# Olfactory Sensitivity, Behaviors, and Transcriptional Profiling by High Fat Diet-Induced Stress in *Drosophila melanogaster*

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## ABSTRACT

Energy homeostasis is essential to most organisms for survival affected by internal state and external factors. Unbalanced nutrient supplies cause obesity and give detrimental effects on a sensory system. In particular, sensory-mediated responses are crucial to maintain energy balance as involved in a metabolic regulation. However, investigating effects and relationship between HFD-induced stress and its effects on the sensory system have yet to be elucidated. To gain

insights into how HFD-induced stress affects olfactory sensitivity and behavioral responses, we used a male *Drosophila melanogaster* model to test this hypothesis involving olfactory and nutrient-related signaling by using physiological, behavioral and transcriptional analysis. Here we demonstrated that lifespan and climbing ability in HFD-treated flies were decreased and moreover olfactory sensitivity and behavioral responses to odorants were modified after HFD treatment. Overall, flies showed reduction of olfactory sensitivity to most odorants after HFD treatment. Interestingly, flies showed increased behavioral attraction to benzaldehyde in both 7-day and 14-day HFD treatment. This behavioral and physiological modulation in HFD-treated flies were accompanied by a significant decreased DmOrco gene expression in antennae organ, suggesting some mediators might regulate olfactory processing through the actions of metabolic and sensory signals. The gene expression profiles of antennae indicated significantly differences in olfactory receptors, odorant-binding proteins, and insulin signaling-related genes. These transcriptional changes affected the genes in functional gene ontology. Taken together, olfactory sensitivity and behavioral responses by HFD-induced stress might be in modulation through olfactory and nutrient-related signaling in Drosophila, reflecting to functional variation of genes and modification the sensory processing from antennae to brains.

**Keywords**: *Drosophila melanogaster*, High fat diet-induced stress, Olfaction, Behavioral response, Transcriptional profiling

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| | |
|---|---|
| **BP** | Biological Process |
| **CC** | Cellular Component |
| **CD** | Control Diet |
| **Chico** | Drosophila Insulin Receptor Substrate |
| **DAVID** | Database for Annotation, Visualization and Integrated Discovery |
| **DEG** | Differentially Expressed Genes |
| **DILP2** | Drosophila Insulin-like Peptide 2 |
| **DILP5** | Drosophila Insulin-like Peptide 5 |
| **DILP6** | Drosophila Insulin-like Peptide 6 |
| **DILP7** | Drosophila Insulin-like Peptide 7 |
| **DILP8** | Drosophila Insulin-like Peptide 8 |
| **EAG** | Electroantennogram |
| **FPKM** | Fragments Per Kilobase of transcript per Million mapped reads |
| **GO** | Gene Ontology |

| | |
|---|---|
| **HFD** | High Fat Diet |
| **InR** | Insulin Receptor |
| **MF** | Molecular Function |
| **OBP** | Odorant Binding Protein |
| **OR** | Odorant Receptor |
| **Orco** | Odorant Receptor Co-Receptor |
| **PTEN** | Drosophila homolog of the human tumor suppressor gene, PTEN |
| **qRT-PCR** | Quantitative Real-Time Polymerase Chain Reaction |
| **RNA-seq** | RNA Sequencing (Transcriptome) |

# Ⅰ. INTRODUCTION

Most organisms maintain energy homeostasis to survive and function effectively under internal state and external factors in response to biological processing for growth, development, metabolism, and aging [1,2]. Many environmental conditions such as nutrients, temperature, and light can influence on food intake and cause health consequences of obesity [3,4]. Obesity contributes to increase the warning of health issues and a myriad of complex burden of diseases, which is likely to be caused by unbalanced energy production [5-7]. Previous study reported that simultaneous processing in pivotal senses for survival, taste and olfaction, were associated with obesity in human [8]. According to this research, food intake controlled by external sensory signals affected consequently energy balance that assessed by internal metabolic signals [9]. Another research demonstrated that many metabolic signaling were involved in the control of physiological responses and integrated in accordance with neurosensory systems by sensory inputs such as visual, acoustic, and olfactory signals [10]. These fundamental responses were made through physiological mechanisms, with mainly metabolic hormones which regulate appetite either stimulation or inhibition [11].

Sensory-mediated responses are thought to be remarkably complex networks [12,13]. In the natural environment, olfaction is related to many aspects of finding food sources, recognizing predators, and locating mate partners [14,15]. In particular, feeding behaviors are initiated with finding food sources in response to sensory modalities that control of olfaction [13]. A previous study revealed that such metabolic regulation in olfactory cortex was changed by internal

metabolic states in mammals. For example, insulin signaling that is an important metabolic cue and its regulation by food intake was attributed to olfaction, showing the increased number of insulin receptor (InR) in olfactory bulb in starved mice [16]. Like mammals, *Drosophila melanogaster*, which is well known for a powerful tool to observe phenotypic changes and research behavioral analysis, has shown evolutionarily conserved pathways [17,18]. In the fruit fly, insulin signaling also provided the evidence of olfactory responses, exhibiting decreased olfactory sensitivity and odor-driven food searching behaviors in fed flies compared to starved flies [19].

Although there are some studies established the regulation of food intake and influence of olfactory responses, little is known about the olfactory signaling and nutrient-related genes by food stress in a main peripheral olfactory organ, the antennae, in *Drosophila melanogaster*. Some attention has been paid to the biological effects of unbalanced diet consumption that modified the metabolic state and sensory signals [20,21]. Another study demonstrated over-weight people were preferred a food-related odorant rather than a non-food related odorant [9]. Given this evidence, extended lifespan is modulated by the olfactory system and food-derived odors with alteration of adult metabolism and enhancement of stress resistance. These findings are likely to be correlated with feeding behaviors under energy balance through sensory processing [22].

To gain insights into how high fat diet (HFD)-induced stress affects olfactory sensitivity and behaviors that mediate nutrient-related signaling, our present study centered on the understanding of behavioral and sensory modification by food stress such as HFD treatment in *D.*

*melanogaster*. To test our hypothesis, we employed lifespan analysis, behavioral tests such as climbing test, a choice test to food-related odorants, and electrophysiological recordings from antennae (electroantennogram, EAG). Moreover, to pinpoint any modifications at molecular level in the main olfactory organ, we examined the expression levels of *DmOrco* gene by quantitative RT-PCR and overall transcriptional profiles of antennal tissues to identify genes including olfactory and nutrient-related signaling genes whose levels were fluctuated by HFD treatment. These approaches provide a general overview of relationships between food stress and sensory modulation machineries in the peripheral olfactory organ based on Gene Ontology (GO).

# Ⅱ. MATERIALS AND METHODS

### *2.1 Drosophila stocks and diet treatment*

The Canton-S wild-type strain of *Drosophila melanogaster* obtained from Bloomington Stock Center (Bloomington, Indiana, USA) was used in this study. All adult flies were maintained at 25℃ with 60% relative humidity and a 12h light:12h dark cycle. Food media for a standard diet was prepared by mixing 10% sugar, 10% yeast extract, 1.5% agar and added 1% Tween 80 (w/v) in case of the toxicity. To make high-fat diet, 2% palmitic acid (w/v) and 1% Tween 80 (w/v) were added into the standard diet according to a previous study [23].

In order to obtain adult flies for the experiments, newly eclosed flies were collected into new bottles and were kept for 2 days on the standard diet. Next day, male and female flies were sorted under $CO_2$ anesthesia and only male flies were placed into the standard diet for one more day. Test male flies were treated with the standard diet and high-fat diet (HFD) for 7 days and 14 days, respectively. Each vial contained 20 male flies.

