

Date: 13 June 2019

Re: Response to the EPA concerns re mosquito landing pressure for field site 2

To whom it may concern:

A meeting was held on Wednesday 5th June 2019 to discuss the study report for “Field evaluation of three topically applied insect repellent products containing IR3535 against mosquitoes in Florida.”

The following people attended the meeting:

- Emma Weeks (University of Florida)
- Robert Jones (ARCTEC, London School of Hygiene and Tropical Medicine)
- Sarah Dewhirst (ARCTEC, London School of Hygiene and Tropical Medicine)
- Kim Davis (Consultant/Agent representing the sponsor)
- Michelle Arling (EPA)
- Eric Bohnenblust (EPA)
- Clara Fuentes (EPA)

In the meeting, the EPA stated that they have assessed the mosquito landing pressure to be insufficient or inconsistent at site 2 on both test day 5 and 6. As a result, they concluded this invalidates the data collected towards the latter stages of testing on these days and complete protection time (CPT) of the products should be 3 hours. The EPA agreed that the study has been done in accordance with the protocol.

Below is a response to the EPA from University Florida and ARCTEC (including Professor James Logan, Director and Head of Department of Disease Control, London School of Hygiene and Tropical Medicine) with regards to the landing pressure and the untreated subject data collected from study site 2 on test days 5 and 6.

In this response we would like to present the following points:

1. Protocol requirements with regards to continued landing pressure on untreated subjects
2. Discussion with University of Florida and the EPA on inadequate landing pressure (Protocol amendment No. 7)
3. Published EPA guidelines (OPPTS 810.3700: Insect Repellents to be Applied to Human Skin)
4. Day 5 untreated subject data
5. Day 6 untreated subject data
6. Field sites, trapping data and mosquito species

Each point is summarized here. Supplementary information to support these points can be found in full at the end of this document.

1. The protocol (v7.0) states “the untreated control subjects will expose the lower leg every 30 minutes for 5 minutes or until 5 landings on the lower leg occur, whichever is sooner”. This protocol was followed.

2. After e-mail and phone discussions with the EPA a protocol amendment (No 7) was prepared to clarify that a testing period with a low landing pressure would be when both of the untreated subjects have fewer than five landings in the five-minute test period. This protocol amendment language was agreed with the EPA. The study was completed according to this agreement, which was signed by the study director and the sponsor and approved by the UF and LSHTM ethics committee.
3. The published EPA guidelines (OPPTS 810.3700: Insect Repellents to be Applied to Human Skin) require landing pressure of the target species to be at least one mosquito landing within one minute. On average one mosquito landing per minute was recorded when the data for the untreated subjects was combined or analyzed separately for the entire test duration. Test day 5 average landings per minute was 1.05 and for test day 6 the landings per minute was 1.73. Please note that this is an estimated rate, since as soon as five landings occurred, regardless of whether five minutes had elapsed, the untreated subject covered the lower leg and recordings of mosquito landings stopped.
4. We conclude that test day 5 was conducted as per study protocol (v 7.0 and amendment No. 7) and sufficient data were collected to show the presence of sufficient landing pressure. Out of 22 time points, there are only two where there is no justification that landing pressure was sufficient (21:56 and 22:29) as neither threshold was reached nor was a CPT recorded. It should be noted that one mosquito did land within one minute, as per the published guidelines, on untreated subject 30 during the testing periods of low landing pressure. Furthermore, an average landing rate of one mosquito per minute was recorded when the data for untreated subjects was analyzed separately or combined (combined average landing rate = 1.05). Although the threshold of five landings in five minutes was not achieved for four of the earlier time points during the test day, importantly this threshold was reached for both of the untreated subjects in the last two testing time points. Despite this high landing pressure at the end of the testing period, six out of 13 treated subjects were yet to reach CPT 14.5 hours after product application. This indicates that the product was repelling mosquitoes far beyond 3 hours post application. To suggest that the test substance provided median complete protection for less than 14 hours would be to ignore the data collected at these later time points.
5. We conclude that test day 6 was conducted as per study protocol (v 7.0 and amendment No. 7) and sufficient data were collected to show the presence of sufficient landing pressure. Overall, there are four time points out of 23, where there is no justification that landing pressure was sufficient (21:42, 23:05, 00:57 and 01:27) as neither threshold was reached nor was a CPT recorded. It should be noted that one mosquito landed within one minute, as per the published guidelines, on one of the untreated subjects during these testing periods. Furthermore, an average landing rate of one mosquito per minute was recorded when the data for untreated subjects was analyzed separately or combined (combined average landing rate = 1.73). Although the threshold was not achieved for four of the earlier time points, importantly the threshold of five mosquitoes in five minutes was reached for both

of the untreated subjects in two of the three last testing time points. Even though landing pressure was high at the end of the testing period, six out of 13 treated subjects were yet to reach CPT at 15 hours after product application. This indicates the product was repelling mosquitoes, providing efficacy, at the end of testing when landing pressure was high. To suggest that the test substance provided median complete protection for less than 15 hours would be to ignore the data collected at these later time points.

