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# Conjugated linoleic acid (CLA) regulates female reproduction via sex pheromone regulation without affecting larval development in *Drosophila melanogaster*



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## Abstract

Knowledge on the potential role of conjugated linoleic acid (CLA) on reproduction and development is currently limited. Therefore, the goal of the current study was to determine the effects of CLA on reproduction and development using the *Drosophila* model. In this study, adult and larva *Drosophila melanogaster* were fed fly food with 0.5% water (blank), CLA (50:50 of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers in triglyceride form) or safflower oil (69% linoleic acid, LA, as control) to examine the effects of CLA on fecundity, sex pheromones, transcription level of oogenesis, larval body composition, and eclosion time. CLA-treated females had lowered brood size without changes in hatchability, along with decreased 7,11-heptacosadiene and 7,11-nonacosadiene, the principle female pheromones, when compared to LA and blank. Moreover, CLA reduced transcription level of *lipid storage droplet-2* (*lzd-2*) compared to LA. CLA did not influence larval composition nor eclosion time. In conclusion, CLA inhibited reproduction capability in *D. melanogaster* in part via reduced fatty acid-derived signaling pheromone and oogenesis modulations. As key biological processes are conserved between humans and flies, knowledge from this research may provide valuable insight into reproduction and development responses to CLA.

**Keywords:** conjugated linoleic acid (CLA), *Drosophila melanogaster*, reproduction, pheromone

## Introduction

Based on its simple anatomy, known genome sequence, and similar metabolic pathways compared to mammals, *Drosophila melanogaster* can be a useful model to evaluate the health effects of food components, including on reproduction and developmental processes (Pandey and Nichols, 2011). The reproduction process of *D. melanogaster* occurs in the sequence of courtship, mating, oogenesis, and ovulation. It is known that polyunsaturated fatty acids (PUFA) exhibit a diverse range of critical functions in reproduction, such as the biosynthesis of hydrocarbons, formation of lipid signaling molecules (e.g., eicosanoids), and lipid deposition during oogenesis (Vrablik and Watts, 2013), which can influence reproduction process of *D. melanogaster* (Marcillac et al., 2005; Ueyama et al., 2005). In particular, the hydrocarbons 7,11-heptacosadiene and 7,11-nonacosadiene, derived from PUFA on female cuticles, function as the principle female sex pheromones to stimulate male

courtship (Antony et al., 1985; Yew et al., 2009) and lead to increased reproduction ability (Chen and Buhler, 1970; Savarit et al., 1999).

Conjugated linoleic acid (CLA), a group of C18:2 fatty acids with conjugated double bonds, has been regarded as a functional food ingredient, especially for its role in lipid metabolism (Kim et al., 2016). Studies in rodents and humans, as well as *Drosophila*, reported reduced body fat with elevated lean body mass after CLA-supplementation (Chen et al., 2018; Kim et al., 2016; Park et al., 1997). Current knowledge on the effects of CLA on development and reproduction is limited and inconsistent. CLA decreased egg production rate and increased embryo mortality in hens and quails (Aydin and Cook, 2004; Leone et al., 2009), which was suggested to be associated with changes in fatty acid composition in egg yolk by CLA; yolks had higher saturated fatty acid and lower monounsaturated fatty acid due to the down-regulation of stearoyl-CoA desaturase

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(Chamruspollert and Sell, 1999; Lee et al., 1998). In addition, CLA influences proteins associated with biological processes of reproduction and development in *Caenorhabditis elegans*, although it did not significantly affect egg laying and hatchability in *C. elegans* (Shen et al., 2018). Another publication reported that CLA decreased larval development and survival rates of European corn borer, *Ostrinia nubilalis* (Gereszek et al., 2008). However, no other adverse effects by CLA were observed on the adult survival, fertility, larval development, and the eclosion of oviposited eggs in a house fly study (Park et al., 2000). Thus, we aimed to determine the effects of CLA on reproduction and development using *D. melanogaster*.

