

Unsaturated Fatty Acid-Rich Diets Modulate Plasticity in Pre-Adult Fitness, Behavior, and Development in *Drosophila melanogaster*

Aradhana Joshi

SASTRA Deemed University

Pooja Ramakrishnan

SASTRA Deemed University

Sunil Kumar Birua

SASTRA Deemed University

Shanmugapriya Chandrasekaran

SASTRA Deemed University

Anusri Nakkeiran

SASTRA Deemed University

Pankaj Yadav

pankajyadav@scbt.sastra.edu

SASTRA Deemed University

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Abstract

Background

Dietary nutrients play a critical role in the development of organisms from the fetal stage to adulthood. High-fat and high-sugar diets impair adult performance and accelerate ageing in mice, fruit flies, and humans. Developmental diet shapes adult energy balance, and unsaturated fatty acids (USFAs) influence membrane lipid composition during pre-adult growth. Here, we hypothesise that USFAs modulate developmental time, as assessed by key larval-to-adult life-history traits in *Drosophila melanogaster*. We subjected fruit flies to various doses of USFA-rich diets from the larval stage.

Results

We assayed larval feeding and motility, pupariation time, pupariation height, pigmentation time, and pre-adult development time. Our results showed that high dietary USFA significantly increased larval feeding and motility, and accelerated development time. Additionally, USFA decreased pupariation height and pre-adult survivorship under various doses, suggesting that development time is neither positively nor negatively correlated with pupariation height and feeding rate. These findings emphasise the differential effects of USFA on pre-adult fitness and behavioural traits.

Conclusion

Accelerated development time in PUFA-fed larvae implies developmental plasticity to the nutrient imbalance, with decoupling of normally linked traits. Nevertheless, whether this plasticity is sustained in later life, with fitness tradeoffs that may not directly correlate with lifespan, and fitness remained to be determined.

1. Background:

Environmental factors such as temperature, humidity [1], nutrition, oxygen levels, hormones [2], and genetics [3] together influence the development of an organism. Sugar and yeast [4, 5] have been shown to influence the development and longevity [6, 7], and organisms are capable of buffering environmental effects during the pre-adult stage [7–9]. Larvae reared on isocaloric diets differing in nutrient composition, particularly those enriched in saturated fatty acids, exhibit developmental plasticity, whereby change in nutrient quality alters growth rate and development time [10]. This highlights the importance of nutrition quality in regulating development. Nutrients are important in development; lipids are essential macronutrients that serve as structural components of cellular membranes, energy reserves and signalling molecules for growth, metabolism and development [11]. Among these, fatty acids are vital for brain development and growth, while a high-fat diet (HFD) reduces the lifespan of an

organism [4, 12]. Given the increased prevalence of obesity and type 2 diabetes [13] has highlighted the importance of understanding the physiological effects of dietary fatty acid quality.

Drosophila is a widely used organism to address the effects of diet on physiology, development and growth [7, 14, 15]. In nature, larvae of fruit flies *Drosophila melanogaster* develop on decaying fruits by feeding on yeast and plant-derived nutrients [16]. The lipid composition of these food sources varies with the fruit species, the ripening stage of fruits, and microbial interaction, exposing larvae to heterogeneous dietary lipid environments [17]. Such natural variations influence development, growth, and membrane composition, favouring evolution of developmental plasticity in larvae to adapt to changes in nutritional conditions. Dietary unsaturated fatty acids (USFA) are obtained from food [18]; variation in diet composition might contribute to energy metabolism and other physiological processes. This ecological relevance supports investigating the effects of diet-enriched unsaturated fatty acids on pre-adult fitness and behavioural traits.

Studies investigating dietary fatty acids have reported a reduction in physical activity, lifespan, and fertility when fed to numerous organisms at different stages or periods of adulthood [4, 5]. The nutritional and dietary intake conditions experienced during development affect the physiology and fitness of *D. melanogaster* [19]. However, there are no clear reports on how unsaturated fatty acids affect fly development and associated traits. To date, only one study by Eickleberg et al [4] reported slight changes in the development rate under unsaturated fatty acids (USFA) and a delayed development rate under saturated fatty acids (SFA). This limited understanding of how USFA influences developmental traits restricts our ability to conclude their role in shaping pre-adult life-history traits. Here, we hypothesise that a diet rich in USFA alters pre-adult developmental traits in *D. melanogaster*.

Pre-adult life-history traits in *Drosophila* are strongly interconnected and shape later adult physiology [19]. Fitness traits such as development, pre-adult survivorship, and pupariation height are highly sensitive to the nutritional environment [4, 7]. Feeding rate strongly drives biomass gain [20] and developmental duration [7, 21], while dietary nutrient composition influences larval foraging and feeding behaviour [7, 20]. In this context, USFA might play an essential role in understanding how lipid quality and quantity modulate feeding behaviour, which in turn can delineate how nutrient intakes are translated into growth, and how body mass reflects the combined outcome during development. Beyond feeding-related foraging, larval motility also reflects energy expenditure through searching and digging [22], making it a sensitive measure of dietary treatment for energy allocation. Adequate feeding enables larvae to attain the critical weight (CW) required for pupariation [23]. Motility rate further supports this process by enabling the wandering and successful transition to a pupariation site [24, 25]. However, the trade-off between the larval feeding rate and pupariation to adult eclosion duration under different fatty acid diets remains poorly understood.

These pre-adult fitness traits are critical, as they dictate survival, pupariation, and adult phenotype. Accordingly, nutritional conditions such as dietary restriction (DR) have been shown to reduce pupariation height [7], and pupariation site choice is influenced not only by larval density, food texture,

temperature, and light, but also by diet [7]. Thus, pupariation height under altered diets (USFA) might reflect variation in resource reservoirs and foraging behaviour. Post-pupariation processes such as pupal development, pigmentation, and the pace of metamorphic transition are regulated by hormonal cues [26], temperature [1], and diet. Diets such as SFA, HSD, and DR have been reported to delay development [4, 7, 27]. Yet explicit studies addressing the effects of USFA on development and the trade-offs between pre- and post-pupariation stages remain limited. Interestingly, both poor and rich nutrient conditions can slow developmental time, but a rich diet shortens lifespan, whereas dietary restriction extends longevity. Moreover, a poor diet slows development due to resource limitations and reduced insulin/TOR signalling [2, 7], while a rich diet causes metabolic stress and insulin resistance [28], both of which shorten lifespan. Notably, the development theory of ageing states that development and ageing are positively correlated [29]. Hereby assessing larval developmental traits with supplementation of a diet rich in USFA may help determine their impact on adult survival and energy allocation. Further research into adult survival following pre-adult USFA feeding and its relation to developmental life-history traits would provide valuable insights into how early exposure to high unsaturated fatty acids influences overall fitness and trade-offs.

Few studies have explored how a high-fat diet affects pre-adult growth or lifelong exposure in *D. melanogaster* [4, 27]. Despite this, limited studies are available on developmental plasticity in relation to behaviour (feeding and motility) under varied nutritional conditions [10].

In the current study, we examined the effects of USFA on pre-adult traits and development pace of fruit flies. The decoupling of normally associated life-history traits under USFA feeding provides the initial evidence for fatty acid-rich nutrition-based developmental plasticity during the pre-adult stage.

2. Methods

2.1 Fly stock and husbandry

A population of the wild-type strain Canton S (CS) of *D. melanogaster* was maintained at a constant temperature ($25 \pm 0.5^\circ\text{C}$) and humidity ($80 \pm 2\%$) on a 21-day discrete generation cycle. The population was housed in a plexiglass cage ($25\text{ cm} \times 20\text{ cm} \times 15\text{ cm}$) containing ~ 1200 adult flies with roughly equal numbers of males and females. Flies were supplemented with a control diet (AL-Ad-libitum). Eggs, larvae, and flies were maintained on USFA-rich (MUFA and PUFA) diets across various doses. Dietary modifications were achieved by supplementing macadamia and walnuts with different concentrations, as shown in Tables 1, 2 and Joshi et. al [30].

Table 1

Dietary composition of AL and unsaturated fatty acids with supplementation of macadamia and walnut powder (MUFA and PUFA, respectively) in 1 litre.

Components	AL	1.25%	2.5%	5%	7.5%	10%
Agar (g)	12	12	12	12	12	12
Corn flour (g)	100	100	100	100	100	100
Sugar (g)	40	40	40	40	40	40
Yeast (g)	40	40	40	40	40	40
Benzoic acid (methyl-p-hydroxybenzoate) (in g/ ml of ethanol)	1.5/15	1.5/15	1.5/15	1.5/15	1.5/15	1.5/15
Propionic acid (ml)	15	15	15	15	15	15
HFD (MUFA /PUFA) (g)	0	12.5	25	50	75	100

Table 2

Overview of total calorie, carbohydrate, protein, sugar, fat, and water intake across each dietary regime under supplementation of fatty-rich diet, as per Food Safety and Standards Authority of India (FSSAI) regulations.