6. Study site selection was as per the protocol (v 7.0); two distinct habitats were identified where the predominant mosquito species differed, and mosquito abundance was demonstrated to be high before the study was initiated. In the trapping conducted before the repellent testing started to estimate mosquito abundance and species diversity, twice as many mosquitoes were caught per night at site 2, the site with lower landing pressure, than site 1, the site that the EPA have determined had sufficient landing pressure on test days. *Aedes albopictus* was the predominant species at site 1 whereas *Culex nigripalpus*, *Ochlerotatus fulvus pallens*, *Psorophora ferox* and *Mansonia dyari* were the predominant species at Site 2, caught during pre-testing trapping and/or landing on subjects. Due to the feeding behavior of the predominant species, *Aedes albopictus*, at test site 1 and overcast weather, no test periods occurred where the threshold for the untreated subject data was not met in test day 1. Although test day 4 was sunnier the aggressive landing behavior of *Aedes albopictus* still resulted in acceptable landing pressure in all but four test periods. In contrast at site 2, where the prominent mosquito species exhibit temporal feeding behaviors and less of a preference for human feeding, recording consistent landing pressure is less likely over a prolonged period of time, i.e.14 hours.
7. Data from site 1, which the EPA accept as sufficient, shows the product lasts for >14 h against *Aedes albopictus*, an aggressive day biting mosquito species with a preference for human blood meals. Furthermore, this species is the known vector of yellow fever and Chikungunya viruses and is capable of becoming infected with Zika virus, although studies with this emerging pathogen are currently ongoing to determine its full potential as a vector. Our data show that even when challenged with a consistent and high landing pressure the repellent is providing protection beyond 14 h post-application against *Aedes albopictus*.

The study has been conducted according to Protocol (v 7.0 and to amendment letter No 7.), in compliance with Good Clinical Practice, was monitored to be compliant to Good Laboratory Practice and a quality assurance statement was signed. The conduct of the study is not under question. The EPA have stated that they would prefer higher or more consistent records of mosquitoes landing on the subjects. However, this conflicts with the levels that were defined and agreed before the study began and are in line with published guidelines. To date we have not been given any information as to what the EPA deems to be an acceptable number of mosquito landings.

In addition to the above points, we would also like to highlight the important ethical implications of the EPA not recognizing the validity of the data collected from site 2. If the data is invalidated by the EPA, then we would have to repeat the study at a third site in order to support 14h CPT shown at site 1 with *Aedes albopictus* and to amend the label claim to reflect this, therefore, putting more subjects at risk of mosquito bites and other adverse events associated with applying a new product and conducting field testing during the night. The study was carried out according to the protocol. The University of Florida and ARCTEC at the London School of Hygiene and Tropical Medicine strongly deem a repeat of the study to be unethical and unjustified.

We welcome the opportunity to discuss all of these points with the EPA on June 17th, 2019.

As a reminder, supplementary information to support these points are found in the following pages.

Yours sincerely

A handwritten signature in black ink, appearing to read 'Emma Weeks', with a stylized, cursive script.

Dr Emma Weeks

1. Protocol requirements with regards to continued landing pressure on untreated subjects.

The protocol agreed with the EPA and HSRB (v7.0, 30-March-2018) states on page 30 and 32:

“10.5.6. Continued landing pressure

After the initial landing pressure monitoring period, before each exposure period starts for the test subjects, **the untreated control subjects will expose the lower leg every 30 minutes for 5 minutes or until 5 landings on the lower leg occur, whichever is sooner.** As soon as 5 landings occur, regardless of whether 5 minutes have elapsed, the control subject can cover the lower leg to minimize the potential for mosquito bites. The time of the landings and when the threshold number of landings occur will be recorded in the study records. Mosquitoes landing on the exposed lower leg of control subjects will be collected by aspiration (before they have chance to probe or bite) for later identification and labelled with the participant number, treatment status, date and time of collection. Mosquitoes landing elsewhere on the body will not be collected. These samples will be pooled by participant and date and tested for pathogen prevalence as described in section 10.2.

If more than 15% of exposure periods (based on the total number of projected exposure periods for the test) have low mosquito pressure measurements on the untreated controls (i.e., low pressure is when mosquito pressure falls <5 landings in 5 minutes), the study should be stopped. Only periods where both test and control subjects are placed in the field and exposed to mosquitoes should be considered exposure periods. Study periods that would have occurred during an intentional delay are not considered exposure periods for the purposes of stopping the study because of low landing pressure. This protocol calls for a maximum of 28 exposure periods (2 exposure periods/hour × 14 hours), with a 2-hour delay after application before testing begins (no landing pressure monitoring will occur during this period). **Therefore, if there is low landing pressure in more than 4 non-consecutive test periods over the course of the 14-hour test period, the test must be stopped. If the test period is shorter than projected, an assessment of whether the landing pressure was sufficient through the duration of the test will be made after the test has been completed.** If a single landing on a test subject during an exposure period is followed by an exposure period with inadequate landing pressure, then the first landing will be treated as a confirmed landing.”

