ESTABLISHING OF THE DISPERSION CAPACITY OF *Simulium ornatum* Meigen, 1818 (complex) LARVAE BY APPLICATION OF METHYLEN BLUE VITAL DYE

„UTVRĐIVANJE KAPACITETA DISPERZIJE LARVI *Simulium ornatum* Meigen, 1818 (complex) KORIŠĆENJEM METILEN PLAVE VITALNE BOJE“

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ABSTRACT: *Identification of the dispersion capacity of S. ornatum late instar larvae, by artificial positioning in the upstream uninhabited sections of the stream, distant from typical breeding zones was the main objective of this study. Further more, the convenience of methylen blue vital marker application as a suitable tool in biological studies of black flies in natural environmental conditions was tested. Larval marking was performed in methylen blue aqueous solution 25 mg/l and 45 minutes exposition. High portion of visually detectible dyed larvae (about 90%) was recorded, no mortality and high persistence of the dye in the larval body all over the 15 days period. Tendency of dispersion and successive inhabitation of downstream sections at increasing distances was expressed in majority of larvae (99,33%). The increase of distances covered in function of time was recorded, starting from 17 m to 93 m after 1 and 15 days, respectively.*

REZIME: *Cilj istraživanja je bilo utvrđivanje kapaciteta kretanja larvi S. ornatum, u kasnim fazama razvića, pri veštačkom pozicioniranju u nenaseljenom delu potoka, uzvodno od zona okarakterisanih kao tipična izvorišta. Takođe se testirala pogodnost primene metilen plave boje u biološkim istraživanjima simulida u prirodnim uslovima, odnosno perzistentnost ovog vitalnog markera u telu insekta. Prilikom bojenja larvi metilen plavom bojom u vodenom rastvoru koncentracije 25 mg/l i pri dužini ekspozicije od 45 minuta, jasno je obojeno 90% larvi, boja se zadržala u telu 15 dana i nije uticala na smrtnost tretirane populacije.*
Većina larvi (99,33%) pokazala je sklonost ka migraciji, pri čemu su sukcesivno naseljavale sve udaljenije nizvodne delove toka. Zapaženo je povećanje dužine pređenog puta larvi u funkciji vremena, od 17 m (prvog dana) do 93 m nakon 15 dana od postavljanja ogleda, što ukazuje na to da dominantna vrsta potoka Fruške gore, S. ornatum ima značajan kapacitet disperzije.

Key words: *Simuliidae, Simulium ornatum, dispersion, methylen blue*
Ključne reči: *Simuliidae, Simulium ornatum, disperzija, metilen plava*

INTRODUCTION

Simulidae, also know as blackflys, are the insects of the order Diptera, suborder Nematocera, family Simuliidae. They are well-studied group of insects, which are given great attention, primarily because of their economic and medical importance. Females are bloodsucking and they feeding on the vertebrates, mostly on mammals and birds. They represent a great danger, for the man and domestic animals, because of their massive attacks and toxic saliva that inject in the hosts body during the stabbing. Female's stings are painful, and may causes strong allergic reactions, especially for sensitive people, which are followed by red swellings around the place of stitches.

Blackflys are molestants, vectors of viruses and protozoa, which are cause various diseases. In tropical areas they transmit nematode *Onchocerca volvulus*, which is cause of

onhocercosa disease and river blindness. They also cause a fever accompanied by headache, depression and lymphadenitis.

Development of Simulida is carried out in the current water, rich with oxygen. Species Simulium ornatum Meigen, 1818 (complex) (Ćupina et al. 2003; Petrić et al. 2006) is the most common species in streams Fruške Gora, especially in the sections of the stream that flows through populated places. This species in our climate conditions preferred feeding on mammals (Ćupina Ignjatovic et al. 2006).