## Materials and Methods

### Materials

*Drosophila* stock bottles, vials, grape agar food, and embryo collection cages were purchased from Genesee Scientific (San Diego, CA, USA). Clarinol G-80 Kosher (composed of triglyceride (TG) form of CLA with 34% of *cis*-9, *trans*-11, 35% of *trans*-10, *cis*-12, and 5% of other CLA isomers, as well as 2% of linoleic, 6% of palmitic, 3% of stearic, and 13% of oleic acids) was from Lipid Nutrition B.V. (Channahon, IL, USA). Safflower oil (composed of 69% of linoleic, 7% of palmitic, 3% of stearic, and 19% of oleic acids) was from California Oil Corporation (Richmond, CA, USA). Standards of 7,11-heptacosadiene (7,11-HD) and 7,11-nonacosadiene (7,11-ND) were purchased from Cayman Chemical (Ann Arbor, MI, USA). 1-Docosene was purchased from Combi-Blocks (San Diego, CA, USA) as an internal standard. High Capacity cDNA Reverse Transcription Kit and StepOnePlus Real-Time PCR System were purchased from Applied Biosystems (Foster City, CA, USA). Other chemicals, reagents, assay kits, and primers were purchased from Thermo Fisher Scientific (Waltham, MA, USA) unless stated otherwise.

### *Drosophila* husbandry and sample treatments

The *Canton-S* strain was used for all experiments. Flies were reared on Jazz-Mix *Drosophila* food in a 12:12 h light/dark cycle at 25°C in a DigiTherm® CircKinetics™ incubator (Tritech Research, Los Angeles, CA, USA). One to two days old of flies were anesthetized with CO<sub>2</sub> before transferring to experimental diets. The experimental diets were prepared by adding 0.5% (w/v) water (as blank), CLA, or safflower oil (as LA

control) to the standard fly food. The concentration of oils was chosen based on the previous publications showing that 0.5% of CLA is effective for body composition regulation in animals (Dilzer and Park, 2012). Oils were stripped in advance by the method of Boon et al. (2008) to remove natural antioxidants, then 0.01% tert-butylhydroquinone were added to all diets to minimize lipid oxidation.

## Reproduction analysis

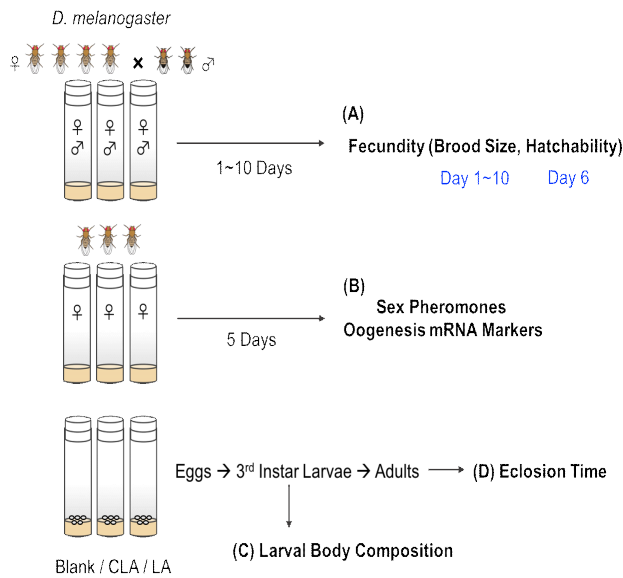
### Fecundity of brood size and hatchability

One to two day old adult flies were treated with experimental diets for 10 days (Tyler, 2000). Flies were transferred into fresh foods every 24 hours, and the accumulated brood size (egg production numbers) from Day 1 to 10 of treatments were recorded. Hatchability was recorded on day 6. Hatchability was calculated by ratio of larva to egg numbers (Galikova et al., 2015). Length of treatment was based on preliminary results that 0.5% of CLA for 5 days is effective for body composition regulation. The simplified experimental scheme was shown in Fig. 1.

### Cuticular hydrocarbon and fatty acids composition

After 5 days of dietary treatments, cuticular lipids were extracted from groups of 12 females with hexane containing 1-docosene as an internal standard, followed by a double hexane rinse (Schal et al., 2001). The resulting extracts were concentrated and analyzed using gas chromatography-mass spectrometry (GC-MS)-QP2010 SE (Shimadzu, Japan) with SUPELCOWAX™ 10 column (100 m, 0.25 mm i.d., 0.25 μm film thickness, Sigma Aldrich, St Louis, MO, USA). The column oven temperature started at 50°C, increased to 200°C at 20°C/min and then to 220°C at 2°C/min and held at 220°C for 162.5 min. The pheromones were identified by comparison of their retention times and quantified using the internal standard and represent as ng per fly.