2. Discussion with University of Florida and the EPA on inadequate landing pressure (Protocol amendment No. 7)

Emma Weeks had a telephone conversation with Eric Bohnenblust and Clara Fuentes on 17th August 2018 to discuss landing pressure on untreated subjects as well as other points that required clarification. Emma followed-up the phone call with an e-mail, summarizing what was discussed. In the e-mail the following point was discussed:

“Finally, no test periods should be skipped for inadequate landing pressure. If both untreated control participants receive less than five landings in five minutes in more than four non-consecutive landing periods, then the study should be stopped. If one of the control participants achieves the threshold of five landings in five minutes this is considered adequate landing pressure and it would not count towards the number of periods with low landing pressure.”

Clara comment: “that is my understanding as well”

Eric comment: “I think this is the best way to proceed. Although I hesitate to commit to saying it would not count toward the number of periods with low landing pressure in an absolute sense. I think we will need to consider based on the whole of the data for a situation whether landing pressure is low, say for instance one control subject continuously has no or few landings vs a situation where they are getting 3 or 4 landings in 5 minutes here and there. So, to allow for some flexibility, i think it might be better phrased as will not count as periods with low pressure for the purpose of stopping the study.”

Emma informed ARCTEC that she “wrote the text as directed by Eric and Clara and they added comments.”

Following on conversation occurred via e-mail and phone between Emma and the EPA with regards to further clarification and if it should be regarded as a protocol amendment or deviation.

A protocol amendment letter (No 7) was written stating the following:

3. Clarification that a testing period with a **low landing pressure will be when both of the control participants have fewer than five landings in the five-minute test period**. If one of the control participants has fewer than five landings, this will not be considered a period of low landing pressure.
4. Clarification that if low landing pressure is recorded with the control participants, the treated participants should not skip the test period; they should exposure their lower leg at this time point in in case complete protection time (CPT) is reached.

On 19-Aug-18 Emma wrote to ARCTEC stating that:

“I talked to the EPA and they agree with an amendment. If you are happy with the attached, please ask the sponsor to sign. They want me to talk to the IRB about the deviation. Although it really doesn’t fit at all into the reportable event category. I will send them an email and see what they say.”

Emma emailed the IRB on 21st August 2018 and asked about the deviations from the protocol, that were mostly due to text that needed to be clarified. After a phone conversation it was agreed that all deviations should be recorded in the deviation log and submitted to the IRB at continuing review. Emma addressed this as appropriate.

The protocol amendment letter (No 7) was approved by the University of Florida and LSHTM ethics review board.

3. EPA published guidelines (OPPTS 810.3700: Insect Repellents to be Applied to Human Skin)

EPA published guidelines state:

“Landing pressure should be measured before treatment and intermittently throughout the course of the test by untreated control subjects. Testing should not be conducted or continued unless landing pressure of the target species is at least one mosquito landing within one minute, or at least one stable fly, black fly, ceratopogonid or tabanid landing within five minutes.”

Protocol (v7, 30-March-2018) requirements are more rigorous than published guidelines as 5 mosquitoes are required, and landing pressure was measured routinely at every exposure rather than intermittently throughout the course of the test.

4. Test day 5 untreated subject data

4.1. Summary of test day 5

On test day 5, the test substance was applied between 16:56 and 17:10 h (14-minute window). Seven subjects achieved Complete Protection Time (CPT) and the test was terminated at around 7:30 h or 14 hours and 30 minutes post-application at which point median CPT is calculable. The remaining six subjects were censored.

Four testing periods occurred where both of the untreated subjects had fewer than five landings in the five-minute test period (Table 1, grey shading). Therefore, these testing periods were determined as low landing pressure, as per protocol amendment letter No 7.

The protocol (v7.0) states that “if there is low landing pressure in more than 4 non-consecutive test periods over the course of the 14-hour test period, the test must be stopped”.

Therefore, the test day was conducted as per study protocol.