### *2.2 Life span and climbing assays*

For life span assays, flies were kept at a density of 20 male flies each vial on the standard diet and HFD. Each group of flies was transferred to a vial with fresh food medium every three

days and dead flies in each group were counted every day. Five replicates were conducted for this experiment.

For climbing assays, we employed a behavioral paradigm reported in the previous study with slight modifications [24]. Twenty male flies were transferred into a 10 cm glass vial and placed at the bottom of the vial by gently tapping the flies down to the bottom. We counted the number of flies that climbed to the top of the vial within 30 seconds. The glass vials were separated from each other and the number of flies in each vial was counted. Experimental trials were repeated five times independently for each group. The climbing index (%) was calculated as a ratio of the number of flies climbing to the top to the total number of flies multiplied by 100 as a percentage. All climbing assay experiments were performed at 25℃ with 60% humidity.

### *2.3 Odor stimulation*

All odorants tested in this study were commercially available at the highest purity (>98%, Sigma-Aldrich, Milwaukee, USA). We used ethyl acetate, isoamyl acetate, pentyl acetate, benzaldehyde, 2-heptanone, 1-hexanol, 3-octanol, 1-octanol, 4-propyl phenol, and 4-methyl phenol. These odorants were dissolved in mineral oil (Sigma-Aldrich-330760, Milwaukee, USA) or ethanol at 1% dilution (v/v). A glass tube was prepared with continuous humidified air. 20 μl of odorant solution were soaked onto a filter paper (4 x 4 mm, Toyo Roshi Kaisha, Japan) that was displaced into 5 ml disposable syringes with the air pressure. These chemicals were used as odorant sources for EAG recordings and for behavioral assays.

### *2.4 Electrophysiological recordings*

We conducted transepithelial electrophysiological recordings (electroantennograms, EAGs) from antennae in male Canton-S flies after individual diet treatment using AgCl-coated silver wire inserted in a glass micropipette filled with 0.1M KCl. The experimental fly was immobilized in a truncated 200 μl plastic pipette tip and trimmed to show an anterior aspect of the fly's head. The third segments of the antennae were exposed for EAG recordings. A reference electrode was inserted into a compound eye and a recording electrode was placed on the dorso-medial surface of the third antennal segment as described previously [25].

Electrical signals were amplified with analog 10x active probe and conveyed to an acquisition system (IDAC4, Syntech, Hilversum, The Netherlands). Signals were then further recorded and analyzed using a EAG Pro software (Syntech). A constant air stream of 40 ml/min was delivered to the fly head by using a stimulus controller (CS-55, Syntech). Pulses of the odorants were produced during a 1 s stimulation through the Pasteur pipette to the syringe pipette. Signal amplitude of olfactory responses (mV) was measured from a baseline before stimulation to a trough of electrical signals after odorant stimulation. Control EAG experiments loaded with no odorant and mineral oil were used. An internal between each odorant stimulus was about 60 s to prevent the adaptation of a test animal to a given odorant.

### *2.5 Quantitative RT-PCR*

Flies were collected according to the procedures presented above for the diet treatment. Total RNA extraction was extracted using RNeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer's protocol. For each treatment, at least three independent extractions were conducted using 100 fly antennae. The quality and quantity of total RNA was measured by Nanodrop 2000 (Thermo Scientific Inc., Wilmington, DE, USA). cDNA was prepared from extracted RNA using the Superscript Ⅲ First-Strand Synthesis kit (Invitrogen Inc., Carlsbad, CA, USA).

Quantitative RT-PCR was performed using a StepOnePlus machine (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's protocol with SYBR green qRT-PCR Master Mix (Fermentas, Ontario, Canada) to measure *DmOrco* gene expression. qRT-PCR measurement for each gene was repeated with three independent biological samples and quantitative analysis was calculated by StepOne plus Software V. 2.0 (Applied Biosystems). The transcript level of the gene was calculated by a standard curve method and normalized to the control gene *ribosomal protein 49 (rp49)* primer that was described in a previous paper [26]. The primer sequences for *DmOrco* were forward: 5'-GGTGGACCATGAGACGAACT-3'; reverse: 5'-CATCACGTCGCATAGATTGG-3'.

### *2.6 Behavioral assay*

Flies were placed under the light $CO_2$ anesthesia. Separated male flies were placed into vials (20 flies per vial) containing each diet treatment. Flies were transferred to new vials every 2 to 3 days and performed on day 7 and 14 after diet treatment. Before one day for the assay, flies were moved into empty vials only containing water on Kimwipes (Kimtech Science, Kimberly-Clark Worldwide, Inc., USA) for at least 15 h to make starved state [27]. For each measurement, the flies were gently tapped to place into a T-maze. The same odorants in EAG were used for this behavioral assay. Each vials at both sides contained two traps; one odor trap and one control trap (water). Flies were allowed to have 30 s to choose one of the sides in T-maze that contained odorant. The attraction index (AI) was calculated as follows: (number of flies moving toward the odor trap – number of flies in control trap) / (total number of flies). Five replicates were performed for each treatment. All T-maze assay experiments were conducted at 25℃ with 60% humidity.

### *2.7 Differentially expressed genes (DEG) analysis of HFD-fed flies*

Approximately 3,000 samples of third antennal segments of male adult flies were prepared to achieve a sufficient representation of genes. The flies in each diet treatment were collected from 11:00 to 14:00 and 16:00 to 19:00 to prevent possible modification of gene expression by circadian rhythms [28]. We incorporated all samples to minimize the random factors other than the treatment that affect gene expression. The Drosophila Genome database (www.flybase.org) was used for differentially expressed genes (DEG) analysis.

The RNA was extracted with same protocol as described above in the quantitative RT-PCR procedure. 4 μg of total RNA were extracted and used for RNA Sequencing experiment. In order to convert mRNA in total RNA into a library of template molecules for subsequent cluster generation, a Illumina ® TruSeq™ RNA sample preparation kit was used according to manufacturer's instructions (Illumina Inc., San Diego, CA, USA). The total RNA sample was used for poly-A mRNA selection using poly-T oligo-attached magnetic beads by two rounds of purification. The resulting mRNA sample from antennae was subjected to thermal mRNA fragmentation using Elute, Prime, Fragment Mix from the Illumina ® TruSeq™ RNA sample preparation kit. The mRNA fragments were reverse transcribed to synthesize the first-strand cDNA using a combination of reverse transcriptase and random primers. mRNA template strand is removed and double-stranded cDNA (ds cDNA) is generated using DNA polymerase Ⅰ. The ds cDNA was purified using Ampure XP beads to separate the ds cDNA from second-strand reaction mix.

The cDNA fragments were then blunt-ended through an end-repair reaction using an End Repair (ERP) mix (Illumina Inc., San Diego, CA, USA). The 3' to 5' exonuclease activity of this mix removed the 3' overhangs and the polymerase activity filled in the 5' overhangs. Next, 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to each other and the ligated to platform-specific double-stranded bar-coded adapters were provided a complementary overhang during the adapter ligation reaction. DNA libraries were sequenced using an Illumina HiSeq2000. A single biological replicate, representing a large sample size was used in the subsequent analysis, generating a series of images each representing a single base extension at a specific cluster.

