American Journal of Entomology
2023; 7(2): 62-69
http://www.sciencepublishinggroup.com/j/aje
doi: 10.11648/j.aje.20230702.13
ISSN: 2640-0529 (Print); ISSN: 2640-0537 (Online)

Human Age and Sex Influences on the Repellent Activity of PMD Towards *Aedes Albopictus*

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To cite this article:

Niry Hasinandrianina Ramarosandratana, Sarah Vonisoa Ralimanana, Miarintsoa Michaële Ranarijaona, Estelle Métay, Voahangy Ramanandraibe, Mbolatiana Tovo Andrianjafy, Marc Lemaire. Human Age and Sex Influences on the Repellent Activity of PMD Towards *Aedes Albopictus*. *American Journal of Entomology.* Vol. 7, No. 2, 2023, pp. 62-69. doi: 10.11648/j.aje.20230702.13

Received: May 4, 2023; **Accepted**: May 18, 2023; **Published**: June 10, 2023

Abstract: Mosquito-borne diseases still remain one of the major public health concerns in Africa, including Madagascar. The development of new repellents has taken an important place for personal protection against mosquito bites to limit the spread of related diseases such as malaria, dengue, chikungunya and zika. However, one of the difficulties of this approach is based on the variability of human attractiveness to mosquitoes due to several factors that cause the bite frequency for each individual to be different. The objective of this article is thus to study the influences of human age and sex on the repellent activity against *Aedes albopictus*. Tests on volunteers inspired by the WHO protocol were conducted. Men and women of different ages from Antananarivo were selected for the experiment and Citriodiol® was used as repellent. Percentage (PI) and duration of protection were measured. We found that age influence repellent activity against *Aedes albopictus.* At the same quantity of compound, seniors are less protected than young volunteers (PI=61% and PI=74%, respectively). Young women show high protection with citriodiol compared to old men and women with PI about 79%. These results could be of interest in the development of an ideal repellent to limit host-vector contact and reduce the transmission of associated diseases.

Keywords: *Aedes Albopictus*, Age, Arboviruses, Sex, Tests on Volunteers, Repellent

1. Introduction

Mosquito-borne diseases still remain one of the major public health concerns in the world, especially in Africa [1]. They have considerable impacts in the socio-economic fields of many countries [2] by causing more than one million deaths per year [3]. When these diseases are present, there are different solutions to combat them. There are the treatments of patients with drugs and the control of the vector population by using the insecticides. Nevertheless, these solutions present their limits. Firstly, repetitive treatment of patient by medicament causes the resistances to pathogens. The massive use of insecticides to control vector mosquitoes also increases the forms of resistance of mosquito populations [4, 5]. In addition, the harmful effects of insecticides on the environment and living beings call into question the ecological aspect and the sustainability of this approach. Indeed, the development of more ecological control is an emergency and a major challenge in the fight against vector-borne diseases (such dengue, chikungunya, zika) transmitted by blood-sucking insects, mosquitoes in general and *Aedes albopictus* in particular.

The use of repellents is a means of protecting oneself individually against the infective bites of mosquitoes, making it possible to limit the transmission of associated diseases. They have already been used for centuries even if their

effectiveness seems to vary greatly from one individual. While, the mode of action of repellents is not yet well defined, as their chemical structures vary widely, some authors have suggested that they disrupt the insect's olfactory system, inhibiting its ability to locate its host by masking or cluttering the kairomones emitted by the latter [6, 7].