Components	AL	Monounsaturated fatty-rich dietary doses				
		M-2.5%	M-2.5%	M-5%	M-7.5%	M-10%
Energy (kcal)	651.21	740.96	830.71	1010.21	1189.71	1369.21
Carbohydrate (g)	141.92	143.67	145.42	148.92	152.42	155.92
Protein (g)	19.2	19.95	20.7	22.2	23.7	25.2
Sugar (g)	39.8	40.375	40.95	42.1	43.25	44.4
Fats (g)	0.44	9.44	18.44	36.44	54.44	76.44
Benzoic acid (methyl-p-hydroxybenzoate) (g/15 ml ethanol)	1.5/15	1.5/15	1.5/15	1.5/15	1.5/15	1.5/15
Propionic acid (ml)	15	15	15	15	15	15
Water (ml)	1000	1000	1000	1000	1000	1000
Components	AL	Polyunsaturated fatty-rich dietary doses				
		P-2.5%	P-2.5%	P-5%	P-7.5%	P-10%
Energy (kcal)	651.21	735.0975	818.985	986.76	1154.535	1322.21
Carbohydrate (g)	141.92	144.446	146.972	152.024	157.076	162.13
Protein (g)	19.2	20.95	22.7	26.2	29.7	33.2
Sugar (g)	39.8	39.8	39.8	39.8	39.8	39.8
Fats (g)	0.44	7.7525	15.065	29.69	44.315	58.94
Benzoic acid (methyl-p-hydroxybenzoate) (g/15 ml ethanol)	1.5/15	1.5/15	1.5/15	1.5/15	1.5/15	1.5/15
Propionic acid (ml)	15	15	15	15	15	15
Water (ml)	1000	1000	1000	1000	1000	1000

2.2 Larval feeding assay

To examine how USFA influences larval feeding behavior, a larval feeding assay was performed as mentioned elsewhere [31, 32] with slight modifications. Firstly, we collected eggs laid over a 2 hr window and transferred them to the respective diets (5 MUFA, 5 PUFA, and 1 Control group = 11 diets). Post-hatching larvae (30 hr, 48 hr, and 72 hr), first (L1), second (L2), and third instar (L3), respectively, were collected in 5 replicates and transferred to 2% agar plates containing 2% yeast solution. For each diet ×

instar stage, five independent vials were assessed. 10 Larvae were sampled per vial (total 50 larvae), and the values were averaged per vial before analysis. Larvae were allowed to habituate to an agar plate for 1 minute, and the number of sclerite extensions per minute was further calculated using a stereomicroscope.

2.2 Larval motility

To study the influence of USFA-rich diets on larval foraging behaviour, larval motility rate was assayed with minor modifications to the protocol, as described [33]. Similar to the feeding assay, we collected larvae from the first (L1), second (L2), and third (L3) instar and transferred them to a 2% agar plate. Once the larvae had habituated to the agar plate, we counted their anterior-to-posterior movement per minute using a stereomicroscope.

2.3 Larval dry body weight

To determine the effects of USFA-rich diets on larval body mass, we measured larval body weight at the third instar (L3) stage. The eggs were collected and transferred to the respective fatty acids. Post-hatching 80 hr, a 20 number of third instar (L3) larvae were washed thoroughly to remove attached food particles on the outer larval body, briefly blotted on tissue paper to remove excess moisture, and transferred to an Eppendorf tube in 3 replicates for each diet. Normal body weight was first measured using a microbalance Shimadzu (precision ± 0.01 mg). Further larvae were dried at 60°C for 24 hr, after which the dry body weight was recorded [34]. Dry body weight is used as a hydration-independent measure of larval body mass under fatty acid-supplemented diets. The mean individual dry body mass was calculated by dividing the pooled dry mass by 20. Each vial represents one replicate ($n = 3$ per diet)

2.4 Development time, pupariation, pigmentation assays

Egg-to-adult development time was assessed in eggs transferred with various USFA diets. Development time assay was performed as per the protocol mentioned elsewhere [7, 32] with slight modifications to the diet. Egg collection was performed from a running culture of CS flies. Before egg collection for the experiment, eggs were collected in a 1 hr window to avoid development time differences. After 1 hr, a fresh food plate of the AL control diet was placed for 2 hr intervals, and eggs laid by flies for 2 hr were collected for the assays. The mid-point of this 2 hr window (i.e., the first 1 hr) was considered as zero hr. The experiments were performed with exactly 30 eggs in each replicate, with a total of 7 replicates for each diet regime ($30 \times 7 = 210$ eggs/ regime). Eggs were collected on an agar block and placed in a vial (18 cm long, containing ~ 6 ml food; 11 diets $\times$ 7 vials = 77 vials in total were assessed). To experiment with pupariation time every 2 hr, the number of pupae formed was recorded until the last larva pupated in a vial. This method was also followed for pigmentation time, and the eclosion time assays (specifically, male and female eclosion time was recorded separately). The recorded raw data were analysed further by taking averages across the replicate vials in a particular diet.

2.5 Pre-adult survivorship assay

Pre-adult survivorship was assessed by recording the number of flies that successfully eclosed on days 8th and 9th at every 2-hour interval, further calculated by dividing the total number of flies eclosed from each vial per diet by the total number of eggs dispensed in each vial in a particular diet (i.e., 30 eggs).

2.6 Pupariation height assay

To determine the effect of USFA diets on pre-adult fitness, pupariation height was examined. Once the development time assay was completed, the vials were left undisturbed for 2 more days to check if any flies eclosed further. Pupariation height was measured according to the established methods [7, 32]. To avoid disturbance due to handling, once the flies had eclosed, we assessed the vials for pupariation height (since the same vial and the experimental setup was used as in the development time assay). Pupariation height (in cm) was calculated as the distance from the food surface to the center of the pupae using the transparent sheet. Pupa on the surface of the food was marked as zero pupariation height. All pupae present within the vial and their heights were measured; each vial was treated as one biological replicate for statistical analysis

2.7 Statistical analyses

The data from the larval body weight, larval feeding, larval motility, pupariation height, development time assay, and pre-adult survivorship were analyzed using one-way ANOVA. Pigmentation time data sets did not meet the normality assumption and were tested for non-parametric analyses. These datasets were therefore analyzed using the Kruskal-Wallis test (diet was taken as an independent factor). Replicates of vial was treated as an independent unit of replication.

To assess the combined effect of diet and larval instar, feeding and motility data were subjected to a two-way ANOVA (Diet and larval instar were independent factors). Based on the p-value showing a significant difference. Tukey's post-hoc test (for parametric data) and Dunn's test (for non-parametric data) were performed using GraphPad Prism 8.0 version. The error bars were plotted around the mean $\pm$ SD. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1 Increased larval feeding rate under various doses of MUFA

To determine whether MUFA supplementation alters larval feeding behavior, we measured sclerite extension rate across first (L1), second (L2), and third (L3) instars under various doses. One-way ANOVA test revealed a significant increase in feeding rate with $p < 0.0001$ across all instars of larvae (Fig. 1A, B, C). Tukey's multiple comparisons showed L1 larvae fed with 1.25%, 2.5%, 5%, 7.5%, and 10% MUFA had higher feeding rates compared to AL-fed larvae ($F_{5,24}=11.31$; $p < 0.0001$; Fig. 1A). At the L2 stage, MUFA-fed larvae, except those at 5% and 7.5% showed a greater sclerite extension rate than AL-fed larvae (Fig. 1B). Notably, 7.5% MUFA-fed larvae showed decreased feeding rate compared to all other MUFA-fed

larvae (1.25–2.5% and 10%; $F_{5,24}=15.78$; $p < 0.0001$ Fig. 1B). Similarly, L3 larvae fed with MUFA doses revealed increased feeding rates against AL-fed larvae ($F_{5,24}=9.287$; $p < 0.0001$; Fig. 1C). Drawing on established findings, we assume that change in feeding behavior can indicate altered developmental timing under USFA [35].

Second-instar larvae show higher feeding rates compared to the L1 and L3 stages [21]. Given that feeding behavior varies across instars, we assessed the effect of larval instar on feeding rate using two-way ANOVA ($F_{12,68}=23.04$; $p < 0.0001$), followed by Tukey's post-hoc test. We observed a similar trend, with second-instar larvae exhibiting a higher feeding rate, except at the 7.5% dose. Larvae fed at 7.5% MUFA showed unaltered feeding rates across three instars. This might be because they showed a higher feeding rate at L1 (Fig. S1A), so it is more likely that they ingested excess food during the first instar stage.