Raw data were extracted as FPKM values for each gene by using Cufflinks software [29]. Quality check, normalization and statistical analysis were performed to develop significantly matched signals. Data of zero FPKM values shown more than one in total number of samples were excluded. Selected gene FPKM value was transformed by logarithm and normalized by the Quantile method. Differentially expressed genes were determined by adjusting |fold-change|≥2 between control flies and HFD-treated flies. The values of fold-change were used to analyze the changes in expression intensity between control and HFD-treated flies. After obtained general information on the gene expression, Gene Ontology (GO) analysis were prepared to establish up- and down- regulated genes in the most significant changes associated with diet treatment. For DEG set, biologically gene functional annotation analysis for DEG list was performed using DAVID tool (http://david.abcc.ncifcrf.gov/) to understand biological meanings. In DAVID annotation system, modified Fisher Exact p value (EASE score) is adopted to measure the gene-enrichment in annotation terms. All data analysis and visualization of differentially expressed genes were conducted using R 2.15.1 (http://www.r-project.org).

### *2.8 Statistical analysis*

Statistical analyses were performed by Student's t-test (SPSS, Version 20, IBM, NY, USA). To determine significance between control diet and HFD treatment, the statistical analysis were performed in lifespan assay, climbing ability, EAGs and behavioral T-maze tests. Data were shown as means ± standard error.

# Ⅲ. RESULTS

### *3.1. Effects of HFD treatment on life span and climbing abilities in male flies*

We hypothesized that high fat diets may contribute to the alteration of physiological states as previously reported [23,30], thus reducing the life span and climbing abilities in flies. To confirm this hypothesis with a slightly modified experimental condition from the previous work [23], we fed control diet and high-fat diet (HFD) *ad libitum* to male Canton-S adult flies 4 days after eclosion for 7 days and 14 days (Fig. 1A). As a result, life span of the flies under the HFD-treated diet conditions was initiated to decrease at day 7 and the survivorship of these flies was significantly decreased by approximately 12% at day 14 after HFD feeding, compared to control diet treatment (Fig. 1B). As consistent with the lifespan analysis, the locomotor activity of the flies also indicated declines after HFD consumption. To test whether the climbing ability by HFD treatment was associated with any changes [24], we measured climbing ability of male Canton-S flies fed with control diet or HFD for 7 days and 14 days. The significant changes in the climbing ability were observed both HFD-treated groups for 7 days and 14 days (Fig. 1C), demonstrating that HFD caused detrimental influences to the fly's mobility.

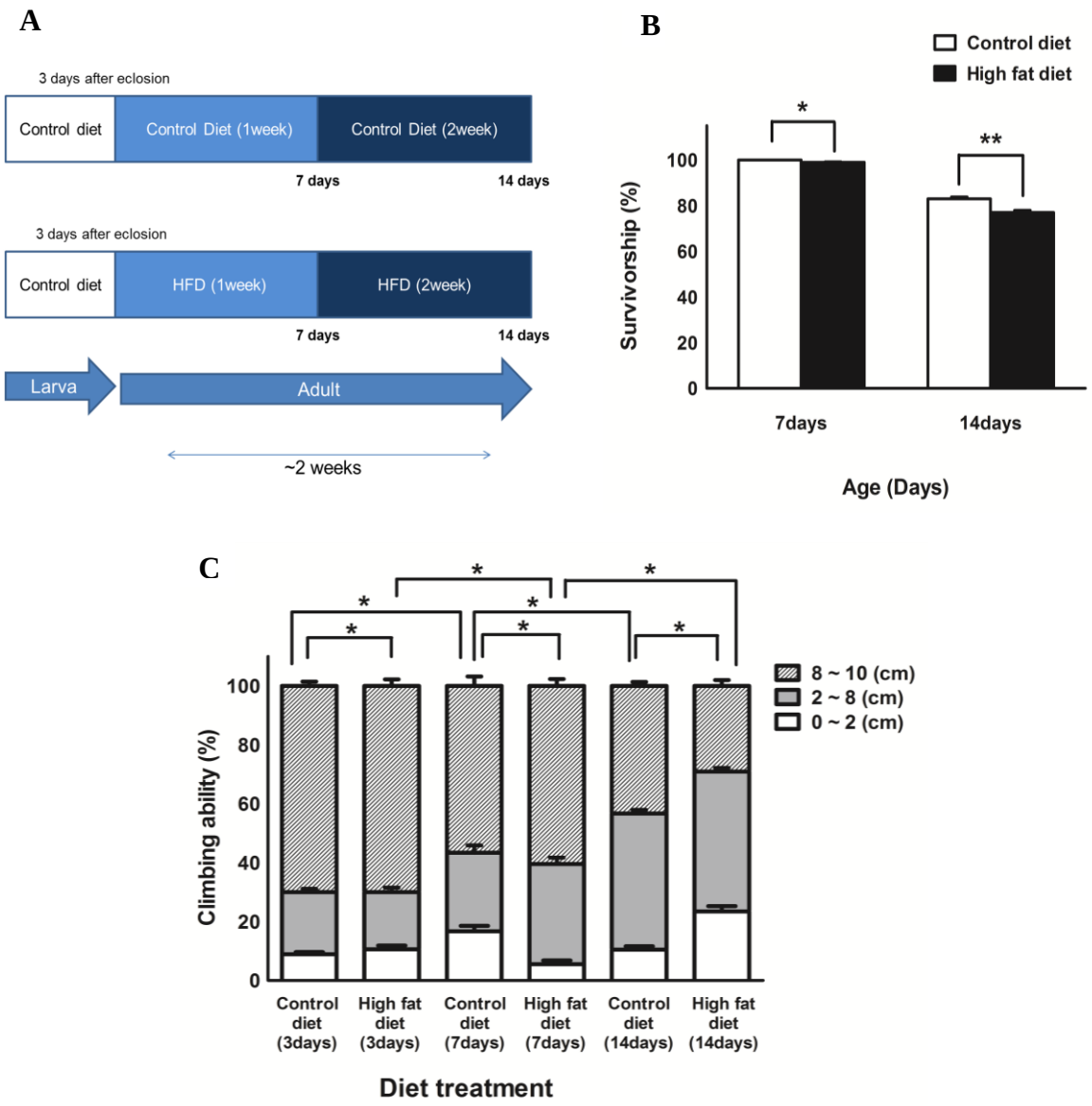


**Figure 1. Physiological effects of HFD intake on wild type Canton-S flies**

(A) Food treatment condition and feeding paradigm. The control diet and HFD were treated *ad lib* for 7 days and 14 days in male Canton-S flies. (B) Lifespan measurement of wild type Canton-S flies fed the control diet and HFD. The lifespan of flies under the treated diet conditions showed initial decrease at day 7 in HFD treatment and the survival of HFD-fed flies was significantly decreased approximately 12% at day 14, compared to control diet-fed flies. (n = 100 - 120 flies, *p<0.05). Error bars represent SEM. (C) Locomotor activity of wild type Canton-S flies fed with control diet and HFD. Locomotor activity of the flies indicated declines after HFD consumption. The significant changes of this locomotor activity were observed both groups at day 7 and day 14. (n = 100 - 120 flies, *p<0.05). Error bars represent SEM.

### *3.2. Modification of olfactory sensitivity by HFD treatment*

Olfactory sensitivity has been reported to be modulated by internal states as well as environment factors [31]. Our main question in the present study was whether HFD consumption in the flies affects olfactory sensitivity to different odorant groups including attractive to food odors such as ethyl acetate, isoamyl acetate, pentyl acetate, 2-heptanone, and benzaldehyde as well as to non-food odors such as 1-hexanol, 3-octanol, 1-octanol, 4-methyl phenol, and 4-propyl phenol [32]. EAG responses demonstrated significant differences after HFD consumption for 7 days and 14 days (Fig. 2A and B). The EAG amplitudes in flies fed with HFD was typically attenuated to most odorants, compared to the flies fed with control diet.