Female mosquitoes transmit pathogens to their host during blood meal and they locate them spatially by detecting a combination of human-derived chemical cues, including carbon dioxide, lactic acid and other volatile organic compounds (more than 300 substances) [8–11]. They detect these compounds through their olfactory system which is located on the antennae and palps. The variability of human attractiveness to mosquitoes is largely due to the individual characteristics of the composition and intensity of the release of these attractants known as kairomones. Indeed, not all humans suffer from mosquito bites with the same frequency or intensity because some individuals attract mosquitoes more than others [12–18]. Bernier et al. 2000 reported that different human individuals typically produce similar types of volatile compounds. However, the amount of compounds released from human skin varies according to the individual [19] due to several factors including metabolic rate, body weight, respiratory activity, and differential skin pH. Other authors have suggested that the volatile compounds released directly through the skin or metabolized by the skin microbiota are the explanations of variations in human attractiveness to mosquitoes [9, 14, 20].

Otherwise, some studies have pointed out that age is also potentially implicated in susceptibility to mosquito bites. The authors found that, the age of the host influenced the mosquito attraction, and the other have reported the contrary [21–23]. Additionally, the influence of the sex of human hosts on mosquito preference is also debated [8]. The influences of age and sex are still unclear, mainly because they are not major determinants of human attractiveness for mosquitoes in some situations [16]. During experimentations about effect of different stereoisomers of para-Menthane-3,8-diol on repulsion toward *Ae. albopictus*, the difference in PMD protection between women and men has been observed [24]. These preliminary results were obtained with a relatively small number of volunteers of similar ages and we decide to perform another study with larger number of volunteers and adding the age parameter. The consideration of these two factors could be crucial for establishing better protection strategies such as the development of an ideal effective, long lasting and specific repellent for individual use. The main objective of this article is therefore to study the influences of human age and sex on the efficacy of a repellent namely citriodiol against *Ae. albopictus* mosquito for Malagasy population. To achieve this, clinical trials using women and men volunteers of different ages were conducted.

2. Materials and Methods

2.1. Mosquito

The mosquito *Ae. albopictus* was selected for laboratory experimentation. The preimaginal stages (larvae and pupae) of this species were collected in bamboo cottages within the Tsimbazaza Zoo and Botanical Park in Antananarivo, Madagascar. Specimens were brought back to the insectary for rearing with the following conditions: 26 ± 3°C temperature, 65 ± 5% relative humidity and 12/12 h photoperiod. The larvae were reared in tank (30 cm x 30 cm x 15 cm) containing breeding ground water. They were fed with dog biscuit powder (Tetramin®) every week. Pupae were sorted and transferred to sail cups to await emergence. The adult males and females were placed together in Gauze cages and they were fed with 10% sucrose solution through cotton. A blood meal was given to adult females once a week. Females obtained from several generations (>F5) of breeding strains were used for all bioassays to have healthy mosquitoes (not carriers of pathogens) and to limit the risk of transmission to volunteers.

2.2. Volunteers

A total of 60 randomly selected adult volunteers from Antananarivo were tested in this study, including 30 men and 30 women belonging to the same ethnic group (Merina, people from highlands) in order to homogenize the population tested. They are divided into two groups according to their age: young people aged 23 to 29 (15 men and 15 women) and older people aged 40 to 64 (15 men and 15 women). The volunteers were informed of the purpose of the tests and the instructions to follow before, during and after the experimentation that included a high probability of them being bitten by mosquitoes. Each volunteer filled out a survey sheet to check their state of health in relation to mosquito bites and repellent products. Individuals who had no history of allergic reactions and were in good health were recruited as volunteers in the clinical trial. Volunteers were prohibited from using aromatic or repellent substances before and during the test to obtain reliable data on the tested product. They signed a volunteer document.

2.3. Bioassay

Direct tests on volunteers according to the WHO protocol [25] were conducted in laboratory. It consists in evaluating the repellent activity of a product impregnated on the skin of the forearms of volunteers towards adult mosquito females. All tests were performed in a room with the following conditions: 26.5 to 30°C of temperature, and 60 to 75% of relative humidity.