The larvaes move downstream several times during their development, while they more remote from the place where the females lay eggs, and place of the larvae's hatching. Factors that cause the movement of larvae's are running from the predators (Simmons, 1982 in the Adler et al., 2004), UV radiation (Kiffney et al., 1997, in Adler et al., 2004; Donahne & Schindler, 1998 in the Adler et al., 2004), the physical and chemical properties of water (Ross & Meritt, 1978 in the Adler et al., 2004).They moving in a loops, which are movements similar to those that performed caterpillars of Lepidoptera: Geometridae or leech (Hirudinea), in which they adhere to the substrate using two adhesive discs. One of those discs is situated at the end of abdominal, and the other is on the pseudopod. Larvae used this way of moving to run across the short distance. Blackflys feed filtrating water with cephalic range and they usually search for the place with the highest content of organic material in water, so they let the water flow position them downstream to point with appropriate conditions. When larvae found optimal conditions for their feeding they adhere to substrate with a long silk thread which exudates by salivary gland, which enables them, contact with the substrate and controlled movement.

Sometimes, larvae are able to move spontaneously using the power of main water flow, which can carries them up to several kilometers what is occurs in major rivers. (Rivosecchi, 1978)

Using vital day, as a marker, is one of the suitable and often used methods by different authors, for monitoring the way of food throught intestinal tract and for the absorption of nutritional substances (Barbosa & Peters, (1970), Wotton, (1992); Petrić et al. (2006).)

Methylene blue is vital dye that is successfully used for monitoring different biological processes in the body of insect in vivo and in vitro, and increasing application of this dye in the environmental studies, related to the movement and distribution of populations of insects, especially vectors of disease (Barbosa & Peters, 1970) .

Petrić and associates (2006) have found extremely low rates of mortality during exposure larvae *S. ornatum* in aqueous solution methylen blue concentration range of 2.5 to 50mg/l dying duration of 10 minutes and 24 hours. The authors have found that the intensity of dye in the applied concentrations of 25 and 50 mg / l was similar, and considering the weak sclerification and transparency of integument, traces of color were easily visible on the level of intestinal tract, as well as in other parts of the body filled chemolimpha. However, the authors did not track the existence of color in the body of larvae in the period more than 4 days.

The aim of the research

Keeping in mind the allegations of previous researchers who have found laravaes preferential habitats in parts of the water course that are under the influence of human activities, the goal of this research was to determine the dispersion capacity of larvae S. ornatum in the late stages of development, in the case of their artificial position in the unhabited part of the flow, upstream from the typical active source zone.

Moreover, the aim of the study was to determine the suitability of applying methylen blue in blackflys biological research in natural conditions and persistent of the dye in the insect body.

METHOD AND MATERIAL

Thousand larvae fourth and fifth stages are originated from Kozarskog streams in Beočin and used in the experiment, which lasted from the 30th September to 15 October. Larvaes stage is determined by visual assessment. Determination larvae samples to species level was performed by Bass key(1998). Larvae were collected together with the substrates that were fixed on (submerged coot grass) and transport in plastic containers, volume 20l, to Stari Ledinaci where equipment is set for dying.

Vital color, methylene blue, is used for dying in aqueous solution concentration of 25mg / l with the exposition of 45 minutes. Coloring process is carried out in two plastic containers with 5 l of water, in which are placed 3 aquatic pumps, which are used for mixing the water and allow feeding larvae and the absorption of color. Before introduction larvae in the new habitat (section Kamenarskog streams upstream from the Starih Ledinaca, in which where not detected blackflys larvae by the previous review), they are transferred from dye solution to clean water, in order to visually determine the color. After that, 50 colored larvae transferred, by the plastic pipette, in each of 6 plastic glasses, volume 2 dl, filled to the edge of the stream water. A few jacks was previously set into glasses to prevent their turnover under the influence of current water which entered from the stream. After the larvaes fixation for the jack, the walls or bottom of the cup, each plastic cup is handover and carefully placed in the appropriate glass cup, which is previously buried in the bottom of the stream and used as additional support. This process provided a minimal disruption of larvae during their transfer and disabled anvil, passive larvae moving (drift). During glasses positioning in water, is take care, that the edge of the cup is below the level of water in the stream, which enables the flow of water in the level of glass edge and ensure the conditions for larvae active emigration from the cup.

