Rev. Brasil. Biol., 23 (2) : 181-187
Agôsto, 1963 – Rio de Janeiro, GB

FLUORESCENT TRACER AND NATURAL FLUORESCENCE IN DIPTERA OF THE GENUS "PHLEBOTOMUS"[1]

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In the beginning of 1960, we were searching for tracer substances in order to study the biology of *Phlebotomus*. We lacked adequate laboratory conditions to work with radioisotopes and besides the apparatus required was too expensive.

We tried to use fluorochromes and got encouraging results in the first experiments we made.

Occasionally, we observed that *Phlebotomus* presented natural fluorescence in several organs. This fact led us into making the preliminary observations that are now being presented.

Acknowledgements — Our thanks are due to Prof. Amilcar Viana Martins for facilities rendered during the time he was director of Instituto Oswaldo Cruz, and to Botanist Dr. Luiz Gouveia Labouriau, who informed us of the usage of fluorescent substances in the tracing of plants to study their physiology.

MATERIAL AND METHODS

We examined about 100 specimens of *Phlebotomus* belonging to three species: *P. bahiensis* Mangabeira & Sherlock, 1961; *P. tupynambai* Mangabeira, 1942, and *P. choti* Floch & Abonnenc, 1941.

As a source of light we used at first a Hannovia pressure mercury vapour lamp of 100 W, that emits Ultraviolet Rays.

The *Phlebotomus,* dead or alive, were directely focused by the Ultraviolet ligth. They were observed either by naked eye or by means of a simple entomological microscope. This method gave us the chance to observe the fluorescent structures through the *Phlebotomus* exoskeleton.

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[1] Received for publication December 18, 1962.

This work was carried out at the Núcleo de Pesquisas da Bahia and received financial support from the Conselho Nacional de Pesquisas (Brazil), Instituto Oswaldo Cruz and Departamento Nacional de Endemias Rurais.

Later on, we used a bacteriological microscope with a supply unit for fluorescence. Then it was easier for us to observe certain peculiarities of the fluorescent structures by means of epi or trans-illumination.

The source of light of this microscope is formed by a maximum pressure vapour lamp, with a filament efficiency of 200 W, and a luminance of about 25,000 Stilb and a Luminous flux of 9,500 Lumens.

The set of exciter and barrier filters that came with the fluorescent unit is explained on Table I, according to directions given by "Zeiss."

Using the three exciter filters, we can get the following spectral transmissions:

a) Ultraviolet excitation for observation of fluorescent light in the range of 4,000 to 8,000 Angstrons long (= 400/800 mμ): filters I and III. All the visible spectrum was observed.

b) Ultraviolet excitation whereby fluorescent light of 4,000/5,000 Angstrons can be observed: filters III and IV or only IV, for observation of blue and green.

c) Blueviolet excitation for observation of fluorescent light, in the range of 5,000 to 8,000 Angstrons long: filters I and II. Yellow and red fluorescences were observed.

In our experiments, the exciter and barrier filters were combined in several ways. On Table II, we present the comparative results. The combinations that are not shown on the Table are the ones that did not give good results.

The technique used in the preparation of *Phlebotomus* was as follows:

a) dissection in saline (0.9% of salt solution);
b) remotion of the saline with filter paper and fixation by Carnoy's liquid, when intended for examination by epi-illumination;
c) the structures in saline were covered with cover-slipers and the edges of these were sealed with paraffin, when intended for trans-illumination examen.

The saline was the liquid that gave the best results in the mounting and observation on insects under Ultraviolet light; because its fluorescence is practically nill. On the other hand, after some time, the fluorescent substances start dissolving in the saline.

We tried other liquids for the mounting of specimens, such as, glycerine, balsam, Zeiss oil. However, they did not give good results, for the simple reason that they spoiled or collapsed the structures of the *Phlebotomus,* when they did not have fluorescence of their own.