2.4. Product Tested

Table 1. *CPG Profile of Citriodiol®.*

Components	Analysis methods	Results % in areas
α-Pinène		0.13
β- Pinène		0.19
β-myrcène		0.07
Limonène		0.17
1,8 Cinéole		0.91
γ-terpinène	LAB-SOP-017	0.07
Terpinolène		0.06
Citronellal		0.02
Néo- Isopulégol		4.13
Isopulégol		7.14
β-caryophyllène		0.80

Components	Analysis methods	Results % in areas
Terpinène-4-ol		0.10
Citronellol		8.21
Cis PMD		44.07
Trans PMD		23.27

The Citriodiol® repellent was used in this study. It is a product of Citrefine obtained from the essential oil of *Eucalyptus citriodora*, hydrated and cyclized. Citriodiol is composed mainly of cis- and trans- of p-menthane-3,8-diol (PMD) (70%), and other natural components (Table 1).

2.5. Test Procedures

A total of 50 female mosquitoes of *Ae. albopictus* aged 5 to 9 days and previously fasted for 12 hours were selected and introduced into a Gauze cage. The test was performed on the volunteer's forearm on a skin surface of approximately 600 cm². Firstly each volunteer's forearm was cleaned with water and then dried with hydrophilic cotton. Then, for the negative or control test, the surface of the skin to be treated was impregnated with 1.5 ml of 90° ethanol using a micropipette. When the skin was dried, the volunteer's forearm was inserted into the cage containing mosquitoes for 3 min. The mosquitoes landed and attempted to bite on the exposed surface of the volunteer's skin were counted. Volunteers wear latex gloves to protect their hands from mosquito bites and their forearm was shaken from time to time to avoid stings during the test. In the case of the product or treated test, the same manipulations were done, but the tested product: 100 mg of Citriodiol® diluted into 1.5 ml of ethanol was impregnated on the skin of volunteers instead of ethanol. The repellent activity of Citriodiol was observed every 30 minutes without re-impregnation. Control tests were conducted in early, in the middle and in the end of the test with the other forearm of the volunteer. Given the activities and aggressiveness of *Ae. albopictus* (daytime mosquito), the tests were carried out during the day from 9 a.m. Our purpose consists to compare the repellent effect of Citriodiol® on the skin of the forearm of the volunteers depending to their ages and sex towards *Ae. albopictus*.

2.6. Data Analysis

The repellent activity was expressed as the percentage of protection (PI) which was calculated by the following formula:

$$PI\ (\%) = [(T-P)/T] \times 100$$

T: number of mosquitoes that attempted to bite on untreated forearm (negative or control) in 5 minutes.

P: number of mosquitoes that attempted to bite on the treated forearm in 5 minutes.

Graphs and statistical analyses were carried out using Graphpad Prism 8.4.2 software. The percentages of protection of the product by volunteer's age and sex were compared by *ANOVA* and the comparison by *post hoc test* was carried out to sort out the difference between the repellent effects. For these analyses, the confidence interval was estimated at 95%. Standard error of the mean (*SEM*) and standard deviation (SD) were calculated and illustrated in the tables and figures.

3. Results

In total, 60 volunteers from different ages were tested, including 30 women and 30 men. Citriodiol had a good repellent effect on all volunteers with a percentage of protection >90% during the first 2 hours. Then, a gradual decrease in the repellent activity is observed as a function of time until it becomes very weak (<20%) after 6 hours.

3.1. Influence of the Age of the Volunteers on the Percentage of Protection of Citriodiol

Overall, focusing on the age parameter, we observed that young volunteers are significantly better protected than older during the six hours-test with the means protections effects of 73 ± 11.5% and 59 ± 12.1% (t=3.7; p=0.0005), respectively.

