

Rotation of male genitalia in various species of phlebotomine sandfly

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Abstract. Phlebotomine sandflies, vectors of *Leishmania* (Kinetoplastida: Trypanosomatidae) parasites that affect millions of people worldwide, breed in terrestrial biotopes. As immature stages are rarely accessible, the detection of their natural breeding sites is primarily based on findings of juvenile males with unrotated external genitalia. In males, permanent 180° rotation on the longitudinal body axis occurs soon after eclosion; however, no study has as yet addressed this aspect in detail. The present study describes the timing and duration of the rotation of male external genitalia in eight highly medically important sandfly species belonging to the genera *Sergentomyia*, *Lutzomyia* and *Phlebotomus* (all: Diptera: Psychodidae), kept under controlled laboratory conditions. The average duration of rotation was species-specific and varied from 12 h in *Sergentomyia schwetzi* to 33 h in *Phlebotomus sergenti*. Significant differences in rotation times were found among species, even between two closely related species of the subgenus *Larrousius*, *Phlebotomus orientalis* and *Phlebotomus tobbi*. The rotation of genitalia in all three studied genera was randomly oriented and similar numbers of clockwise and counter-clockwise events were observed. The study also addresses the effects of some external factors. In all species studied, rotation was not affected by the time of day of eclosion. Similarly, no differences in total rotation time were found between *Phlebotomus papatasi* males maintained at 25 and 20 °C, respectively. The present findings will assist in the search for natural breeding sites and in studies aimed at elucidating strategies for integrated sandfly and leishmaniasis control.

Key words. *Lutzomyia*, *Phlebotomus*, *Sergentomyia*, biology and behaviour, laboratory colony, leishmaniasis.

Introduction

Leishmaniasis is an important vector-borne protozoal disease that endangers more than 350 million people in almost 100 countries worldwide (Alvar *et al.*, 2012). Its causative agents, parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), are transmitted exclusively by blood-sucking females of species of phlebotomine sandfly (Diptera: Psychodidae). The vectors of human leishmaniasis belong to two genera, *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Species of an Old World genus *Sergentomyia* transmit *Leishmania* spp. in reptiles and are suspected as a vector of human leishmaniasis in some foci (reviewed by Sadlova *et al.*, 2013).

Adult sandflies are tiny nocturnal insects that normally do not fly very far and remain within several hundred metres of their breeding sites. Because of their limited flight range, the transmission of harboured pathogens is often geographically discontinuous, with characteristically small and separate foci (reviewed by Killick-Kendrick, 1999).

Leishmaniasis control strategies focused on the disease vectors are subject to considerable difficulties. Sandflies, unlike mosquitoes, do not breed in water. Only small numbers of larvae have been recovered from natural habitats and knowledge of the ecology of immature stages in field conditions and the distribution of sandfly breeding sites is still insufficient. Sandfly females oviposit in specialized ecological niches in which larvae

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feed on composting organic matter of animal and plant origin (Killick-Kendrick, 1999; Feliciangeli, 2004). Searches for sandfly larvae in appropriate microhabitats are seldom successful; therefore, the most common approach to the identification of breeding sites involves the use of emergence traps (reviewed by Moncaz *et al.*, 2014a, 2014b). This method was designed to capture sandfly adults emerging from their putative breeding sites in suspect habitats. However, sandflies captured by emergence traps cannot automatically be considered as newly emerged individuals; they may also represent resting adults. The only possible method of distinguishing sandfly resting sites from their breeding sites is by grading the ages of captured sandfly males. As age-grading based on histology (Schlein & Gratz, 1972) is not suitable for this purpose, the method of choice is based on assessing the rotation of external genitalia in males (Davis, 1967). Sandfly males have relatively large terminalia on the end of the abdomen. This clasping structure is composed of a large basal coxopodite (coxite) and a smaller citron-shape stylopodite (style) with conspicuous spines. The surstylus (surstyle) and paramere lie in opposition.