Table 1: Test Day 5 untreated subject data

| Start Time | Untreated control subject 6 | | | | | | | Untreated control subject 30 | | | | | | | CPT |
|------------|-----------------------------|-------|-------|-------|-------|-------------------|----------------------------------|------------------------------|-------|-------|-------|-------|-------------------|----------------------------------|-----|
| | Time of Landings | | | | | Threshold reached | Time between start and threshold | Time of Landings | | | | | Threshold reached | Time between start and threshold | |
| | 1 | 2 | 3 | 4 | 5 | | | | | 1 | 2 | 3 | | | 4 |
| 18:57 | 18:57 | 18:57 | 18:57 | 18:58 | 19:01 | 19:01 | 00:04 | 18:57 | 18:57 | 18:57 | 18:58 | 18:59 | 18:59 | 00:02 | |
| 19:34 | 19:34 | 19:34 | 19:35 | 19:35 | 19:35 | 19:35 | 00:01 | 19:34 | 19:36 | 19:36 | 19:37 | 19:37 | 19:37 | 00:03 | |
| 20:14 | 20:15 | 20:15 | | | | | | 20:14 | 20:15 | 20:15 | 20:15 | 20:16 | 20:16 | 00:02 | |
| 20:49 | 20:49 | 20:49 | 20:49 | 20:51 | 20:52 | 20:52 | 00:03 | 20:53 | 20:54 | | | | | | |
| 21:23 | 21:23 | 21:23 | 21:24 | 21:24 | 21:24 | 21:24 | 00:01 | 21:27 | 21:28 | 21:28 | 21:28 | 21:28 | 21:28 | 00:05 | |
| 21:56 | | | | | | | | 21:56 | 21:57 | 22:00 | 22:00 | | | | |
| 22:29 | 22:29 | 22:31 | 22:31 | | | | | 22:29 | 22:31 | 22:31 | | | | | |
| 23:04 | | | | | | | | 23:04 | 23:04 | 23:04 | 23:05 | 23:05 | 23:05 | 00:01 | |
| 23:39 | 23:39 | 23:39 | 23:39 | 23:44 | 23:44 | 23:44 | 00:05 | 23:42 | 23:42 | 23:42 | | | | | |
| 00:32 | 00:32 | 00:32 | 00:34 | 00:34 | 00:34 | 00:34 | 00:02 | 00:34 | | | | | | | |
| 00:59 | 00:59 | 00:59 | 01:01 | 01:01 | 01:01 | 01:01 | 00:02 | 00:59 | 01:02 | 01:02 | 01:02 | 01:02 | 01:02 | 00:03 | |
| 01:39 | | | | | | | | 01:39 | 01:44 | 01:44 | 01:44 | 01:44 | 01:44 | 00:05 | |
| 02:12 | | | | | | | | 02:12 | 02:13 | 02:13 | 02:15 | 02:15 | 02:15 | 00:03 | |
| 02:44 | 02:47 | 02:47 | 02:49 | | | | | 02:44 | 02:47 | 02:47 | 02:47 | 02:47 | 02:47 | 00:03 | |
| 03:21 | | | | | | | | 03:21 | 03:21 | 03:23 | 03:24 | 03:24 | 03:24 | 00:03 | |
| 03:54 | | | | | | | | 03:54 | 03:58 | | | | | 1 CPT | |
| 04:33 | | | | | | | | 04:33 | 04:37 | | | | | 1 CPT | |
| 05:11 | 05:11 | 05:11 | 05:15 | 05:15 | 05:15 | 05:15 | 00:04 | 05:13 | | | | | | 3 CPTs | |
| 05:42 | | | | | | | | 05:42 | 05:42 | 05:42 | 05:44 | 05:46 | 05:46 | 00:04 | |
| 06:19 | 06:22 | 06:24 | | | | | | 06:19 | 06:19 | 06:19 | 06:19 | 06:19 | 06:19 | 00:00 | |
| 06:48 | 06:49 | 06:52 | 06:52 | 06:52 | 06:52 | 06:52 | 00:04 | 06:48 | 06:48 | 06:49 | 06:49 | 06:50 | 06:50 | 00:02 | |
| 07:20 | 07:21 | 07:22 | 07:22 | 07:22 | 07:23 | 07:23 | 00:03 | 07:20 | 07:20 | 07:20 | 07:21 | 07:21 | 07:21 | 00:01 | |

4.2. Complete protection time (CPT) for test day 5

Complete protection time for test day 5 was determined as 14 h 6 min.

Despite low landing pressure being determined for two time points later in the test (03:54 and 04:33), CPTs were recorded indicating mosquitoes were present and landing although not being recorded at 5 mosquitoes per 5 minutes on either of the untreated subjects (Table 1). At time point 05:11, one untreated subject reached threshold and 3 CPTs were recorded again implicating that sufficient mosquitoes were present.

In addition, landing pressure was demonstrated to be sufficient at the end of testing as the threshold was reached for both of the untreated subjects at the last two testing time points (06:48 and 07:20). Six subjects were yet to reach CPT at 14.5 hours after product application, indicating the product was repelling mosquitoes at the end of testing when mosquitoes were landing in numbers above the threshold.