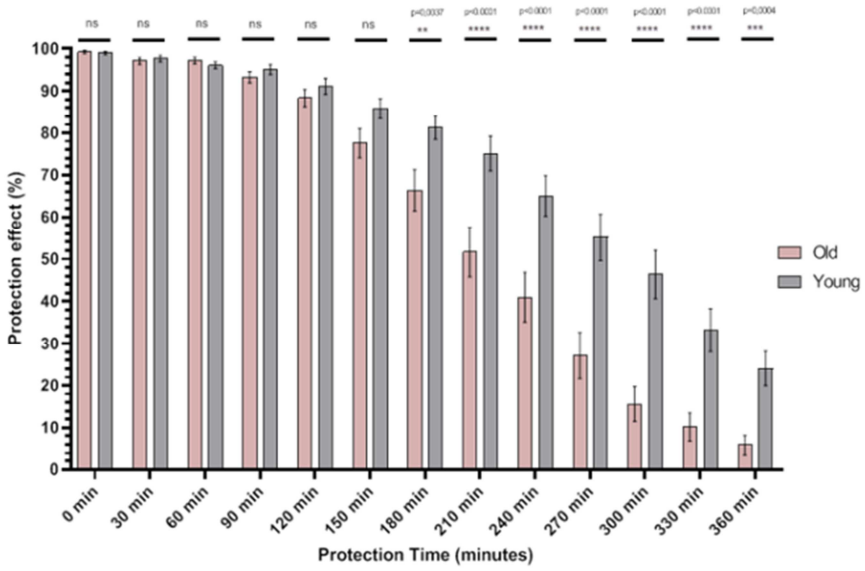

Figure 1. *Mean of protection effect (SEM) of Citriodiol® on old and young volunteers depending on time.*

For each observation interval of 30 min, the repellent activity of citriodiol® on old and young volunteers is the same at the start of the experiment until the 150th minute. Beyond this period, we gradually observe significant differences between the protection percentages of older and younger volunteers. The young volunteers were better protected than the older ones from 180 minutes until the end of the test. The statistical analysis showed the difference significantly on the young volunteers and elderly for each thirty minutes (Figure 1).

3.2. Influence of the Sex of the Volunteers on the Percentage of Protection of Citriodiol

The comparison of the means of the protection effects between men and women volunteers was done. In generally, there are no significant differences between them (t= 2.021; p= 0.052). They present the same protection effect up to 60%.

By analyzing the data as a function of time, Citriodiol® has similar effect on men and women volunteers during the first hour of the test (60min). From 90min, the percentages of protection in men stand out from those of women decreasing much more if we refer to the trend of the curves (Figure 2). However, statistical analyzes showed that significant differences were only reported at the 210th and 270th minute during which women are clearly better protected than men.

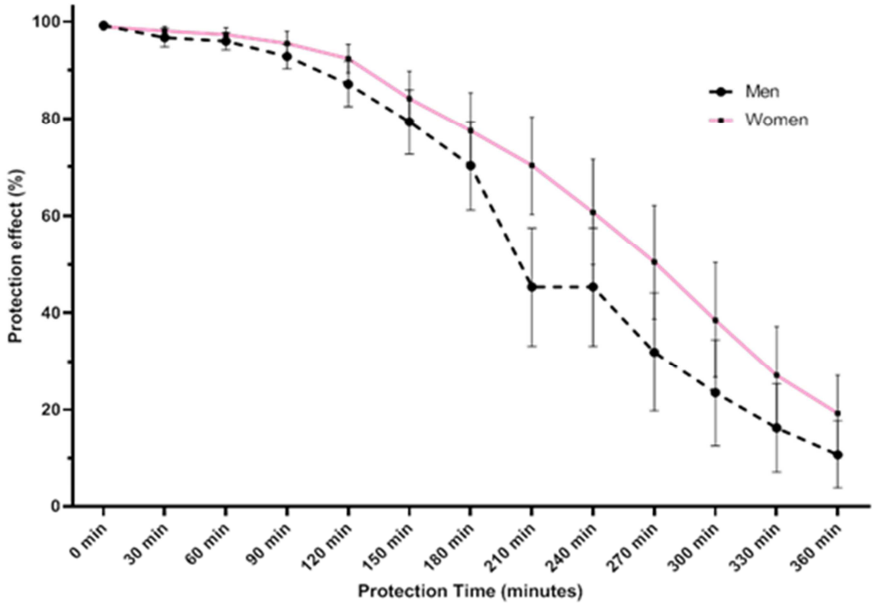

Figure 2. *Mean of protection effect (SEM) of Citriodiol® on men and women volunteers depending on time.*

