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UNIVERSIDAD DE ZARAGOZA

FACULTAD DE VETERINARIA

Departamento de Patología Animal

"Fototropismo en dípteros del género *Phlebotomus*"

Proyecto de Fin de Grado en Biotecnología dirigido por

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ABBREVIATIONS

ANOVA: analysis of variance

CDC: Center for disease control

cm: centimeter

g: gram

LED: light-emitting diode

l: liter

L1-3: larval instars 1 through 3

mg: milligram

mm: millimeter

NJLT: New Jersey light trap

PCR: polymerase chain reaction

***spp*:** multiple species

Th: T helper (lymphocyte)

V: Volt

W: Watt

ºC: degrees Celsius

1. Abstract

Phlebotomus papatasi (Diptera: Psychodidae) es el flebotomo vector de de la leishmaniasis cutánea zoonótica causada por *Leishmania major* (Trypanosomatidae: Kinetoplastida), una enfermedad parasitaria causada por un protozoo prevalente en la mayoría de regiones mediterráneas, siendo considerada en muchos casos un problema de salud grave tanto animal como humano.

En este trabajo se estudió el tropismo positivo de individuos adultos de esta especie, confinados en un recinto experimental tubular desarrollado durante el transcurso del mismo, hacia la luz emitida por dispositivos LED (diodo emisor de luz) de tres colores principales, el verde, rojo y azul, además de varias pruebas adicionales con luces violeta y amarilla. Se empleó la luz ultravioleta procedente de trampas CDC como control positivo y un control negativo en ausencia de luz para todas las pruebas. Los individuos proceden de una colonia de flebotominos (*P. perniciosus* y *P. papatasi*) ubicada en el departamento de patología clínica veterinaria de la universidad de Zaragoza.

Los resultados indican que existe una atracción elevada hacia la luz ultravioleta en los casos estudiados, mientras que las luces led no generan una atracción muy significativa. Específicamente la luz verde dio lugar a un grado menor de atracción en los casos estudiados. Tanto en las pruebas con machos aislados como con una combinación de machos y hembras, los machos resultaron ser más atraídos por las luces que las hembras. Estos resultados podrían resultar de utilidad en el diseño de nuevas trampas para el control o monitoreo de flebotominos y en el desarrollo de sistemas de iluminación menos peligrosos para animales domésticos.

1. Abstract

Phlebotomus papatasi (Diptera: Psychodidae) is a sandfly that acts as a vector of the zoonotic cutaneous leishmaniasis caused by *Leishmania major* (Trypanosomatidae: Kinetoplasmida), a parasitic disease caused by a protozoan that is prevalent in most Mediterranean regions, and that is considered a serious threat to the health of both humans and livestock.

In this paper the attraction towards light emitting diodes (LEDs) experienced by adult individuals of the aforementioned species was tested within a tubular experimental circuit constructed specifically to this end. The experiments focused on three main colors; green, red and blue, and additionally yellow and purple lights were also tested for. Ultraviolet lighting from CDC insect traps was used as the positive control, and every experiment was also accompanied by a lightless negative control. All sandfly specimens used were from a phlebotomine colony (that features both *P. papatasi* and *P. perniciosus*) located in the department of clinical pathology of the University of Saragossa.

The results indicate that there exists a strong attraction towards ultraviolet light in the experimental conditions, whereas LED lighting does not generate a strong tropism. Green light specifically appeared to show the lowest degree of attraction. Males were more attracted to the lights than females in all the tests, both in a same sex group and combined with females. These results could prove useful in the design of new insect traps for pest control or phlebotomine population monitoring, as well as in the development of safer lighting systems for livestock.

2. Introduction

2.1 Main characteristics of sandflies

Sandflies (Phlebotominae) are a subfamily of insects belonging to the order Diptera and specifically to the family Psychodidae. Amongst this group, only two genera function as vectors of leishmaniosis, *Lutzomyia* in the new world, and *Phlebotomus*, which is the focus of this paper, in Europe, Africa and Asia **[1]**. In Spain, the genus is represented by 13 species but only those belonging to the subgenus *Larrossius* are capable of transmiting the disease **[2]**. This paper has its focus centered upon the species *P. perniciosus* and *P. papatasi*, the most important vectors in the Spanish interior **[3]**.

These dipterans may be distinguished by the naked eye thanks to their small size of between 2 and 3 mm, very long legs, two lanceolate wings that are held open in a V-like shape when at rest, and a color that varies between light brown and beige. They are nocturnal insects, with a peak in their activity that starts at dusk and continues until midnight. As for their habitat, sandflies are to be found in natural environments such as animal nests and at the foot of trees and bushes, but also in man-made areas like sewers, gardens, farming land, etc **[2]**.

The full life cycle of the sandfly lasts about 45 days in optimal conditions. The animal completes 4 developmental phases: egg, larva, pupa and an imaginal or adult stage. It is not possible to give more precise time periods for each stage because their length varies significantly depending on air temperature and humidity **[1]**.

Ideal growth conditions include a temperature of 17-30ºC, the exact value depending on the species, and a humidity level of 80%. **[2, 4]**. A lower temperature would increase the duration of the cycle, whereas temperatures above 40ºC and low humidity levels can kill the sandflies **[2, 5]**. In laboratory conditions, the eggs take 7-10 days to hatch. The larvae, worm-like in appearance, take about 3 weeks to pupate and go through 3 molts that separate the 4 larval stages, termed as instars, and referred to as LI-IV. The adult sandflies finally emerge from the pupae after about 10 days, with males emerging earlier than females **[1]**.

Nourishment depends on the stage of the insect. Larvae have chewing mouth parts and feed off organic matter. Adults bear cutting-sucking mouth parts that allow them to cut through skin and sever capillaries to let the blood flow out, which they then directly ingest **[2]**. However, the adult insects also feed on the nectar of flowers, ripe fruits and other plant sources of sugar, as well as aphids. The ingestion of blood is a needed requirement for the female to be able to develop eggs. Only the females have sufficiently developed mouth parts to be able to cut through skin, so that they are the sole vectors of leishmaniasis. On occasion blood-filled males may be observed, but in these cases it is assumed that they have obtained it from an already open wound **[1]**. Throughout their life females may bite between 3 and 5 times, although most of them only bite once in their life time. The number of eggs laid is directly correlated to the quantity of blood ingested, and may reach 200. Most species, among them those mentioned in this paper are also gonotrophic, meaning they lay their eggs after each blood ingestion**[1, 2]**.

2.2 General aspects of leishmaniosis

2.2.1 Introduction

Leishmaniasis in human medicine, or leishmaniosis in a veterinarian context, refers to a disease caused by all the tripanosomatidean protozoa belonging to the genus *Leishmania*. It is a vector disease that is always transmited by the bite of a sandfly **[6]**. It may be zoonotic, when the reservoir is wild animals or livestock, or anthroponotic when the reservoir is human **[1]**. The first depictions of human leishmaniasis trace back to the year 650 b.C. in Babylon **[7]**. Currently 88 countries are affected, especially those located in tropical and subtropical areas, and it is associated with about 70000 deaths every year **[8]**. The clinical manifestations can be more or less severe, giving name to three distinct types of leishmaniasis: cutaneous, mucocutaneous and visceral **[6]**.