Recently, assessment of the rotation of external genitalia in males was successfully used to search for breeding sites of two important sandfly vectors of human leishmaniasis, *Phlebotomus sergenti* in Israel (Moncaz *et al.*, 2012) and *Phlebotomus orientalis* in Ethiopia (Moncaz *et al.*, 2014a), and also to search for *Sergentomyia* spp. breeding sites in Ethiopia (Moncaz *et al.*, 2014b). Males of *Lutzomyia longipalpis* with unrotated genitalia captured by emergence traps are also mentioned in the study by Casanova *et al.* (2013) conducted in Brazil.

The sandfly male ecloses from the pupa with unrotated genitalia, like other dipterans in which two primary types of rotation have been described: a permanent 360° circumversion in Cyclorhapha and a 180° inversion in Nematocera. The most plausible evolutionary explanation for the rotation of male genitalia is associated with a male-to-female mating position that requires abdominal twisting and flexing. Independent shifts in mating positions between male-dominated mating stances (with the male above the female) and tail-to-tail configurations have led to a number of apomorphic changes in the male terminalia, including their rotation (Sinclair *et al.*, 2013). Among the Nematocera, the 180° inversion may be temporary (e.g. in Tipulidae, Chironomidae and Ceratopogonidae), occurring only during copulation in the end-to-end position, or permanent (e.g. in Culicidae), occurring shortly after adult emergence (Griffiths, 1972). In phlebotomine sandfly males, permanent rotation occurs; the external genitalia rotate on the longitudinal body axis to reach their mature, 180° rotated position (Griffiths, 1972; Just, 1973). Therefore, males with unrotated or partially rotated external genitalia can be considered to have been captured during their first night of activity as adults and thus the location of their capture can reveal their breeding site (Moncaz *et al.*, 2012, 2014a, 2014b).

The time of initiation and overall duration of the rotation of genitalia may vary according to insect species and external conditions (Provost *et al.*, 1961); however, as yet no study has systematically addressed this topic in sandflies. Knowledge of the timing of rotation in sandfly males may help in the search for breeding sites and contribute towards the development of integrated sandfly and leishmaniasis control strategies. In the

Table 1. Genera, subgenera and species of sandfly males in which the timing of genitalia rotation was analysed.

Genus	Subgenus	Species	Origin of colony
<i>Lutzomyia</i>	<i>Lutzomyia</i>	<i>L. longipalpis</i>	Brazil, Jacobina, 1991
<i>Sergentomyia</i>	<i>Sergentomyia</i>	<i>S. schwetzi</i>	Ethiopia, 2012
<i>Phlebotomus</i>	<i>Adlerius</i>	<i>P. arabicus</i>	Israel, 2002
	<i>Larrousius</i>	<i>P. orientalis</i>	Ethiopia, 2008
		<i>P. tobbi</i>	Turkey, Adana, 2005
	<i>Euphlebotomus</i>	<i>P. argentipes</i>	India, 2008
	<i>Paraphlebotomus</i>	<i>P. sergenti</i>	Turkey, Sanliurfa, 1998
	<i>Phlebotomus</i>	<i>P. papatasi</i>	Turkey, Adana, 2005

present study, the timing and duration of the rotation of the external male genitalia in eight medically important sandfly species were analysed. In addition, the influence of ambient temperature and time of day of adult emergence on this rotation were studied.

Materials and methods

Males of eight sandfly species belonging to three genera were analysed. These included *L. longipalpis*, *Sergentomyia schwetzi* and six species of the genus *Phlebotomus* belonging to five subgenera (Table 1).

Colonies were maintained under standard conditions as previously described (Volf & Volfova, 2011). The same temperature (25 ± 1 °C), photoperiod (LD 12:12 h) and constant access to cottonwool soaked in a 50% sugar solution were used in studies on genitalia rotation. In the single experiment focused on the influence of ambient temperature, pupae and adults were maintained at either 25 ± 1 or 20 ± 1 °C.

In each experiment, 50 pupae were individually placed into glass vials (25 mm in diameter, 55 mm in height) with a folded filter paper and a permeable lid. The vials were maintained in a humid chamber as described by Killick-Kendrick & Killick-Kendrick (1991). To reduce the time pupae spent in vials, 'old' pupae with darker bodies and silvery wing rudiments were selected from the breeding pots. To increase the proportion of males among emerging individuals, pupae with narrower and more bent abdomens were selected. The experiment was repeated twice or three times for each sandfly species.