3.3. Comparison Between the Four Groups of Volunteer

Volunteers can be best classified into four groups: young men, young women, old men and old women. During six hour, the means of percentage of protection of Citriodiol between these volunteers were compared and significant differences are observed (F=6.57; p=0.0007).

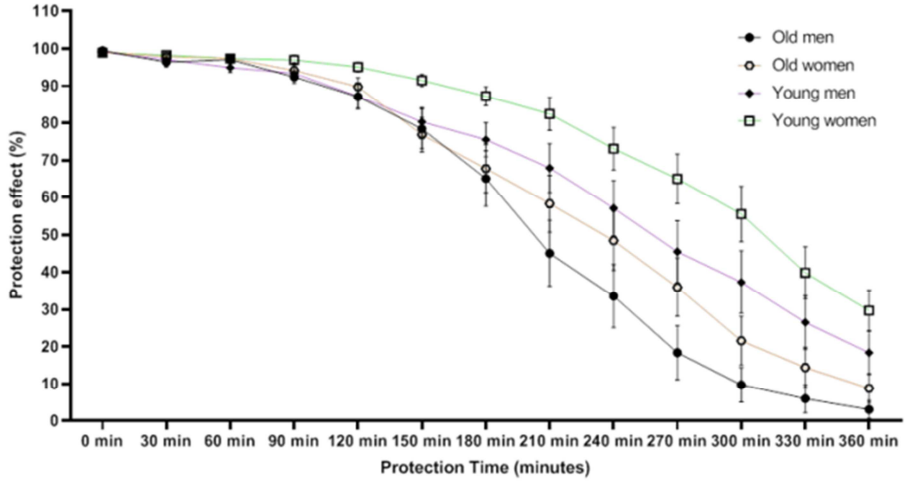

Figure 3. *Comparison of the means of protection effect of Citriodiol® on four groups of volunteers: old men, old women, young men and young women.*

More importantly, depending on the duration of application of Citriodiol, the percentages of protection of the four groups of volunteers remained stable (PR> 80%) throughout the 150 minutes (Figure 3). During this period, these percentages of protection of the product had the same, there is no significant difference of the protection between the groups of the volunteer. From the 180th minute, the protective effects begin to gradually decrease but this decrease varies according to the groups. Citriodiol confers better protection in young women compared to other volunteers until the end of the experiment, followed by young men and older women. Conversely, the repellent loses its effectiveness more quickly in older men as shown in Table 2.

Table 2. *Protection effect of Citriodiol® for each group of volunteer at 180 to 360 minutes.*

Times (minutes)	Percentage of protection (± SEM) of Citriodiol ® for each group of volunteer			
	Young women	Young men	Old women	Old men
180	87.2 ± 2.5	75.5 ± 4.6	67.7 ± 6.5	65.1 ± 7.5
210	82.4 ± 4.5	67,.9 ± 6.6	58.3 ± 7.6	45.1 ± 8.8
240	73.1 ± 5.7	57.1 ± 7.5	48.4 ± 7.9	33.6 ± 8.4
270	64.9 ± 6.7	45.5 ± 8.2	36 ± 7.8	18.4 ± 7.3
300	55.5 ± 7.4	37.3 ± 8.3	21.6 ± 6.5	9.7 ± 4.7
330	39.9 ± 6.9	26.5 ± 7.2	14.4 ± 5.5	6 ± 3.8
360	29.7 ± 5.5	18.4 ± 5.8	8.8 ± 3.9	3.1 ± 2.4

