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ORIGINAL ARTICLE

BLOOD SUPPLEMENTATION WITH VITAMINS INCREASES THE FERTILITY OF *ANOPHELES DARLINGI* (DIPTERA: CULICIDAE)

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ABSTRACT

Anopheles darlingi is one of the main vectors of human malaria in Brazil. Female mosquitoes use blood from vertebrates to produce their eggs and larvae. Blood composition, including vitamins, may alter fecundity and fertility, impacting mass production in the laboratory. Thus, the objective of this work was to evaluate the effect of blood supplementation with vitamins on the reproductive parameters of *An. darlingi*. Mosquito females were collected in a rural area of Porto Velho, and a blood meal was given in the field, adding different amounts of multivitamins in concentrations between 1 and 0.01%. The number of engorged mosquitoes and, subsequently, other variables such as survival up to oviposition, proportion of mosquitoes laying eggs, number of eggs and larvae produced were recorded. Engorgement, survival, and proportion of females laying eggs, as well as egg production, except in females supplemented with 1% of the multivitamin, were not altered by supplementation. However, the number of larvae produced increased significantly (about 20%) in females supplemented with 0.01% compared to control (no vitamins added). The present results suggest that general supplementation by vitamins increases the fertility of *An. darlingi*.

KEY WORDS: Malaria; breeding; hematophagy; mosquito.

INTRODUCTION

Anopheles darlingi is one of the main vectors of human malaria parasites in the Amazonian regions of South American countries such as Bolivia, Colombia, Guiana, Peru, Venezuela, French Guiana, Suriname and Brazil (Hiwat & Bretas, 2011). This species is highly anthropophilic and transmits malaria both intradomiciliary and peridomiciliary (Forattini, 2002).

Mosquito transmitted diseases are related to the blood feeding habit of these insects, since blood is important to trigger vitellogenesis and continue oogenesis (Clements, 1992). Moreover, the reproductive capacity of

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mosquitoes is affected by the blood nutritional status, including micronutrients, e.g., vitamins, metals and minerals in both larval and adult stages (Rivera-Pérez et al., 2017).

The blood that mosquitoes ingest is a mixture of various components such as proteins, lipids, carbohydrates, salts, vitamins and minerals (Krebs, 1950).

Many vitamins are essential for the successful breeding of mosquitoes as reported in classical works on the rearing of immature mosquitoes, e.g., *Culex pipiens* (Kleinjan & Dadd, 1977), *Aedes aegypti* (Akov, 1962; Golberg et al., 1945), *Anopheles stephensi* (Rosales-Ronquillo et al., 1973), but data on direct vitamin supplementation of blood and its effect on mosquito fecundity and fertility were not found. Despite that, Phasomkusolsil et al. (2014, 2015) related the use of vitamin supplementation for anopheline females during sugar feeding, prior and after the blood feeding in their methods. Recently, Tan et al. (2016) related that females of *Culex quinquefasciatus* fed with sucrose solutions supplemented with 1% vitamin B complex after the blood meal increased the number of egg rafts and also viable larvae. Thus, the objective of the present work was to evaluate the effect of multivitamin and mineral supplementation within different biological parameters important in the reproductive capacity of the *An. darlingi* and its potential for increasing larval production of this species.

MATERIAL AND METHODS

Mosquito collection, feeding and experiment design

The collection and feeding of the females were carried out in a rural property (Sítio do Bigode), located 31 km from the city of Porto Velho, Rondônia, at coordinates 08 ° 39.145 'S/063 ° 56.155' W, BR 319, Ramal Santo Expedito.

The collections of the *An. darlingi* females were carried out from 6:00 p.m. to 8:00 p.m., using human landing catches. Ten wild female mosquitoes were separated and distributed in cages (300 mL disposable cups), transparent with screened tops. Subsequently, the mosquitoes were fed human blood using an artificial feeding device adapted from Rutledge et al. (1964), i.e., a 50 mL disposable cup containing the blood source on the bottom wrapped in polytetrafluoroethylene tape and filled with warm water ~ 37 to 40 °C, placed on the top of the cages during 20 minutes. The blood source was supplemented with vitamin and mineral salts (1%, 0.1% and 0.01% multivitamin based on Vytinal® multivitamin - Table) and non supplemented blood was used as a control (n=9 per treatment).

