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Effect of different food and sugar sources on the larval biology and adult longevity of *Anopheles darlingi* (Diptera: Culicidae)

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Abstract. *Anopheles darlingi* is the main vector of the disease in the Amazon, and several studies on its ecology and behavior have been conducted. Although the basic nutritional requirements of insects are well known, quantitative needs with respect to food components and their balance vary among insect species; therefore, such information is needed in order to improve insect rearing for mass production. The present work evaluated the effect of different animal diets on larval biology and the utilization of different sugar sources in *An. darlingi* adults. First instar larvae obtained from wild-captured mosquitoes were reared in plastic trays containing 1 L of water and fed daily according to their larval instar stage with different commercial diets for fish, reptiles, and dogs, as well as a manipulated diet including Macapo. Larval mortality was recorded daily, and development time was calculated as the time required for 50% of the larvae to reach the last larval instar. Pupae and adults produced from larvae fed with different animal diets were also counted daily. Adults were fed with 10% sugar solutions of honey, sucrose, and sugar cane molasses, and longevity was recorded. Larval mortality was reduced for larvae feeding on fish food (higher protein content) compared to those fed with dog food (lower protein content) in later instars; the first and third instars presented a higher mortality than other instars, regardless of the food provided. Larval development time was reduced in larvae fed with fish food compared with dog food. The average daily production of pupae and adults were significantly higher in the treatment using fish food than in all other diet treatments. In general, adults from larvae fed on fish food and those that ingested honey as an adult sugar source lived longer than those reared on other treatments. Fish food, i.e., Tetramin Tropical Flakes, for larvae and honey, as a carbohydrate source for adults, seem to better support the rearing of *Anopheles darlingi* under our experimental conditions.

INTRODUCTION

Mosquitoes are vectors of several important diseases, such as yellow fever, zika virus, dengue fever, chikungunya, and malaria. Among these diseases, malaria stands out with more than 220 million cases per year and 429 thousand deaths globally (WHO, 2017).

In South America, specifically in Brazil, the main malaria vector is *Anopheles darlingi* (Sinka, 2012) comprising up to 99% of all anopheline species captured in Rondonia, Western Amazon (Gil, 2003 and 2007).

Colonization of *Anopheles darlingi* was previously reported by Villarreal-Treviño *et al.* (2015), who reared *An. darlingi* larvae with a commercially available rodent food containing 23.0% protein, 4.5% fat, and 6.0% crude fiber. However, testing different combinations of nutrients from different sources might improve mosquito mass production for experimental purposes. Insect breeding conditions such as nutrition, both in the larval stage and in adults, as well as the

quality and quantity of food affect (i) reproductive capacity (Richards *et al.*, 2012); (ii) growth (Manorenjitha *et al.*, 2012); (iii) development and pupation (Joy *et al.*, 2010); (iv) survival (Vrzal *et al.*, 2010); (v) oviposition (Yoshioka *et al.*, 2012); and (vi) vector capacity (Araújo *et al.*, 2012).

Mosquito larval nutrition was intensively studied during the 40's, 50's, and 60's (e.g., Golberg *et al.*, 1945; Singh & Brown, 1957; Akov & Guggenheim, 1963), mostly for *Aedes aegypti*, using chemically defined diets. Nevertheless, rearing of several mosquito species relies on animal diets, e.g., dog (Asahina, 1964) and fish (Somda *et al.*, 2017).

Kivuyo *et al.* (2014) concluded that diet composition greatly affected the survival, pupation, and sex ratio of *Anopheles gambiae* and highlighted that nutritional quality and availability are essential for good colony maintenance.

The use of manufactured foods for animals to feed mosquito larvae is an alternative to more expensive and laborious preparations of chemically defined diets containing several different substances. Nevertheless, differences in the concentration and quality of major components, e.g., protein and/or amino acids, lipids, and vitamins from manufactured animal diets may vary and, therefore, affect larval biology.