3.2 Increased larval feeding rate under various doses of PUFA

One-way ANOVA revealed a significant difference across all the instars of PUFA-fed larvae ($p < 0.0001$; Fig. 1D, E, F), where larvae at the L1 stage showed a higher feeding rate from 1.25% to 10% as compared to that of AL-fed larvae ($F_{5,24}=7.842$; $p = 0.0002$; Fig. 1D). The feeding rate was unaltered by second instar larvae fed at 5% and 7.5% PUFA as compared to that of AL-fed larvae. However, the feeding rate of 1.25%, 2.5%, and 10% PUFA fed larvae was significantly increased as compared to that of AL, 5%, and 7.5% fed PUFA ($F_{5,24}=13.51$; $p < 0.0001$; Fig. 1E). Similarly, L3 fed at various doses of PUFA showed a higher feeding rate than AL-fed larvae ($F_{5,24}=9.746$; $p < 0.0001$; Fig. 1F).

Larval instar was treated as an independent variable to examine stage-specific feeding behavior across PUFA. Two-way ANOVA revealed a significant effect ($F_{17,68}=22.50$; $p < 0.0001$; Fig. S1B) followed by Tukey's post-hoc test. Feeding rate remained higher at the L2 stage than at the L1 and L3 stages across PUFA doses. At 5% PUFA, larval feeding rate did not differ across L1, L2, and L3 stages. This observation of uniform feeding at 5% PUFA suggests larvae might have met growth demand, which may help them develop faster. Thus, results indicate that USFA feeding enhances feeding behavior in a stage-specific manner.

3.3 Increased larval motility rate under various doses of MUFA

To examine the role of USFA on larval behavior, we carried out larval motility using body wall contraction from anterior to posterior. One-way ANOVA revealed a significant increase in larval motility rate across all the instars (Fig. 2A, B, C). L1 larvae fed with 1.25%, 2.5%, 5% and 10% were more motile than AL-fed larvae. Tukey's multiple comparisons showed that larvae fed 1.25% MUFA had significantly higher motility rates as compared to those of 7.5% MUFA-fed larvae ($F_{5,24}=6.049$; $p = 0.0009$; Fig. 2A). However,

motility at 2.5%, 5%, and 10% MUFA did not differ significantly from 7.5% MUFA group, but they remained more motile than AL-fed larvae.

At the L2 stage, except for 2.5% MUFA-feeding, larvae raised under all the other doses of MUFA showed an increased motility rate compared to that of AL-fed larvae. Tukey's multiple comparison revealed that 10% of MUFA-fed larvae had higher motility rates than those of 2.5%, 5%, and 7.5% of MUFA-fed larvae ($F_{5,24}=11.28$; $p < 0.0001$; Fig. 2B). Similarly, L3 MUFA-fed larvae, when compared to AL-fed larvae, showed a high motility rate across all MUFA doses, except 2.5% and 7.5%. The comparison among MUFA doses revealed that 1.25% of MUFA-fed larvae had significantly higher motility rate than that of 2.5% and 7.5% fed larvae ($F_{5,24}=8.928$; $p < 0.0001$; Fig. 2C). It is interesting to note that, at the L3 stage, larvae fed with 2.5% MUFA showed reduced motility relative to those fed with 1.25% MUFA (Fig. 2C). Across-stage comparison of larval motility using Two-way ANOVA revealed that larvae at the L3 stage were less motile than that at the L1 stage under higher doses of MUFA and AL ($F_{17,68}=12.34$; $p < 0.0001$; Fig. S2A). MUFA increased larval motility but did not change the normal development process leading towards pupariation.

3.4 Increased larval motility rate under PUFA fed at the L2 and L1 stages

L1 larvae fed with 1.25% and 2.5% PUFA showed a high motility rate compared to AL-fed larvae when tested by one-way ANOVA ($F_{5,24}=4.501$; $p = 0.0049$; Fig. 2D). Following Tukey's multiple comparisons revealed that 7.5% fed PUFA were less motile than 1.25% and 2.5% (Fig. 2D). L2 stage larvae likely showed a higher motility rate, except for 2.5% against AL-nurtured larvae. Larvae fed with 5% and 10% PUFA had the highest motility rate as compared to that of 1.25%, 2.5%, and 7.5% ($F_{5,24}=26.18$; $p < 0.0001$; Fig. 2E). Likewise, L3 showed a high motility rate when fed with various doses of PUFA ($F_{5,24}=5.100$; $p < 0.0001$; Fig. 2F). These data suggest that larval motility can influence development time at various doses of PUFA which might be plausibly by energy allocation and altering attainment of critical weight [34]. Two-way ANOVA across instar suggests that higher doses of PUFA (5% and 10%) increased larval motility rate at the L2 stage. Whereas, at low doses of PUFA (1.25%, 2.5%) and an AL diet, L1 larvae show the highest motility ($F_{17,68}=13.14$; $p < 0.0001$; Fig. S2B). This implies that PUFA modulates larval activity, and larvae manage their stage-specific foraging or energy distribution, which is driven by dietary fatty acid levels.

3.5 Shorter pupariation duration at 10 percent dose of PUFA

Pupariation time is a key life-history trait, which is influenced by nutrition during growth and development of flies [22], making it suitable to examine the effects of MUFA and PUFA doses on the development time. We examined the duration taken by larvae to pupate at various doses, here we do not find any significant difference in pupariation time under various doses of MUFA (Fig. 3a). Similarly, One-way ANOVA followed by Post-hoc Tukey's HSD test revealed that larvae fed at 10% PUFA (~ 100 hr) showed

shorter pupariation time than that at 5% PUFA (~ 107.157 hr; $F_{5,32} = 3.574$, $p < 0.0100$; Fig. 3B). Thus, PUFA-fed larvae at the higher dose have a significant effect on pupariation time. Alongside, the pupariation time and feeding rate showed no correlation with both MUFA ($R^2 = 0.002296$; $p = 0.7632$, Fig. S3A) and PUFA diets ($R^2 = 0.01008$; $p = 0.5270$; Fig. S3B). These results suggest that feeding is not correlated with pupariation time.

3.6 Reduced pupariation survival under 7.5% PUFA

Besides testing their pupariation time, we further assessed the number of eggs dispensed in vials that developed into the pupal stage. Larvae fed at 7.5% PUFA, had reduced survival to the pupal stage as compared with those fed 1.25%, 10% PUFA and AL-diets ($F_{5,36} = 3.440$, $p < 0.0121$; Fig. 3D). Further, ANOVA revealed no significant difference in the MUFA-fed pupal survival rate (Fig. 3C). This suggests PUFA compromised survival, whereas MUFA managed to undergo metamorphosis like AL. The PUFA feeding at 7.5% might impose greater metabolic stress.

3.7 MUFA and PUFA shorten the pupariation height

To determine whether USFA supplementation influences pupariation height as a marker for energy allocation. One-way ANOVA revealed that 1.25% (6.3597 cm) and 2.5% (7.70831 cm) fed larvae pupated at a shorter distance against AL (10.13 cm), and 5%, 7.5%, and 10% (~ 10 cm) MUFA-fed larvae ($F_{5,36} = 15.43$; $p < 0.0001$; Fig. 4A). Likewise, one-way ANOVA revealed pupariation height of larvae fed at 1.25%, 2.5%, 5%, and 7.5% (6.3 cm -7.1 cm) have shorter distance against AL, and 10% (10.206 cm) of PUFA-fed larvae ($F_{5,36} = 8.033$; $p < 0.0001$; Fig. 4B). Thus, indicating that USFA has a significant effect on the pupariation height of the flies. Pearson's correlation analysis reveals that no correlation exists between pupariation height and either motility rate ($R^2 = 0.07744$; $p = 0.0743$, Fig. S3C) or development time ($R^2 = 0.04504$; $p = 0.1772$, Fig. S3E) under MUFA conditions. Similarly, upon assessing the PUFA group, a significant correlation was neither observed between pupariation height and larval motility rate ($R^2 = 0.02460$; $p = 0.3213$, Fig. S3D) nor between development time ($R^2 = 0.01117$; $p = 0.5053$, Fig. S3F). This suggests that pupariation site choice is neither regulated by motility nor by development pace under USFA

3.8 7.5% PUFA delays the wing pigmentation duration

Kruskal-Wallis test revealed that the duration taken by pupae to attain the wing pigmentation state is significantly different at PUFA-fed larvae ($H(5) = 23.30$; $p = 0.0003$; Fig. 5B). Multiple comparison using Dunn's test revealed that 7.5% PUFA significantly delayed wing pigmentation time (~ 172.786 hr) to that of AL (167.486), 2.5% (~ 168.386 hr), and 10% (~ 156.657 hr) pupae. In contrast, pupae at various doses of MUFA didn't differ in pigmentation time (Fig. 5A). Following pigmentation time, the percentage of pupae pigmented did not differ between MUFA and PUFA-fed larvae. Thereby, it reveals that PUFA significantly varies its effects on pigmentation time, which is not the same as MUFA-raised pupae.