4.3. Untreated subject 6

- 10 out of 22 time points reached threshold (5 mosquitoes landing in 5 min, Table 1)
- Threshold was reached within 3 min for 6 out of 10 time points indicating landing pressure was higher than required for these time points.
- Out of the 12 time points that did not reach threshold, 0 mosquito landings were recorded in 8 of the time points. Two of the time points recorded 2 landings within 5 min, and 2 of the time points recorded 3 landings within 5 min. This may indicate that this subject was not as attractive as untreated subject 30 but mosquitoes were present.
- Estimated average landing pressure on untreated subject 6, throughout the study, was 1.14 mosquitoes per minute. Please note that this is an estimated rate, since as soon as five landings occurred, regardless of whether five minutes had elapsed, the untreated subject covered the lower leg and recording of mosquito landings stopped.

4.4. Untreated subject 30

- 14 out of 22 time points reached threshold (5 mosquitoes landing in 5 min, Table 1)
- Threshold was reached within 3 min for 11 out of 14 time points indicating landing pressure was higher than required for these time points.
- Out of the 8 time points that did not reach threshold, 0 or 1 mosquito landing was recorded in 2 of the time points. Three time points recorded 2 landings within 5 min, 2 time points recorded 3 landings within 5 min and 4 mosquitoes landed in one time point, therefore, indicating mosquitoes were present.
- Estimated average landing pressure on untreated subject 6, throughout the study, was 1.71 mosquitoes per minute. Please note that this is an estimated rate, since as soon as five landings occurred, regardless of whether five minutes had elapsed, the untreated subject covered the lower leg and recordings of mosquito landings stopped.

4.5. Untreated subject data per published guidelines (OPPTS 810.3700: Insect Repellents to be Applied to Human Skin)

As stated in 4.1, four testing periods (21:56, 22:29, 03:54 and 04:33) occurred where both of the untreated subjects had fewer than five landings in the five-minute test period (Table 1). During these testing periods, one mosquito landed within one minute, as per published guidelines (see section 2) on untreated subject 30 and in one of the testing periods (22:29) on untreated subject 6.

4.6. Conclusion of test day 5 untreated subject data

We conclude that test day 5 was conducted as per study protocol (v 7.0 and amendment No. 7) and sufficient data were collected to show the presence of sufficient landing pressure. Out of 22 time points, there are only two where there is no justification that landing pressure was sufficient (21:56 and 22:29) as neither threshold was reached nor was a CPT recorded. It should be noted that one mosquito did land within one minute, as per the published guidelines, on untreated subject 30 during the testing periods of low landing pressure. Furthermore, an average landing rate of one mosquito per minute was recorded when the data for untreated subjects was analyzed separately or combined (combined average landing rate = 1.05). Although the threshold of five landings in five minutes was not achieved for four of the earlier time points during the test day, importantly this threshold was reached for both of the untreated subjects in the last two testing time points. Despite this high landing pressure at the end of the testing period, six out of 13 treated subjects were yet to reach CPT 14.5 hours after product application. This indicates that the product was repelling mosquitoes far beyond 3 hours post application. To suggest that the test substance provided median complete protection for less than 14 hours would be to ignore the data collected at these later time points.

5. Test day 6 untreated subject data

5.1. Summary of test day 6

On test day 6, test substance was applied between 16:50 and 16:57 h (7-minute window). Seven subjects achieved CPT and the test was terminated at around 8:00 h or 15 hours post-application at which point median CPT is calculable. The remaining six subjects were censored.

Four testing periods occurred where both of the untreated subjects had fewer than five landings in the five-minute test period (Table 2, grey shading). Therefore, these testing periods were determined as low landing pressure, as per protocol amendment letter No 7.

The protocol (v7.0) states that “if there is low landing pressure in more than 4 non-consecutive test periods over the course of the 14-hour test period, the test must be stopped”.

Therefore, the test day was conducted as per study protocol.

5.2. Complete protection time (CPT) for test day 6

Complete protection time for test day 6 was determined as 15 h 10 min.

At three time points, 03:12, 05:40 and 07:52, one untreated subject reached the threshold and of five landings in five minutes and five CPTs were recorded in total implicating that sufficient mosquitoes were present.

In addition, landing pressure was demonstrated to be sufficient at the end of testing as this threshold was reached for both of the untreated subjects in two of the three last testing time points (06:54 and 07:16). Six subjects were yet to reach CPT at 15 hours after product application. Therefore, despite there being a high landing pressure 15 hours after product application, the test substance was providing protection against mosquitoes indicating the product was repelling mosquitoes at the end of testing when mosquitoes were present in numbers above threshold.