In regard of the duration of HFD treatment, we also found that olfactory sensitivities were affected differently. Flies treated with HFD for 14 days showed that olfactory sensitivities were mostly decreased in all tested odorants such as ethyl acetate, pentyl acetate, 1- hexanol, and 3-octanol (Fig. 2B). However, HFD treatments for 7 days described that olfactory responses to several odorants such as ethyl acetate, isoamyl acetate, 2-heptanone were increased (Fig. 2A). Furthermore, olfactory sensitivity to benzaldehyde and 1-hexanol were substantially increased in flies fed with HFD for 7 days. In particular, olfactory responses to pentyl acetate were only significantly decreased (Fig. 2A).

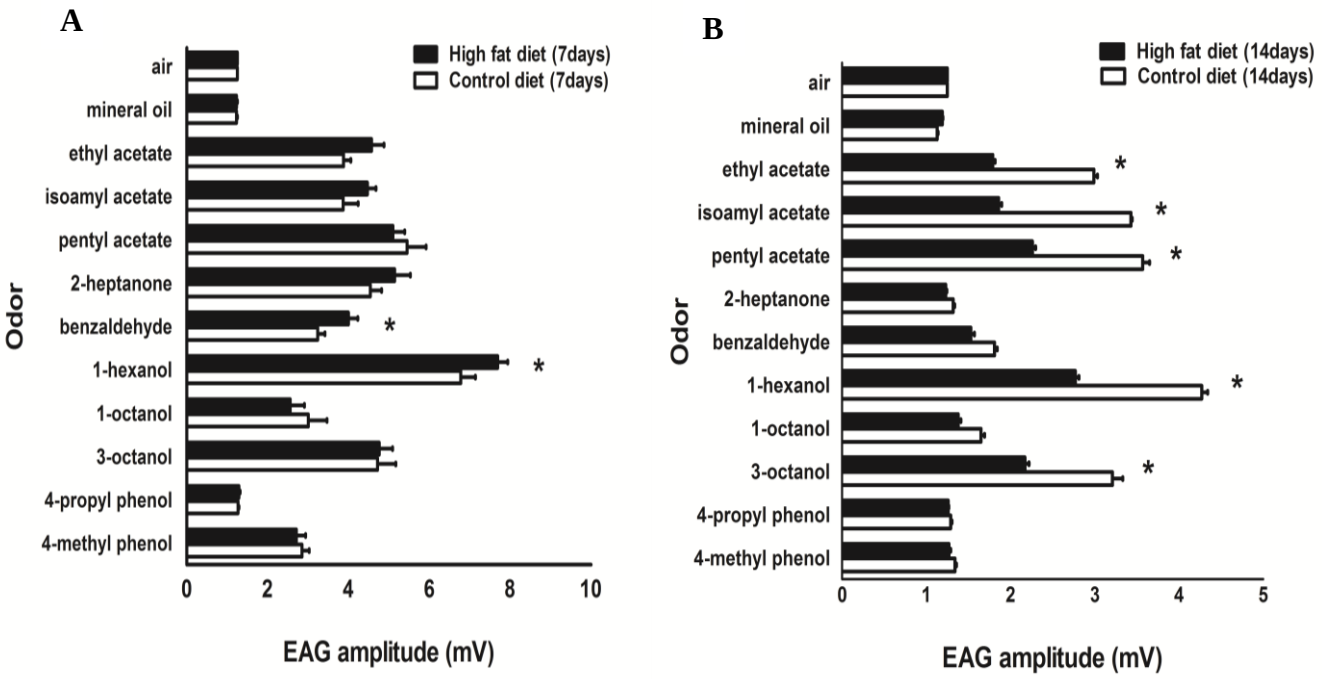


**Figure 2. Olfactory responses of wild type Canton-S flies fed with control diet and HFD for 7 days and 14 days**

(A) Flies fed with HFD for 7 days showed reduction of olfactory responses to several odors. The EAG amplitude of flies fed with HFD was typically smaller than flies fed with control diet. HFD-fed flies for 7 days demonstrated that olfactory responses were increased to certain odorants such as benzaldehyde and 1-hexanol. (n = 6 - 10 flies, *$p<0.05$). Error bars represent SEM. (B) Diet treatment for 14 days, demonstrated that the olfactory sensitivity was mostly decreased to most odorants. Flies fed with HFD for 14 days showed the decline of olfactory responses to several odors such as ethyl acetate, isoamyl acetate, pentyl acetate, 1-hexanol and 3-octanol. (n = 6 - 10 flies, *$p<0.05$). Error bars represent SEM.

### *3.3 Reduction of DmOrco gene expression in the fly antenna after HFD treatment*

We next asked whether the expression levels of the odorant receptor co-receptor gene of *D. melanogaster* (*DmOrco*), which is expressed in most olfactory receptor neurons (ORNs) [31], may be then affected by HFD treatment. *DmOrco* expression levels from control diet- or HFD-fed flies were analyzed by quantitative RT-PCR (qRT-PCR), showing that the level of *DmOrco* transcripts was significantly reduced in HFD-treated flies by approximately 70% at 7 days. In contrast, about 47% reduction of *DmOrco* gene expression was detected 14 days after HFD treatment (Fig. 3A and B). Notably, strong down-regulation of the *DmOrco* gene in fly antennae after HFD treatment for 7 days rather than 14 days was examined, demonstrating that *DmOrco* gene expression was significantly decreased.

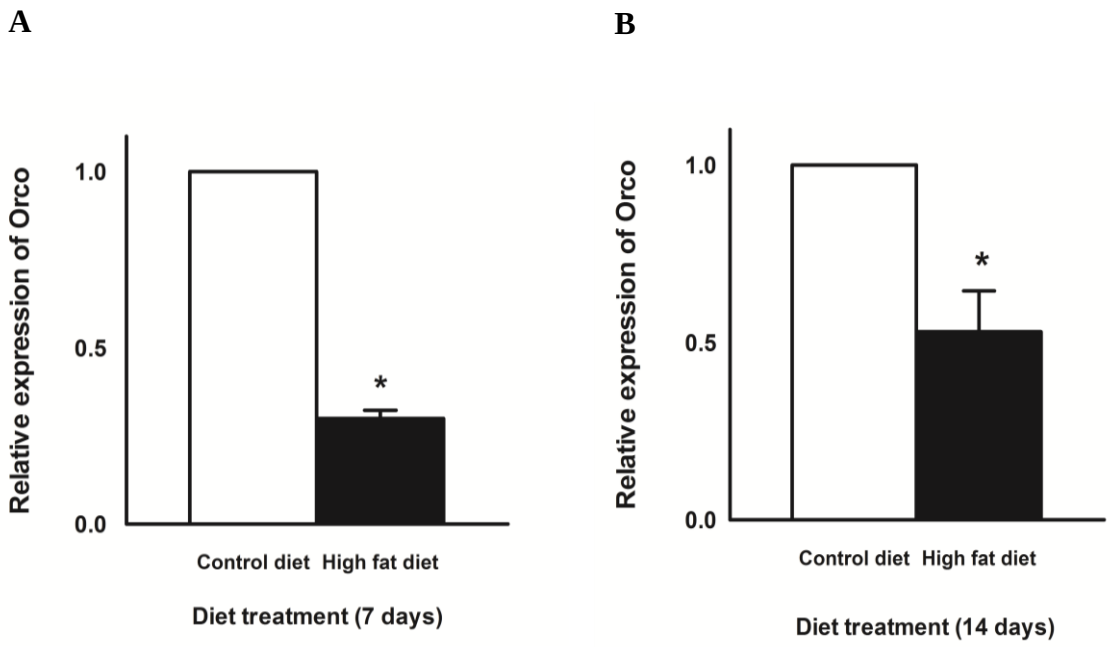


**Figure 3. Relative expression of DmOrco in antenna of wild type Canton-S flies fed control diet and HFD for 7 days and 14 days**

(A) Relative *DmOrco* mRNA transcript levels after different food treatment for 7 days. The level of *DmOrco* transcript was significantly reduced in HFD-fed flies 7 days after food treatment, showing approximately 70 percents decrease, compared to control flies. (B) Relative *DmOrco* mRNA transcript levels after different food treatment for 14 days. *DmOrco* gene expression was significantly decreased approximately about 47% in HFD-fed flies, compared to control flies. All qRT-PCRs were done in triplicate (*$p<0.05$). Error bars represent SEM.