After removal of cuticular hydrocarbons, flies were homogenized and lipids were extracted by the Folch method (Folch et al., 1957) and transmethylated with 3 N methanolic HCl at 55°C for 40 min to prepare fatty acid methyl esters (FAMES) (Park et al., 2001). The FAMES were analyzed using GC-MS with the same method described above. The FAMES were identified by the National Institute of Standards and Technology (NIST) mass spectrum library and by retention time of



**Fig. 1. Experimental scheme of reproduction and development study in *Drosophila melanogaster*.** One to two days old of flies were treated with experimental diets of Jazz-Mix *Drosophila* food with 0.5% (w/v) water (as blank), 0.5% CLA (triglyceride form), or 0.5% safflower oil (as LA control). Flies were transferred into fresh foods every 24 hours, and accumulated brood size from the first to tenth days and hatchability on day 6 were recorded as fecundity (A). Cuticular hydrocarbons (sex pheromones) and mRNA gene expressions of oogenesis were analyzed from females after 5 days of treatment (B). Untreated eggs were grown in the treatment foods, and the body compositions of fat and protein content were measured in third instar larvae (C). Eclosion time was recorded as the duration from egg to adults from the pupal cases (D). CLA, conjugated linoleic acid; LA, linoleic acid.

the standards. Fatty acid composition was determined from three biological replicates and reported as a percentage of the total identified fatty acids.

### Quantitative analysis of mRNA transcripts

Quantitative real-time PCR (qRT-PCR) was conducted according to Sun et al. (2016) with minor modifications. After 5 days of dietary treatments, total RNA was extracted from 8 flies in TRizol reagent, and reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit. qRT-PCR was performed using TaqMan Gene Expression Master Mix and primers on the StepOnePlus Real-Time PCR System using the following cycling conditions: 1 cycle of 50°C for 2 minutes, 1 cycle of 95°C for 10 minutes, and 40 cycles of 95°C for 15

seconds with 60°C for 1 minute followed by a dissociation curve analysis. Primers of *lipid storage droplet-2* (*lsd-2*, Dm01838905\_m1), *midway* (*mdy*, Dm01791906\_m1), and *actin-42A* (*act42A*; a reference gene; Dm02362162\_s1) were used.

### Larval development

Untreated females laid eggs on fly foods containing 0.5% water, CLA, or safflower oil, then third instar larvae grown from the treatment foods were collected to measure the protein and TG content as body composition. The larvae were rinsed with water, and homogenized with phosphate buffered saline with Tween-20 (PBST), and protein and TG levels were measured using Pierce BCA protein assay kit and Infinity triglycerides reagent, respectively. The protein and TG contents were quantified by the standard curves of bovine serum albumin and glycerol, respectively (Tennessee et al., 2014). Eclosion time was measured as the duration (days) from egg to adult eclosed from pupal cases (Reis, 2016).

### Statistical analysis

Data were presented as means with standard error of the mean (SE). Comparisons of various treatments were conducted by the Statistical Analysis System version 9.4 (SAS Institute, Cary, NC) using one-way analysis of variance with post-hoc analyses using Tukey's test. *p*-values less than 0.05 were considered statistically significant.

### Results

After 5 days of treatments, CLA intake was confirmed by detecting 3.8% of *c*-9,*t*-11 and 2.6% of *t*-10,*c*-12 CLA isomers in CLA-fed female flies (Table 1). Female flies typically showed peak egg laying behavior on day 4-6 (age of 6-8 days old) (Fig. 2A). However, after 5 days of treatments, the brood sizes were decreased by 32% in LA group and 43% in CLA group (*p*=0.0027 and 0.0011, respectively) compared with the blank group. Over 10 days of treatment, CLA-fed females had significantly lower accumulated brood sizes by 36% (*p*=0.0016) and 25% (*p*=0.0183) compared with the blank group and LA group, respectively. However, there were no significant effects on the hatchability between dietary treatments (Fig. 2B).