Table. General multivitamin and mineral* composition provided in different concentrations during the blood feeding of *Anopheles darlingi* (Diptera: Culicidae).

Vitamin/mineral	1% (ug/mL)	0,1% (µg/mL)	0,01% (µg/mL)
Vit A (retinol)	1.2	0.12	0.012
Vit B1 (Thiamine)	3.6	0.36	0.036
Vit B2 (Riboflavine)	3.9	0.39	0.039
Vit B3 (Niacine)	48	4.8	0.48
Vit B5 (Pantothenic acid)	15	1.5	0.15
Vit B6 (Pyrodoxine)	3.9	0.39	0.039
Vit B9 (Folic acid)	0.72	0.072	0.0072
Vit B12 (Cobalamin)	0.0075	0.00075	0.000075
Vit D	0.015	0.0015	0.00015
Vit C	135	13.5	1.35
Vit E	20,1	2,01	0,201
Vit H (Biotin)	0.09	0,009	0,0009
Vit K	0,195	0.0195	0.00195
Calcium	750	75	7.5
Copper	1.35	0.135	0.0135
Chromium	0.054	0.0054	0.00054
Iron	24.3	2.43	0.243
Phosphorous	525	52.5	5.25
Iodine	0.099	0.0099	0.00099
Magnesium	300	30	3
Manganese	3.6	0.36	0.036
Molybdenum	0.069	0.0069	0.00069
Selenium	0.06	0.006	0.0006
Zinc	21	2.1	0.21

*Based on Vytinal® composition

After feeding, the non-engorged mosquitoes were discarded from the next stages of the experiment. From that moment on, the engorged mosquitoes were placed in a Styrofoam box, and a cotton swab soaked with 10% sucrose was placed on the top of the mosquito cages, which were also covered in plastic and a moist tissue while inside the box, and transported to the Insect Bioecology Laboratory - LABEIN/UNIR, and kept for three days (for egg maturation) at 25 - 28 °C and a photoperiod of 12 hours.

On the third day after the field experiments, mosquitoes were identified at the species level using the Consoli & Lourenço-de-Oliveira (1994) identification key and induced to oviposition after the removal of one of each female's wings using entomological tweezers under a stereomicroscope.

Ethical issues

The collection of blood and mosquitoes used in the experiments was authorized by the Research Ethics Committee of the Federal University of Rondônia CAAE: 53016315.9.0000.5300.

Evaluation of biological and reproductive parameters

The parameters evaluated in this study were: number of engorged mosquitoes, survival to oviposition, number of eggs (fecundity) and number of larvae produced (fertility). Mosquitoes that contained the abdomen visibly distended after the blood repast were recorded as engorged mosquitoes.

The survival ratio up to oviposition was calculated by dividing the number of live females, after the third day of blood meal, by the number of engorged females. The proportion of females that laid eggs was obtained by dividing the number of females that laid eggs by the number of surviving females up to oviposition in each treatment. The number of eggs (fecundity) was obtained counting the eggs present on the moistened filter paper at the bottom of cages containing a single female with the use of a stereomicroscope. The eggs were transferred to 100 mL disposable cups containing tap water for larval hatching. On the third day the larvae were counted to evaluate larval production (fertility).

Statistical analysis

Data analysis was evaluated by ANOVA using the Prism 6 program (Graph Pad Inc.) at a significance level of 5%.