Thirty species are known within the genus *Leishmania*, out of which about twenty are known to be pathogenic to humans **[9]**. In Europe, and more specifically in the Mediterranean region, *L. infantum* is the main causative agent of the disease, which causes cutaneous and visceral forms in humans as well as in dogs, its other main reservoir **[10]**.

2.2.2 Biological cycle of *Leishmania*, mecanism of pathogeny and clinical profile

Leishmania presents two stages, the stage present depending on the host: in sandflies it is present as a promastigote, the elongated extracellular flagellated form, and in its vertebrate hosts it is found as an amastigote, oval in shape, much smaller and located within the phagolisosomes of mononuclear phagocytic cells **[11]**.

The cycle begins when a female sandfly feeds on the blood of a mammal with amastigote-infected macrophages. These amastigotes turn into promastigotes in the sandflies' intestine and quickly rise in number as they traverse a series of non-infective middle stages. On reaching the stage of metacyclic promastigotes, the protozoans are already infective and extend a phlagellum that allows them the mobility to migrate to the sandflies' proboscis. During the sandflies' next blood ingestion the promastigotes are regurgitated and penetrate the wound caused during the bite. In the vertebrate host they are engulfed by macrophages and other phagocytes, then transform into amastigotes and are included into a parasitophorous vacuole that constitutes the phagolysosome. There the protozoan reproduces without being detected until it causes its host cell to lysate. Once free in the blood vessels it can again be phagocyted by other cells, completing the cycle **[11]**.

In dogs the mechanism of patogenicity adjusts to the following description. When the promastigotes are inoculated into the mammalian host a local inflammatory response is triggered that attracts neutrophils, eosinophils, natural killer cells, and later on macrophages. The antigens carried by *Leishmania* are phagocyted by dendritic cells or are freely transported by way of the lymphatic ganglia, where they are presented to activate T lymphocytes. This initially triggers a Th2 type response that leads to the release of various cytokines. Depending on the cytokines released the Th1 or the Th2 type response will prevail, which in turn

determines whether the individual is susceptible or resistant to the disease, respectively. In the Th1 response, activated Th1 lymphocytes release cytotoxic NO that causes the death of the parasite, and at the same time the proliferation of B lymphocytes is activated so that immunoglobulins are secreted that encourage resistance to intracellular microorganisms, and the destruction of infected macrophages occurs through Fas-dependent apoptosis. In the Th2 response however, the parasite causes a polyclonal stimulation of B lymphocytes that causes an excess in immunoglobulins. The high concentration of circulating immune complexes that results is the direct cause of the pathologies related to this disease **[12]**.

The clinical profile of leishmaniasis in humans, as well as in dogs, depends on a variety of factors that determine the immune response: parasite genotype, size of the inoculum, zone of inoculation, number of bites received, the sandflies' saliva, certain genetic traits of the host, the existence of concomitant infections, etc. This originates three types of leishmaniasis. In the first one, the cutaneous type, the parasites remain isolated in the macrophages of the area that was bitten as a result of a strong cellular immune response. This causes the appearance of erythematose papules in the site of the bite that grow and ulcerate, generating scabs of dry exudate that may heal by themselves leaving behind hypopigmented scars. In the mucocutaneous form, the parasites spread towards the mucous membrane and can potentially destroy and disfigure it, especially the nasal and buccal mucosa. Finally, in its visceral form, the parasites spread into inner organs as a result of a failed cellular response and cause fever, weight loss, hepatosplenomegaly, glomerulonephritis, anemia and kidney failure, among other alterations **[13]**. It should be noted that specifically canine leishmaniasis is frequently both cutaneous and visceral **[14]**.

2.2.3 Diagnosis and treatment

Diagnosis, in humans as well as in animals, can be achieved through microscopy, examining the presence of the parasite in dyed blood films from splenic biopsy, bone marrow and lymph nodes, in skin extracts and tissue biopsies. If the infection is of a low degree, parasite detection should be accomplished by isolating it *in vitro* or *in vivo* or with a polymerase chain reaction (PCR). Since the differences between species are few, any isolated *Leishmania* must be identified by molecular, biochemical and immunological methods **[15]**.

The treatment depends on the location, the type of *Leishmania* and the seriousness of the disease. The first line of treatment consists in the administration of pentavalent antimony derivates: N-metilglucamine antimoniate, generally in Europe, and sodic estibogluconate, normally in America. More and more resistances to these drugs are appearing, however. Noteworthy second line pharmaceuticals include Amphotericin-b, that can cure visceral leishmaniasis in spite of its toxicity and, more recently, Miltefosine, a new drug that has proven itself safe and effective in the short term, but also has teratogenic properties. In addition to these, levamisol and the Calmette and Guerin bacillus are used as coadjuvants. Despite these medical approaches, the death rate among patients suffering from visceral leishmaniasis remains high even with a proper treatment **[13]**.

2.3 Control and prevention of leishmaniosis

No effective vaccine against leishmaniasis exists currently **[15]**. For this reason, control measures for the disease focus on the early detection and treatment of cases, and on the elimination of vectors and reservoirs.

The sacrifice of seropositive dogs is an option hardly considered because of ethical and social reasons. Despite this, a study was conducted in Brazil where this prophylactic measure was taken, and it was observed that a reduction in seropositive dogs does not correlate with a decrease in the prevalence of human leishmaniasis **[16]**. On the other hand, the use of insecticide-impregnated collars for dogs has been suggested. Experimental results indicate that this could significantly lower infection in humans and dogs, but more studies on the subject are still needed **[17]**.

Treatment of infected animals has also been attempted as a control method. However, in a study conducted in Italy where all asymptomatic and oligosymptomatic dogs were treated and symptomatic ones were suppressed, it was concluded that prevalence of the disease did not vary much either **[18]**.

Finally, fighting off the vectors has been tried, spraying the walls of houses and animal nests such as rabbit holes and termite mounds with insecticidal agents. However, more studies of this kind are required, and also a better knowledge of the feeding, resting, and reproductive habits of the various sandfly species so as to characterize them before insecticide implementation. There is also evidence that insecticide-resistant sandfly populations are starting to arise **[17]**.

2.3.1 The use of light traps in sandfly control

Within the context of leishmaniasis control programmes, it is important to have methods of vector monitoring. One of the most frequently used is the capture of sandflies with CDC light traps **[1]** the history of which will be briefly outlined.