Since 7,11-heptacosadiene (7,11-HD) and 7,11-nonacosadiene (7,11-ND) on female cuticles function as the principle aphrodisiacs to stimulate male courtship and corresponding egg production (Antony et al., 1985), 7,11-HD and 7,11-ND were

**Table 1.** Fatty acid composition of female *Drosophila melanogaster*

	Blank	CLA	LA
C12:0	5.2±0.3 <sup>a</sup>	4.5±0.03 <sup>b</sup>	3.6±0.2 <sup>c</sup>
C14:0	22.4±1.4 <sup>a</sup>	20.0±0.2 <sup>a</sup>	15.6±1.3 <sup>b</sup>
C16:0	20.0±0.6 <sup>a</sup>	20.1±0.3 <sup>a</sup>	18.8±0.2 <sup>b</sup>
C16:1 $\Delta^7$	1.4±0.1	1.4±0.01	1.6±0.1
C16:1 $\Delta^9$	19.0±0.9 <sup>a</sup>	15.4±0.4 <sup>b</sup>	11.6±0.5 <sup>c</sup>
C18:0	1.2±0.1 <sup>b</sup>	1.6±0.1 <sup>a</sup>	1.6±0.1 <sup>a</sup>
C18:1 $\Delta^9$	18.9±0.3	19.5±0.5	18.5±0.9
C18:2 $\Delta^9,12$	11.0±1.6 <sup>b</sup>	10.5±0.3 <sup>b</sup>	28.0±0.7 <sup>a</sup>
C18:3	0.8±0.2	0.6±0.1	0.6±0.1
C18:2 $\Delta^9,11$	ND	3.8±0.3	ND
C18:2 $\Delta^{10,12}$	ND	2.6±0.4	ND
Desaturation index <sup>1)</sup>	1.78±0.1 <sup>a</sup>	1.61±0.1 <sup>b</sup>	1.48±0.1 <sup>c</sup>

Flies were reared on standard food with 0.5% water (as blank), 0.5% CLA, or 0.5% safflower oil (as LA control) for 5 days. The values (% of total fatty acids) were measured by GC-MS and expressed as means±SE (n=3).

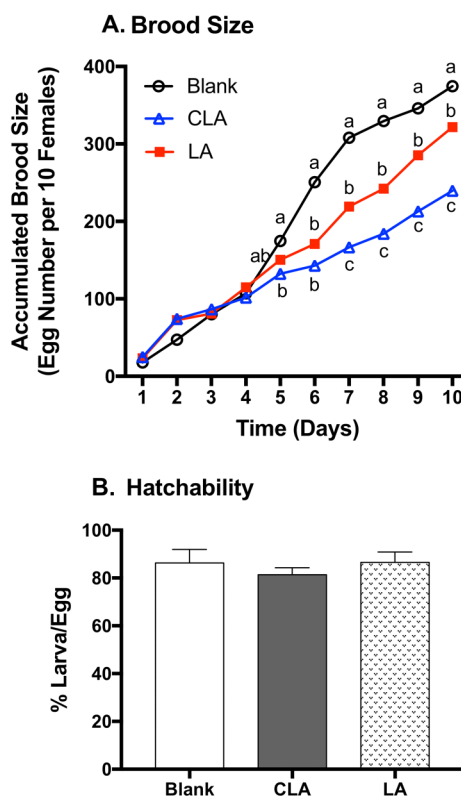
<sup>1)</sup> Desaturation indices were calculated by sum of C16:1 and C18:1 over sum of C16:0 and C18:0.

<sup>a-c</sup> Means with different letters are significantly different ( $p < 0.05$ ) in each row.

CLA, conjugated linoleic acid; LA, linoleic acid; ND, not detected.

quantified in female flies (Fig. 3). LA significantly decreased 7,11-HD and 7,11-ND by 43% ( $p < 0.0001$ ) and 41% ( $p = 0.0004$ ) when compared to the respective blanks. CLA significantly decreased 7,11-HD and 7,11-ND by 65% ( $p < 0.0001$ ) and 70% ( $p < 0.0001$ ) when compared to the respective blanks. Moreover, CLA-fed flies lowered 7,11-HD (39%,  $p = 0.0011$ ) and 7,11-ND (50%,  $p = 0.0027$ ) compared to LA groups. As 7,11-HD and 7,11-ND play significant role in courtship stimulation and reproduction behavior, the reduction of 7,11-HD and 7,11-ND by LA and CLA may correspond to the reduction of brood size (Antony et al., 1985).