### *3.4 Modification of odorant choice behaviors by HFD treatment*

HFD-fed flies showed different responses to attraction and repulsiveness to the experimental odors compared to the control diet. Attractive behavioral responses to ethyl acetate, isoamyl acetate, and 2-heptanone in food odors had tendency in attenuation, while repulsive behaviors to 1-hexanol were increased in both flies fed with HFD for 7 and 14 days (Fig. 4A and B). Whereas, behavioral responses to benzaldehyde and pentyl acetate showed transition from repulsive to attractive behaviors upon HFD feeding in flies. In detail, attractive behavioral responses were shown to benzaldehyde in flies fed with HFD for 7 and 14 days, and attractive behaviors to pentyl acetate were disappeared in flies fed with HFD for 7 days.

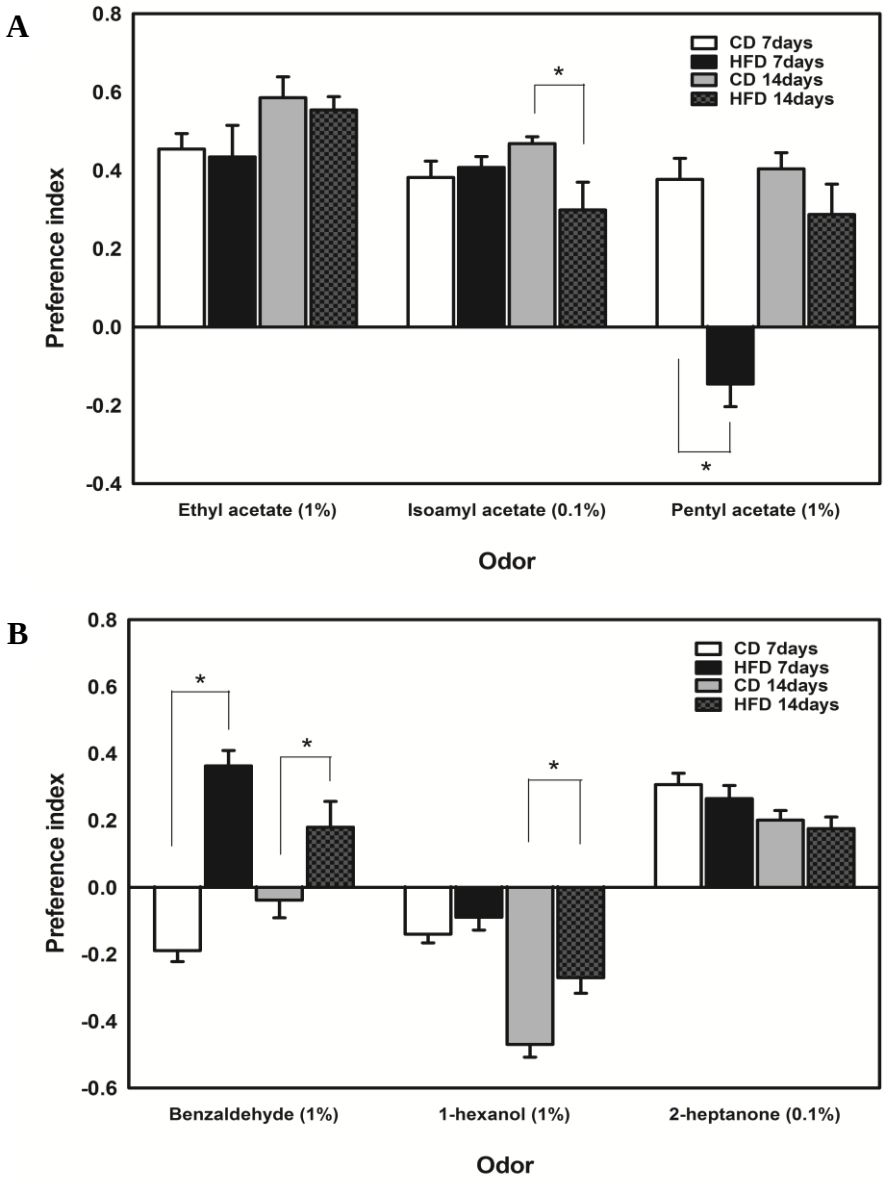


**Figure 4. Alteration of attraction behavioral responses of wild type Canton-S flies fed with control diet and HFD for 7 days and 14 days to several odors in a T-maze**

Flies fed with HFD showed different responses to attraction and repulsiveness to odorants compared to the control diet. In responses to ethyl acetate, isoamyl acetate, 2-heptanone in food-related odors had tendency to decline attraction behaviors to these odorants. In contrast, repellent behaviors to 1-hexanol were increased in both fly groups fed with HFD for 7 and 14 days. (n = 5 – 7). 20 male flies were tested in each trial. The numbers of flies trapped in the control trap were indicated on the right side, those in the test trap on the left side of T-maze.

### *3.5 Alteration of gene expression profiles by HFD treatment*

Together with the modification patterns of olfactory sensitivity and behaviors to odorants, we next examined the gene expression levels in the antenna samples from flies fed with HFD for 14 days (Table 1). We displayed that genes were regarded to express in antennae of control and HFD-treated flies by indicating as “Present” to the detected genes by statistics analysis. After mapping reads encoding a transcripts in flies, 9,610,945 (72%) were expressed in the control diet-treated flies while 7,488,727 (67%) were shown as “Present” in HFD-treated flies (Fig. 5). To sum up, over two-thirds of the *Drosophila melanogaster* genome data was expressed in both control and HFD-treated flies, showing the similar observation of gene expression in other insects as well [28]. Based on these values, it seemed that HFD might bring about a reduction of gene expression.

A total number of 7,950 genes indicated the change in gene expression level in which 4,009 genes were up-regulated while 3,941 genes were down-regulated after HFD treatment. We identified genes with 2-fold change in the expression level by HFD treatment. In this criterion, only 361 genes showed up-regulation and 321 genes were down-regulated. Among these genes, we obtained a wide understanding of olfactory genes to HFD and the small expression changes in insulin signaling pathway that might be involved in olfaction [19]. Thus, we studied to distinguish particular groups of genes including olfactory receptor, odorant binding protein and insulin signaling based on the detected expression data.