Net with openings of 1 mm in diameter is used for presentation of larvae drift and for holding substrate and it was set to a distance of 1 m from the first the glasses and fully dam stream.

The hunting PVC tapes, dimensions 30 x 4 cm, were fixed for the stream coast with rope, at a distance of 2 m, 5 m, 10 m, 20 I 40 m from the first posted glasses, crosswise in relation to the stream flow.

In every distance is set to 10 bars, except at a distance of 2 m, where is because of less wide stream set only 6 bars. Then, the rest of colored larvae with the substrate are slowly released in a stream in the zone directly in front of net.

Larval dispersion in the stream is controlled by their counting, on the artificial objects in the stream (in the glasses and the hunting tapes) and on the plant substrate (the submerged leaves, branches, ridicule, branch various plants such as blackberry, grass, nettle, etc.) where the branches counting by segments of 10 cm if they are longer then 10 cm to standardize number of larvae per sample.

Control of the larvae position was carried out in five terms after 1, 3, 7, 10 and 15 days of the colored larvae release in the stream.

In every sampling time is precisely measure maximal distance on which last colored individual was found and only visually colored larvae and pupae are counting.

Experiment lasted for 15 days and the temperature of water in the brook, in this period was quite balanced and moved in the range from 12-14oC.

Water flow is varied within the limits from 0035 to 0048 m3/s, providing water flowing and plenty of suitable substrates for the larvae positioning in each section of followed part of the flow Kamenarskog stream.

On the seventh day after experiment setting there was a stream dimling, because of less rainfall the previous day.

The results are displayed graphically and in tables, and then they are analyzed and compared by days and intervals of distances from the larvae releasing point.

Indices of intensity dispersion is calculated for each distance interval, which covered the area between the horizontal bar, by the formula

I = N x D where

I - Index of dispersion intensity

N - average number of individuals per sample in a certain interval of distance;

D - mean value of the distance interval

The number of founded larvae and pupas was multiply with the middle of the distance interval length to compensate significantly smaller number of larvae founded on greater distance from the place of larvae releasing. After that, dispersion intensity is compared for every sampling day.

RESULTS AND DISCUSSION

Most determinate larvae belonged to the type *of Simulium ornatum*, and some individuals samples are identified as the type of *Simulium aureum* Fries, (1824), and *Simulium erythrocephalum* De Geer (1776).

After larvae coloring in the aqueous solution of methylen blue, concentration 25 mg/l, is visually determinate excellent larvae color, as at the level of intestinal tract and at the level of chemolympha, and their 100% vitality.

In the first part of experiments, when dominated the population of larvae (from first to seventh day) finds the highest proportion of colored larvae, which was close to 90% (Fig. 1.).

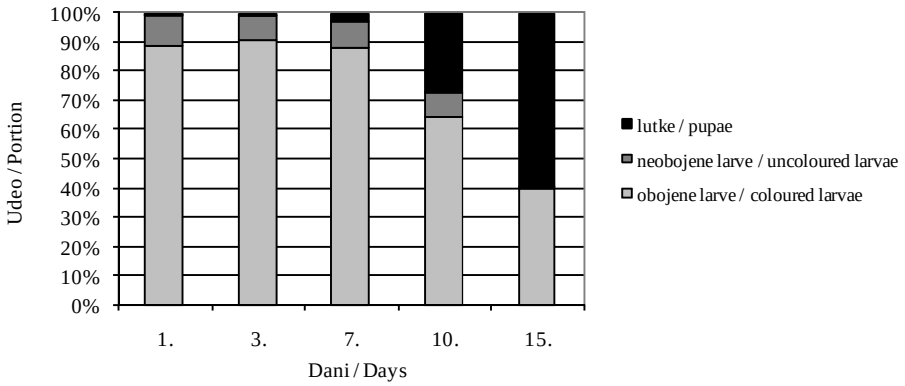

Figure 1. Different categories of black fly immature stages during the trial

On the tenth and the fifteenth day, is finding a growth pupaes population. Every day, except the fifteenth, percent of uncolored larvae was similar.