During oogenesis, fly mothers synthesize numerous lipid droplets in the nurse cells of the egg chambers, which are transferred to the oocyte to provide energy for the developing embryo (Kuhnlein, 2012). *Lipid storage droplet 2 (lsd2)* and *midway (mdy)* are both particularly abundant in ovaries; *lsd2* encodes lipid storage droplet proteins, which regulates transferring lipids to developing oocyte, whereas *mdy* encodes *Drosophila* acyl Coenzyme A: diacylglycerol acyltransferase



**Fig. 2.** Effects of CLA on fecundity in *Drosophila melanogaster*. One to two days old of 10 females and 5 males were placed in a vial with standard food with 0.5% water (as blank), 0.5% CLA, or 0.5% safflower oil (as LA control). Flies were transferred into fresh foods every 24 hours, and the brood size (expressed as cumulated egg laying number per 10 females) from the 1<sup>st</sup> to 10<sup>th</sup> days (A) and hatchability (ratio of larva to egg number) on day 6 (B) were recorded. Data are means±SE (n=3-4). Means with different letters are significantly different at each time point at  $p < 0.05$ . CLA, conjugated linoleic acid; LA, linoleic acid.

(DGAT), which catalyzes the conversion of diacylglycerol into TG and promotes storage fat deposition within the ovary (Arrese and Soulages, 2010). To evaluate the effects of CLA on oogenesis, mRNA levels of *lsd2* and *mdy* were measured (Fig. 4). CLA showed lower *lsd2* gene expression (19% reduction,  $p = 0.0205$ ) compared to LA-treated flies, while there were no differences between CLA and the blank on *lsd2*. There were no significant changes in *mdy* gene expression between treatments, suggesting the TG biosynthesis during oogenesis remained unchanged. Altogether, CLA may lower the brood size in part through downregulating *lsd2*, leading to lower lipid deposition in the eggs in flies.

To study the developmental effects of CLA, larval body

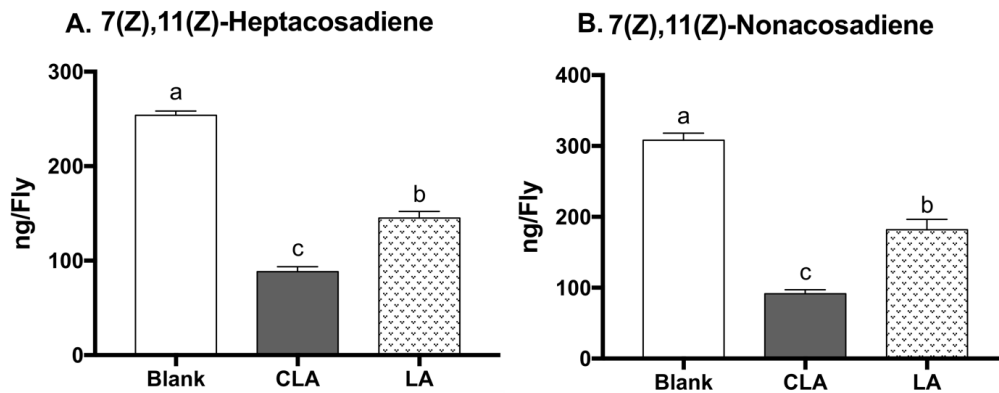


Fig. 3. Effects of treatments on females on dominant cuticular hydrocarbons. Twenty females were reared in standard food with 0.5% water (blank), 0.5% CLA, or 0.5% safflower oil (as LA control). After 5 days, females were collected for cuticular hydrocarbons analysis using GC-MS and 7(Z),11(Z)-heptacosadiene (C27:2) (A) and 7(Z),11(Z)-nonacosadiene (C29:2) (B) were quantified. Data are means  $\pm$  SE (n=3). Means with different letters are significantly different at  $p < 0.05$ . CLA, conjugated linoleic acid; LA, linoleic acid.