Table 2: Test Day 6 untreated subject data

| Start Time | Untreated control subject 35 | | | | | | | Untreated control subject 48 | | | | | | | CPT |
|------------|------------------------------|-------|-------|-------|-------|-------------------|----------------------------------|------------------------------|-------|-------|-------|-------|-------------------|----------------------------------|--------|
| | Time of Landings | | | | | Threshold reached | Time between start and threshold | Time of Landings | | | | | Threshold reached | Time between start and threshold | |
| | 1 | 2 | 3 | 4 | 5 | | | 1 | 2 | 3 | 4 | 5 | | | |
| 18:51 | 18:51 | 18:51 | 18:51 | 18:51 | 18:51 | 18:51 | 00:00 | 18:53 | 18:53 | 18:53 | 18:53 | 18:53 | 18:53 | 00:02 | |
| 19:24 | 19:24 | 19:24 | 19:25 | 19:25 | 19:25 | 19:25 | 00:01 | 19:24 | 19:25 | 19:25 | 19:25 | 19:25 | 19:25 | 00:01 | |
| 20:05 | 20:05 | 20:05 | 20:05 | 20:05 | 20:07 | 20:07 | 00:02 | 20:06 | 20:06 | 20:09 | | | | | |
| 20:36 | 20:36 | 20:36 | 20:36 | 20:36 | 20:36 | 20:36 | 00:00 | 20:36 | 20:38 | 20:38 | | | | | |
| 21:12 | 21:13 | 21:13 | 21:15 | 21:15 | | | | 21:12 | 21:12 | 21:13 | 21:13 | 21:14 | 21:14 | 00:02 | |
| 21:42 | 21:43 | 21:43 | | | | | | 21:42 | 21:42 | | | | | | |
| 22:24 | 22:24 | 22:24 | 22:24 | 22:25 | 22:27 | 22:27 | 00:03 | | | | | | | | |
| 23:05 | 23:05 | | | | | | | 23:07 | 23:07 | 23:09 | | | | | |
| 23:41 | | | | | | | | 23:41 | 23:41 | 23:41 | 23:41 | 23:41 | 23:41 | 00:00 | |
| 00:22 | 00:26 | | | | | | | 00:22 | 00:22 | 00:23 | 00:23 | 00:24 | 00:24 | 00:02 | |
| 00:57 | 00:59 | 01:00 | 01:00 | | | | | 00:57 | 00:58 | | | | | | |
| 01:27 | 01:32 | 01:32 | | | | | | 01:27 | | | | | | | |
| 02:07 | 02:07 | 02:07 | 02:09 | 01:09 | 02:09 | 02:09 | 00:02 | 02:11 | 02:11 | | | | | | |
| 02:39 | 02:42 | 02:43 | 02:43 | 02:43 | 02:43 | 02:43 | 00:04 | 02:39 | 02:40 | 02:40 | 02:40 | | | | |
| 03:12 | 03:13 | 03:14 | 03:15 | 03:16 | 03:16 | 03:16 | 00:04 | 03:12 | 03:13 | 03:14 | | | | | 1 CPT |
| 03:44 | 03:44 | 03:44 | 03:44 | 03:44 | 03:44 | 03:44 | 00:00 | 03:47 | | | | | | | |
| 04:24 | 04:24 | 04:29 | 04:24 | 04:24 | 04:24 | 04:24 | 00:00 | 04:26 | 04:26 | | | | | | |
| 05:00 | 05:01 | 05:02 | 05:02 | 05:02 | 05:02 | 05:02 | 00:02 | 05:00 | 05:09 | | | | | | |
| 05:40 | 05:40 | 05:40 | 05:40 | 05:40 | 05:40 | 05:40 | 00:00 | | | | | | | | 3 CPTs |
| 06:16 | 06:19 | 06:19 | 06:19 | 06:20 | 06:20 | 06:20 | 00:04 | 06:16 | 06:16 | 06:20 | | | | | |
| 06:54 | 06:54 | 06:54 | 06:55 | 06:55 | 06:55 | 06:55 | 00:01 | 06:54 | 06:54 | 06:54 | 06:54 | 06:54 | 06:54 | 00:00 | |
| 07:16 | 07:18 | 07:18 | 07:18 | 07:18 | 07:19 | 07:19 | 00:03 | 07:16 | 07:16 | 07:16 | 07:18 | 07:18 | 07:18 | 00:02 | 2 CPTs |
| 07:52 | 07:52 | 07:52 | 07:54 | 07:54 | 07:55 | 07:55 | 00:03 | 07:52 | 07:52 | | | | | | 1 CPT |

5.3. Untreated subject 35

- 17 out of 23 time points reached threshold (5 mosquito landings in 5 min, Table 2)
- Threshold was reached within 3 minutes for 13 out of 17 time points indicating landing pressure was higher than required for these time points.
- Out of the 6 time points that did not reach the threshold, 0 or 1 mosquitoes landing was recorded in 3 of the time points. Two time points recorded two landings within 5 min, and 3 time points recorded 3 landings within 5 min, therefore indicating mosquitoes were present.
- Estimated average landing pressure on untreated subject 35, throughout the study, was 2.28 mosquitoes per minute. Please note that this is an estimated rate, since as soon as five landings

occurred, regardless of whether five minutes had elapsed, the untreated subject covered the lower leg and recordings of mosquito landings stopped

5.4. Untreated subject 48

- 7 out of 22 time points reached threshold (5 mosquito landings in 5 min, Table 2)
- Threshold was reached within 3 minutes for all 7 time points, indicating landing pressure was higher than required for these time points.
- Out of the 16 time points that did not reach the threshold, 0 or 1 mosquito landings was recorded in 4 of the time points. Six of the time points recorded 2 landings within 5 min, and 5 of the time points recorded 3 landings within 5 min and 4 mosquitoes landed in 1 time point. This may indicate that this subject was not as attractive as untreated subject 35 to the mosquitoes present at the time.
- Estimated average landing pressure on untreated subject 48, throughout the study, was 1.37 mosquitoes per minute. Please note that this is an estimated rate, since as soon as five landings occurred, regardless of whether five minutes had elapsed, the untreated subject covered the lower leg and recordings of mosquito landings stopped.