3.9 PUFA doses influence the egg-to-adult developmental duration

Dietary ingestion modifies development duration in fruit flies; nutrient-rich diets are proven to delay development, but their influences on pre-and post-pupariation tradeoff remain scarce. We investigated whether MUFA and PUFA affect the egg-to-adult stage development duration and their associated tradeoff. One-way ANOVA revealed that there is no significant variation in development time when fed with doses of MUFA as compared to AL ($F_{5,36} = 1.050$; $p = 0.4041$; Fig. 5C). However, two-way ANOVA revealed a sex-based difference in pre-adult development time at 10% MUFA ($p < 0.0022$; Fig. S4A), indicating sex-dependent differences in development time under higher doses of MUFA.

In contrast, one-way ANOVA followed by multiple comparisons using Tukey's HSD test ($F_{5,36} = 15.84$; $p < 0.0001$; Fig. 5D) suggest that flies at 10% PUFA (~ 166.657 hr) develop faster as compared to AL (~ 173.7433 hr), 1.25% (~ 183.13 hr), 2.5% (~ 187.563 hr), and 7.5% of PUFA (~ 182.786 hr). Flies' emergence from 5% of PUFA (~ 167.381 hr) had shortened development time as compared to that of 2.5% and 7.5% PUFA-fed flies. No sex-based difference was observed in development time (Fig. S4B).

Correlation analysis showed no significant relationship between feeding rate and development time under different doses of MUFA ($R^2 = 0.02485$; $p = 0.3187$; Fig. S3G) and PUFA ($R^2 = 0.1681$; $p = 0.0070$; Fig. S3H). However, MUFA-fed larvae showed a positive correlation between pupariation time and development time ($R^2 = 0.05437$; $p < 0.0001$; Fig. 5E), as well as between wing pigmentation time and development time ($R^2 = 0.7598$; $p < 0.0001$; Fig. 5G). Additionally, positive correlations were observed between development pace and pupariation time ($R^2 = 0.1458$; $p < 0.0126$; Fig. 5F) and between development time and pigmentation time under PUFA diet ($R^2 = 0.5478$; $p < 0.0001$; Fig. 5H). Thus, PUFA and MUFA fed larvae strongly establish a correlation between the pace of different pre-adult stages including that of pupariation, pigmentation, and adult eclosion suggesting that the altered development time of flies is a response to their dietary polyunsaturated fatty acids accelerated at a higher dosage (Fig. 5D), and monounsaturated fatty acids showed sex-specific variation, where males delayed development (Fig. S4A), thereby showing differential development time due to ample nutrition provided.

3.10 Pre-adult survivorship altered at PUFA doses

To determine larval viability and survival success under USFA, we assessed egg-to-adult survivorship. ANOVA followed by Tukey's multiple comparisons revealed pre-adult survivorship (percent of adult emergence from egg stage) reduced at 7.5% compared to 2.5% of PUFA ($F_{5,36} = 2.651$; $p = 0.0386$; Fig. 6B). One-way ANOVA revealed no significant variation in pre-adult survivorship across MUFA doses (Fig. 6A), suggesting that PUFA alters the survival to pupariation stage at 7.5%, while MUFA remains ineffective in terms of pre-adult survivorship. Pre-adult survival success to pupate and develop implies that the larval food was non-toxic, whereas reduced pre-adult survivorship at PUFA-7.5% against lower dosage suggests dose-dependent stress. Although juvenile survivorship is measured numerically, adult

survival and physiological robustness may vary because they depend more on the development rate rather than the diet alone.

3.11 Larval dry body weight remains unchanged under MUFA and PUFA

Dry body weight serves as a reliable measure of dietary effect on energy storage, independent of water content. One-way ANOVA revealed no significant change in the body weight across MUFA ($F_{5,12}=1.231$, $p < 0.3536$) and PUFA doses ($F_{5,12}=2.121$, $p < 0.1328$), even though we visually observe some variation (Fig. 6C, D). Thus, indicate that excessive food consumption is strategically utilized by larvae across various behavioral and fitness traits of the pre-adult stage.

4. Discussion

Dietary nutrients have been shown to influence the development of *Drosophila* [4, 7, 27]. Studies on the impact of fatty acids on *Drosophila* development have been accelerated recently [4, 27]. *Drosophila* is naturally dependent on olfactory and gustatory chemical cues to guide feeding, development, and fulfill nutritional demands [36]. Studies have shown that larvae sense fatty acids [37], although specific receptors involved remain unidentified. Flies expressed ionotropic co-receptor IR25a in larval and adult chemosensory organs [38], and in adults, fatty acids detection depends on IR56d and IR25a [39], indicating that fatty acids are detected across various life stages in fruit flies. Carvalho et al. suggest that dietary plant-rich USFA reshape the *Drosophila* lipidome, suggesting a role of lipids in membrane lipid composition during development stages [40]. However, limited studies have explored the impact of HFD (USFA) on *Drosophila* development traits. In our study, we report for the first time that polyunsaturated fatty acids modulate the overall development of fruit flies.

4.1 The feeding rate of USFA-fed larvae increased with different dosages

In flies, diet is crucial for regulating both pre-adult development and larval feeding behavior [7, 27], hence exhibiting a positive correlation [35]. In the present study, we found an increase in feeding rate at the L1, L2, and L3 stages when larvae were fed with MUFA and PUFA (Fig. 1). For the first time, Musselman et al. reported that HSD (High sugar diet) reduces sclerite extension rate and induces hyperglycemia, while testing larval feeding rate with HFD revealed a minor increase in feeding rate but statistically remained insignificant. Their findings indirectly support the observation, but the variations in results actually stem from their focus only on L3 stage larvae and differences in both HFD composition and percentage. Herby, we chose to assess feeding behavior across all instars, as larvae continuously feed during the L1 and L2 for growth and energy storage [21, 27]. Whereas the L3 stage shows wandering behavior, which in turn helps in understanding the post-feeding physiology [27]. Further, the L2 stage manifested a higher feeding rate than L1 and L3 (Fig. S1A, B), except at MUFA-7.5%, and PUFA-5%, following the trend shown

by previous studies with respect to the stages of feeding [21]. The difference noted at MUFA 7.5% could be because they were fed at a higher rate at the L1 stage (Fig. S1A), thereby they might have adapted a compensating and managing mechanism to feed the earlier stage and remain unchanged, at L2, supporting the presence of behavioral plasticity [41]. Following PUFA 5% had insignificant feeding behavior at L2 and L3 stages, but significantly showed delayed developmental duration (Fig. 5D), implying that feeding PUFA 5% for a longer period causes lengthened developmental duration. These results lead us to hypothesize that PUFA influences the pre-adult development time. As earlier studies have established a correlation between feeding rate and development time [35]. The mechanical approach for increased feeding rate observed in USFA-supplemented larvae might be driven by enhanced lipid sensing and NPF/sNPF appetite signaling [42], metabolic demand due to accelerated growth, and excess feeding, which in turn modulate the growth and development plausibly mediated by endocrine regulators such as insulin-like peptides and TOR signaling [2].

4.2 Larval motility at L1, L2, and L3 stages

At the L3 stage, larvae undergo cessation of feeding, move away from the food source, and select a site for pupariation. This transition is regulated by the circadian clock, which influences the feeding rate [43], which in turn correlates with pupariation duration (development) [35]. Additionally, dietary nutrients affect larval motility rate [34]; we observed increased larval motility rates (L1, L2, and L3) when fed with MUFA and PUFA compared to AL. Suggesting that larvae might exhibit rover-like foraging behaviour by crawling longer distances. In earlier studies, rover-type larvae were found to pupate over a longer distance, while sitter larvae pupate close to the food or fruit [44, 45], suggesting that the rover larvae have higher pupariation height [46]. Debatably, we observed larvae to pupate at shorter distances mainly due to the lower concentrations of fatty acids (Fig. 4A, B). Apart from these, larvae might choose to pupate near the food surface by saving energy (extra weight gain), where higher humidity helps them survive more [1]. Larvae at lower doses of MUFA and PUFA might influence rover foraging behaviour to act as a sitter or vice versa, since rover larvae have been proven to have behavioural plasticity in response to the food environment [47].