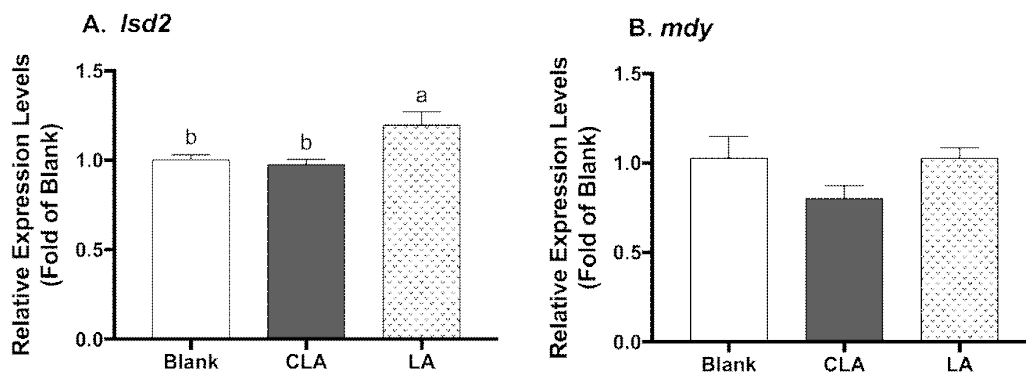


Fig. 4. Effects of CLA on *lipid storage droplet-2 (Isd-2)* and *midway (mdy)* mRNA levels in *Drosophila melanogaster*. *Isd2* (A) and *mdy* (B) gene expressions were measured from 2-3 days old adult flies fed with 0.5% water (as blank), 0.5% CLA, or 0.5% safflower oil (as LA control) in standard fly food for 5 days. Data are means  $\pm$  SE (n=5). Means with different letters are significantly different at  $p < 0.05$ . CLA, conjugated linoleic acid; LA, linoleic acid.

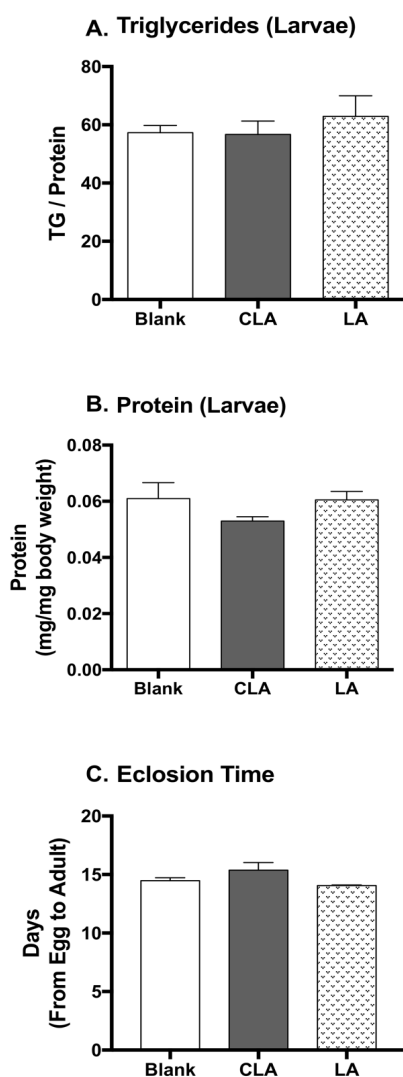
composition and the eclosion time were measured (Fig. 5). CLA and LA did not show impacts on larval protein level, triglyceride level, nor eclosion time when compared to the blanks.

## Discussion

The current results showed that supplement of CLA decreased fecundity in part through decreasing pheromone in the female adults, without affecting the hatchability and larval development in *D. melanogaster*. To the best of our knowledge, the current study is the first to determine the effects of CLA on fecundity, pheromone, and oogenesis in this model.