Adult male and female mosquitoes, on the other hand, usually feed on natural sugar sources containing different carbohydrates such as glucose, fructose, sucrose, maltose, and melizitose, which are ingested a few hours after emergence. In temperate and subtropical regions, several species ingest sugars every 2–5 days. Sugar concentration is also related to longevity, and lower concentrations, i.e., 0.5–1%, appear to create a greater energy deficit. Furthermore, under field conditions it is likely that adults not feeding on sugar will rarely reproduce (Foster, 1995).

Adult mosquitoes reared in a laboratory are usually fed with sucrose solutions, but the qualitative composition of different sugar sources, e.g., honey and molasses, may affect the overall survival of mosquitoes (Vrzal *et al.*, 2010).

Therefore, the present work investigated the effect of different animal diet sources on mosquito biology, i.e., larval mortality in different instars, development time, pupa and adult production, and the effect of alternative sources of sugars on longevity in adult *Anopheles darlingi*.

MATERIALS AND METHODS

Mosquito capture and breeding

Adult mosquitoes were collected at two different locations in the State of Rondônia, at a rural site of Porto Velho city (08°38'00.3"S, 63º55'51.9"W) and Nova Mutum (9°18'55.51"S, 64°32'44.96"W), using the modified BG-Sentinel trap (Gama *et al.*, 2013). The collected mosquitoes were fed with mouse blood (CEUA 2015/1) and transported to the Laboratory of Insect Bioecology of the Federal University of Rondônia. Oviposition was induced in captured mosquitoes by removal of one of the wings 3 days after blood feeding. Eggs were obtained and placed in plastic trays (25 x 15 x 6 cm) containing 1 L of dechlorinated tap water to allow the larvae to hatch. Species identification of mosquitoes was performed at the moment of oviposition, using a dichotomic identification key (Consoli and Lourenço de Oliveira, 1994).

Larval mortality, development time, pupation rate, and adult emergence of Anopheles darlingi reared on different animal diets

Larvae (100/tray) were reared on four different animal diets: (i) TetraMin Tropical Flakes® Fish Feed; (ii) Royal Canin Dog Canine Hepatic®; (iii) Nutricon Pet Reptiles®; and (iv) Macapó-based diet (Moreno *et al.*, 2014). All larval food sources were prepared by grinding the food flakes/grains using a mortar and pestle and the resulting powder passed through a 60-mm sieve before use. The experiments were replicated five times.

Larvae were fed daily according to instar stage: (i) once a day for L1 (2.0 mg/100), (ii) twice a day for L2 (6.0 mg/100), and (iii) three

times a day for L3 and L4 (10.0 mg/100) (Araújo *et al.*, 2012).

Larval development time was estimated as the time required for 50% of the larvae to reach the last larval stage (L4) (Bayoh and Lindsay, 2003). Mortality was recorded daily for each larval instar, i.e., L1-4, during larval development for each treatment. Pupae produced were counted and transferred to cages to allow adults to emerge. The adults produced were also counted and kept in cages to be used in subsequent experiments. The numbers of pupae and adults were recorded daily during the course of the experiment and expressed as daily means.

Longevity of Anopheles darlingi adults fed with different carbohydrate sources

Anopheles darlingi adults (n = 20) obtained from larvae reared on different animal diets were fed different carbohydrate sources and divided into three groups: (i) 10% sucrose, (ii) 10% honey, and (iii) 10% sugar cane molasses. Honey and molasses are complex sources of nutrients, which include vitamins, minerals, and small amounts of proteins and amino acids (Binkley and Wolfrom, 1953; Camargo *et al.*, 2006), but provide lower quantities of total sugars than sucrose, i.e., 80% of total sugars as fructose and glucose and 10% as sucrose and maltose. They also contain water, proteins, enzymes, organic acids, minerals and a low amount of vitamins. Molasses has a lower sugar content but a higher concentration of other nutrients than honey (Binkley and Wolfrom, 1953; Camargo *et al.*, 2006). The adults were placed in screened cages. A cotton swab soaked in sugar solution was placed on the top of the cages and replaced daily.

Longevity, i.e., the total lifetime after adult emergence, was monitored, and mortality was recorded daily. All adults were maintained under controlled laboratory conditions (12-hour photoperiod, 29 ± 2°C and 70%–80% RH).

