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Larval diet influences development, growth, and survival of mosquitoes in artificial rearing condition

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Abstract
The rate of development and survival of insects depend on many biotic and abiotic factors. One of the factor is availability of nutrient at early developmental stages. We showed that these diets differentially influence the speed of larval development, growth and survival in *Culex quinquefasiatus* mosquito. Our focus was on soybean powder, commercial Protein supplement powder, a mixture of soya bean powder + protein supplement powder (1:1), aquarium fish feed, dog biscuit, dry fish powder. Lowest larval length was observed in dry fish followed by soybean powder diet and highest in protein supplement and dog biscuit diet. We found that percentage of adult emerged after egg hatching had improved with protein supplement, soybean powder and in the mix diet. Carbohydrate to protein ratio and percentage of adult emerged is significant. Development of gut microbiota in mosquito larvae depends on the source and nature of the diet. The effect of larval diet on larval development reported in this article could be mediated by the differences in microbiota load and/or composition.

Keywords: Larval diet, mosquito rearing, gut microbiota, mosquito nutrition, *Culex quinquefasiatus*

1. Introduction
The larvae of mosquito exhibit different ecological niche than the adults that are responsible for disease transmission. In wild condition, the types of soil and food richness in the larval breeding sites are known to influence larval development [1, 2]. There are studies showing the influence of diet (or detritus) type on mosquito performance [3–5]. Many studies also showed that nutrient richness at the larval stage influences the size and metabolism of adult mosquitoes [6], they influence the microbiota composition in larvae and adults and permissiveness to *Plasmodium* infection [7]. Moreover, for many holometabolous insects, larval ecology can influence the adult life-history traits [8]. There are other studies that described the effect of quality and quantity of protein diet on growth, the efficiency of food utilization and metabolic rates in *Spodoptera eridania* larvae [9]. There are reports on *Schistocera americana*, described that poor diets at larval stage could not be compensated for by superior diet quality in adulthood and individual fed on a high nutrient diet as larvae were found to be significantly larger and contained better stored nutrients at adulthood [10].
Maintaining of mosquitoes in the laboratory is critical for the fundamental and applied research and new product developmental activities. In the past many years, scientists used various food materials as a larval diet for mosquito colony maintenance. There is literature [11] describing the food material and feeding procedures for mosquito larvae. Other study showed the various modes of feeding, feeding mechanisms and the effect of different nutrients on the growth of the mosquito larvae [12]. A proper food for the mosquito larvae ideally includes carbohydrates, fats, minerals (calcium), protein (at least nine amino acids are needed) and lipid (cholesterol) as growth promoters, the source of the vitamin is B-complex (especially biotin and others).
We studied the relative impact of larval diet of mosquito on the feeding, egg hatching, development and survival in artificially reared condition. Focusing on soybean powder, commercial Protein supplement powder, a mixture of soybean powder and Protein supplement powder (1:1), aquarium fish feed, dog's biscuit, dry fish powder, we showed that these diets differentially influence the speed of larval development and survival.

In this report, we highlight our findings and demonstrate how important the role of dietary composition in maintaining a sustainable mosquito colony.

2. Materials and methods

2.1 Mosquitoes and Larval Diet

We used *Cx quiquefasciatus quinquefasciatus* mosquito taken from Godrej Consumer Products Ltd. permanent insectary for the study. Food materials used as larval diet for this study are (i) Protein supplement powder procured from local medical shop (vegetable food source); (ii) Soybean powder (as referred in this paper), prepared by grinding soya chunks, a fermented products made from soybean, purchased from local grocery shop (vegetable food source); (iii) mixture of Soybean powder: Protein supplement powder in the ratio 1:1 (vegetable food source); (iv) Dry Fish was purchased from local fish market that is composed of a variety of small fishes and we grounded this to powder (animal food source); (v) Dog biscuit was purchased from local grocery shop (animal food source); (vi) Aquarium fish feed was purchased from local market (animal food source). Vitamin B-complex capsules (Bcosule) were purchased from the local pharmacy.

2.2 Mosquito Colony Maintenance

We performed the experimental work on *Cx quiquefasciatus quinquefasiatus* mosquito taken from the permanent lab colony of Godrej Consumer Products Ltd., Mumbai (India), which is maintained on artificial blood at 27 °C and 70 ± 10% relative humidity. We collected adults daily, transferred to cloth cages, and provided them 10% sucrose solution. The larval generation prior to those obtained for dietary treatments was fed standard laboratory mice feed mixed with vitamin B-complex (100g mice feed mixed with 1.6g B-complex).