4.3 Variation in egg to pupa survival without altering pupariation time

The relative consistency in pupariation time across the doses of MUFA and PUFA (Fig. 3A, B) compared to the control suggests that dietary USFA do not significantly regulate the initial developmental stage. However, an intermediate dose of PUFA (5%) delayed the maturation compared to that at the 10% dose (Fig. 3B), implying a dose-specific effect at the early development stage. Our results were similar to a previous study that showed no difference in pupariation time when larvae were fed on HFD, while showing accelerated metamorphosis compared to those fed on a high-sugar diet and a high-protein diet [27]. In our study, dose-dependent variation in pupariation timing is sensitive to the amount of PUFA supplemented.

In *Drosophila*, the initiation of maturation requires sufficient fat stores [27] and steroid prohormone ecdysone from the prothoracic gland-PG [48]. Carreno et al. further demonstrated that lipid carrier apolipoprotein in fat cells and *Sema1a* in the neuroendocrine PG are required for sensing fat and signaling. The absence of *Apolpp* and *Sema1a* does not initiate metamorphosis, despite fat storage and body weight gain [49]. We assume that this could be proven true in our current study with larvae at 7.5% of PUFA showing reduced survival percent to AL, 1.25%, and 10% (Fig. 3D). Further, it needs to be tested if *Apolpp* and *Sema1a* are involved in reduced survival rates.

4.4 Shorten pupariation height when fed with USFAs

Pupariation height helps determine the fitness of an organism, and it is shown that the greater the pupariation height, the higher the survival success [50]. Our study revealed reduced pupariation height at lower doses of MUFA 1.25% and 2.5% (Fig. 4A), and PUFA fed with 1.25%, 2.5%, 5%, and 7.5% (Fig. 4B). Dietary intake studies revealed pupariation height depends on larval mean critical weight (CW). After achieving CW, larvae initiate wandering and further pupate [23]. Ormerod et al [34] showed that differences in crawling speed varied by the dry mass of the larva. We assumed there might be a difference in larval body weight at various doses of fatty acids. However, we do not find a significant difference in dry body weight, although a slight increase was noted at low doses of MUFA 1.25% and 2.5% (Fig. 6C) and PUFA fed with 1.25%, 2.5%, 5%, and 7.5% (Fig. 6D), which is not evident at MUFA 5%, 7.5%, 10% (Fig. 6C), and PUFA 10% (Fig. 6D).

Further, motility and pupariation height are not significantly correlated (Fig. S3C, D). We believe that larvae at low doses delay wandering and prolong food ingestion [48], and larvae subsequently choose to pupate on a humid surface, which in turn results in slight weight gain and prevents them from crawling greater distances from food sources, as discussed earlier under motility [1, 24]. Despite higher feeding, unaltered dry body weight at higher doses, the increased pupariation height is likely an escape behavioral response, prompting larvae to move farther from food. This pattern at PUFA-10% may be a reallocation of available energy towards a longer crawling distance (Fig. 2E, F). Additionally, due to the accelerated development (Fig. 5D), the excess energy storage might have been restricted, which in turn can account for unchanged body weight (Fig. 6D). A comparable trend was noted under a low protein diet, where DR (10%) larvae pupate at shorter height due to reduced energy usage [7].

4.5 Larvae fed with PUFA doses differ in pigmentation time

The transition of mature pupae to wing pigmented pupae is delayed at 7.5% compared to AL, 1.25%, and 10% of PUFA-fed larvae (Fig. 5B). Larvae at the highest dose of PUFA showed a faster development rate than 5% and 7.5%. Studies are available on diet and pigmentation time, but also, limited resources are available on genes that regulate wing pigmentation [51]. Further, we see no difference in the survival percentage of pupae to wing pigmentation (data not shown).

4.6 The altered egg to adult development duration under various doses of PUFA

We observe higher dose of PUFA (10%) showed early eclosion compared to AL, 1.25%, 2.5%, and 7.5%. Further, eggs dispensed at 5% PUFA develop faster than 7.5% and 2.5%. Earlier studies reveal that dietary nutrients influence the development of *Drosophila* [7, 12, 27, 34]. Eickleberg et al showed delayed development when fed with SFA. Also, Musselman et al HFD-fed larvae did not differ in development from egg to adult stage; as previously mentioned, their results differ from our present study due to the dosage supplementation. Additionally, eggs dispensed on MUFA did not differ in development time (Fig. 5C). This could be because they have reduced pupariation height, but such assumptions could not be proved true only by showing correlations (Fig. S3E, F). MUFA at higher dosage delayed males' eclosion over females (Fig. S4A). To the best of our knowledge, for the first time, we report sex-specific effects on egg to adult development under higher doses of MUFA. Although larvae fed more and showed higher motility, their body weight remained unchanged, indicating that the energy was allocated across different development traits. In line, we observed no existence of positive or negative correlation between feeding rate and pupariation height (Fig. S3A, B) or development time (Fig. S3G, H) when raised with USFA. Therefore, one can suggest that increased feeding alone does not translate to faster or slower development under USFA. Previous studies report positive correlations between feeding rate and development duration [7, 21]. In contrast, this tradeoff is decoupled under nutrient rich condition, reshaping pre-adult plasticity. This demonstrates that larvae can buffer the developmental traits of ingesting high dietary nutrients using developmental plasticity.

4.7 Pre-adult survivorship was altered when fed with PUFA

Pre-adult survivorship was reduced at an intermediate dose of PUFA diet (7.5%), which imposes a detrimental effect on the pre-adult stage of flies. Earlier studies have reported that larvae can continue feeding and growing, but fail to successfully transition to the pupal stage and eventually die within the food source [27, 49]. Similarly, in our study, we report that 7.5% of PUFA-fed larvae showed reduced pupariation percent and pre-adult survivorship (Fig. 3D; 6B).

The observation of larvae under 7.5% at the L2 stage with premature initiation of wandering behavior could be due to hypoxia [52]. Following that, the black debris at the L3 stage near the food (inside the food surface and /or on the food surface) with an unaltered larval weight suggests that the larvae on 7.5% continued to feed and grow, but could not transit to the pupariation and died inside the food. Suggesting that larvae under a 7.5% dose might experience developmental stress, leading to compromised survival at pre-adult stages. In contrast, a higher dose (10%) did not affect the survivorship at the early stage. Together, these observations indicate a non-linear, dose-specific effect of PUFA, with the intermediate dose being partially detrimental.

4.8 Larval body weight during development

Unaltered body weight at L3 suggests that under USFA (Fig. 6C, D), energy is efficiently utilized for motility (Fig. 2), growth, and development (Fig. 5C, D) rather than excess storage of energy. This pattern may contribute to early metabolic balance that can influence health later in adult physiology [53].

Conclusion

The findings of the current study suggest that unsaturated fatty acids strongly affect various pre-adult development traits such as feeding behavior, motility rate, pupariation time, pigmentation time, pupariation height, development time, and pre-adult survivorship. PUFA feeding accelerates development and reduces pre-adult survivorship. MUFA caused no major developmental changes, whereas PUFA-fed larvae exhibit pronounced alterations, demonstrating how fatty acids have varied biological effects. Taken altogether, this is the first study to report a lack of correlation among specific developmental traits exposed under USFA-rich diets, suggesting that USFA-rich diets feeding led to developmental plasticity. Further understanding of such dietary fatty acid variation in development may reveal some adaptive strategies of adult flies to balance the growth, energy usage, and survival under nutrient-rich conditions.

Limitations

In our current study, dietary treatments consist of walnut and macadamia powder rather than purified unsaturated fatty acids. As nuts contain bioactive components, macronutrients including vitamins and fiber, the observed effect likely reflects their combined nutritional effects. The current study addresses USFAs and not macadamia and walnut, as these nuts are specifically rich in MUFAs and PUFAs. This approach is analogous to dietary protein restriction studies, in which protein intake is manipulated by the source of yeast in the diet.

Experimental approach: Feeding assay using 2% yeast solution is adapted from earlier studies. Future investigation using purified fatty acids may further distinguish the specific role of individual USFAs on feeding rate and preadult fitness.

Declarations

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interest

The authors state that they have no conflicts of interest to declare.

Availability of data and materials

Data supporting the findings of this study will be available upon request to the corresponding author

Author information

Contributions AJ: writing the original draft, methodology (Larval feeding, motility, dry body weight, pupariation time, pigmentation time, development time, pre-adult survivorship, and pupariation height), data curation, investigation, conceptualisation, visualisation, reviewing and editing the draft, Validation, and Resources. PR: Methodology (Pupariationtime, pigmentation time, and development time). SKB: Methodology (Pupariationtime, pigmentation time, and development time). SP: Methodology (Dry body weight), AN: Methodology (Dry body weight). PY: Writing the original draft, methodology, conceptualisation, visualisation, data curation, reviewing and editing the draft, Validation, Resources, Supervision, Project administration, Investigation, and Funding acquisition.

Supplement

Electronic supplementary material is available online.