RESULTS AND DISCUSSION

The present concentrations of vitamins and minerals added to blood does not seem to affect the percentage of engorged mosquitoes (Figure 1), but according to Hosoi (1959), high concentrations of salts in the blood can be rejected by mosquitoes. In addition, solutions with osmotic pressure divergent from that of the blood can reduce the engorgement and distention of the abdomen of these insects. Despite that, concentrations up to 1% of the multivitamin in the blood did not affect *An. darlingi* engorgement ($F = 1.63$, $P = 0.2$) and sucrose solutions containing up to 5% of multivitamin solution are routinely used to feed anophelines (Phasomkusolsil et al., 2014, 2015).

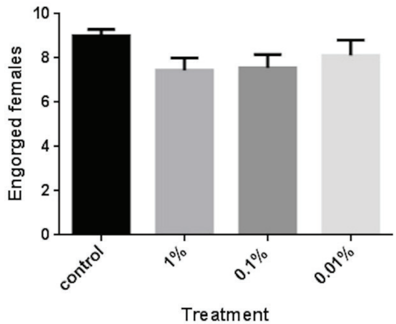

Figure 1. Mean number of engorged *Anopheles darlingi* fed with blood containing different concentration of multivitamins.
Legend: Control= non-supplemented blood; 1%= Blood+ 1% multivitamin (v/v); 0.1% =Blood+ 0.1% multivitamin (v/v); 0.01%= Blood+ 0.01% multivitamin (v/v).

Most of the engorged females survived up to oviposition in all the groups and no significant differences (F = 1. 61, P = 0.21) were found (Figure 2). Although the absence of vitamins in general in artificial diets reduced the survival of adults of the dipterous *Dacus oleae* (Tsiropoulos, 1980), the amount of vitamins in the blood seems sufficient for the survival of adult *An. darlingi* in the observed time interval. In addition, higher concentrations of multivitamin, i.e., 5%, were added to sugar solutions offered to anophelines by Phasomkusolsil et al. (2014) suggesting that there are no toxicity problems associated with supposedly high vitamin concentrations.

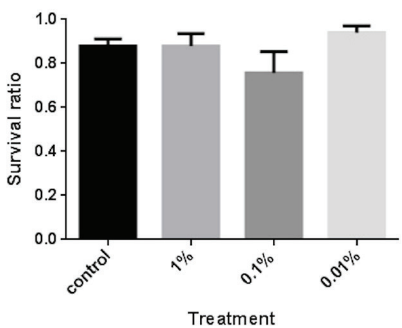

Figure 2. Mean survival ratio of *Anopheles darlingi* fed with blood containing different concentration of multivitamins.
Legend: Control= non-supplemented blood; 1%= Blood+ 1% multivitamin (v/v); 0.1% =Blood+ 0.1% multivitamin (v/v); 0.01%= Blood+ 0.01% multivitamin (v/v).

The mean ratio of females that laid eggs did not differ significantly between groups (F = 0.2, P = 0.89) and ranged from 74 to 81% (Figure 3), suggesting that there was no alteration in the gonotrophic cycle of *An. darlingi* females.

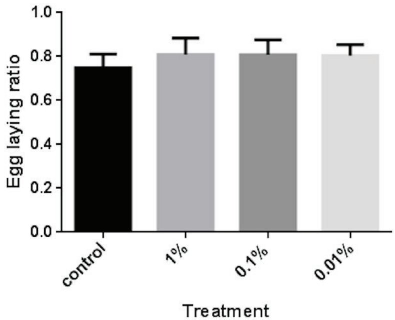

Figure 3. Mean ratio of *Anopheles darlingi* that laid eggs after feeding on blood containing different concentration of multivitamins

Legend: Control= non-supplemented blood; 1%= Blood+ 1% multivitamin (v/v); 0.1% =Blood+ 0.1% multivitamin (v/v); 0.01%= Blood+ 0.01% multivitamin (v/v).

Egg production, on the other hand, was significantly (F = 4.31, P = 0.0058) lower in mosquitoes fed with 1% multivitamin compared to control and to mosquitoes fed multivitamin at 0.01% (Figure 4). Differently from other variables, the amount of vitamins seems to adversely affect *An. darlingi* fecundity at 1% concentration and none of the concentrations evaluated increased fecundity as also related for *Ae. aegypti* fed on a medium containing sheep washed erythrocytes supplemented with vitamins (Greenberg, 1951).