Pupa's body color could be seen in the case when they were younger, because their body integument was lighter.

There isn't possibility for visual color determination in pupa's body, because they are situated in cocoon

Reviewing of the stream before the experiment, established complete black fly absence in the stream section where experiment was posted and in sections about 1 km upstream from this zone, so it can be claimed, with great confidence, that all registered pupas origin from larvae which were colored and introduced in the stream.

Following dispersion of larvae which are placed in glasses, found that the majority of larvae (99.33%) shows a tendency to leaving plastic cup (Fig.2).

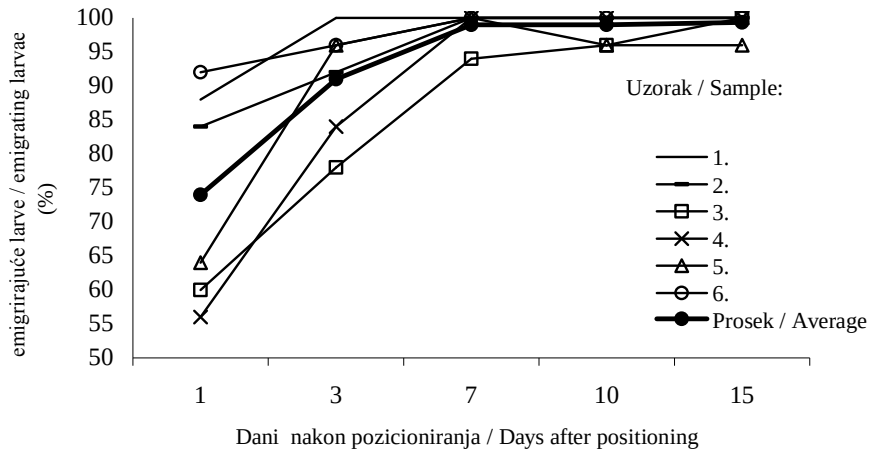

Figure 2. Black fly larval emigration dynamics from glasses positioned into the stream

Namely, to the fifteenth day, in the five repeat, all larvae left the original substrate, and in only one repeat is registered the presence of smaller number of individuals.

During the first, and then on the third day after experiment setting, the percentage of larvae that have left the original position varied considerably. Generally is recognized that the larval emigration intensity was most intensive between 1. and 3. day, slightly lower between the 3rd and 7. day, while after the 7.day the most larvae leave the cup.

Analyzing the distance of larval migration in particular time period (Fig. 3), it can be see that on 1st sampling day the highest average number of individuals per sample was located in the interval up to 2 m.

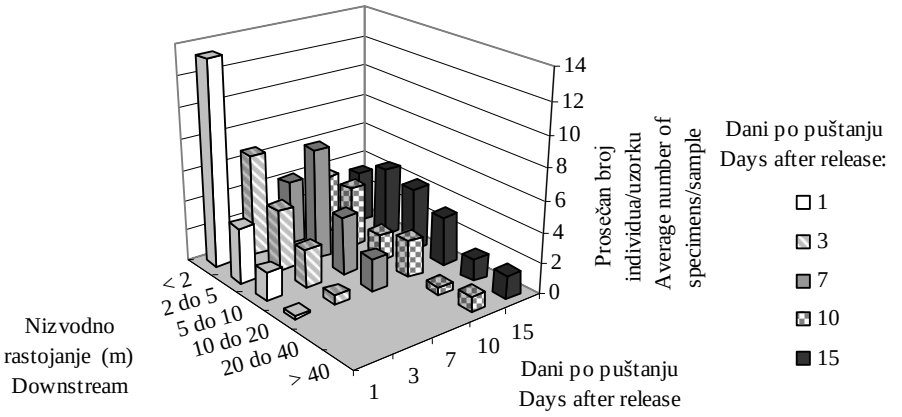

Figure 3. Dynamics of blackfly dispersion at different distance intervals downstream from the release point

With increasing the distance interval is observed a tendency decreasing number of individuals and in intervals greater than 20 m is not found any individuals.