Each experiment lasted at least 96 h. During this period, pupae and subsequently emerged males were checked regularly in glass vials under the stereomicroscope at 1-h intervals during the day and 2-h intervals at night. Four developmental categories were classified and recorded (Fig. 1): time of eclosion (the genitalia are unrotated); initiation of rotation; rotation of genitalia by 90°, and rotation of genitalia by 180° (termination of rotation). Only data for males with fully rotated genitalia, for which information on all four of these stages was available, were included in the final statistical analysis. Analyses were conducted using one-way analysis of variance (ANOVA) in STATISTICA Version 6.1 (StatSoft, Inc., Tulsa, OK, U.S.A.).

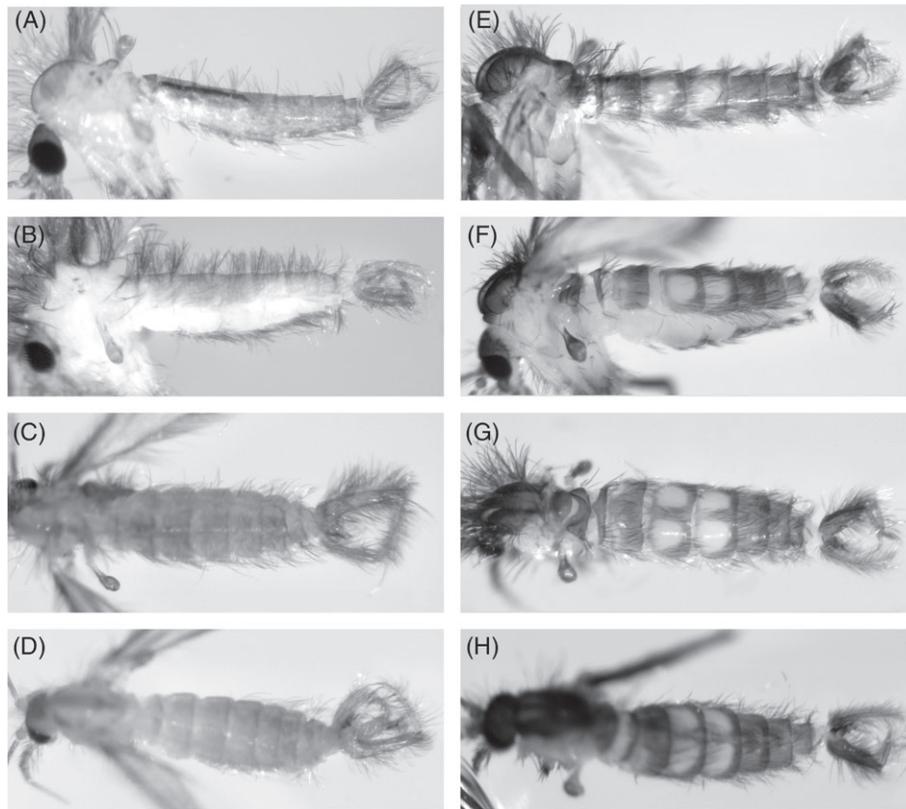


Fig. 1. Rotation of sandfly male genitalia in (A, B) *Phlebotomus orientalis*, (C, D) *Phlebotomus papatasi* and (E–H) *Lutzomyia longipalpis*. Three developmental categories were classified and recorded: unrotated genitalia at the time of eclosion (B, F); genitalia rotated 90° counter-clockwise (C, G) or clockwise (D, H), and the termination of rotation with genitalia rotated 180° (A, E).

For all specimens, the time of day of eclosion was recorded in order to analyse whether genitalia rotation is affected by the time of day at which the male hatches from the pupa. In addition, the influence of ambient temperature on genitalia rotation was investigated in a selected species, *Phlebotomus papatasi*. Two different temperatures were chosen: (a) $25 \pm 1^\circ\text{C}$ as an optimal temperature for sandfly development, and (b) $20 \pm 1^\circ\text{C}$ as a temperature at the lower edge of the range for rearing conditions.