The first prototypes of light traps were the New Jersey traps during the 1920s **[19]**. The NJLT uses a 25 W incandescent light bulb as a means to attract numerous mosquito species, and it is still in ample use as an element in mosquito population assessment. Later on, the need for smaller and more portable traps led to the development of CDC type traps **[20]**, which use an incandescent lamp powered by a 6 V battery and are very often supplemented with a CO_2 emitting agent. In the last 70 years, investigators have tried a wide variety of traps that incorporate artificial light of different colors, intensity and/or frequencies in an attempt to improve the effectiveness of the present capture systems **[21, 22, 23]**.

The color of the trap itself (reflected light) and the color of the lamp are among the most studied stimuli in the attempt to increase the efficacy of traps for the capture of mosquitoes, sandflies, and other biting Diptera **[22]**.

3. Aims

The main aim of this paper is to add new data regarding the response shown by species of the genus *Phlebotomus* towards monochromatic light emitting diodes (LEDs). To this end, the following specific objectives are set:

1. To contribute to the maintenance and management of a laboratory-reared colony of the species *P. papatasi* and *P. perniciosus,* and becoming familiar with their life cycle and requirements.

2. To establish a self-developed protocol of exposure to a selected range of LED colors as well as other kinds of light.

3. To determine the characteristics of light-induced behavior upon exposure to green, red and blue lights of young adult individuals of the species *P. papatasi* in same-sex groups as well as in mixed groups.

4. Materials and Methods

The procedures described in this paper structure themselves around the following sequence: larval substrate preparation, procurement of wild-caught individuals, establishment and maintenance of colonies, setup of the enclosure used for the study of phototropism and finally, the phototropism studies themselves in *Phlebotomus papatasi* and analysis of the data obtained.

4.1 Species used

The colony currently holds two species, *P. perniciosus* and *P. papatasi*. Rearing requirements of both species have been studied, whereas the light attraction studies were performed on *P. papatasi*.

4.2 Rearing of *Phlebotomus spp*

4.2.1 Preparation of larval substrate

The first step of this endeavor was the preparation of the substrate required to feed the larvae of *Phlebotomus spp* that would normally be obtained subsequently. Said substrate was elaborated starting with a mixture composed of 50% rabbit feed and another 50% in rabbit pellets, dried for at least 7 days. The feed and the dry pellets were ground with a coffee grinder until a fine dust was obtained. Distilled water was added, mixing it in by hand until a thick dough-like texture was achieved, and then the mix was introduced into the heater, set at 35ºC, in 28,5x17x15 cm bins covered with aluminum foil. Rapid fungal growth was observed on the surface of the mix, forming a crust that was mixed back into the inner mass by hand until no traces remained and put back into the heater on a weekly basis so that a new layer of mold could form. This same procedure was repeated until mold growth failed to occur and the substrate released a fertilizer-like smell, which took about two months to occur. This process was further reinitiated every 2 months approximately, starting with 2300 g of each component (rabbit feed and pellets). The already elaborated feeding substrate was introduced into plastic bins of the same characteristics as the former, leaving a side of the lid open, and was stored at room temperature, about 23ºC.

4.2.2 Obtention of live material

The capture of wild sandflies was accomplished through the placement of traps consisting in an empty plastic 2L soft drink bottle, washed, cut and bound with tape so that the bottleneck would work as a funnel. This device was placed in a number of about 60 in total inside rabbit holes in an almond tree orchard located in the “La Cartuja” area near Saragossa. The presence of rabbits living in specific holes was easily assessed through observation of recent almond shells and remains of dug up soil. Two traps were placed for each rabbit hole so that they stuck out slightly in order to facilitate their future extraction. The rest of the rabbit holes was sealed with plastic bags to encourage the sandflies to fly directly into the trap. Traps were set during the afternoon, at about 15:00 and were collected on the following day at 7:30 at the very latest.

Newly collected traps were carried to the laboratory in a dark plastic bag, where they were visually inspected one at a time. Possible *Phlebotomus* were isolated from a diversity of other invertebrates by means of a manual insect aspirator. This would be the origin of all adults reared in the colonies.

4.2.3 Colony establishment

The *Phlebotomus* obtained were introduced into small vials equipped for the establishment of new colonies in the following way: a thin mesh cloth cover, cotton impregnated in a 90% sucrose solution and a naked base made of plaster to allow possibly gravid females to lay their eggs. The containers with the collected *Phlebotomus* were placed in plastic bins to preserve humidity and were then introduced in a heater with the optimal growth and reproduction yield conditions: 35ºC temperature and a fluorescent light adapted to a 16:8 circadian cycle.

Once the females had laid their eggs and all adults present were dead, the bodies were manually extracted, placed on a slide and directed to Javier Lucientes to have them identified at the subgenus and species level.

4.2.4 Colony maintenance

Maintenance was performed in circular-based containers, 12 cm in diameter and 10 cm in height, covered with a thin mesh cloth adjusted with rubber bands. On top of the cloth a piece of cotton imbibed in sucrose solution was placed, which would be changed on a daily basis. A cut was also executed on the cloth to allow entry to the insect aspirator, and at all other times it would be kept tightly plugged with a piece of cotton.

Starting with a container were there would be eggs about to hatch, substrate began to be administered in low quantities with a teaspoon, insisting on the edges where more eggs would be expected. The newly born L1 larvae kept being fed during the next 20-22 days that their growth through all three instars lasts (L1, L2 and L3), increasing the quantity administered in accordance to larval development. Containers tended in these conditions were kept in the heater in groups of 4 or 5 inside closed plastic bins, the inner surface of the bottom of the bins being lined with damp plastic tissue to preserve humidity. Local humidity was maintained at a level of 90-100% in this way.

When the larvae had already become adults, and these had reached an age of about 7 to 10 days they were transferred to a new plastic container of the same dimensions and characteristics as the former (cloth cover, plaster, etc.), with the added feature of having 4 cuts in the cloth cover through which the legs of an anesthetized hamster could fit.

The source of blood for the female sandflies were “panda” hamsters kept in the same laboratory, which would be anesthetized with 0,6 mg of ketamine (50 mg/L, hydrochloride) administered as an intramuscular injection, so that they could then be placed on the cloth covering allowing the females to gain access to the legs and soft parts. The time of exposure of the females to the hamster lasted 60 minutes. These lab bred hamsters were used in 4 different occasions each, after which the animal was discarded and sacrificed.

Another, analogous, feeding option that was also employed was placing the anesthetized hamster inside a cubic iron-frame cloth mosquito cage, 10 cm high, into which the sandlflies would then be released.

Once the feeding was done the females would be transferred to containers with a plaster basing but without any larval substrate, the ideal number of nourished females transferred being about 150 and an additional 20% of males. These insects would, as usual, have access to the usual sucrose-imbibed cotton on the cover, but taking additional care to avoid any drops falling on the plaster below to avoid fungal growth.

After 9 days in these conditions the dead females were discarded using a natural-hair paintbrush and the maintenance of newly born larvae was resumed in this new container, where the females would have been expected to have oviposited.