2.3 Analysis of nutritional values of larval diet

We analysed the nutritional values (total Carbohydrate, Fat and Protein content) of all the larval diets studied here. We followed protocols as described by Bureau of Indian Standards (BIS: http://www.standardsbis.in/Gemini/home/Home.action).

Table 1 represents the nutritional values and BIS protocol references.

2.4 Preparation of diet

The soya bean chunks used in this study were initially crushed with the help of a mortar and pestle and then it was subjected to rigorous grinding until it becomes a fine powder. Similarly, aquarium fish feed, dog biscuit, and dry fish powder were prepared. Soybean powder: Protein supplement powder was mixed in the ratio of 1:1. The food material was diluted in water before providing it to the mosquito larvae so that the larvae can take it up easily.

2.5 Feeding Procedure

Individual *Cx quiquefasciatus* egg rafts were collected from the previous generation from the insectary. Each of the six egg rafts was isolated individually in six different plastic containers containing 100mL aged tap water free from chlorine. Each egg raft was allowed to hatch at optimum temperature and humidity (temperature: 27 °C; relative humidity: 70%). After hatching of the egg rafts, six different diet materials were given individually in six containers in such a way that the final concentration of the food in 100mL of the water is 0.22mg/ml. Here

The rearing water was changed when these larvae reach its 2nd instar. Again new batch of same food was provided with the same concentration. As soon as the larvae reached 3rd instar, we transferred them to large containers (3000 mL capacity) containing 1500 mL water. The containers were designed in such a way that it has an air supply point for proper aeration for the larvae to survive and the upper part of the container is capped with netted cloth so that other impurities and organisms does not disturb the setup. Each of the food materials was given to the respective mosquito larvae in the containers. The adults were collected in six different cages with respect to the food materials provided.

2.6 Data Recording and Analysis

All experiments were carried out up to F3 generation and all data presented in this manuscript were based on all four generations (F0 – F3). Larval and pupal periods were determined and time spent from 1st instar to pupation was recorded for all hatched larvae. We measured the larval length by taking 20 larvae from each larval diet after 7 days of egg hatching and placed into individual wells of a 96-well plate for imaging at 5X magnification. The whole body length was measured from top of the head to the tip of the abdomen using Fiji program [13]. Adult mosquitoes were counted immediately after emergence and survival rate was measured by comparing the number of eggs hatched. The adult life expectancy was calculated by counting the number of mosquitoes that died after 7 days of emergence across all larval diet and compare it with the number of mosquitoes emerged. This is one of the indicators of the effect of nutrient during immature stage on the life expectancy of mosquitoes.

The Stats Direct statistical software v2.7.9 (07/09/2012) was used to analyze the data. Data were subjected to suitable statistical analysis (Correlation, Kruskal-Wallis test, Ducan's multiple comparison test etc.).

3. Results and Discussion

In Table 1 the nutritional values of all the larval diet tested is summarized. We have summarized larval growth, emergence, adult survival and sex distribution in emerged adults in Table 2 and Fig. 1-3. We analysed the correlation between nutrient values and developmental parameters (Table 3). Larval growth was measured by measuring the larval length and we found lowest growth in dry fish followed by soybean powder diet and highest in protein supplement and dog biscuit diet (Table 2 and Fig. 1). The percentage of adult mortality found to be higher on aquarium fish-feed diet compared to both protein supplement and soybean powder (Table 2). We found that percentage of adult emerged after egg hatching is better on protein supplement, soybean powder and the mix diet, which also indicated there was less larval or pupal mortality in these diets (Table 2). We also found that sex of emerged mosquitoes in filial generations is significantly different (p<0.05) across larval diet (Table 2 and Fig. 4).

We tested six different diets (both vegetable and animal source) and measured the larval growth, adult emergence, survivability, and sex distribution. Lowest growth was found in dry fish followed by soybean powder diet and highest in

protein supplement and dog biscuit diet (Table 2 and Fig. 1). Larval growth is positively correlated with carbohydrate & fat content in the larval diet, however negatively correlated with protein. Previous studies suggested that larval development in wild is influenced by the amount and quality of food present in the water [14, 15]. It was also found that the larval diet had an impact on their development, growth rate and survival in the laboratory. Mosquito larvae require a minimum amount of nutrition to trigger hormonal development cascade to progress to the adult [16]. One critical parameter is the impact of larval nutrition (i.e. quality of larval diet) on adult survival. This parameter was calculated by counting the number of mosquitoes that died after 7 days of the first adult emerged in a larval diet and calculated the survival percentage of total emergence. The protein supplement and soybean powder showed decreased adult mortality compared to aquarium fish food (Table 2). Total adult emergence (production of mosquito) had improved (i.e., less larval or pupal mortality) with protein supplement, soybean powder and in the mix diet (Table 2). The carbohydrate to protein ratio and the percentage of adult emerged are correlated in a very significant manner ($P<0.0001$; t = 12.298 with df = 10). Larval mortality rates differ significantly across diet (Kruskal-Wallis = 11.9; $P = 0.035$). We also found that sex distribution of emerged mosquitoes is significantly different ($P<0.05$) across larval diet (Table 2 and Fig. 4), but we do not observe any pattern here. Therefore, it remains inconclusive about any possible role of diet in sex determination. The development times from hatch to pupation were found to be 10.5 days across diet and there is no significant difference in pupation time due to diet.