**Table 1. Number of genes differentially expressed after 14 days HFD treatment**

| | 14 days HFD treatment | |
|---|---|---|
| | **Upregulated** | **Downregulated** |
| **Antennae** | 4009 (361) | 3941 (321) |

(fold-change>2)

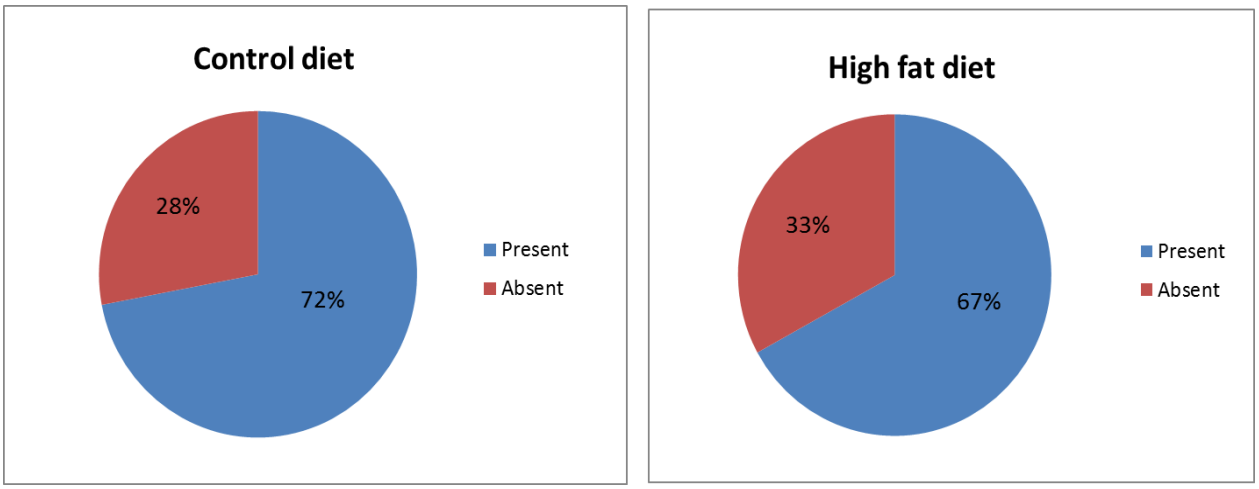


**Figure 5. The male antenna RNA-seq mapping data from the antennae after HFD and control diet treatment in male Canton-S flies**

Percentage of genes that show expression under the control and HFD treatment in antennae, measured as the percentage of genes that showed expression. The gene was regarded to express in antennae of control and HFD-treated groups when it showed as "Present" to detected genes by statistics analysis. n = 3,000 third antennal segments.

Table 2 summarizes the results identified gene expression of olfactory receptors (ORs). Of OR genes regulated by HFD treatment, 26 ORs and odorant receptor co-receptor (Orco) were shown to be down-regulated (Table 2A) while only 7 ORs were associated with up-regulation (Table 2B). As clearly seen from the table, Or9a, Or19a, Or19b, Or23a, Or33a, Or33b, Or35a, Or42a, Or42b, Or43a, Or45a, Or47a, Or47b, Or49b, Or56a, Or65b, Or65c, Or67a, Or67b, Or69a, Or85a, Or85e, Or88a, Or92a, Or98a, Or92a, and Orco were down-regulated. On the other hand, Or1a, Or22a, Or22b, Or65a, Or67c, Or83c, and Or85f were up-regulated. Similarly, odorant binding proteins (OBPs) exhibited modified expression in response to HFD treatment as the same pattern of ORs expression, presenting 2l OBPs of down-regulation (Table 3A) and 6 OBPs of up-regulation (Table 3B). Of OBP genes, OBP 8a, OBP18a, OBP19a, OBP19b, OBP19c, OBP49a, OBP51a, OBP56c, OBP56e, OBP56f, OBP56g, OBP56h, OBP57c, OBP57e, OBP58b, OBP58c, OBP58d, OBP59a, OBP83g, OBP99b, and OBP99d were decreased while OBP50a, OBP50e, OBP56a, OBP56d, OBP83ef, and OBP99c were increased. Specifically, OBP 51a, OBP56g and OBP56h genes in this group were shown the significant declines.

Notably, most of genes associated with insulin signaling pathways in the antennae displayed fluctuation as well as effects on the olfaction (Table 4). Among 8 genes in this group, DILP6, chico and InR genes demonstrated up-regulation after HFD treatment while the expression of DILP2 gene was significantly decreased in DEG analysis.

**Table 2. Olfactory receptor genes: down- and up-regulated after 14 days HFD treatment**

| Gene symbol | Flybase Idx | Expression level (Control diet) | Expression level (High fat diet) | Fold-change |
|---|---|---|---|---|
| **ORs** | | | | (downregulated) |
| **Orco** | FBgn0037324 | 125.928 | 142.808 | -1.084 |
| **Or9a** | FBgn0030204 | 5.460 | 4.624 | -1.565 |
| **Or19a** | FBgn0041626 | 0.501 | 0.643 | -1.143 |
| **Or19b** | FBgn0062565 | 3.173 | 2.590 | -1.629 |
| **Or23a** | FBgn0026395 | 2.807 | 0.166 | -3.978 |
| **Or33a** | FBgn0026392 | 1.398 | 0.649 | -1.906 |
| **Or33b** | FBgn0026391 | 0.237 | 0.229 | -1.135 |
| **Or35a** | FBgn0028946 | 3.734 | 1.188 | -2.920 |
| **Or42a** | FBgn0033041 | 0.167 | 0.229 | -1.041 |
| **Or42b** | FBgn0033043 | 6.763 | 5.374 | -1.658 |
| **Or43a** | FBgn0026389 | 4.500 | 5.172 | -1.223 |
| **Or45a** | FBgn0033404 | 0.009 | 0.014 | -1.003 |
| **Or47a** | FBgn0026386 | 7.944 | 3.780 | -2.561 |
| **Or47b** | FBgn0026385 | 17.574 | 9.875 | -2.210 |
| **Or49b** | FBgn0028963 | 1.041 | 1.306 | -1.201 |
| **Or56a** | FBgn0034473 | 3.576 | 2.483 | -1.837 |
| **Or65b** | FBgn0041624 | 5.650 | 4.988 | -1.508 |
| **Or65c** | FBgn0041623 | 0.788 | 0.570 | -1.451 |
| **Or67a** | FBgn0036009 | 1.672 | 1.619 | -1.419 |
| **Or67b** | FBgn0036019 | 2.156 | 2.075 | -1.441 |
| **Or69a** | FBgn0041622 | 0.561 | 0.471 | -1.308 |
| **Or85a** | FBgn0037576 | 3.591 | 1.825 | -2.247 |
| **Or85e** | FBgn0026399 | 0.088 | 0.047 | -1.078 |
| **Or88a** | FBgn0038203 | 5.801 | 6.051 | -1.304 |
| **Or92a** | FBgn0038798 | 12.727 | 4.816 | -3.151 |

| Gene symbol | Flybase Idx | Expression level (Control diet) | Expression level (High fat diet) | Fold-change |
|---|---|---|---|---|
| **ORs** | | | | (upregulated) |
| **Or1a** | FBgn0029521 | 0.041 | 0.128 | 1.042 |
| **Or22a** | FBgn0026398 | 2.790 | 5.481 | 1.233 |
| **Or22b** | FBgn0026397 | 4.810 | 7.035 | 1.022 |
| **Or65a** | FBgn0041625 | 3.838 | 6.005 | 1.060 |
| **Or67c** | FBgn0036078 | 0.682 | 3.238 | 1.845 |
| **Or83c** | FBgn0037399 | 0.558 | 1.258 | 1.106 |
| **Or85f** | FBgn0037685 | 0.454 | 0.876 | 1.015 |