*Drosophila* encodes two desaturase genes, *desat1* and *desat2*

(Dallerac et al., 2000). The biosynthetic pathway of 7,11-dienes (female sex pheromones) in *D. melanogaster* generally begins from C16:0 fatty acid (Marcillac et al., 2005); *Desat1* introduces a first double bond on the carbon  $\omega$ -7, then the second double bond on carbon  $\omega$ -11 is added on elongated mono-unsaturated precursors, C20:1 fatty acid, followed by several elongation steps coupled with a final decarboxylation step to form 7,11-dienes (Marcillac et al., 2005). Previously, it was reported that CLA inhibits delta-9 desaturase (SCD) in mammalian tissues (Smith et al., 2002). However, CLA treated flies showed greater desaturation index (1.61) compared to the LA group (1.48) in the current study (Table 1). Thus, it is unlikely CLA inhibited desaturases that led to reduced 7,11-



**Fig. 5.** Effects of CLA on development in *Drosophila melanogaster*. Protein content (A), triglycerides level (B), and eclosion time (C) were measured from 3<sup>rd</sup> instar larvae grown in 0.5% water (as blank), 0.5% CLA, or 0.5% safflower oil (as LA control) in standard fly food. Data are means  $\pm$  SE (n=3-4). TG, triglyceride; CLA, conjugated linoleic acid; LA, linoleic acid.

dienes in this model. As previously reported, CLA reduced total body fat in female flies, which is likely led to reduced 7,11-dienes production due to limited substrate availability, rather than inhibition of desaturases, in this model (Chen et al., 2018).

Previous studies suggested that decreased desaturation indices of fatty acid composition in bird egg yolk by CLA lead to reduced egg production and increased embryo mortality (Aydin and Cook, 2004; Leone et al., 2009). However, there was no

effect of CLA on desaturation index nor significant difference in hatchability in the current study. The decreased brood size by CLA may be in part due to the reduction of aphrodisiac pheromones 7,11-HD and 7,11-ND, which are known to promote egg production in female flies. Another study reported that CLA did not change the brood size in *C. elegans* study (Shen et al., 2018). This inconsistency may be due to the difference in reproductive processes between species; only insect species rely on sex pheromones in the reproduction process. Therefore, the implication of this finding regarding sex pheromone in reproduction to human is relatively limited. More research is needed to determine the working mechanisms of CLA on pheromone biosynthesis pathway.

Lipid metabolism plays an essential role in oogenesis and embryo development. *Drosophila* DGAT, encoded by the *midway* (*mdy*) gene with function in storage lipid deposition from diacylglycerol to TG during oogenesis, is expressed mainly in the fat body and ovaries (Buszczak et al., 2002). In addition, *lsd2* is also important in TG accumulation during mid-oogenesis (Teixeira et al., 2003). Overall, CLA attenuated *lsd2*-associated lipid deposition without influencing *mdy* gene expression during oogenesis. With limited influence on oogenesis, we did not further examine the effects of CLA on eicosanoids, another key mediator in follicle maturation (Tootle and Spradling, 2008).

Additional factor may be involved in the reproduction changes by CLA. Juvenile hormones, synthesized and secreted by the corpora allata of brain, regulate courtship and mating behaviors in females and males (Bilen et al., 2013). During mating, juvenile hormone is stimulated by sex peptide and further induces onset of pheromone production, including 7,11-dienes, and yolk protein uptake in oogenesis in female flies (Flatt et al., 2005; Moshitzky et al., 1996). In addition, female pheromone profiles and attractiveness are also regulated by insulin and the Target of Rapamycin (TOR) signaling (Kuo et al., 2012). Hence, further studies in these fields would be needed to understand additional effects of CLA on reproduction.

## Conclusion

As PUFAs play an essential role in the fecundity, mating behavior, and oogenesis, this study evaluated the effects of CLA on reproduction and development in *D. melanogaster*. In conclusion, the current study showed that CLA decreased brood

size, potentially *via* reduction of sex pheromone and lipid deposition in oogenesis. Meanwhile, CLA did not alter the hatchability and larval development in *D. melanogaster*. By understanding the effects of CLA on reproduction and development in *D. melanogaster*, we will be able to gain better knowledge of the potential role of CLA in development.

### Conflicts of Interest

The authors declare no potential conflict of interest.

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### Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants

### Author Contributions

Conceptualization: Chen PB, Park Y

Data curation: Chen PB

Formal analysis: Chen PB

Methodology: Chen PB, Young L, Kim JH, Qi W, Clark JM

Software: Chen PB

Validation: Qi W, Kim JH, Clark JM

Investigation: Chen PB, Park Y

Writing - original draft: Chen PB, Park Y

Writing - review & editing: Chen PB, Young L, Kim JH, Qi W, Clark JM, Park Y

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