This study showed that the mosquito larval diet influences development, growth, and survival of mosquitoes. Adult survival rate for larvae feeding on protein supplement and soybean powders were found to be higher than those larvae fed on fish feed, dog biscuit and dry fish. Another study on *Manduca sexta* suggested that protein to carbohydrate ratios (nutrient ratios) affect the growth of larvae, where protein consumption had a greater effect on growth than carbohydrate consumption [17]. However, we could not find this correlation in mosquitoes, rather we observed that higher larval length with dog biscuit might be attributed to the higher fat content and balanced protein to fat ratio (Table 1 and 2). Some study [7] have also reported the impact of larval growth using various diet and hypothesized that effect of larval diet on larval development may be mediated by the observed differences in microbiota load and/or composition. Many studies suggested that microbiota participates in macromolecule breakdown [18], synthesize nutrients such as vitamins and essential amino acids [19] and promote growth and development via the insulin pathways [20, 21]. However, we must acknowledge that the information on detailed composition, energetic value, vitamin content for the diets we used is missing in order to analyse these parameters. It is reported that the development of gut microbiota in mosquito larvae varies with the variation of food [7], therefore, vegetable and animal food source, which we used for this study, will create different sets of microbiota in the mosquito gut.

Differential larval growth, development, survival reported in the current study may be linked to this differential microbiota development in the gut of mosquito larvae. However, a detailed study is required to conclude this hypothesis. We can conclude with this study that source of larval diet impacts on the development and survival rates of the mosquito and using this diet will be helpful for artificial rearing of *Cx quiquefasciatus quinquefasciatus* mosquitoes.

Table 1: Nutritional values of larval diet tested following Bureau of Indian Standard methods.

Parameters	Nutrient Values (g/100g)						
	Protein Supplement	Soybean Powder	Soybean Powder + Protein Supplement	Dog Biscuit	Dry Fish	Fish Feed	Reference Test Methods
Carbohydrate	53.23	36.97	40.56	57.65	2.1	58.14	IS:1656-2007 (RA: 2009)
Fat	1.25	0.28	0.17	7.22	1.75	2.62	IS:7874 (Part 1)-1975 (RA:1990)
Protein	32.68	48.94	46.06	20.5	65.05	21.05	IS:7874 (Part 1)-1975 (RA:1990)
Carbohydrate to Protein Ratio	1.63	0.76	0.88	2.81	0.03	2.76	
Carbohydrate to Fat ratio	42.58	132.04	238.59	7.98	1.20	22.19	
Protein to Fat ratio	26.14	174.79	270.94	2.84	37.17	8.03	

Table 2: Showing larval growth, adult survival and adult emergence data

Larval Diet	Average Larval Length (mm)				Average % of Adult Died Post 7 days of emergence	Average % of Adult Emerged	Average % of Male	Average % of Female
	1st Instar	2nd Instar	3rd Instar	4th Instar				
Protein Supplement	2.01	4.1	6.16	7.28	16.2	86.4	61.8	38.2
Soybean Powder	1.96	4.09	5.81	6.22	16.4	80.5	55.5	44.5
Soybean Powder + Protein Supplement	1.99	3.99	5.87	6.51	21.2	87.7	38.6	61.4
Dog Biscuit	2.14	4.24	6.37	7.75	24.6	69.2	53.6	46.4
Dry Fish	1.87	3.37	5.3	6.11	21.9	73.5	58.8	41.2
Fish Feed	1.94	3.41	5.2	7	33.5	48.7	40.5	59.5