A similar tendency of larvae location occurs on the third day from the experiment setting, which is expressed by decreasing number of registered individuals which are founded in the intervals nearest to the initial point.

Significant changes of individuals position occurs on seventh day, when the largest number of larvae and the pupas was found in the interval from 2 to 5 m and when comes to the significant movements of individuals to the intervals of 5 to 10 mi of 10 and 20 m.

The tenth and fifteenth day after experiment setting is occurred appearance of individuals at the same time in the intervals from 20 to 40 m and more than 40 m and equal number of larvae in each interval.

In the intervals from 10 to 20 m, 20 to 40 me and more than 40 m in function of time comes to increasing number of larvae and pupas, which proves there active movement.

The greatest number of larvae is moved in distance from 2 to 20 m during period of 15 days.

On the basis of the greatest record distance in which are found colored individuals in certain day (Fig. 4.), can be noticed weaker larvae moving from the first to seventh day (17 to 21.5 m).

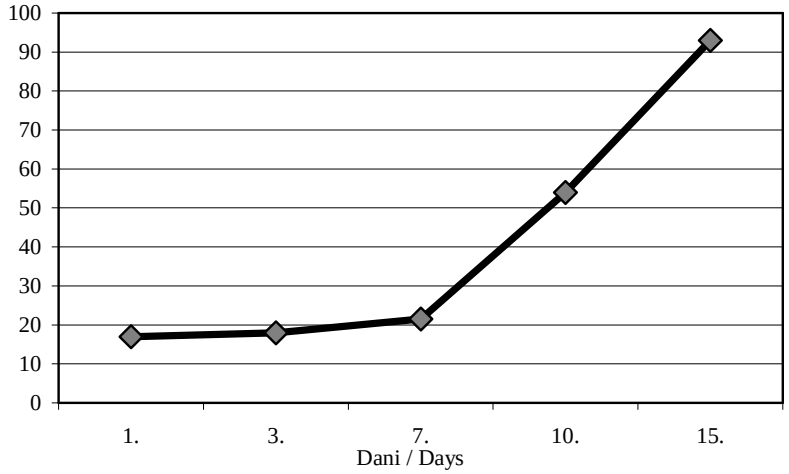

Figure 4. Maximal downstream distance of blackfly immature stages in different days

Increasing larval mobility is recorded after the seventh day, so larvae are founded in a distance between 54 m and 93 m, which indicates increasing larval moving in the last stage of development, before transformation into a pupa.

Suddenly increasing the length of the traveled path could be in connection with mild water dimling. Namely, Rivosecchi (1978) considered that water dimling is one of the important factors that initiate larval mobility.

Maximal distance on which individuals was found is 93 m and it registered on 15^{th} day after experiment setting, which indicates that black flies types which can be found in our streams have considerable dispersion capacity.

This result, which is obtained in the stream, could be in conformity with the allegations of Rubtsova (quote from Adler, 2004), who found that downstream larval movement in large rivers is about 100 m to several kilometers, and Wotton-a (quote from Adler , 2004), who considered that the individuals migration which happens in small waterfowls is in small range.

Comparing the dispersion intensity indices at different distance from the releasing point and by days (Tab. 1), can be observed its continuous value decreasing in the nearest interval (up to 2 m), a continuous increasing indices value in the interval from 10-20 m, 20-40 me more than 40 m, while in the intervals from 2 to 5 m and 5 to 10 m leads to its value variations in the function of time, which can be explained with larval immigration from upstream zone and their emigration to downstream flow parts in the same time.

Table 1.