Nonetheless, a 1% multivitamin supplementation may exceed the concentration of B-complex vitamins in the blood (Krebs, 1950) up to 700-fold. The use of 5% multivitamins in sugar solutions was offered to other anophelines during the development of their eggs as a routine method (Phasomkusolsil et al., 2014). In fact, the use of sucrose solutions supplemented with vitamins of the B complex seems to increase the reproductive capacity of *Cx. quinquefasciatus* (Tan et al. 2016).

Fertility in females fed multivitamins was significantly affected (F = 3.98, P = 0.008) (Figure 5). Tsiropoulos (1980) also reported significant changes in the fertility of dipterous *D. oleae* when different vitamins were omitted. In addition, there was a 40% decrease in fertility in these insects fed with artificial diets without vitamins.

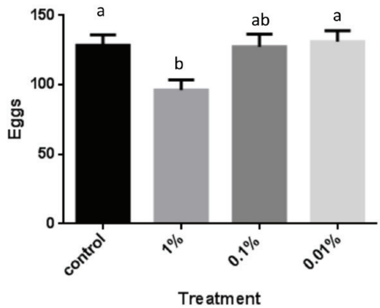

Figure 4. Mean egg number of *Anopheles darlingi*, fed with blood containing different concentration of multivitamins.

Legend: Control= non-supplemented blood; 1%= Blood+ 1% multivitamin (v/v); 0.1% =Blood+ 0.1% multivitamin (v/v); 0.01%= Blood+ 0.01% multivitamin (v/v). Different letters indicate significant differences (p<0.05)

There was an increase in the fertility of the *An. darlingi* females, which received blood supplemented with 0.01% of the multivitamin compared to the others, except at the concentration of 0.1%. The increase over the non-supplemented control was about 20% (Figure 5), suggesting that only low blood vitamin supplementation for the purpose of increasing *An. darlingi* immature production is required. The present data corroborate the use of vitamins to increase the number of viable larvae as recently related by of *Cx. quinquefasciatus* (Tan et al. 2016).

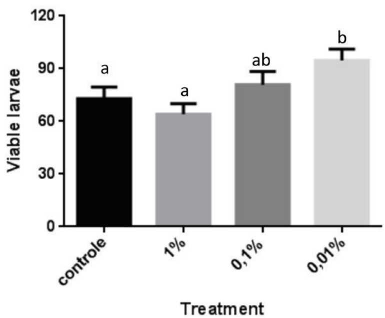

Figure 5. Mean viable larvae produced by *Anopheles darlingi*, fed with blood containing different concentration of multivitamins.

Legend: Control= non-supplemented blood; 1%= Blood+ 1% multivitamin (v/v); 0.1% =Blood+ 0.1% multivitamin (v/v); 0.01%= Blood+ 0.01% multivitamin (v/v). Different letters indicate significant differences (p<0.05).

The basic qualitative need for vitamins and minerals for the development of insects has long been known (House, 1962; Dadd, 1973), but the optimal concentrations, the effect of low concentrations or absence of some of these, and their impact on the survival and development of immature individuals of different species of mosquitoes, e.g., *Ae. aegypti* (Singh & Brown, 1957), *Cx. pipiens* (Kleinjan & Dadd, 1977) vary. Thus, a more detailed study of the effect of some vitamins on blood supplementation, especially the B complex (Fraenkel & Blewett, 1943), should be investigated in order to improve *An. darlingi* immature production.

In conclusion, blood supplementation with several vitamins and minerals in concentrations up to 1% did not alter the engorgement with the food, the survival and the ratio of females laying eggs. However, fecundity (eggs) and fertility (larvae) were affected, indicating that general supplementation can increase vitamins and minerals at the lowest tested concentration, i.e., 0.01%, increased the larval production of *An. darlingi* by up to 20%.

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