Data analysis

The effect of the independent variables 'larval stage' and 'food source' on the dependent variable 'larval mortality' (mean number of dead larvae) and the effect of the independent variables 'sugar source' and 'food source' on the dependent variable 'longevity' (mean number of days from adult emergence to death) of *An. darlingi* were analyzed by two-way ANOVA, and the differences between groups were analyzed by the Tukey test at a significance level of 0.05.

The effect of the independent variable 'larval food source' on the dependent variables larval development time, pupation rate, and adult emergence were analyzed using one-way ANOVA and the differences between the groups by the Tukey test at a level of significance of 0.05. All analyses were performed using the software Prism 6 (GraphPad Inc).

RESULTS

Larval mortality and development time

Overall, the first and third instars had the highest larval mortalities ($F=23.46$; $P<0.0001$). The food source affected larval mortality significantly ($F=3.48$; $P=0.0017$) when larvae were in the first, third and fourth instars, but no general effect of the food source on larval mortality was observed ($F=2.01$; $P=0.12$) (Table 1) (Figure 1).

Larval development time on differed diets tended to increase in the following sequence: fish food < Macapo < reptile food < dog food, and the development time of larvae fed on fish food was significantly ($F_{df\ (3),\ n\ (5)}=9.21$; $P<0.0009$) higher than that of those fed on dog food (Figure 2).

Pupation and adult emergence

The mean daily production of pupae was significantly higher ($F_{df\ (3);\ n\ (5)}=7.61$; $P=0.0022$) in the treatments that used fish food compared to the others (Figure 3).

Overall, the emergence of adults from pupae was higher when larvae were fed with fish food compared with other food sources ($F_{df\ (3);\ n\ (5)}=6.65$; $P=0.03$) (Figure 4). Therefore, the adult number produced daily was very similar to the daily pupa production.

Table 1. Summary of two-way ANOVA analysis for larval instar and food source effect in *Anopheles darlingi* larval mortality

Source of variation	% of total variation	df	F value	P value
Interaction	18.02	9	3.42	P=0.0017
Instar	41.09	3	23.46	P<0.0001
Food	3.52	3	2.03	P=0.121

Larval instars: L1, L2, L3, and L4. Food: fish, macapo, reptile, and dog.

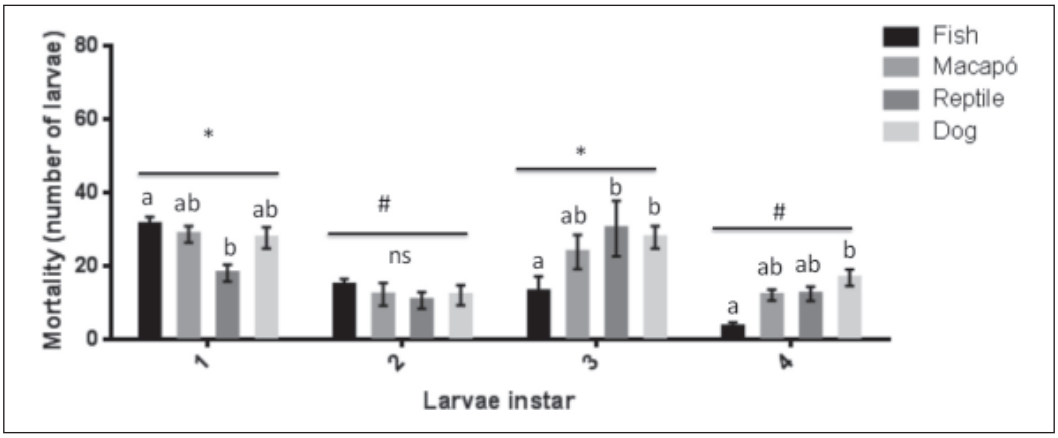

Figure 1. Larval mortality (mean +/- standard error) of different instars of *Anopheles darlingi* reared on different animal diets. Fish: Tetramin Tropical Flakes; Macapó: fish meal, wheat flour, soy flour, maca flour, corn starch, and fuba (Moreno *et al.*, 2014); Reptile: Nutricon Pet; Dog: Royal Canin Canine Hepatic. Lines with different symbols (# and *) indicate significant differences (P<0.05) between the instars for the same animal diet; Different letters indicate significant differences (P<0.05) between different animal diets for the same instar.