For fluorochrome, we used the Berberin sulphate in water solution of 0.1%. We mixed it with the larva culture medium (ox dung with a small amount of yeast) or injected 0.5 ml of it in washed raisins, on which the adults were fed.

TABLE I

Explanation of the filters used according to the directions given by "Zeiss"

EXCITER FILTERS (in the lamp)

Number	Aproximate Spectral Transmission
I	3,000 to 5,500 Å
II	3,000 to 5,500 Å
III	2,500 to 4,000 and 7,000 to 9,200 Å
IV	2,000 to 4,500 and 6,500 to 9,500 Å

BARRIER FILTERS (in the extension tube)

UPPER FILTER DISC

Number	Barrier Filter For	Spectral Transmission
I	Red	3,200 to 6,500 Å
II	Blue	4,800 to 9,000 Å
III	Blue	5,200 to 9,000 Å

LOWER FILTER DISC

I	U. V.	3,600 to 9,000 Å
II	Blue	5,200 to 9,000 Å
III	Blue	5,800 to 9,000 Å
IV	Neutral gray filter	

TABLE II

Filters used and comparative results obtained

Filters and type of combinations			Comparative Results	Fluorescence color of *Phlebotomus*	Color of the field
Of excitation	Of barrier				
	Upper	Lower			
I	II	II	Very good	Yellowish	Dark
I	III	I	Very good	Yellowish green	Dark
III	I	II	Good	Greenish	Blue
IV	I	II	Regular	Green	Dark
I+ II	III	I	Good	Green	Dark
I+III	II	I	Regular	Yellow	Dark
II+III	IV	I	Good	Green	Violet
III+ IV	I	I	Good	Green	Grey
I+II+III	II	I	Regular	Greenish-white	Dark
I+II+III+IV	IV	I	Regular	Greenish-white	Dark

OBSERVATIONS

1 — *Phlebotomus* collected in nature, which were not fed in laboratory, when examined under the Ultraviolet Light of the Hannovia lamp, presented a blue-gray fluorescence of low intensity in their eyes.

2 — *P. bahiensis* and *P. tupynambai,* collected in nature, when not fed in laboratory, and examined still alive, under Ultraviolet Light, presented a yellowish fluorescence at the level of the four abdominal segments.

3 — The testicles and seminal vesicles of those *Phlebotomus*, dissected and observed under the fluorescent microscope, presented high index of fluorescence of a yellowish color. The same color was observed for the pompette and spicules. Their Malpighian tubes showed a low blue-gray fluorescence.

4 — Some testicles and seminal vesicles of *P. bahiensis* and *P. tupynambai* were examined under Ultraviolet Light. We concluded that the fluorescence in those organs came from a substance that existed in the seminal vesicle and not from the spermatozoon, as we first thought.

We came to that conclusion because we verified that some testicles, full of spermatozoa, did not show any fluorescence. After pressing the coverslips on those organs, they became fluorescent because the vesical liquid would get into them.

5 — The spermatozoa of *Phlebotomus* were kept alive for more than one hour when examined under a standard microscope. Under Ultraviolet Light, they lost their mobility after a period of 10 to 20 minutes.

6 — Some females of *P. bahiensis* and *P. tupynambai,* collected in nature, presented fluorescent spermathecae while others did not.

We cannot say for sure why fluorescence was not seen in those females. We guess that one of the reasons was lack of technical skill. Another reason could be the fact that those females had not yet been copulated. The other organs had the same characteristics both in the male and females.

7 — Males and females of *P. bahiensis, P. tupynambai* and *P. choti,* collected in nature, were fed on raisins containing fluorochrome. They were taken alive to be observed under Ultraviolet Light. They showed fluorescence in the thorax, abdomen, genitalia, which changed on intensity on the various days of observations. After those phlebotomi had been dissected and examined on consecutive days, we observed:

a) on the first day, there was an intense yellowish fluorescence in the stomach and esophagean diverticula;

b) on the second day, the structures above had a less intense yellowish fluorescence;

c) after two or three days, the phlebotomi showed intense fluorescence in the thorax that was seen through the chitin. We could verify that those fluorescence came from the thoracical muscular tissue.