Results

Duration of genitalia rotation

A total of 530 laboratory-reared males of eight sandfly species belonging to three genera (Table 1) were statistically analysed for genitalia rotation. Means, medians, variance (and minimal and maximal durations) and standard deviations (SDs) were calculated for the three stages of genitalia rotation [initiation, 90° rotation and termination (180° rotation)] (Table 2). Data on the most important parameter, the termination of rotation, are also summarized in Fig. 2.

Sandfly species differed significantly in all parameters studied. In some species, rotation was initiated quite early post-eclosion (*S. schwetzi*, 2.2 h; *P. orientalis*, 2.3 h; *P. papatasi*,

2.4 h), whereas in others the initiation of rotation was postponed (*L. longipalpis*, 3.1 h; *P. sergenti* and *Phlebotomus argentipes*, 4.3 h; *Phlebotomus tobbi*, 4.9 h) (Table 2). The longest mean duration of genitalia rotation, 32.9 h after emerging from pupae, was recorded in *P. sergenti*; the shortest mean rotation, which represented less than half of this time (12.0 h), was found in *S. schwetzi*. Within the genus *Phlebotomus*, the shortest duration of genitalia rotation (14.9 h) was found in *P. papatasi* (Table 2, Fig. 2).

Rotation was randomly oriented; similar numbers of clockwise and counter-clockwise rotation events were observed in members of all three genera studied (Fig. 1).

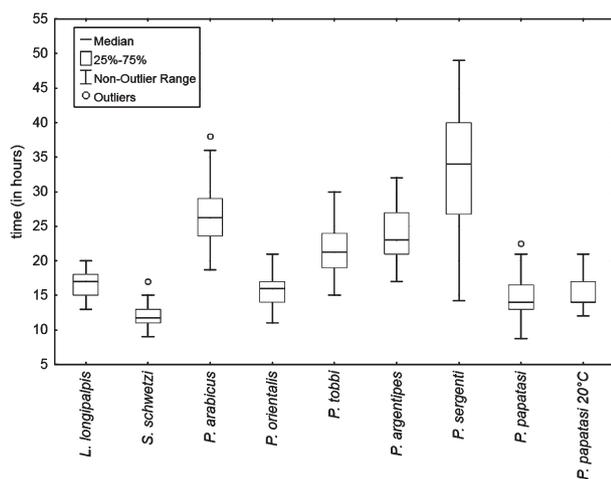
Effect of ambient temperature on genitalia rotation

The effect of ambient temperature on the duration of genitalia rotation was studied in *P. papatasi* maintained at 20 ± 1 and $25 \pm 1^\circ\text{C}$ (Table 2, Fig. 2). In sandfly males maintained at the lower temperature, the initiation of rotation was significantly postponed: rotation commenced at 3.2 h after emergence in $20 \pm 1^\circ\text{C}$ and at 2.4 h after emergence in $25 \pm 1^\circ\text{C}$ (ANOVA $F_{(1,110)} = 21.12$, $P < 0.01$). These males showed less variance in total rotation time (variance of 5.1 h in $20 \pm 1^\circ\text{C}$ and 11.5 h in $25 \pm 1^\circ\text{C}$), but, importantly, did not differ in the main variables

Table 2. Timing and duration (in hours, h) of male genitalia rotation in eight sandfly species of the genera *Lutzomyia*, *Sergentomyia* and *Phlebotomus* for three stages of rotation: initiation of rotation after emergence from pupa; genitalia rotation to 90°, and genitalia rotation to 180° (termination of rotation).