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Figures

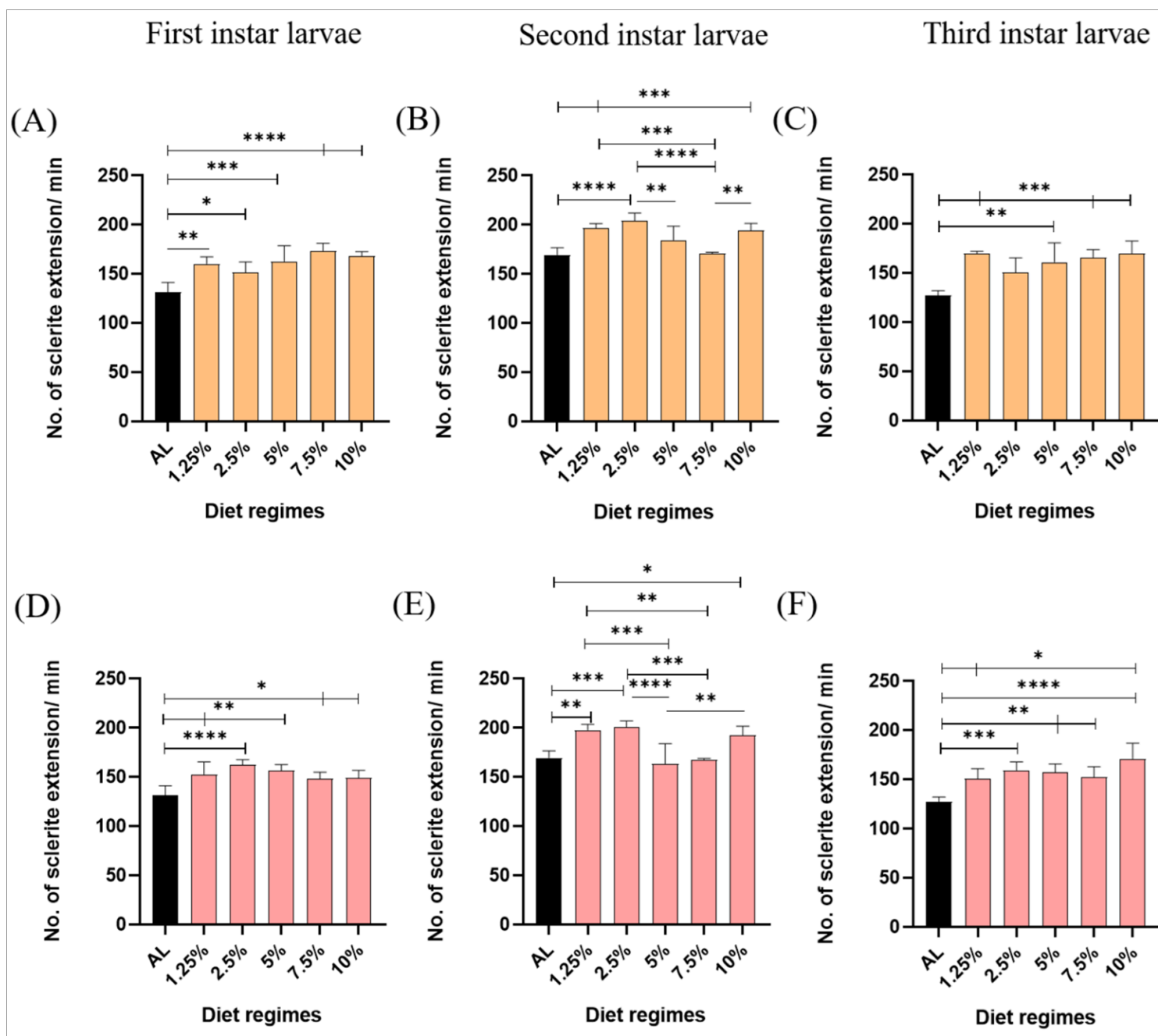


Figure 1

Increased larval feeding rate under different quality of USFA. Different doses of MUFA enhanced larval feeding rate at the first instar stage (L1) (A), second instar stage (L2) (B), and third instar stage (L3) (C). Dietary PUFA fed at various doses showed an increased feeding rate. First instar stage (L1) (D), second instar stage (L2) (E), third instar stage (L3) (F). N = 5 (10 larvae per replicate = 50 larvae/diet regime, where the x-axis indicates diet regimes and the y-axis denotes the number of sclerite extension rate/minute. Error bars are mean $\pm$ SD. * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.00001$

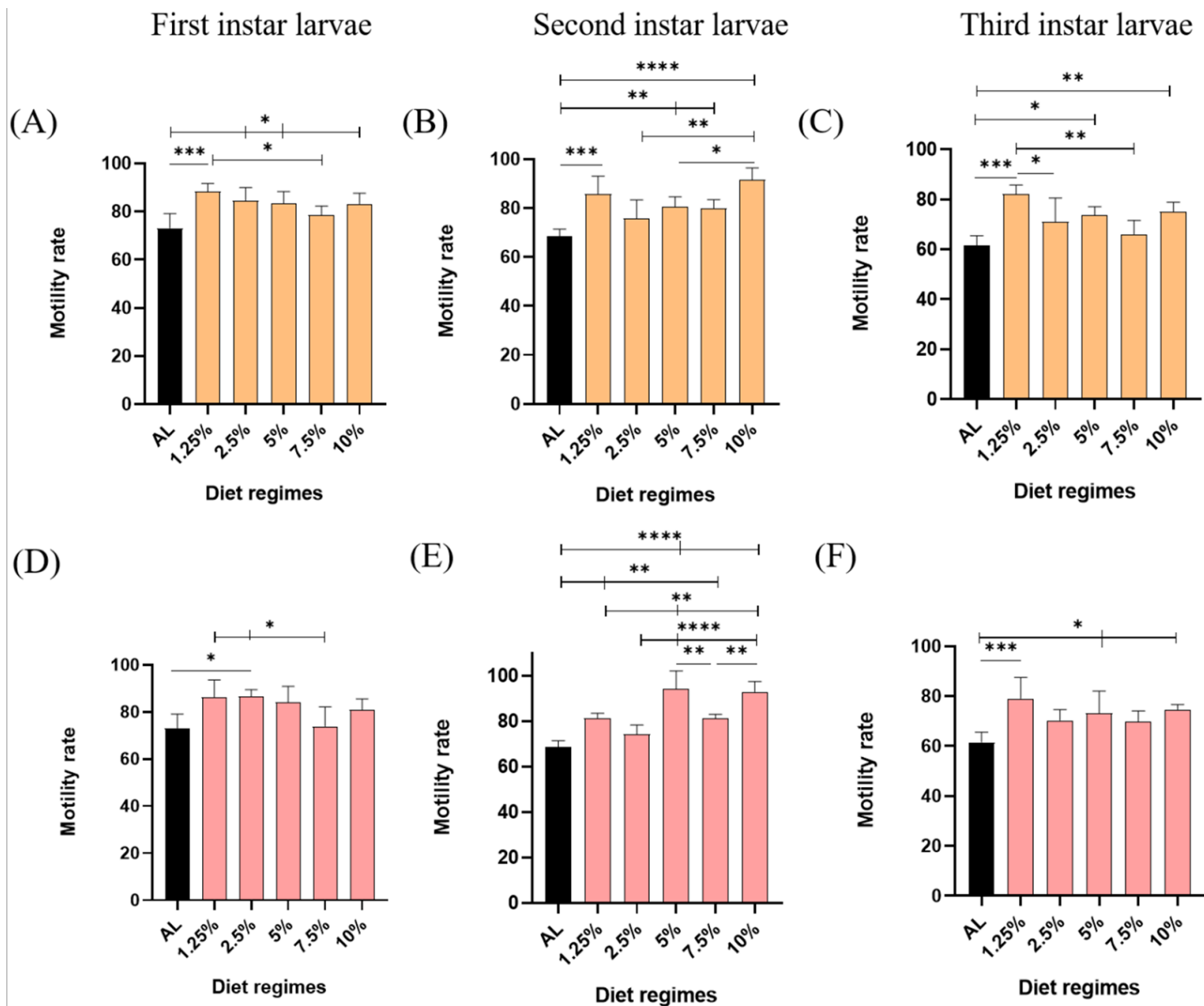


Figure 2

Increased larval motility rate under higher fed USFA. Different doses of MUFA-fed larvae had higher motility rate at the first instar stage (L1) (A), second instar stage (L2) (B), and third instar stage (L3) (C). Dietary PUFA fed at various doses showed increased motility. First instar stage (L1) (D), second instar stage (L2) (E), third instar stage (L3) (F). N = 5 (10 larvae per replicate = 50 larvae/diet regime, where the x-axis indicates diet regimes and the y-axis denotes the motility rate (number of anterior to posterior movement/minute)).