Table 3: Correlation between nutrient values and developmental parameters

Nutrients	Statistical Parameters	Larval Length (mm)				Adult death after 7 days of emergence	Adult Emergence
		First Instar	Second Instar	Third Instar	Fourth Instar		
Carbohydrate	Pearson Correlation	0.700	0.489	0.470	0.797*	0.289	-0.245
	# Significance, P value	0.061	0.162	0.174	0.029	0.290	0.320
	Covariance	1.337	3.922	4.606	10.878	39.096	-75.113
Fat	Pearson Correlation	0.700	0.203	0.385	0.761*	0.428	-0.457
	# Significance, P value	0.061	0.350	0.225	0.039	0.199	0.181
	Covariance	0.165	0.201	0.466	1.282	7.157	-17.320
Protein	Pearson Correlation	-0.689	-0.303	-0.358	-0.888**	-0.504	0.484
	# Significance, P value	0.065	0.279	0.243	0.009	0.154	0.165
	Covariance	-1.085	-2.006	-2.895	-9.998	-56.374	122.556

Significant Correlation is indicated by * (P<0.05) and ** (P<0.001)

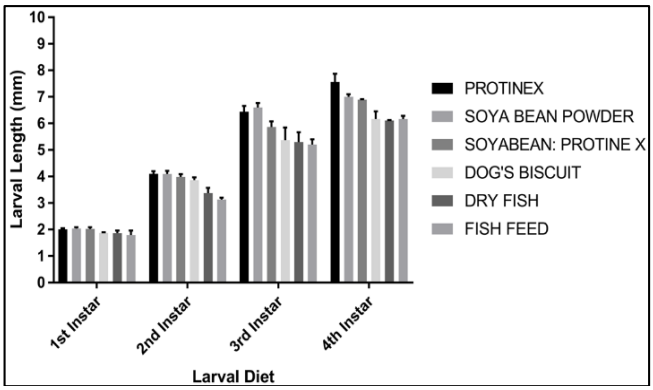

Fig 1: Larval length at different instar in six larval diets

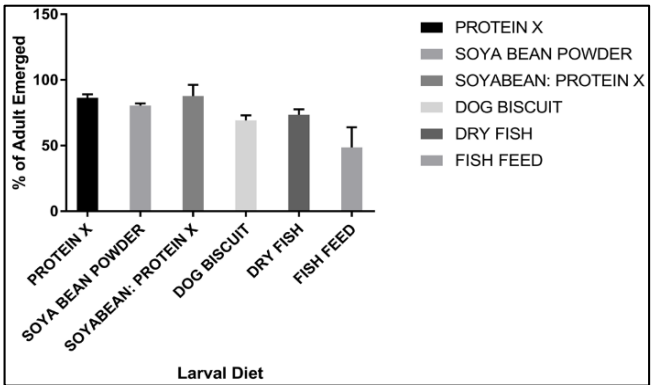

Fig 2: Adult emergence rate in different larval diets

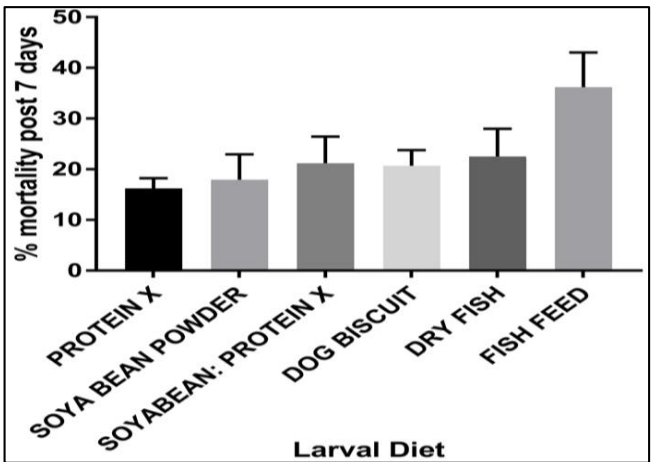

Fig 3: Percentage of adults diet post seven days of emergence in different diets

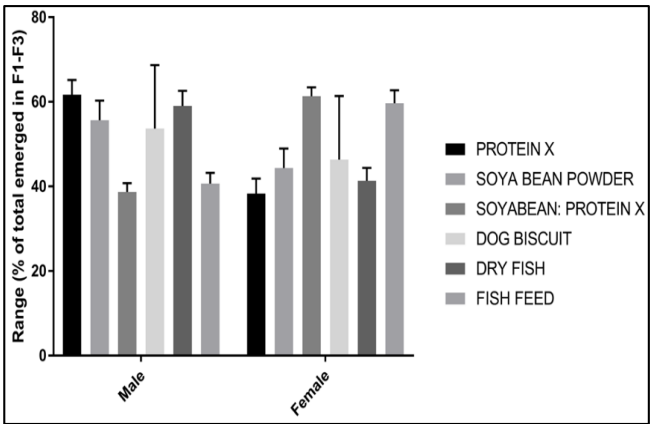

Fig 4: Sex distribution of emerged adults from different larval diets

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