4.3 Study of the phototropism of *P. papatasi*

4.3.1 Design of the experimental enclosure

In order to perform the analyses of the phototropism of *P. papatasi* two distinct experimental enclosures were designed sequentially.

The first model for an experimental enclosure consisted in a system formed by three large cloth iron-framed mosquito cages kept inside cardboard boxes of a size that fit each cage, connected with each other in 10 cm gaps with methacrylate tubes. The methacrylate tubes, 28 cm in length and 7 cm in diameter, entered the boxes in their middle part, about 10 cm above ground level. The closure of the cloth cages and these tubes was kept tight with the help of rubber bands. Inside each box and under the cage a piece of wet tissue was placed. Finally, the assembled system was covered with a piece of black cloth that kept outside light from interfering.

In this system, the presence of each individual in the distal chamber, which would be the nearest one to the source of light, was considered to be a positive entry, whereas any other location was valued as a negative entry.

The second enclosure consisted in methacrylate tubes of the same characteristics as the former coupled with a 12 cm long and 4.5 cm wide plastic tube equipped with a sliding plastic pane, that allowed the isolation of two distinct chambers: the one delimited by the methacrylate cylinder and the initial antechamber. Both ends of this system were isolated from the outside with a parafilm membrane reinforced with rubber bands to allow repeated use.

In the middle of the parafilm surface that covered the plastic end of the system (that is, the antechamber), a small cut was performed with a pair of scissors and a plug made of clumped cotton was put in place.

For this system the definition of a positive entry would cover all sandflies located within a margin of 20 cm of the distal end of the methacrylate tube, that is, close to the light source. Any other situation was classified as a negative entry.

The model was placed as described horizontally on a table in quadruplicate. Three of the tubes were set up to receive direct light while the fourth one was left isolated and would serve as a negative control.

The system was covered with two layers of the same black cloth that was referred to for the first model. Individual tubes were also isolated from each other, and all access of light to the negative control was blocked. All experiments were performed with the main lighting switched off, so that the only possible sources of light are the ones that follow.

The lighting equipment consisted of three main elements:

-An ultraviolet light tube from an insect trap, powered by a lead battery and located at the distal edge of the experimental setup, in the case of the second setup; perpendicular to the three tubes and 3 cm away from the limit of the methacrylate tubes. Light irradiated all three experimental tubes in an approximately equivalent manner, but not the control tube, which remained isolated by two folds of opaque cloth.

-The second was an incandescent commercial light bulb, powered by the same lead battery as the above ultraviolet source. The bulb was used in the initial stages of this work using the initial experimental setup based on three boxes, being also in this case located at the distal end of the last cage, exactly in the middle of the distal face of the same. Suspension of the bulb was made possible thanks to a 13x13 cm wooden plank and secured with duct tape.

-The third was a commercial LED strip, equipped with a remote control that allowed regulation of light intensity, color, and different intermittent on and off patterns, as well as sustained light. Colors available were red, green, blue, and a series of 9 additional intermediate colors, which were evenly distributed along the strip. The light used was always in the sustained mode with maximum intensity, with a special emphasis on green, blue and red light. The location of the LED source within the setup was analogous to that of the ultraviolet tube, the element which it substituted during the corresponding tests. The strip was powered by the local electrical current, and not by a lead battery as in the previous cases.

4.3.2 Phototropism tests

Apart from light intensity and color, the other experimental parameter that was controlled was the temperature. The experiments were all carried out at room temperature, measured with a thermometer that was placed between the two middle tubes of the experimental enclosure, and that read 23ºC in all recorded experiments. The sandflies enjoyed a period of adaptation to this new temperature of about 10 minutes before being exposed to the corresponding light.

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The animals were introduced, as well as extracted, from the enclosure with an insect aspirator. The candidates for each experiment were selected manually from containers that were part of the colony and isolated in an intermediate container. Once the desired number of individuals was reached, they were all introduced into the antechamber (the plastic cylinder) at the same time through the small hole in the parafilm that was normally covered by a cotton plug. The plastic slide isolated this side of the enclosure from the other one, keeping the sandflies all together in this space.

All four tubes were loaded in this manner, and only once all four of them were properly isolated from the outside and from each other was the light turned on and the slides lifted manually as quickly as possible. At this moment the period of light exposure was considered to have begun.

Once the time of light exposure, fixed in 30 minutes for each case, was finished, the cloth was lifted and the counting of positive and negative entries was performed with the light still on. Once this was done the experiment was concluded and the tubes taken apart in order to extract the specimens with the insect aspirator.

The animals were not discarded once the process was over, but instead were immediately returned to their container of origin and were reincorporated into the process of colony maintenance.

4.3.3 Analysis of obtained data

The data obtained in the way outlined above were compared graphically using the StatSoft software product Statistica.

Statistica uses ANOVA (analysis of variance), a collection of statistical models used to analyze the differences between group means and their associated procedures **[24]**.

Means of each set of three simultaneous experiments were obtained and these were used to plot the results. Plots were obtained that covered the most important features of this study, which pertain to the response of young adult individuals in same sex and mixed groups to a range of colored lights as well as to insect trap ultraviolet light, representing the data obtained for each individual experiment in the form of a percentage of light response plotted versus the two possible outcomes of the test: the sandflies had either generated a positive entry or a negative one. This was done for each individual light source for the different conditions that were tested.

The data were additionally condensed in one last graph that would include all light sources tested (not the negative control) to more easily outline the differences between male and female behavior.

5.1 Results for the first experimental enclosure

The first experiments that were performed used the first described enclosure, which consisted in three cages linked by tubes. Several repeats were carried out with the incandescent light bulb and with the ultraviolet light, yielding a negative response from the sandflies, which all remained in the first cage, with isolated individuals reaching the tubes or the intermediate cage on occasion.

The vast majority of the specimens tended to perch in the upper corners of the cage, where they remained oblivious to the possible attraction exerted by the light.

The groups that were tested for were comprised of about fifteen individuals each and without making any sex distinctions, since these tests were performed in the initial phase where a valid enclosure model was still being sought for.

Since this first model was clearly inadequate, it was discarded and a second model was constructed. Because the sandflies appear to congregate and get stuck in any crevices the space may have, such as the corners of the cages, it was clear that the second model would have to take the form of a smooth cylinder or a funnel.

5.2 Results for the second experimental enclosure

Initial testing with this enclosure showed that it was clearly adequate, as opposed to the first model, because in this case a response by the sandflies was clearly recorded and all specimens were equally under the influence of the light, there being no corners or darker areas where they could preferentially congregate.

The results shown here refer to groups of young adult individuals, as a few tests were carried out using newly hatched adults and even a few representatives of *P. perniciosus*, but these did not make out the bulk of the experiments and the data were deemed quantitatively insufficient to be worth treating further.