	<i>L. longipalpis</i>	<i>S. schwetzi</i>	<i>P. arabicus</i>	<i>P. orientalis</i>	<i>P. tobbi</i>	<i>P. argentipes</i>	<i>P. sergenti</i>	<i>P. papatasi</i>	<i>P. papatasi</i> (at 20 °C)
Males, <i>n</i>	55	60	50	85	55	55	55	60	55
Initiation	Mean, h	3.1	2.2	3.8	2.3	4.9	4.3	4.3	3.2
	Median, h	3.0	2.0	3.5	2.0	5.0	4.0	4.0	3.0
	Variance, h	0.9	0.8	1.3	0.5	2.6	1.1	1.3	0.9
	SD, h	0.9	0.8	1.1	0.7	1.6	1.1	1.2	0.9
90°	Mean, h	8.4	5.2	10.6	6.8	10.6	9.9	10.7	6.1
	Median, h	8.0	5.1	10.1	6.9	11.0	10.0	11.0	6.0
	Variance, h	2.7	1.4	7.8	2.2	8.0	5.3	15.2	3.9
	SD, h	1.7	1.2	2.8	1.5	2.8	2.3	3.9	1.9
180°	Mean, h	16.7	11.9	26.6	15.6	21.8	24.1	32.9	14.9
	Median, h	17.0	11.8	26.3	16.1	21.3	23.2	34.0	14.2
	Variance	4.1	2.6	20.9	7.5	10.1	17.5	84.7	11.5
	(min–max), h	(13–20)	(9–17)	(19–38)	(11–21)	(15–30)	(17–32)	(14–49)	(9–28)
SD, h	2.0	1.6	4.6	2.7	3.2	4.2	9.3	3.4	2.3

SD, standard deviation.

**Fig. 2.** Comparison of male genitalia rotation to 180° (representing the termination of rotation) in eight sandfly species.

studied, 90° rotation [5.7 h vs. 6.1 h (ANOVA $F_{(1,110)} = 0.49$, $P = 0.49$)] and termination of 180° rotation [15.2 h vs. 14.9 h (ANOVA $F_{(1,110)} = 0.35$, $P = 0.58$)] (Table 2, Fig. 2). Thus, the overall duration of rotation was the same in males tested in both ambient temperatures.

Effect of daytime eclosion on genitalia rotation

Emerging males were divided according to time of emergence within the 24-h cycle based on assumed circadian rhythm into four groups categorized by 6-h intervals: two light intervals (06.00–12.00 hours, 12.00–18.00 hours) and two dark intervals (18.00–00.00 hours, 00.00–06.00 hours). None of the three stages of genitalia rotation studied (initiation, 90° rotation,

180° rotation) differed significantly among males hatching in different intervals of the 24-h cycle in any of the sandfly species studied: *P. papatasi* (ANOVA $F_{(16,13)} = 1.43$, $P = 0.26$); *Phlebotomus arabicus* (ANOVA $F_{(19,10)} = 0.28$, $P = 0.99$); *P. orientalis* (ANOVA $F_{(18,11)} = 1.89$, $P = 0.14$); *P. tobbi* (ANOVA $F_{(15,11)} = 1.03$, $P = 0.493$); *P. argentipes* (ANOVA $F_{(17,10)} = 4.77$, $P = 0.11$); *P. sergenti* (ANOVA $F_{(16,17)} = 0.63$, $P = 0.823$); *S. schwetzi* (ANOVA $F_{(16,13)} = 0.97$, $P = 0.60$), and *L. longipalpis* (ANOVA $F_{(18,10)} = 1.63$, $P = 0.22$).

Discussion

The duration of male external genitalia rotation differed significantly among sandfly species. According to the total rotation interval, the eight sandfly species studied could be divided into two categories: (a) species in which rotation takes on average more than 20 h after the fly hatches from the pupa (*P. tobbi*, *P. argentipes*, *P. arabicus* and *P. sergenti*), and (b) species in which the process of rotation is terminated within the first 20 h of adult life (*S. schwetzi*, *P. papatasi*, *P. orientalis* and *L. longipalpis*). The slow rotation of genitalia in *P. sergenti* males was also referred to by Moncaz *et al.* (2012), who reported that the first males to achieve fully rotated genitalia did so at 25 h post-eclosion.

The overall duration of the rotation of genitalia and the timing of rotation did not demonstrate any association with sandfly genus as the durations of rotation in *S. schwetzi* and *L. longipalpis* were similar to those among members of the genus *Phlebotomus*. In addition, significant differences in rotation times were found between two closely related species belonging to the subgenus *Larrousius*, *P. orientalis* and *P. tobbi* (total rotation times were 15.6 h and 21.8 h, respectively).