**Table 3. Odorant-binding protein genes: down- and up-regulated after 14 days HFD treatment**

| Gene symbol | Flybase Idx | Expression level (Control diet) | Expression level (High fat diet) | Fold-change |
|---|---|---|---|---|
| **OBPs** | | | | (downregulated) |
| **Obp8a** | FBgn0030103 | 17.758 | 7.317 | -2.923 |
| **Obp18a** | FBgn0030985 | 12.889 | 16.751 | -1.003 |
| **Obp19a** | FBgn0031109 | 1565.008 | 1140.013 | -1.382 |
| **Obp19b** | FBgn0031110 | 22.246 | 21.034 | -1.324 |
| **Obp19c** | FBgn0031111 | 1.007 | 0.795 | -1.466 |
| **Obp49a** | FBgn0050052 | 29.605 | 16.060 | -2.232 |
| **Obp51a** | FBgn0043530 | 12.346 | 0.175 | -13.327 |
| **Obp56c** | FBgn0046879 | 0.490 | 0.248 | -1.410 |
| **Obp56e** | FBgn0034471 | 16.794 | 13.856 | -1.537 |
| **Obp56f** | FBgn0043533 | 0.401 | 0.539 | -1.109 |
| **Obp56g** | FBgn0034474 | 51.047 | 4.431 | -12.843 |
| **Obp56h** | FBgn0034475 | 9.533 | 0.252 | -10.081 |
| **Obp57c** | FBgn0034509 | 511.622 | 438.961 | -1.179 |
| **Obp57e** | FBgn0050145 | 1.995 | 1.228 | -1.833 |
| **Obp58b** | FBgn0034768 | 9.845 | 8.554 | -1.497 |

| | | | | |
|---|---|---|---|---|
| **Obp58c** | FBgn0034769 | 0.088 | 0.116 | -1.024 |
| **Obp58d** | FBgn0034770 | 2.660 | 3.741 | -1.086 |
| **Obp59a** | FBgn0034766 | 469.444 | 386.799 | -1.259 |
| **Obp83g** | FBgn0046875 | 0.436 | 0.471 | -1.188 |
| **Obp99b** | FBgn0039685 | 91.138 | 49.329 | -2.205 |
| **Obp99d** | FBgn0039684 | 28.822 | 27.569 | -1.291 |

| Gene symbol | Flybase Idx | Expression level (Control diet) | Expression level (High fat diet) | Fold-change |
|---|---|---|---|---|
| **OBPs** | | | | (upregulated) |
| **Obp50a** | FBgn0050067 | 0.184 | 0.492 | 1.098 |
| **Obp50e** | FBgn0033931 | 0.164 | 0.324 | 1.023 |
| **Obp56a** | FBgn0034468 | 25.125 | 61.964 | 1.936 |
| **Obp56d** | FBgn0034470 | 2389.382 | 3098.572 | 1.405 |
| **Obp83ef** | FBgn0046876 | 0.401 | 2.210 | 1.732 |
| **Obp99c** | FBgn0039682 | 603.275 | 740.023 | 1.176 |

**Table 4. Insulin signaling genes: down- and up-regulated after 14 days HFD treatment**

| Gene symbol | Flybase Idx | Expression level (Control diet) | Expression level (High fat diet) | Fold-change |
|---|---|---|---|---|
| **DILP2** | FBgn0036046 | 3.578 | 1.047 | -3.003 |
| **DILP5** | FBgn0044048 | 1.534 | 0.891 | -1.801 |
| **DILP6** | FBgn0044047 | 0.863 | 2.314 | 1.285 |
| **DILP7** | FBgn0044046 | 0.272 | 0.247 | -1.157 |
| **DILP8** | FBgn0036690 | 1.161 | 1.512 | -1.188 |
| **chico** | FBgn0024248 | 0.376 | 1.208 | 1.255 |
| **InR** | FBgn0013984 | 0.034 | 0.112 | 1.037 |
| **Pten** | FBgn0026379 | 2.464 | 1.489 | -1.925 |

Normalization of qRT-PCR from DEG analysis was performed four reference genes which were involved in insulin signaling genes to increase accuracy. The normalization of qRT-PCR data may have some changes according to analysis approaches. In this regards, randomly selection of reference genes in Table 3 was shown gene expression for validation of DEG results. Here, we measured the transcript variation of DILP2, DILP3, DILP5, and InR genes for data normalization (Fig. 6). The relative levels DILP2, DILP3, DILP5 expression show no significant differences. However, InR expression indicates a significant decrease by qRT-PCR.

To find out the main functional process by HFD treatment, we categorized the genes whose expression exhibited the greatest changes after HFD treatment (Table 5A, B and C). We analyzed the genes with more than two fold changes ($p<0.05$) in up- and down-regulation in expression. These data were established according to functional groups of Gene ontology (GO) groups including biological process (BP), molecular function (MF), and cellular component (CC). Certain classes of genes were enriched in our DEG analysis. In particular, several categories of genes based on GO biological process represented the largest number of genes; neurological system process, cognition, and sensory perception that suggest the relevance to olfactory responses in both peripheral and central nervous system [31]. Furthermore, genes of defense response and immune response were associated with response to HFD treatment.