It is worth mentioning that the phlebotomi that where not fed with fluorochrome did not show fluorescence in the thorax.

8 — The phlebotomi that were in close contact with sugar substances containing fluorochromes, had the last tarsal segments markedly fluorescent, due to the fact that remains of Berberin Sulphate were stuck on them.

9 — We put some Berberin Sulphate in the food of the larvae of *P. bahiensis* and *P. choti*. After a day or two, those larvae were observed under the Hannovia lamp; they showed fluorescence in the exoskeleton, probably due to the direct contact they had with the fluorochrome that was in the culture medium.

Some of those larvae were smashed on filter paper which then showed yellowish fluorescence, when examined under Ultraviolet Light.

10 — Larvae of the kind described above, which had no contact with fluorochrome, were observed under Ultraviolet Light, alive or smashed on filter paper. Fluorescence was not seen on the former. On the latter, we observed weak blue and yellow fluorescence, different from that observed on the larvae fed with fluorochrome.

11 — Larvae that were fed on substances containing Berberin Sulphate grew to the pupa stage. We observed some of those pupae under Ultraviolet Light. The skin of larvae on the last stage that were attached to the pupae showed fluorescent remains.

12 — Inside the body of the pupae mentioned above, we observed some indication of fluorescence inner structures that could be seen through the chitin. When their digestive tubes were taken out, we noticed evidence of yellow fluorescence on the remains of the food which had been swallowed during the larval stage.

13 — The Malpighian tubes of the pupae that had no contact with fluorochrome showed a slight whitish fluorescence, different from the one observed previously.

COMMENTS AND CONCLUSIONS

The bibliography on insect fluorescence is very extensive. Many authors presented encouraging results based on the experiments they performed with natural fluorescence and in the usage of fluorochromes to mark insects.

They mention several advantages in the use of fluorochromes to mark insects. It ought be remembered that those substances should be used without putting researchers in danger and without altering the vitality of insects, an inconvenience presented by radioisotopes.

On many circumstances the fluorochromes can be used to study the biology of insects. For example, the marking and recovery for observations, on flight range, longevity in nature, and measurement of field population.

We think that this method will explain many facts in the field of biology, ecology and even insect physiology.

In the few experiments we have made we observed that:

1 — *Phlebotomus* have natural fluorescence in the eyes.

2 — *P. bahiensis, P. tupynambai* and *P. choti* present natural fluorescence in the seminal vesicles, testicles and Malpighian tubes.

3 — The fluorescence of genital organs is determined by substances that are found in the seminal vesicles, and not in the spermatozoa.

4 — The spermatozoa of phlebotomi have good mobility in saline solution that last for more than an hour; they lose it when they are focused under Ultraviolet Light for ten or twenty minutes.

5 — The males and females of the phlebotomi described above have identical fluorescence in the organs that are similar in both sexes.

6 — Those females sometimes show fluorescent spermathecae and sometimes do not. The Authors think it is possible that the fluorescent spermathecae belong to the class of females which had already been copulated.

7 — The three species above mentioned, both males and females, feed on sugar substances containing fluorochromes. This can be seen throughout their digestive tubes when they are examined under ultraviolet light.

8 — As days went by, the fluorescence in the food they are disappeared little by little from their stomachs and was intensified in the esophagean diverticula, Malpighian tubes and thoracical muscles. This could very well indicate a process of absorption, incorporation and elimination of food, although it is possible that it only occurs with the fluorochrome.

9 — The phlebotomi present a superficial fluorescence on the parts of the body that were in contact with fluorescent substances.

10 — The larvae of *P. bahiensis* and *P. tupynambai* present a slight natural fluorescence and feed on substances containing fluorochromes that can be seen under Ultraviolet Light.

11 — The larvae that were fed on substances containing fluorochromes have a natural evolution until they reach the pupa stage.