Daily dispersion intensity indices at different distances from the release point

Interval udaljenosti (m)/ Distance interval (m)	Indeks intenziteta disperzije / Dispersion intensity index				
	1. dan/day	3.dan/day	7.dan/day	10.dan/day	15.dan/day
< 2	13.38	6.66	4.14	3.8	3.37
2-5	12.495	14	25.165	13.825	15.855
5-10	**13.575**	**18.075**	27.9	13.425	31.05
10-20	3.15	8.55	**30.75**	36.3	47.85
20-40	0	0	0	15	43.2
> 40	0	0	0	**64**	**93.44**

Comparing the maximal daily values of dispersion intensity indices, can be observed its gradual moving towards to greater distances intervals. Namely, on the first and third day after larval releasing into the stream, the maximal index value is observed in the interval of 5-10 m downstream, after the seventh day it was recorded in the interval from 10 to 20 m, and after the tenth and fifteenth day in the interval more than 40 m.

Analysis of the maximal daily larval dispersion indices, points to the existence of increasing linear trends of indices value in function of time, with significant high value of coefficient correlation (Fig. 5.).

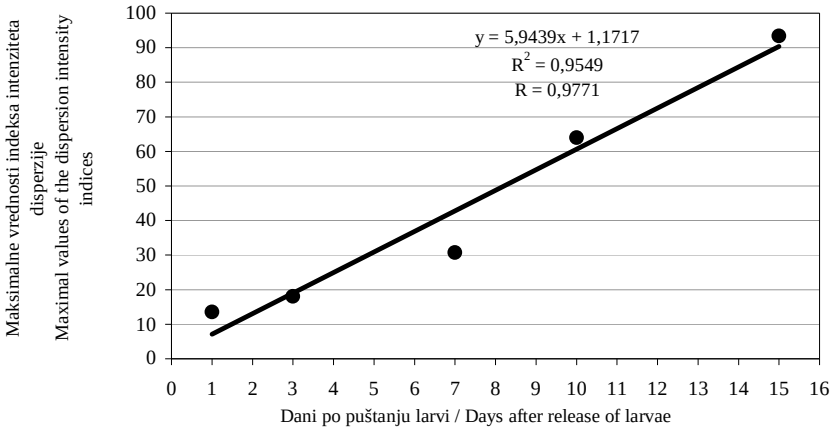

Figure 5. Increasing dinamics of the maximal dispersion intensity indices values

The results of the research indicate on the existence of expressed capacity of larval dispersion , that could be seen even in such a short tracking period which lasted for 15 days.

CONCLUSIONS

Staining larvae species *S. ornatum* with methylen blue, the exposition of 45 minutes and the concentration of 25 mg / l achieved a good body color, visible naked eye and the full specimens vitality. Color is persistent and was noticeable in the colored individuals in the period of 15 days, and traces of color could be more or less visible after their shift in the stage of pupae.

The specified method of marking can be used with success for easy tracking development of biology and behavior of the population simulida in natural habitats, where the colored and non-ferrous individuals visually easy to distinguish.

Larvae *S. ornatum* showed tendency to downstream movement and active search for the optimum ecological niche. Most larvae in the period of 15 days showed tendency to migration (99.33%), and the majority of larvae leave the original artificial substrate after 7 days, which is probably the consequence for the appropriate natural base.

Larvae, which are fixed on plant material, also show dispersion tendency, while after the first and third days after introduction majority of larvae remained in the section of the stream up to 2 m downstream from the initial point of release. Later is evident successive winning further parts of the course, with the achievement of uniformly distribution. The largest crossed distance is gradually increased from 17 m, after the first day, to 93 m on fifteenth day.

Decreasing tendency of migration intensity is recorded in interval which is nearest to the initial point (up to 2 m) and increasing tendency of migration intensity is founded in the two farthermost intervals (from 20-40 m and over 40 m).

In the middle part of the followed course dispersion intensity is varied, which is probably a consequence of simultaneous immigration larvae from upstream points and emigration part of population into downstream points. Between the migration intensity and time period after larval introduction in the stream there was a positive correlative relation and significant linear dependence.

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