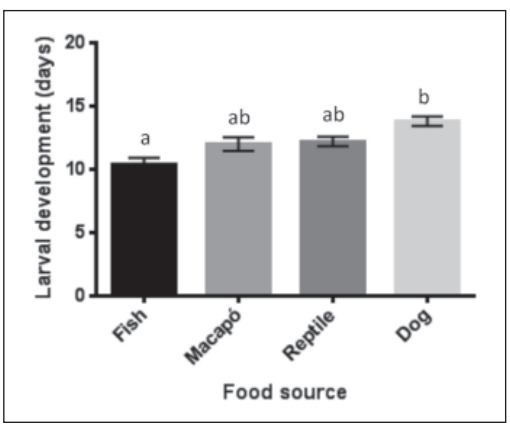

Figure 2. Development time (mean +/- standard error) of *Anopheles darlingi* larvae reared on different animal diets. Fish: Tetramin Tropical Flakes; Macapó: fish meal, wheat flour, soy flour, maca flour, corn starch and fuba (Moreno *et al.*, 2014); Reptile: Nutricon Pet; Cachorro: Royal Canin Canine Hepatic. Different letters indicate significant differences (P<0.05) between treatments.

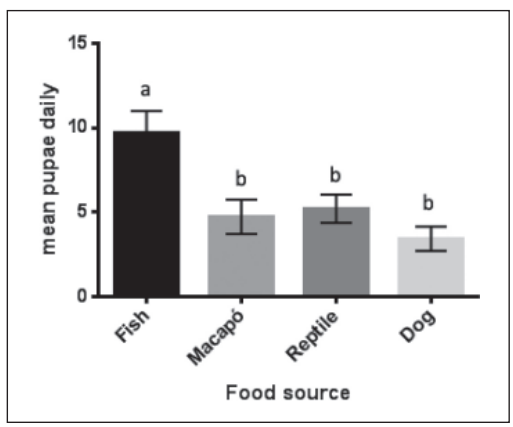

Figure 3. Daily pupae production (mean +/- standard error) of *Anopheles darlingi* from larvae fed with different animal diets. Fish: Tetramin Tropical Flakes; Macapó: fish meal, wheat flour, soy flour, maca flour, corn starch and fuba (Moreno *et al.*, 2013); Reptile: Nutricon Pet; Dog: Royal Canin Canine Hepatic. Different letters indicate significant differences (P<0.05) between the treatments.

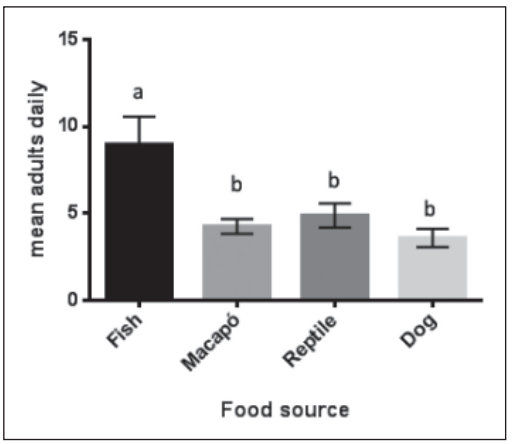

Figure 4. Daily adult production (mean +/- standard error) de *Anopheles darlingi* originated from larvae raised on different animal diets. Fish: Tetramin Tropical Flakes; Macapó: fish meal, wheat flour, soy flour, maca flour, corn starch, and fuba (Moreno *et al.*, 2013); Reptile: Nutricon Pet; Dog: Royal Canin Canine Hepatic. Different letters indicate significant differences (P<0.05) between the treatments.

Adult longevity

Larval food source (F=19.44; P<0.0001) and adult food source (F=3.55; P<0.031) significantly affected adult longevity. In general, adults originating from larvae fed on fish food and honey lived longer than adults originating from larvae fed on other animal diets, except for those fed on Macapo (Table 2) (Figure 5).