5.2.1 Young males and young females in same-sex groups

Young individuals would be of ages comprised between 3 and 5 days after emergence. This is a representative age for tropism studies, since young, as yet unfed individuals disperse at a higher rate **[25]**. Experiments carried out using same-sex groups formed by ten individuals each yielded the plot shown in **figure 5.1**.

The data used for this graph belonged to six different repeats for each individual color in the cases of red, green and blue, including one negative control for each one of these (see tables A1 and A2). Data for white, yellow and purple were not as abundant, originating from only three repeats. The mean of these groups, each composed by three tubes, was obtained and plotted.

5. Results

The results point towards similarities between the control and the main colors studied. Among the colors that received a more comprehensive treatment, red, while not being overly attractive does seem to stand out for males while green showed the lowest levels of attraction. Yellow and white seemed to be more attractive to males, but more experiments should be conducted to prove this.

Differences between the sexes were outlined comparing overall performance of males as compared to females, as can be seen in **figure 5.2**.

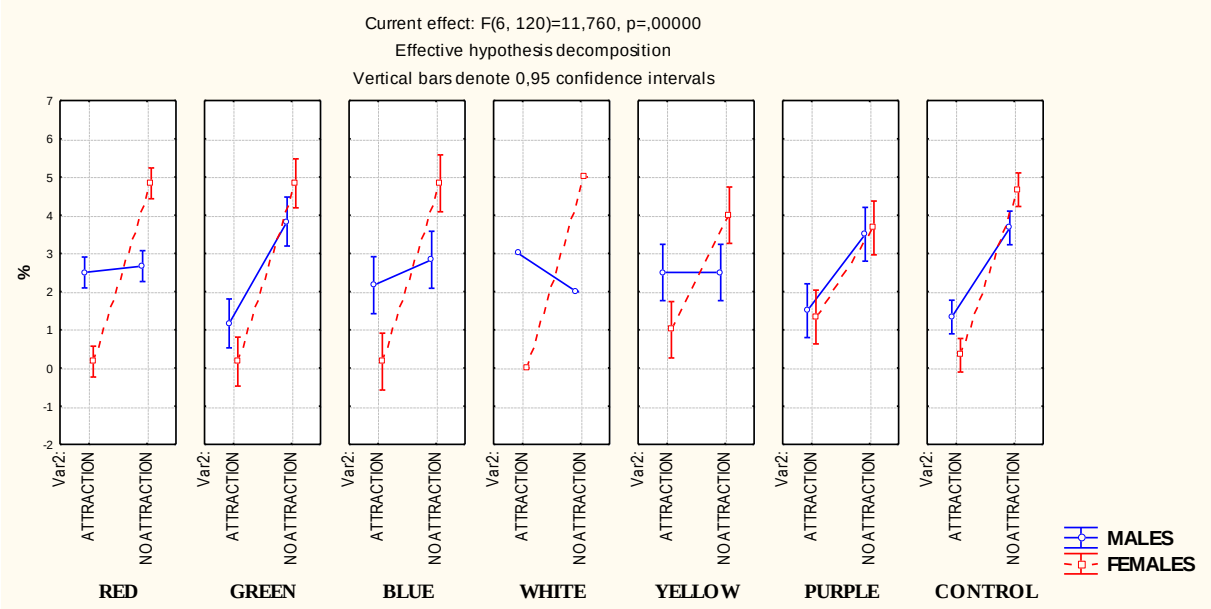

Figure 5.1 Male or female response to individual colors: *Results for males and females are expressed in percentages of attraction and non-attraction related to each individual group. Red line depicts female tendencies for each color and blue line depicts those of the male.*

There was a clear bias in the performance of males and females in isolation from each other, with males showing a much higher tendency to move towards the light than females. The negative control already showed a slight imbalance with more males than females reaching the end of the tube though, so these results may not necessarily indicate that the males are drawn to the light. The discrepancy was however further increased by the presence of the light.

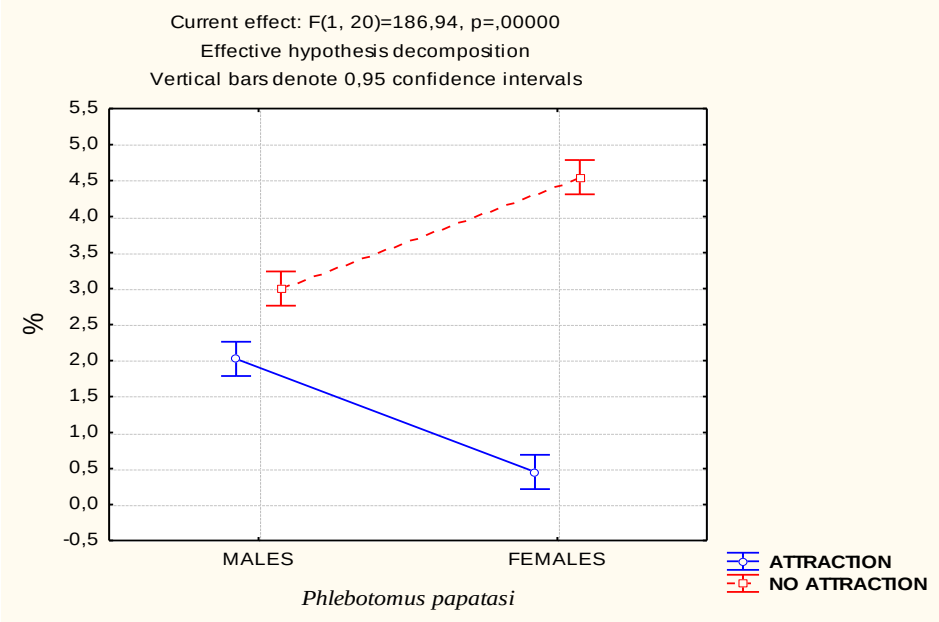

***Figure 5.2 Male or female response to all colors**: Results for males and females are expressed in percentages of attraction (blue) and non-attraction (red).*

Response to ultraviolet light was also tested in these same conditions, and gave the results summarized in **figure 5.3**. Data for this type of light stemmed from three repeats like the ones described above (see tables A5 and A6). This was sufficient to demonstrate a clear tropism towards this kind of light in the conditions imposed by the experiment, with males still showing higher values than females