4. Discussion

Para-menthane-3,8-diol (PMD), a natural molecule derived from the *Eucalyptus citriodora* plant, is known for its repellent activity against mosquitoes [26]. This compound has shown significant efficacy against different species of mosquitoes such as *Ae. aegypti* [27], *Culex quinquefasciatus* [28], *Ae. albopictus* [29], *Anopheles* [30]. The results of our study confirm the repellent effect of PMD against *Ae. albopictus* with a percentage of protection greater than 90% for 150 minutes, from the 330th minute, the PR was less than 20%. This implies that the product gradually lost its effectiveness over time. Borrego et al. found similar results when studying the effects of PMD diastereoisomers on *Ae. albopictus* but the durations of protection are different. In this study, (1R)-(+)-cis- PMD was the best of the isomers, it had a PR greater than 90% during 90min but a weak effect (PR<20%) was recorded from the 150th minute [24]. In our case, the major product in the solution tested is also the racemate cis of PMD, but in the presence of other compounds also known to have repellent effects: citronellal [31], citronellol, limonene, pinene, isopulegol [32]. We suppose that there were synergistic effects between these constituents leading to an increase in the efficacy and protection time of the mixture.

By focusing on the age parameter, the results showed that young volunteers were more protected by Citriodiol than older volunteers against *Ae. albopictus* during the 6 h test. This observation is probably due to the difference in attractive odors or kairomones released on the skin of the two groups of volunteers. These odors come from a combination of sweat gland secretions and volatile substances released as by-products of metabolism by volunteer's skin microflora [14, 20]. Studies have shown that these volatile compounds including lactic acid, 2- methylbutanoic acid, CO_2, octanal, etc. are closely associated with increased attraction to adult females looking for blood meal [33]. These kairomones are used by mosquitoes to locate their hosts and also play a role in their trophic preference. Haze et al. reported that substances responsible for body odor also increased with age [34]. Indeed, this supposes that in our case that the kairomones released by the elderly volunteers are more intense than those of the young. The repellent, by gradually volatilizing, is no longer able to mask these attractants and loses its protective activity. Thus, the attractive odors of older volunteers are much more exposed to the olfactory system of mosquitoes and this is why they are less protected than young volunteers. Similar observations have been reported by some authors with other mosquito species such as *Anopheles gambiae* [35], *Anopheles farauti* [36]. Observations on biting habits of *Anopheles albimanus* on family groups in Jamaica showed that this species bit the young people much less than the adults [37]. Thomas and colleagues also suggested that adults are more attractive than children to *Anopheles gambiae* [38]. Similarly, studies have found that adults are three times more attractive than children to the same species [35]. All of these studies have determined that age is an important factor affecting attraction. The results of this experiment show us that the age of the host significantly influences the effectiveness of a repellent applied to the skin and confirms all previous observations.

The effectiveness of Citriodiol is maximum and remains more or less stable in the two types of volunteers: young and old during the 150 minutes. This observation can be explained if we assume that the ratio of the concentrations of repellent/attractant on the skin of the volunteers is high. That is to say, the kairomones emitted by the skin are always outclassed from Citriodiol during this period. Indeed, mosquitoes do not detect the host's kairomones at all or only slightly in the presence of the repellent. Beyond the 150th minute, the repellent activity of Citriodiol for the young and old volunteers decreases progressively as a function of time, but that of the old volunteers decreases much more quickly. This implies that the ratio of repellent/attractant concentrations gradually tends towards 0. The explanation for this result lies in the volatilization of part of the repellent on the host's skin leading to the exposure of the host's kairomones which are released in a stable manner as a function of time for

each individual [13, 16]. It can be hypothesized that the longer the repellent volatilizes, the more kairomones emitted from the host's skin are exposed and detected by mosquitoes because the repellent acts as a barrier between the kairomones and the insect's olfactory system. Consequently, the repellent confers less and less protection, especially in elderly volunteers who have a higher concentration of kairomones [34] than young volunteers. It can be deduced that the effectiveness of the repellent as a function of time depends closely on the ratio of the repellent/attractant concentrations on the skin of the host. In addition, the volatility of a repellent product also plays a key role in its effectiveness especially in its duration of protection. A repellent compound or formulation with a slower evaporation rate will certainly confer a longer duration of protection. These results could be useful in the research and development of effective topical repellents against mosquito bites, taking into account that the elderly are generally less protected than the young.

