning to the surface, <10 cm below the surface interface) was on average 24.6 s, most times no longer than 60 s and with a maximum time of 140 s. This is consistent with reports from Workman and Walton (2003) where the duration of deep diving of *C. quinquefasciatus* lasted on average 30 s in a low food availability environment. However, in that work, dive duration was significantly shorter, approximate-

**Table 2** Values of the self-similarity parameter ($\alpha$) for windows smaller than 60 s, in *Culex quinquefasciatus* larvae from the control and treated groups: *Lippia polystachya* essential oil at concentrations of 20, 40 and 80 ppm (POL 20, 40 and 80, respectively) and *Lippia turbinata* essential oil at concentrations of 20, 40 and 80 ppm (TUR 20, 40 and 80, respectively)

| Treatment | Alpha | *n* |
|---|---|---|
| Control | $0.99\pm0.04^{a}$ | 7 |
| POL 20 | $0.97\pm0.03^{a,b,*}$ | 9 |
| POL 40 | $0.85\pm0.02^{c}$ | 7 |
| POL 80 | $0.83\pm0.04^{c}$ | 9 |
| TUR 20 | $0.66\pm0.13^{c}$ | 9 |
| TUR 40 | $0.69\pm0.12^{c}$ | 8 |
| TUR 80 | $0.84\pm0.05^{b,c,*}$ | 7 |

Values are mean±standard error. *Z* and *p* values were estimated with a proportions test between the treated and control groups in the proportion of animals with an $\alpha$ value of DFA approaching 1. Treatments without a common letter differ significantly ($p<0.05$)
*n* sample size
$^{*}p=0.07$

ly 3.5 s in food enriched environments. Much longer diving times were found in three species of *Anopheles*, for instance 30 min in *Anopheles funestus*, when animals were forced to submerge (Tuno et al. 2007). Thus, the duration and number of dives reported depends not only on inter-specific differences but also on the nature of the tests, amount of food, light intensity, etc. In the context of our experiment, it is likely that diving of the control animals represents a search for food because food is frequently found at a higher density on the bottom in shallow water (Tuno et al. 2007). Animals treated with essential oils of the POL 80, TUR 40 and TUR 80 ppm groups tended to decrease diving duration, dived significantly fewer times and a smaller percentage of animals dived compared with the control.

Insecticidal action on *C. quinquefasciatus* of both POL and TUR has been shown (Gleiser and Zygadlo 2007). Mortality of larvae exposed to doses of 80 ppm or higher concentration of either oil were significantly higher than the control mortality since the first hour post-treatment. Our behavioural analyses showed that 80 ppm and a lower dose of 40 ppm of POL and TUR significantly affect locomotion because they decreased ambulation speed and time ambulating. Distance moved decreases with TUR 40 and 80 ppm and tends to decrease with POL 80 ppm. Toxicity of both oils may be attributed to their main constituents α-Thujone and Carvone (Gleiser and Zygadlo 2007). α-Thujone has been shown to be an inhibitor of the $GABA_A$ receptor in humans (Ratra et al. 2001) and mice (Hold et al. 2000), which could explain the decrease in the locomotor activity of the larvae. This contention is supported by evidence that, in mice, the toxicity of α-Thujone is alleviated by diazepam, phenobarbital and ethanol (Hold et al. 2000). In addition, *Drosophila* with a single point mutation in the Rdl GABA receptor subunit of Ala302 to Ser conferring resistance to dieldrin (ffrench-Constant and Roush 1991; ffrench-Constant et al. 1993) is also resistant to α-Thujone, albeit to a lesser degree (Hold et al. 2000).

A more detailed analysis of the percentage of time ambulated was carried out by sorting the assay in 5-min intervals. This showed that animals treated with POL 80 ambulated less than the control since the beginning of the trial, while TUR 80 decreased ambulated time after 10 min, and became practically 0% after 25 min. The significant difference found between POL 80 and TUR 80 in the first 5 min of the trial indicates that high doses of POL produce an immediate and faster reaction on the organism than TUR. In POL and TUR at 40 ppm, the decrease in time ambulated compared to the control was detected later than at 80 ppm, after 25 and 20 min, respectively.

When the temporal organisation of ambulation is analysed, it is first observed that POL 80, TUR 40 and TUR 80 ppm decrease the number of ambulation events, but interestingly, the number of immobility events was not significantly affected with either POL or TUR. Second, doses above 40 ppm of POL and of TUR decreased average distance moved per ambulatory event and average duration of ambulatory event (it should be considered that this two variables are independent of the immobility period). Third, DFA of the locomotor activity of all but two of the larvae tested showed long-range correlations consistent with a fractal locomotor pattern. In other words, the locomotor behaviour at one moment is statistically correlated with the locomotor behaviour at a relatively remote time, and its influence decays in a scale-invariant, fractal manner. Long-range autocorrelations were also found in minnow reproductive behaviour (Alados and Weber 1999), chimpanzee social behaviour (Alados and Huffman 2000), domestic fowl walking and vigilant behaviour (Rutherford et al. 2003; María et al. 2004), Japanese quail home cage ambulatory behaviour (Kembro et al. 2007, 2008a,b) and particularly interesting for the present study in locomotor (flying) behaviour of adult *Aedes aegypti* mosquitoes (Kembro, Marin and Gleiser, unpublished data). Moreover, these correlations are susceptible to change when larvae are exposed to sublethal and lethal doses of the essential oils. The control and POL 20 ppm groups presented $\alpha$ values close to 1 (1/*f*-like noise) for windows smaller than 60 s, which could be interpreted as a "compromise" between the complete unpredictability of white noise ($\alpha$=0.5) and the much smoother "landscape" of Brownian noise ($\alpha$=1.5; Peng et al. 2000). POL 40, POL 80, TUR 20 and TUR 40 showed significantly lower values of $\alpha$. At the same time, the $\alpha$ value of TUR 80 was significantly lower than the control and tended to be lower than POL 20 ppm. In all, these results clearly suggest that treatment with POL and TUR alters the behaviour of the larvae increasing the complexity of their locomotion pattern (making it a more unpredictable pattern). Interestingly, for POL 20 ppm, an increase in complexity was observed while no changes in general activity were detected, suggesting that fractal analysis may be more sensitive to detect behavioural changes produced by the toxicity of the essential oils than conventional behavioural analyses.

In conclusion, our results not only support a neurotoxic effect on mosquito larvae of the EO of *L. polystachya* and *L. turbinata* but also highlight the usefulness of fractal analyses as a complementary tool to detect behavioural changes. Considering that larvae in the field may also be exposed to sublethal doses of larvicides, such as the ones used in this study, changes in the pattern of behaviour resulting from exposure to those doses may compromise the survival of the larva (for example, while avoiding predation or flushing). Studies aiming to evaluate the influence of an altered complexity of the locomotor behaviour (predictability of its pattern) on the survival of the larvae are currently underway.

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