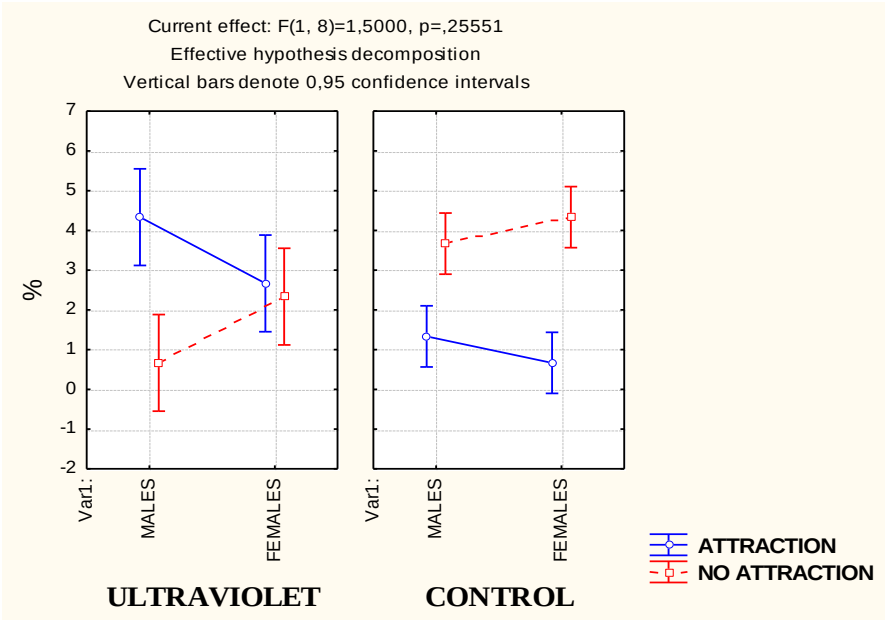

***Figure 5.3 Male or female response to ultraviolet light:** Both males and females are shown to develop an increased attraction, in blue, and a lower attraction, in red, to ultraviolet light as compared to the control. Data are expressed as percentages of attraction in each repeat.*

5.2.2 Young males and young females in combined groups

Combined groups composed of males and females were also tested. In this case four repeats of three tubes and an accompanying negative control each were tested (see tables A3 and A4). Group composition included 5 males and 5 females for each tube. Results are shown in **figure 5.4**.

Red again stood out in comparison to blue and green, albeit with rather modest results. Specifically males seemed to be attracted the most to this type of light. Green showed the most negative results. The combination of males and females did not seem to exert a noticeable influence over their response to the light.

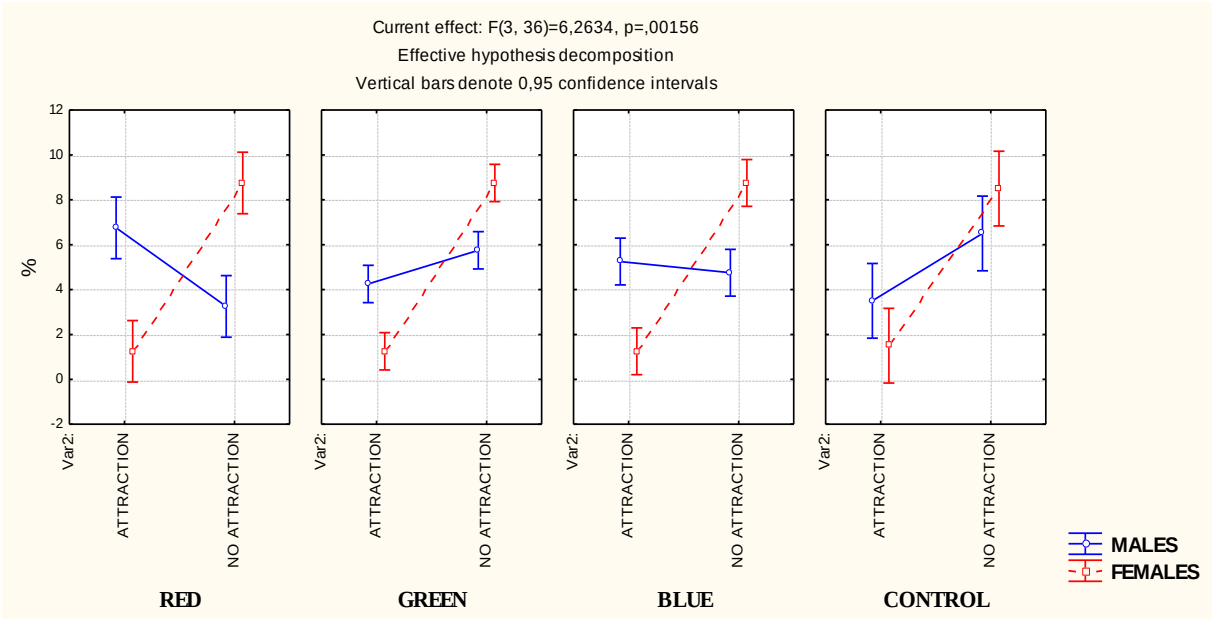

Figure 5.4 Male and female response to individual colors: *The colors tested were red, green and blue. Percentages of attraction and non-attraction are shown for males in blue and for females in red.*

Male and female behavior was also summarized in **figure 5.5**, which shows results analogous to those from **figure 5.2**. The same concept applies for the tests run with ultraviolet light (see tables A5 and A6), **figure 5.6**, where the results clearly resemble those from **figure 5.3**. In conclusion, the presence or absence of members of the opposite sex does not seem to influence phototrophic behavior in experimental conditions.

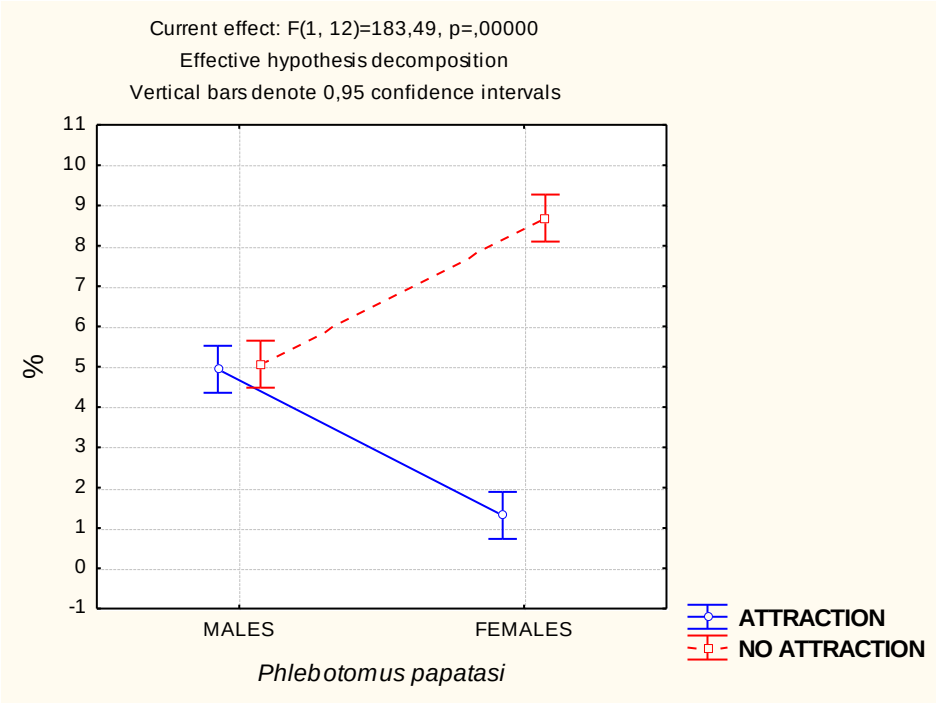

Figure 5.5 Male or female response to colors: *Attraction, in blue, and non-attraction, in red, are shown as percentages for each repeat. Lights included here are red, green and blue as per figure 5.4.*