Depending of the sex, the efficacy of Citriodiol against the mosquito *Ae. albopictus* in human subjects of different sexes was evaluated. During the first part of the experimentation, the results showed that men are as protected as women by the repellent. In particularly, as a test progresses, we found that the women are better protected than men.

The influence of human host sex on mosquito attraction has been debated for years [8, 16]. According to Carnevale et al, 1978, sex does not influence mosquito attraction while other authors have reported the opposite. Muirhead-Thomson, 1951 observed that the mosquito *Anopheles albimanus* was more attracted to adult men [37]. Similar observations have been reported by Gilbert *et al*. with the *Ae. aegypti* mosquito. They found that this species was more attracted to man than woman volunteers based on the number of mosquitoes trapped in an olfactometer [22]. Other studies have also reported similar results with *Anopheles gambiae* [35] and *Anopheles stephensi* [39]. It is suggested that this phenomenon is attributed to the composition of kairomones emitted by the two sexes, which is not significantly different. Bernier et al, 2000 found that different human individuals generally produce similar types of volatile compounds (attractants). Qiu et al, 2006 also reported that no difference was found between the skin fumes of different sexes. Thus, it is assumed that mosquitoes probably detect male and female volunteers in the same way in the presence of Citriodiol.

Conversely, other authors have observed that the repellent activity of PMD varies according to the sex of the volunteers [24]. They found that better efficacy and longer duration of protection were recorded in women. These authors suggested that the difference in concentration of volatile organic compounds released on the skin of both sexes is the likely explanation for their observation. However, the study of the interaction quantity or concentration of kairomones-sex of the host and attraction of mosquitoes should be made in order to clarify several points because it is difficult to understand the difference in quantity of the attractants in both sexes for the time being, although studies have shown that the quantity of compounds emanating from human skin varies according to the subjects [19]. Moreover, it has been reported that the attractant compounds released vary considerably between individuals and that this variability at least partly explains the preference of mosquitoes [9, 15].

Previous studies against *Ae. aegypti* have shown that when all individuals have been impregnated with the same amount of DEET, the protection time is longer in women than in men. This is also the case for our result if we refer to the trend of the curve (Figure 2). This shows that Citriodiol has a longer duration of protection in women than in men volunteers. On the other hand, by applying a repellent formulation containing DEET against *Anopheles stephensi*, Golenda and his collaborators were able to prove that over time, men are more protected than women [39]. However, the methods used in these studies are different, as are the species of mosquito tested. This study made it possible to establish a Citriodiol efficacy scale according to the groups of volunteers: young woman > young man > elderly woman > elderly man.

5. Conclusion

To conclude, this study made it possible to highlight the influence of the two parameters sex and age of the host on the effectiveness and duration of protection of repellent against mosquitoes. Tests performed on the forearms of volunteers show that Citriodiol has generally similar efficacy at the beginning of the test in both sexes: men and women. However, its duration of protection is different in which it lasts longer in women than in men. This experiment also elucidates that age is an important factor influencing repellent activity. As a result, Citriodiol confers both higher efficacy and significant protection in young volunteers compared to elderly volunteers. Nevertheless, the product had better durability to the young compared to elderly volunteer. The concentration's ratio of odor emitted (kairomones) to repellents is an essential element which could be the probable explanation of these observed differences. Thus, knowledge of the influence of the sex and age of the host on the effectiveness of a repellent could be an important key in the development of an ideal repellent in the context of individual protection against mosquito-bites.

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