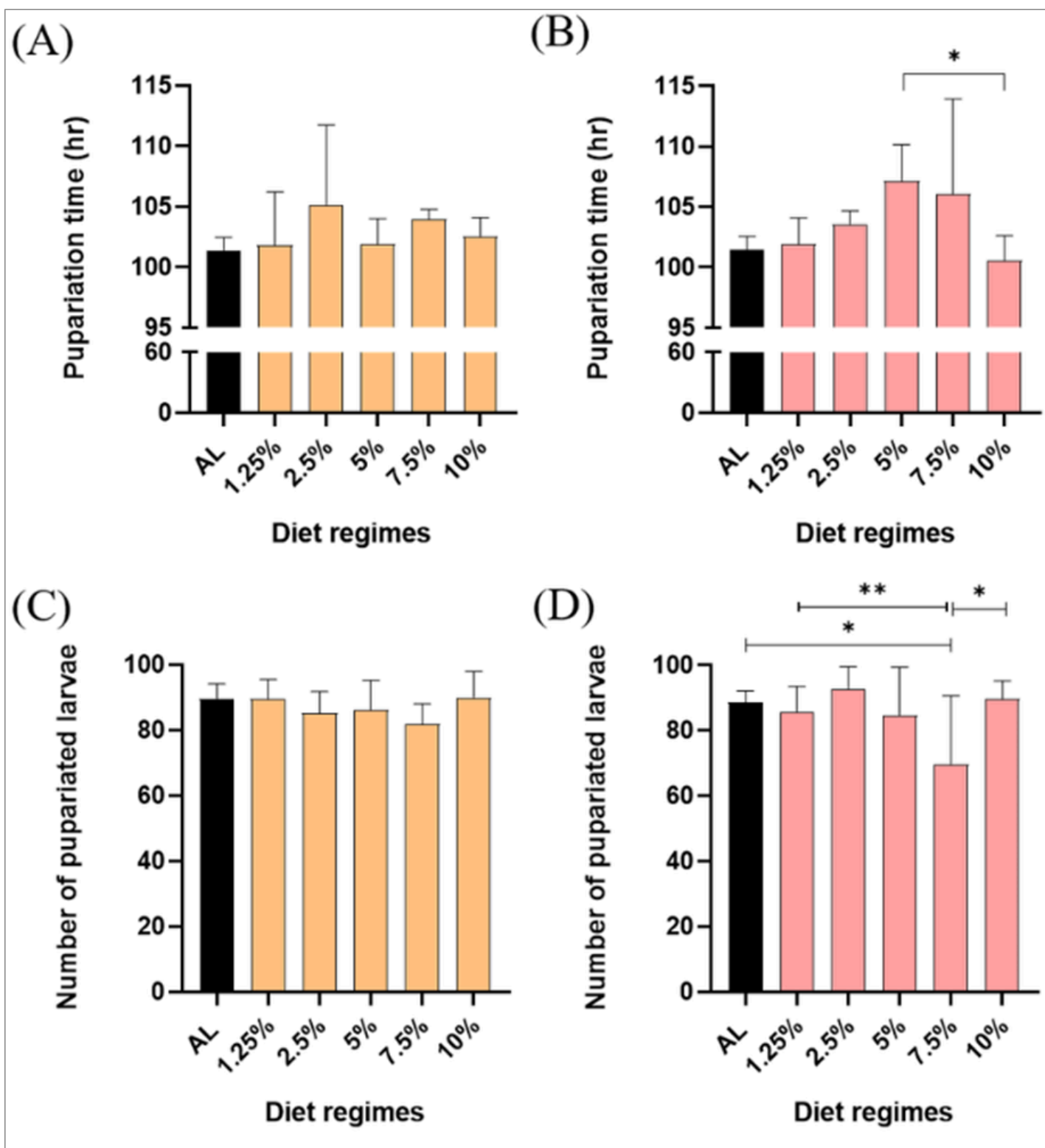


Figure 3

Delayed larval metamorphosis at PUFA 5%. Pupariation time of egg dispensed under MUFA (A) and PUFA (B), where the x-axis indicates diet regimes and the y-axis denotes the pupariation time (in hr). Survival percentage of larvae that transit into the pupal stage at MUFA (C) and PUFA (D). N = 7 (30 larvae per replicate = 210/diet regime).

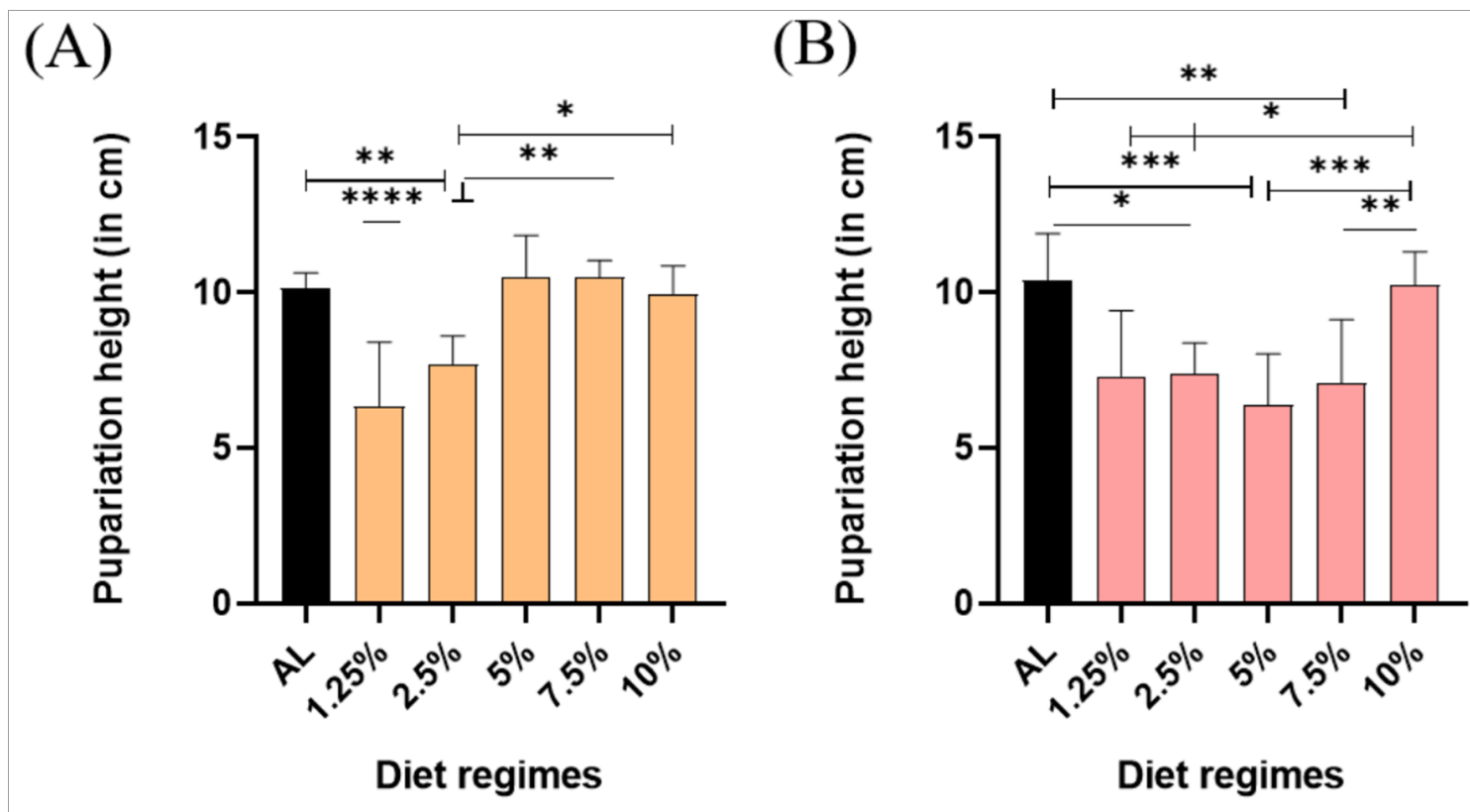


Figure 4

Pupariation height of larvae fed varying doses of unsaturated fatty acids. Pupariation height under multiple doses of MUFA (A) and PUFA (B), where the x-axis indicates diet regimes and the y-axis denotes the pupariation height (in cm); N = 7 (30 pupae per replicate = 210/diet regime).