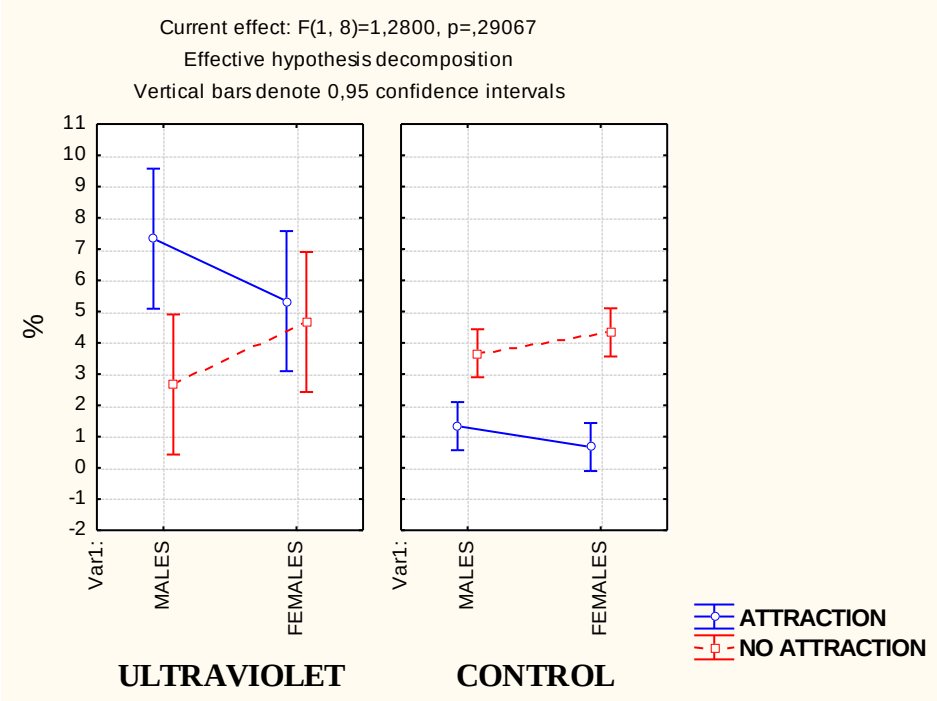

Figure 5.6 Male or female response to ultraviolet light: *Attraction, in blue, and non-attraction, in red, are shown as percentages for each repeat for ultraviolet light and its corresponding negative controls.*

6. Discussion

6.1 Influence of the experimental enclosure

It was confirmed that a member of the phlebotomines, *P. papatasi*, does not show an elevated attraction towards light emitting diodes, to which it was exposed in groups of between 5 and 20 colony-bred individuals in the above experiments.

Even upon exposure to the ultraviolet lamp, attraction was not unanimous with a significant number of specimens, especially females, staying away from it. This last result is partially attributable to the design of the experimental enclosure where the insects were located during the tests, as was shown by their behavior in the initial three cage system, and the fact that much higher levels of attraction were achieved keeping an undetermined but high number of insects inside the glass cylinder of the aspirator, a space with a 1 cm diameter.

Differences between the aspirator and the experimental enclosure which could help explain this change are available space, number of individuals (a lot higher in the aspirator and hence a higher density), and the sex ratio, unknown in the aspirator but specified for the experiments.

6.2 Light as the only stimulus

Studies performed by Müller and Hogsette show that, in field conditions, which necessarily differ from those found in the laboratory, light is not the determining stimulus in the tropism of the *P. papatasi* captured near the Dead Sea **[26]**. In one of these studies, traps without attractive stimuli were presented in addition to different combinations of CO_2, light, attractive colors, temperature, humidity and chemical attractants. CO_2 turned out to be the main attractant, followed by ultraviolet light, which has also exerted an undeniable effect on the animals in our experiment, with the effect of the other stimuli being lower. It was also discovered that there exists a synergistic effect between these stimuli.

Many other studies referred to *Phlebotomus* and other genera also imply that attraction towards ultraviolet light is the highest, followed by incandescent light **[26]**, but the presence of animal odors in the form of octanol, CO_2, caproic acid or the presence of avian livestock **[27]**, is a more powerful factor in itself than light, synergistic in the cases studied, and probably necessary to achieve an elevated positive tropism, which could add to explain why the levels of attraction in our tests were far from maximal.

This also concurs with the experiments conducted by Kirstein and Faiman **[28]**, where the traps that were not equipped with chemical lighting actually yielded a higher number of representatives of the genus as compared to CO_2 traps additionally equipped with lights.

6.3 Effect of the diodes

Light emitting diodes only began to be employed in light traps for insects a few years ago **[29]**, and thus far most of the work has been focused on the capture of Culicidae. Studies that used traps equipped with diodes of various colors (blue, green, yellow, orange, red and infrared) have proven useful for the capture of several species of *Anopheles, Culex, Culiseta, Ochlerotatus* and *Psorophora*, with the most effective colors being blue and green, which equaled incandescent light in effectiveness measured as the number of mosquitoes lured in one night. Burkett also proved that these culicids are more readily attracted to reflected light than to emitted light, a point that has not been covered in this study, but that also holds true for *P. perniciosus* **[30]**.

The differences in the effect exerted by the different LED colors in our study were not very high, but there was a sustained tendency towards a lower attraction for green diodes. In a study conducted by Hoel and Butler in Egypt, CDC traps were modified for their use with red, green and blue diodes, resulting in high numbers of captured *P. papatasi* **[30]**. In this case the red lights attracted more than twice as many specimens as the incandescent controls and more than four times those lured by the blue and green lights. These results would suggest that red light could be an adequate substitute for conventional traps, with a higher and more specific yield and also a reduced energy budget since the light comes from diodes.

Although in our study, much more limited in space and subjected to the conditions imposed by the experiment, such a high difference between the performance of individual colors was not noted, there was a distinctly lower attraction towards green lights as compared to red or blue for the young adults of this species, but the effect of the red light remains small nevertheless, with the only clearly positive response being exerted by the ultraviolet light, a fact that is already well known and covered in studies for many kinds of dipterans, and the basis of how CDC traps and others work.

Even though light emitting diodes have been successfully deployed to capture culicids, our result suggests that the same doesn't necessarily hold true for *P. papatasi*. For the same reason, lighting systems that employed diodes instead of incandescent light bulbs, perhaps with the exception of red light in view of the field results from Hoel and Butler and the slight inclination of our own specimens in comparison to blue or green, could be more adequate in a situation of risk of *Leishmania* transmission to humans and livestock.