Temperature affects many processes in sandflies (Benkova & Volf, 2007), but there have been no studies on the effect of

temperature on genitalia rotation. In mosquitoes, Provost *et al.* (1961) demonstrated that lower temperatures postpone the overall duration of genitalia rotation in *Stegomyia taeniorhynchus* (= *Aedes taeniorhynchus*) (Diptera: Culicidae). This is by contrast with the findings of the present experiments, in which only the initiation of genitalia rotation in *P. papatasi* males was delayed at the lower ambient temperature and the overall duration of rotation was not affected.

Although sandfly larvae were maintained in non-transparent breeding pots lined with plaster of Paris (for details, see Volf & Volfova, 2011), the photoperiod (LD 12 : 12 h) in the insectaria and the unavoidable manipulation of the pots in light may affect the perception of immature stages and circadian rhythms may develop. Nevertheless, in the present experiments, males of the eight species studied hatched continuously throughout the whole of the 24-h cycle, which corresponds with results previously published on *P. argentipes* (Dinesh *et al.*, 2009). The time of day at which eclosion occurred did not have any effect on genitalia rotation in any of the species studied.

All of the findings reported herein are crucial to any application connected with the search for sandfly breeding sites. However, it should be noted that the times of initiation and duration of genitalia rotation do not necessarily correlate with the probability of finding males with unrotated genitalia on breeding sites. In addition to overall rotation time, the chances of capturing newly emerged males using emergence traps is markedly influenced by another aspect of the sandfly's life: the mobility of the male within the 24 h after its emergence from the pupa. For example, males of the species *S. schwetzi*, which demonstrated quick rotation, were very active from the beginning of adult life. These males hopped and flew within the glass vials, even at 2 h post-eclosion, which makes inspections under the stereomicroscope very difficult. By contrast, males of the species *P. orientalis*, which demonstrated slow rotation, stayed relatively calm until rotation was terminated. These observations complicate a possible discussion about the importance of the speed of male genitalia rotation in the selection of mating sites and in the epidemiology of leishmaniasis. For example, it might be speculated that species which demonstrate faster rotation may be more likely to mate with females in the breeding site; however, given the differences in the behaviour of males in the hours post-eclosion, there is probably no direct correlation between rotation speed and the choice of place for copulation.

Assuming that similar behaviour occurs in nature, the likelihood that unrotated males can be captured in emergence traps is higher in *S. schwetzi* than in *P. orientalis*, although the duration of genitalia rotation is much longer in the latter species. The present observations in laboratory-reared sandfly males of both species fully correspond with the results obtained in field studies in Ethiopia. The fact that males that had not yet achieved full genitalia rotation represented 20–50% of *Sergentomyia* spp. males captured by emergence traps in the study of Moncaz *et al.* (2014b) indicates their high level of activity during the first night of their adult lives. Meanwhile, the proportion of 'lazy' unrotated males of *P. orientalis* was significantly lower (Moncaz *et al.*, 2014a).

The orientation of genitalia rotation after eclosion from the pupa was observed in approximately a dozen males of each of five sandfly species belonging to three genera: *L. longipalpis*,

S. schwetzi, *P. papatasi*, *P. orientalis*, and *P. sergenti*. Similar numbers of clockwise and counter-clockwise rotation events were observed in all five species. These results correspond with findings by Chevone & Richards (1976), who described two pairs of opposing muscles in the yellow fever mosquito *Stegomyia aegypti* (= *Aedes aegypti*), which allow the genitalia to rotate in a random direction.

In summary, this study describes for the first time details of the timing of male genitalia rotation in eight medically important sandfly species belonging to three genera, *Phlebotomus*, *Lutzomyia* and *Sergentomyia*. The duration of rotation is species-specific and varies from an average of 12 h in *S. schwetzi* to an average of 33 h in *P. sergenti*. Rotation intervals are not affected by lowering the ambient temperature by 5 °C or by the particular time of male eclosion within the 24-h cycle. The knowledge gained from this study may help support the search for sandfly breeding sites and may facilitate the application of larval source reduction as a component within integrated sandfly and leishmaniasis control strategies.

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