DISCUSSION

Effects of food on larval biology

Currently, several researchers who investigate mosquitoes use manufactured foods (Araújo *et al.*, 2012), mixtures of different manufactured foods and other ingredients (Manorenjitha *et al.*, 2012), or just selected ingredients (Moreno *et al.*, 2014) to rear immature stages.

The use of commercial animal diets provides general data about the nutritional value, protein content, fat, fiber, and other ingredients. Thus, foods with varying nutritional composition were used in the present study, with the protein content ranging from 47% (fish) to 12% (reptile) and the fat content ranging from 14% (dog) to 3% (reptile).

Larval mortality in *An. darlingi* was affected by the animal diet provided to certain larval instars (Figure 1) and was lower for larvae fed with fish food (Tetramin Tropical Flakes). Kivuyo *et al.* (2014) also reported lower larval mortality of *Anopheles gambiae* fed on the same fish food (Tetramin Tropical Flakes) compared with other animal diets. Moreover, Araújo *et al.* (2012) also fed *An. darlingi* with Tetramim Tropical Flakes, but they related a lower general larval mortality than the present study.

Larval mortality varied for different larval instars of *Anopheles darlingi* and was higher in the first and third instars (Figure 1). In contrast, Araujo *et al.* (2012) reported higher mortality rates for the fourth larval instar and pupal stages of this mosquito species. Bergo *et al.* (1990) reared *An. darlingi* with diferent food sources based on manipulated ingredients and also reported that diet composition affected larval instars differently.

Nutrients such as proteins can greatly impact larval development, and *Aedes aegypti* larvae deprived of protein do not reach the second instar. Moreover, increasing the protein concentration of artificial diets

Table 2. Summary of two-way ANOVA analysis for the effect of larval food source and adult source on longevity in *Anopheles darlingi*

Source of variation	% of total variation	df	F value	P value
Interaction	2.59	6	1.05	P=0.39
Larval food	23.86	3	19.44	P<0.0001
Food	2.91	2	3.55	P=0.031

Adult food: sucrose, honey, and molasses. Larval food: fish, Macapo, reptile and dog.