6.4 Differences in the response of males and females

The clearest bias in this study was originated by differences in the response of males and females. Males generated a clearly higher number of positive results in all the conditions that were tested for.

There are differences in the distances covered by sandflies, mainly related to their sex, physiological status and type of habitat. Generally it is young females that travel the greatest distances in search of a source of blood, while males lack such a need and disperse more slowly as a result **[25]**. However, it has also been observed that, in adults of *P. perniciosus* that enter homes right after sunset, the males are the first ones to appear, followed by the females after an interval of approximately an hour and a half **[31]**.

These observations illustrate that there are differences in movement and dispersal in field conditions, explained mainly by the need for blood of females.

The relation of these differences in dispersal to phototropism is hard to establish, and it doesn't seem to carry much validity in the conditions established in our experiments with the diodes due to the restrictions in space and time available to them.

The differences in dispersion capacity have been tentatively explained through reproductive behavior, whereby males would tend towards aggregation on domestic animals, though not sucking blood and they would be the ones exerting attraction on the females by means of secreted pheromones. In this way, the males would be the ones to find a suitable location where they would form "mating swarms" and contribute to the approach of females **[31]**. This behavior was observed in our case during the times of feeding females, where males also perched in high numbers on the hamster.

This effect of males over females has been used to optimize light traps, with the conclusion that small groups of males used as bait in these traps increase the number of captured females in comparison to baitless traps **[32]**.

However, the explanation of the differential results found for males and females in this paper is not clearly feasible due to the restrictions imposed by the space where the experiments took place and the time they lasted, and it will suffice to say that there has been a clear bias in the behavior of the sexes, not necessarily related to the light since it also happened to an extent for the negative controls.

7. Conclusions

From the data obtained in this paper, the following conclusions may be drawn:

1. The rearing of *Phlebotomus spp* in laboratory conditions is a viable process and a useful one to obtain live material for behavior studies.

2. The data obtained in the experiments suggest that *Phlebotomus* sandflies do not experience a strong attraction towards the monochromatic light emitted by LED devices, in clear contrast with a strong attraction towards ultraviolet light. This idea could find its importance in the development of lighting systems that are less prone to attract potential vectors of *Leishmania*, consequently safer for domestic animals.

3. There don't seem to be very significant differences between the different light colors, with a slightly lower response to green light. In all the cases males are the more mobile specimens and more readily drawn to the light, which has its implications since females are the sole vector of the disease.

A partir de los datos obtenidos en este trabajo pueden extraerse las siguientes conclusiones:

1. El mantenimiento de colonias de *Phlebotomus* es un proceso viable en el laboratorio y útil para disponer de material vivo para realizar estudios de comportamiento.

2. Los datos obtenidos en los experimentos con luz sugieren que los *Phlebotomus* no experimentan una atracción muy intensa por la luz monocromática emitida por los dispositivos LED, en contraposición con una atracción muy intensa por la luz ultravioleta. Esta idea podría encontrar su importancia en el desarrollo de sistemas de iluminación menos propensos a la atracción de potenciales vectores de *Leishmania*, consecuentemente más seguros para animales domésticos.

3. No parece haber grandes diferencias entre los distintos colores de luz, con una respuesta ligeramente más baja a la luz verde. En todos los casos son los machos los individuos más móviles y más prontamente atraídos por la luz, lo cual tiene sus implicaciones porque las hembras son el vector de la enfermedad.

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9. Annex of Tables

♂ only	Red+	Red-	Green+	Green-	Blue+	Blue-
a	4	6	4	6	5	5
a	4	6	3	7	4	6
a	4	6	2	8	4	6
b	6	4	3	7	3	7
b	3	7	4	6	4	6
b	4	6	3	7	4	6
c	6	4	2	8	2	8
c	5	5	1	9	4	6
c	4	6	3	7	4	6
d	6	4	3	7	5	5
d	3	7	2	8	5	5
d	6	4	5	5	4	6
e	2	8	2	8	3	7
e	5	5	3	7	2	8
e	4	6	2	8	3	7
f	4	6	2	8	4	6
f	6	4	4	6	4	6
f	5	5	2	8	2	8

***Table A1- Male only data**: Data collected for groups of ten males using the second experimental model.*

♀ only	Red+	Red-	Green+	Green-	Blue+	Blue-
a	1	9	0	10	0	10
a	0	10	1	9	1	9
a	0	10	0	10	1	9
b	1	9	0	10	0	10
b	2	8	2	8	0	10
b	1	9	0	10	0	10
c	0	10	2	8	2	8
c	0	10	1	9	1	9
c	0	10	2	8	0	10
d	2	8	0	10	2	8
d	3	7	0	10	0	10
d	1	9	0	10	1	9
e	0	10	1	9	2	8
e	0	10	0	10	0	10
e	1	9	0	10	2	8
f	2	8	2	8	2	8
f	0	10	3	7	1	9
f	2	8	2	8	0	10

***Table A2- Female only data:** Data collected for groups of ten females using the second experimental model.*

9. Annex of Tables

♂ with ♀	Red+	Red-	Green+	Green-	Blue+	Blue-
a	2	3	2	3	3	2
a	2	3	2	3	2	3
a	1	4	1	4	2	3
b	3	2	2	3	1	4
b	2	3	2	3	2	3
b	3	2	2	3	2	3
c	4	1	1	4	1	4
c	3	2	3	2	2	3
c	3	2	2	3	2	3

***Table A3- Male and female data**: Data collected for 5 males in the presence of 5 females.*

♀ with ♂	Red+	Red-	Green+	Green-	Blue+	Blue-
a	0	5	0	5	0	5
a	0	5	1	4	1	4
a	0	5	0	5	0	5
b	1	4	0	5	0	5
b	0	5	0	5	0	5
b	1	4	0	5	0	5
c	0	5	0	5	0	5
c	0	5	1	4	0	5
c	0	5	1	4	2	3

***Table A4- Female and male data**: Data collected for 5 females in the presence of 5 males.*

♂	UV+ alone	UV- alone	UV+ +♀	UV- +♀
a	6	4	4	1
a	7	3	3	2
a	6	4	3	2
b	8	2	4	1
b	6	4	4	1
b	8	2	3	2
c	7	3	2	3
c	7	3	4	1
c	6	4	4	1

***Table A5- Male ultraviolet data**: Data collected for 10 males in the second experimental model.*

9. Annex of Tables

♀	UV+ alone	UV- alone	UV+ +♂	UV- +♂
a	6	4	3	2
a	5	5	2	3
a	6	4	3	2
b	5	5	3	2
b	4	6	4	1
b	7	3	3	2
c	7	3	3	2
c	6	4	2	3
c	6	4	3	2

Table A6- Male and female ultraviolet data*: Data collected for 5 males in the presence of 5 females.*