5.5. Untreated subject data per published guidelines (OPPTS 810.3700: Insect Repellents to be Applied to Human Skin)

As stated in 5.1, four testing periods (21:42, 23:05, 00:57 and 01:27) occurred where both of the untreated subjects had fewer than five landings in the five-minute test period (Table 2). During these testing periods, one mosquito landed within one minute, as per published guidelines (see section 2) on either of the untreated subjects (21:42, 23:05 and 01:27 on untreated subject 30; 00:57 on untreated subject 48).

5.6. Conclusion of test day 6 untreated subject data

We conclude that test day 6 was conducted as per study protocol (v 7.0 and amendment No. 7) and sufficient data were collected to show the presence of sufficient landing pressure. Overall, there are four time points out of 23, where there is no justification that landing pressure was sufficient (21:42, 23:05, 00:57 and 01:27) as neither threshold was reached nor was a CPT recorded. It should be noted that one mosquito landed within one minute, as per the published guidelines, on one of the untreated subjects during these testing periods. Furthermore, an average landing rate of one mosquito per minute was recorded when the data for untreated subjects was analyzed separately or combined (combined average landing rate = 1.73). Although the threshold was not achieved for four of the earlier time points, importantly the threshold of five mosquitoes in five minutes was reached for both of the untreated subjects in two of the three last testing time points. Even though landing pressure was high at the end of the testing period, six out of 13 treated subjects were yet to reach CPT at 15 hours after product application. This indicates the product was repelling

mosquitoes, providing efficacy, at the end of testing when landing pressure was high. To suggest that the test substance provided median complete protection for less than 15 hours would be to ignore the data collected at these later time points.

6. Field sites, trapping data and mosquito species

6.1. Protocol

Protocol (v7.0) states on page 21:

10.1.1. Site monitoring

The field study will be conducted in the spring and summer in Florida when mosquito activity is normally high. Mosquito trapping at each possible field site will begin in spring and trap catch and observations of mosquito activity will be made. **The study will be initiated once mosquito abundance is high.**

Field tests for mosquito repellents will be conducted in at least **two distinct habitats**, most likely a forest or wetland and an urban environment, **where the predominant mosquito species differ**. The tests likely will be conducted in Putnam and Alachua counties, Florida, USA. This area is outside the current hotspot of ZIKV transmission but in an area of high mosquito abundance and diversity. However, **efforts will be made to include a site where *Aedes albopictus* is present.**

And on page 22:

10.2. Test Insects

Mosquito tests will be conducted where **more than one species are present**. At least **one site** will be selected that has an **abundance of ZIKV vectors (*Aedes albopictus*)**, but both sites will have no previous history of transmission.

6.2. Study site selection and mosquito abundance before study initiation

The two study sites were selected from the five sites with nine sampling locations due to trapping abundance and diversity. The mosquito captures per night were highest at these two sites compared to all other sites and locations with 91 and 185 mosquitoes caught per night for site 1 and site 2, respectively. It is important to note that more than twice as many mosquitoes were caught per night at site 2, the site with lower landing pressure on the repellent testing days.

The species diversity was relatively equal across the sites, ranging between 21 species at site 1 to 26 at site AC (an unused site). See page 25, Table 2 of the final report for a summary of species captured during trapping for site 1 and 2. Site 2 had 24 species represented. However, it is important to note that at site 1, 62% of the mosquitoes captured were *Aedes albopictus*. At site 2 the most predominant species was *Culex*

nigripalpus at 32% followed by *Ochlerotatus fulvus pallens* at 25% and *Psorophora ferox* at 20%. Site 1 and 2 were selected due to these differences in species diversity and representation in trap catches.

6.3. Mosquito species landing on subjects

Looking at the test day mosquito collection data, it is not surprising to note that *Aedes albopictus* makes up 75% of the captures from test day subjects in site 1. It is interesting to note that this species makes up 22% of the captures from test day subjects in site 2. *Aedes albopictus* was not a common species in site 2, making up just 0.6% of total captures, such is its predisposition for feeding on human beings. The most common species on subjects at site 2 was *Mansonia dyari*, a species that made up just 1.24% of captures in traps at this site. Of the most common species in traps at site 2, only *Psorophora ferox* was recorded during the landing catches, making up 3.7% of captures on subjects. These data suggest that even when targeting an area with a diverse mosquito fauna you will likely be relying on a few individual species with human feeding preferences to sustain the landing pressure.