12 — After the pupation residues of the food swallowed during the larval stage remain in the digestive tract of the pupa.

13 — After the larvae had contact with fluorochrome alive, the larval exuvia shows fluorescence. This fact may be used to observe the changes in evolutional stages, by speading a fluorochrome solution over the larva by means of brush.

14 — There are internal structures in the pupa that shows natural fluorescence. It seems that fluorescence later on will be observed in the adult phlebotomi.

RESUMO

Os Autores apresentam os resultados de observações feitas em 1960, sôbre fluorescência natural e traçadores fluorescentes em *Phlebotomus*. Observaram cêrca de 100 exemplares, compreendendo 3 espécies: *P. bahiensis* Mangabeira & Sherlock, 1961; *P. tupynambai* Mangabeira, 1942 e *P. choti* Floch & Abonnenc, 1941.

Descrevem as técnicas e o material utilizado nas observações. Empregaram uma lâmpada a pressão de vapor de mercúrio e um microscópio equipado com dispositivo para fluorescência. Como fluorocromo utilizaram o Sulfato de Berberina em solução aquosa a 0,1%, o qual era misturado ao meio de cultura

para as larvas, ou injetado em passas escaldadas, com as quais os adultos se alimentavam.

Salientam serem essas observações preliminares e obtiveram os seguintes resultados:

1 — Os flebótomos têm fluorescência natural nos olhos.

2 — *P. bahiensis, P. tupynambai* e *P. choti,* apresentam fluorescência natural amarelada na vesícula seminal, testículos e tubos de Malpighi.

3 — A fluorescência dos órgãos genitais é devido a substâncias existentes na vesícula seminal e não aos espermatozóides.

4 — Os espermatozóides dos flebótomos têm boa mobilidade em sôro fisiológico, durante mais de uma hora e perdem a mobilidade ao serem focalizados pela luz U.V., durante 10 a 20 minutos.

5 — Os machos e as fêmeas dos flebótomos acima, têm fluorescência igual, nos órgãos que são comuns para ambos os sexos (tubos de Malpighi, olhos, etc.).

6 — As fêmeas dêsses flebótomos, às vêzes, mostram espermatecas fluorescentes e às vêzes não. Os Autores julgam que há possibilidade de serem as espermatecas fluorescentes, as de fêmeas já copuladas.

7 — As 3 espécies citadas, tanto machos como fêmeas, se alimentam com substâncias açucaradas contendo fluorocromo, o que pode ser depois visualizado ao U.V., em todo o tubo digestivo.

8 — Poude-se constatar que à medida que passavam os dias, a fluorescência do alimento ingerido, ia desaparecendo do estômago e se intensificava no divertículo esofageano, tubos de Malpighi e, por último, na musculatura torácica. Sugere isso um processo de absorção, aproveitamento e eliminação do alimento, embora haja a possibilidade de que êsse processo ocorra sòmente com o fluorocromo.

9 — Os flebótomos apresentam fluorescência superficial nas porções do corpo que mantiveram contacto com substâncias fluorescentes.

10 — As larvas de *P. bahiensis* e *P. tupynambai* têm leve fluorescência nos órgãos internos.

11 — Essas larvas alimentam-se de substâncias impregnadas por fluorocromo, as quais são posteriormente vistas fluorescentes, à luz U.V.

12 — As larvas alimentadas com substâncias contendo fluorocromos evoluem normalmente até pupas.

13 — Após as larvas terem contacto com fluorocromos, as exúvias larvais apresentam fluorescência. Isto pode ser utilizado para a observação de mudança de estádio, pincelando-se a larva com solução de fluorocromos.

14 — Após a pupagem, permanecem restos alimentares fluorescentes, ingeridos durante o estádio larval, no tubo digestivo da pupa.

15 — Existem órgãos internos, na pupa, com fluorescência natural, o que os Autores supõem corresponder as estruturas que se mostram fluorescentes no adulto.