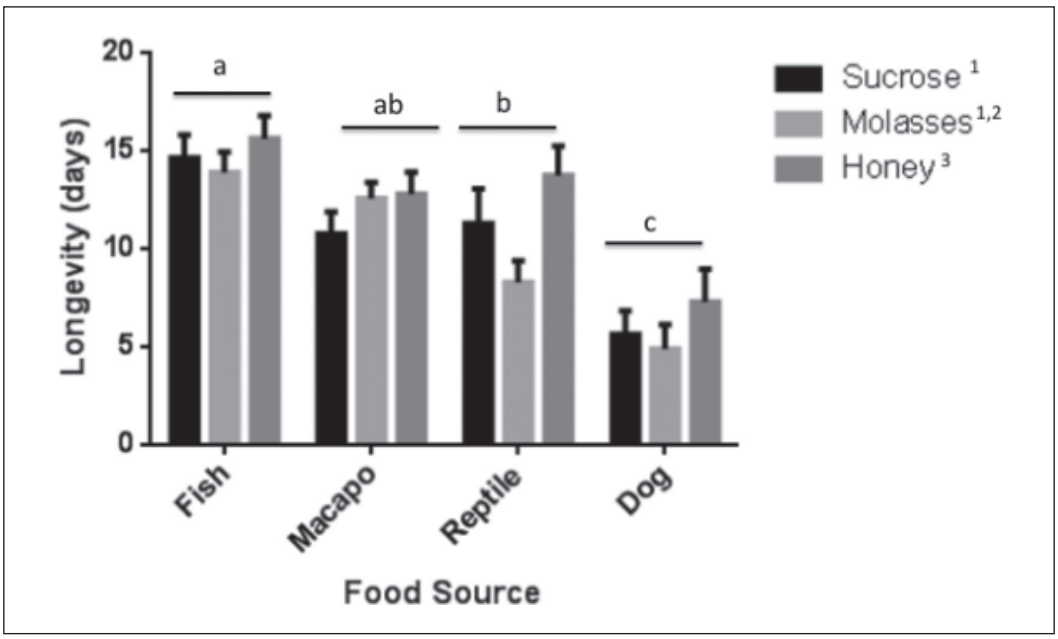

Figure 5. Longevity (mean +/- standard error) of *Anopheles darlingi* adults originated from larvae raised on different animal diets and fed on different sugar sources, i.e., sucrose, molasses and honey. Fish: Tetramin Tropical Flakes; Macapó: fish meal, wheat flour, soy flour, maca flour, corn starch and fuba (Moreno *et al.*, 2013); Reptile: Nutricon Pet; Dog: Royal Canin Canine Hepatic. Different letters indicate significant differences (P<0.05) between the larval animal diets provided. Different numbers indicate significant differences (P<0.05) between the adult animal diets provided.

significantly increased the number of second instar larvae and the number of mosquitoes produced (Golberg and Meilon, 1948).

Although a clear comparison with manipulated food (Macapo) cannot be made, fish food provided higher protein content (47%) and positively affected later *An. darlingi* larval instars (Figure 1), in contrast to dog food, which was the lowest in protein.

However, House (1969) points out that once the essential nutrients are present, their proportion can contribute to nutritional quality more than the absolute amount. Thus, the proportion of other ingredients in the animal diets used may be related to the mortality observed.

Kivuyo *et al.* (2014) reported that *Anopheles gambiae* larvae fed with the same fish food (Tetramin Tropical Flakes) and larval number per tray (100 larvae) completed its development in approximately 10 days, in agreement with our observations on *Anopheles darlingi* (Figure 2).

Araújo *et al.* (2012) also fed *An. darlingi* with the same fish diet (TetraMin Tropical Flakes). However, they reported a much longer development time (i.e., 29 days), at 27°C in the insect-rearing chambers, whereas in the present work, the rearing temperature was set to 29 ± 2°C. Bayoh and Lindsay (2003) reported that even increases of just 2°C usually resulted in differences in longevity of *Anopheles gambiae* larvae. In addition, the shorter time of development may have affected the critical mass accumulation needed for pupation. Telang *et al.* (2007) observed higher pupation and hatching rates for *Ae. aegypti* when the time available for feeding was increased.

The daily pupa and adult production (Figure 3 and 4, respectively) were notably higher in the treatments using fish food (47% protein). Hood-Nowotny *et al.* (2012) found that the highest percentage of N in the larval diets were significantly correlated with a higher production of adults of *An. arabiensis*.

Adult longevity

In the present study, we used a 10% concentration of all the sugar sources, which was sufficient to prevent energetic deficits caused by weak concentrations from 0.5% to 1% (Foster, 1995). Overall, adult mosquitoes that emerged from larvae fed with fish food and those fed with honey had the highest longevity (Figure 5).

Sugar is the basic food of adult mosquitoes and may be ingested for between 2 and 5 days in temperate and subtropical regions. Furthermore, sugar consumption is common after emergence, although it tends to decline with age (Foster, 1995).

The main sugar sources in nature are floral nectars, which usually contain sucrose, fructose, and glucose at concentrations between 20% and 50%, in addition to some amino acids and lipids (Foster, 1995).

The presence of amino acids, originating from nectars used to produce honey, might notably increase the lifespan of mosquitoes. Actually, honey and molasses are more complex sources of nutrients and include vitamins, minerals, and small amounts of proteins/amino acids (Binkley and Wolfrom, 1953; Camargo *et al.*, 2006) related to sucrose solutions. According to Vrzal *et al.* (2010), the addition of aminoacids to the diet of *Culex quinquefasciatus* adults increased survival by 5%.

CONCLUSION

The larval food source affected several biological variables of *Anopheles darlingi*, e.g., mortality, development time, and pupal and adult production, and the adult sugar source affected mosquito longevity. Fish food, i.e., Tetramin Tropical Flakes, for larvae and honey, as a carbohydrate source for adults, seemed to better support the rearing of *Anopheles darlingi* under our experimental conditions. Future investigations on the effects of non-energetic nutrients in mosquito physiology should be carried out for a better understanding of the ecophysiology of mosquitoes (Rivera-Pérez *et al.*, 2017).

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