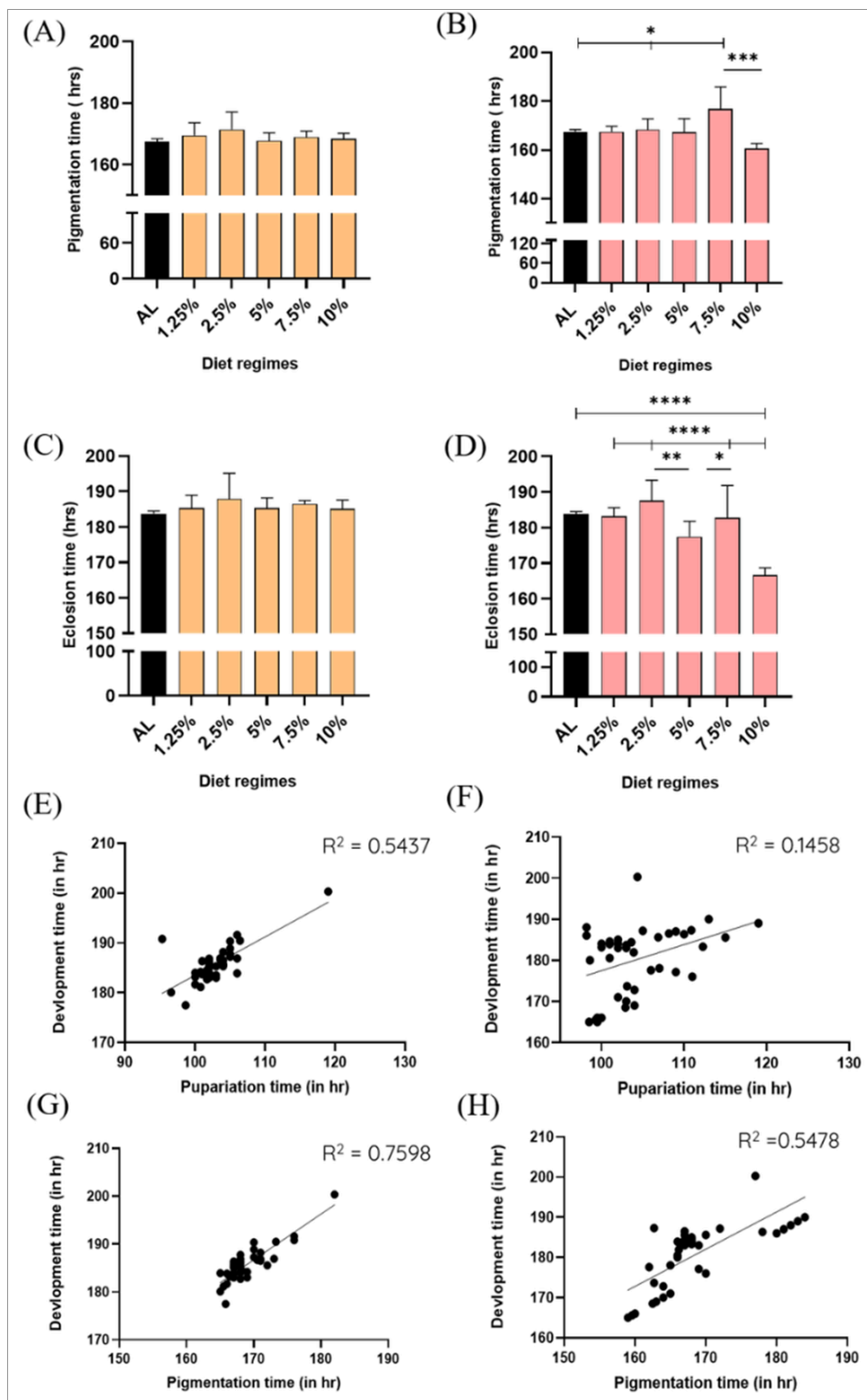


Figure 5

Pupae transited into the pigmented pupal stage when eggs were dispensed with various doses of MUFA (A) and PUFA (B). The x-axis indicates diet regimes, and the y-axis denotes the pigmentation time (in hr). Flies emerged from eggs dispensed at various concentrations of MUFA (C) and PUFA (D), where the x-axis indicates diet regimes and the y-axis denotes the development time (in hr); N = 7 (30 larvae per replicate = 210/diet regime). Positive correlation shared by development time with pupariation time (E)

and pigmentation time (G) at various doses of MUFA. No correlation is shared by development time with pupariation time (F) and pigmentation time(H) at different doses of PUFA.

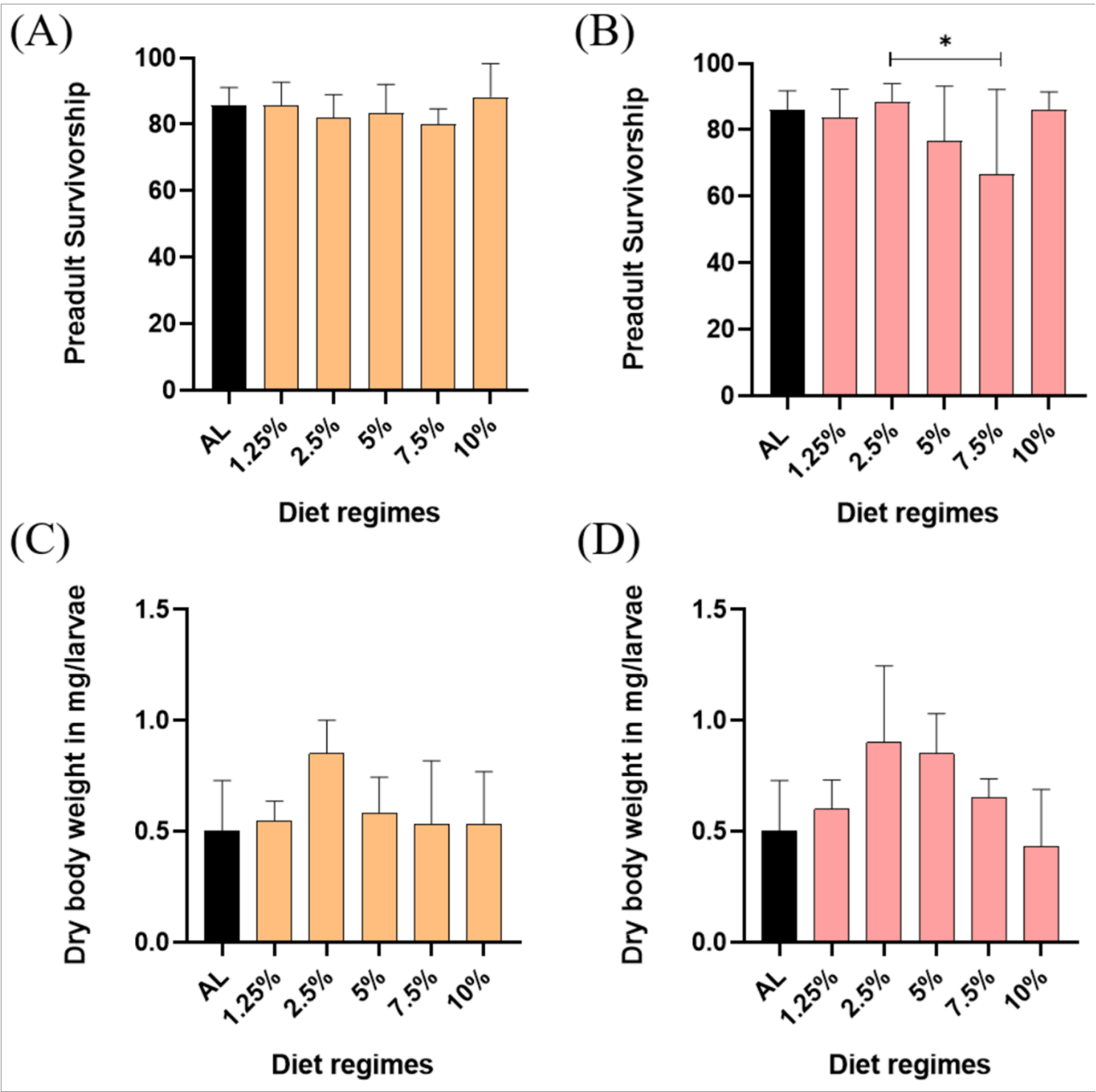


Figure 6

Pre-adult survivorship of eggs dispensed under USFAs. (A) Percentage of adults emerging from eggs dispensed at various doses of MUFA (A) and PUFA (B). Where the x-axis denotes diet regime, and the y-axis shows the per-adult survivorship; N = 7 (30 larvae per replicate = 210/diet regime); **Dry body weight of larvae (L3) Stage.** Dry weight of larvae fed with various MUFA (C). Dry weight of larvae fed with

various PUFA (D). N = 3 (20 larvae per replicate = 60/diet regime). Where the x-axis denotes diet regime, and the y-axis shows the dry body weight in mg/larvae.

Supplementary Files

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