6.4. Effect of mosquito species on landing pressure

Aedes albopictus was predominately collected from site 1 where median CPT of 14 h and 8 min and > 16 hours was recorded. The EPA have determined that sufficient landing pressure was maintained throughout testing. *Aedes albopictus* are a medically important opportunistic feeder with a preference for human blood meals. Also, female *Aedes albopictus* are aggressive biters during daylight hours and prefer to feed outdoors. Due to the feeding habits of the predominant species, *Aedes albopictus*, at test site 1 the landing pressure was considered valid by the EPA (test days 1 and 4). Even so the landing pressure was lower on test day 4 (compared to test day 1) most likely due to the sunnier conditions, the maximum light intensity was 576 lum/ft on test day 4 compared to 192 lum/ft on test day 1. Therefore, on test day 1 at site 1, the behavior of *Aedes albopictus* and the overcast conditions, resulted in no test periods where the threshold for the untreated subject data was not met. On test day 4 at site 1, there were four periods where the threshold was not met, three of these were at the end of the day. This is related to the abundance of day biting mosquitoes at this site and relative lack of nocturnal feeders. Florida is known for its abundance of mosquitoes and other biting insects. However, even so, in Florida *Aedes albopictus* typically would not exhibit activity over the whole testing period i.e. 14 hours, due to the heat in summer (when abundance is also at its peak). Avoidance of activity during the sunny hot weather reduces the risk of desiccation and death. However, we were fortunate that on both test days 1 and 4 the weather remained overcast allowing for prolonged activity throughout the duration of the test.

With regards to test site 2, due to protocol restrictions, a different habitat, where the predominant mosquito species differ to *Aedes albopictus* was to be located. The challenges of finding two sites of high mosquito abundance and varying mosquito diversity between the two sites as recommended in the guidelines should not be underrated. Especially if one must also locate a site with mosquitoes with a preference for feeding on human beings to an extent high enough to maintain landing pressure for 14 hours when very few mosquito

species would be active for 14 hours consecutively. Previous research detailing diel activity patterns have recorded that, in general, host-seeking activity peaks last for four hours at most for each species (Jaenson 1988, Caglar et al. 2003, Veronesi et al. 2011, Barnard et al. 2014, Montarsi et al. 2015).

Culex and *Mansonia* species bite during the night. To compensate for this, field testing start time was moved from a daytime test to ensure the total night was in the testing period, but these species still normally have a temporal blood feeding rhythm, i.e. *Culex* may feed more during twilight than in the middle of the night and *Mansonia* are known to be particularly aggressive at sunset but activity dies down after a few hours. Therefore, inconsistent landing pressure during a prolonged period of time, i.e. 14 hours, in test site containing less anthropophilic biters should be expected. Different species are expected to be active at different times of the day and may show different host preferences. It is therefore natural that one untreated (or indeed treated) subject may be more attractive than another, and as a consequence over the course of a day or night there may be periods of lower landing pressure, but also fluctuations between which of the untreated subjects receives more landings. In addition, behavior of the mosquitoes was observed to be different by the study director and her staff between day (site 1) and night (site 2) tests. During the dark hours mosquitoes were clearly using their olfactory senses more than their visual senses and it appeared that they moved more slowly and often in a wave towards one person. Therefore, it is more likely that stronger differences in preference will be observed by nocturnal mosquitoes that are mainly relying on olfaction compared with diurnal species that utilized vision in addition to other sensory cues.

6.5. Conclusion of field sites, trapping data and mosquito species

Study site selection was as per the protocol (v 7.0); two distinct habitats were identified where the predominant mosquito species differed, and mosquito abundance was demonstrated to be high before the study was initiated. In the trapping conducted before the repellent testing started to estimate mosquito abundance and species diversity, twice as many mosquitoes were caught per night at site 2, the site with lower landing pressure, than site 1, the site that the EPA have determined had sufficient landing pressure on test days. *Aedes albopictus* was the predominant species at site 1 whereas *Culex nigripalpus*, *Ochlerotatus fulvus pallens*, *Psorophora ferox* and *Mansonia dyari* were the predominant species at Site 2, caught during pre-testing trapping and/or landing on subjects. Due to the feeding behavior of the predominant species, *Aedes albopictus*, at test site 1 and overcast weather, no test periods occurred where the threshold for the untreated subject data was not met in test day 1. Although test day 4 was sunnier the aggressive landing behavior of *Aedes albopictus* still resulted in acceptable landing pressure in all but four test periods. In contrast at site 2, where the prominent mosquito species exhibit temporal feeding behaviors and less of a preference for human feeding, recording consistent landing pressure is less likely over a prolonged period of time, i.e.14 hours.

7. References

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