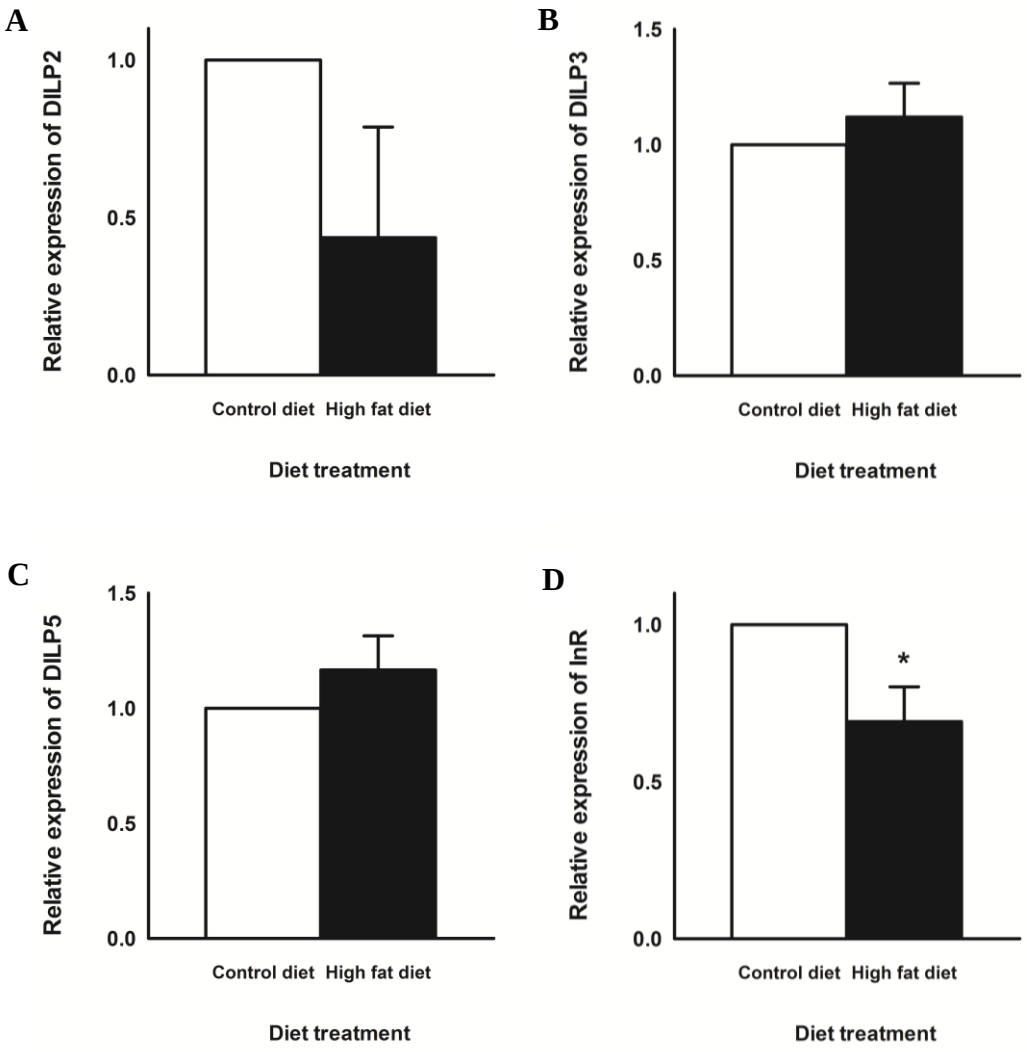


**Figure 6. The gene expression levels of some target genes related to insulin signaling identified by DEG analysis**

(A) Relative DILP2 mRNA transcript levels after different food treatment for 14 days. The level of DILP2 transcript was significantly reduced in HFD-fed flies 14 days after food treatment, showing approximately 50% decrease, compared to control flies. Relative DILP3 (B) and DILP5 (C) mRNA transcript level after different food treatment for 14 days. Both gene expressions were increased approximately 10% in HFD-fed flies, compared to control flies. (D) Relative InR mRNA transcript levels after different food treatment for 14 days. InR gene expression level was significantly decreased approximately 30%, compared to control flies. All qRT-PCRs were done in triplicate (*$p<0.05$). Error bars represent SEM.

**Table 5. Gene ontology (GO) groups based on biological process (A), molecular function (B), cellular component (C) that showed the greatest changes after 14 days HFD treatment ($p < 0.05$)**

**(A) Biological process (BP)**

| Gene ontology | # of genes |
|---|---|
| **Biological process (BP)** | |
| Antibacterial humoral response | 8 |
| Defense response | 20 |
| Innate immune response | 13 |
| Response to bacterium | 11 |
| Immune response | 17 |
| Defense response to bacterium | 10 |
| Humoral immune response | 11 |
| Antimicrobial humoral response | 10 |
| Defense response to Gram-negative bacterium | 5 |
| Amine biosynthetic process | 10 |
| Cellular amino acid derivative metabolic process | 9 |
| Biogenic amine metabolic process | 6 |
| Cellular amino acid derivative biosynthetic process | 5 |
| Biogenic amine biosynthetic process | 4 |
| Coenzyme metabolic process | 9 |
| Cofactor metabolic process | 10 |
| Coenzyme biosynthetic process | 6 |
| Folic acid and derivative biosynthetic process | 3 |
| Cofactor biosynthetic process | 7 |
| Cellular amino acid biosynthetic process | 6 |
| Sulfur amino acid metabolic process | 4 |
| Sulfur amino acid biosynthetic process | 3 |
| Cellular acyl-CoA homeostasis | 3 |
| Homeostatic process | 13 |
| Protein-chromophore linkage | 4 |

| | |
|---|---|
| Detection of external stimulus | 8 |
| Detection of abiotic stimulus | 7 |
| Phototransduction | 6 |
| Nucleic acid transport | 7 |
| RNA transport | 7 |
| Establishment of RNA localization | 7 |
| 'de novo' IMP biosynthetic process | 3 |
| IMP metabolic process | 3 |
| IMP biosynthetic process | 3 |
| Neurological system process | 38 |
| Cognition | 25 |
| Sensory perception | 19 |
| Protein stabilization | 3 |
| Transmembrane transport | 7 |
| Nitrogen compound biosynthetic process | 24 |
| Ribonucleotide metabolic process | 12 |
| Purine ribonucleotide metabolic process | 11 |
| Ribonucleotide biosynthetic process | 11 |
| Purine nucleotide metabolic process | 12 |
| Nucleotide biosynthetic process | 13 |
| Purine ribonucleotide biosynthetic process | 10 |
| Nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process | 13 |
| Nucleobase, nucleoside and nucleotide biosynthetic process | 13 |
| Purine nucleotide biosynthetic process | 11 |
| Protein import into nucleus, translocation | 3 |
| Coenzyme metabolic process | 9 |

**(B) Molecular function (MF)**

| Gene ontology | # of genes |
|---|---|
| **Molecular function (MF)** | |
| Structural constituent of ribosome | 21 |
| Structural molecule activity | 32 |
| Acyl-CoA binding | 4 |
| Fatty acid binding | 4 |
| Carboxylic acid binding | 8 |
| Diazepam binding | 3 |
| Monocarboxylic acid binding | 4 |
| G-protein coupled photoreceptor activity | 4 |
| Photoreceptor activity | 4 |
| Carboxylic acid binding | 8 |
| Enzyme inhibitor activity | 12 |
| Odorant binding | 12 |
| Vitamin binding | 9 |
| mRNA 3'-UTR binding | 4 |

**(C) Cellular component (CC)**

| Gene ontology | # of genes |
|---|---|
| **Cellular component (CC)** | |
| Extracellular region | 36 |
| Ribosomal subunit | 21 |
| Mitochondrial ribosome | 13 |
| Organellar ribosome | 13 |
| Ribosome | 22 |
| Mitochondrial lumen | 17 |
| Mitochondrial matrix | 17 |
| Mitochondrial large ribosomal subunit | 8 |
| Organellar large ribosomal subunit | 8 |
| Large ribosomal subunit | 12 |
| Mitochondrion | 40 |
| Small ribosomal subunit | 9 |
| Ribonucleoprotein complex | 28 |

# Ⅳ. DISCUSSION

### *4.1 A high-fat diet leads to olfactory dysfunction on homeostatic processing in Drosophila*

We have demonstrated a direct relationship between high-fat diet treatment and olfactory dysfunction in the fruit fly. The excessive stress by high-fat diet has a crucial impact on olfactory responses at the receptor level. HFD decreased olfactory sensitivity and altered behavioral responses to odors. Olfaction is one of the fundamental senses to maintain energy homeostasis under nutritional status [20,22]. By exhibiting reduction of life span and loss of climbing ability after HFD treatment, these phenomena implied that HFD gave detrimental effects to internal state and consequently led to sensory dysfunction, suggesting that external sensory signals are correlated with internal metabolic processing.

According to several researches on regulation of food intake and olfactory responses, olfactory signaling and nutrient-related genes by food stress were still needed to be more studied. A previous study suggested that unbalanced diet consumption showed alteration of metabolism and sensory systems, implying diet treatment is correlated with feeding behaviors that have fundamental determinants of energy balance from sensory processing in peripheral organs to the brain circuits of central nervous system [20,21]. Moreover, obese people were preferred a food-related odorant rather than a non-food related odorant, assuming that these people might not be possible to regulate food intake by internal state through appropriate odor processing [9].

Overall, HFD treatment has a considerable impact on behavioral responses to the odors on attraction as well. A previous report demonstrated that human in disposition to obesity were more sensitive to benzaldehyde (food odor) compared to n-butanol (non-food odor) than normal weight subjects, which influenced by external factors more than metabolic changes [9]. Likewise, we found that HFD-treated flies were less sensitive to food odors overall, except the benzaldehyde odor in both HFD-fed flies for 7 and 14 days. Rather, the flies were more responsive to benzaldehyde odor in electrophysiological and behavioral experiments, assuming several functional ORs (*or10a, or43a, or67b*) in the benzaldehyde odor among the genes involved in olfactory reception might have mediated to olfactory responses. More interestingly, a previous study demonstrated that misexpression of the *or43a* caused a reduction of behavioral avoidance responses to benzaldehyde [33].

By showing 26 ORs and Orco genes (fold change >2) in our DEG analysis, however, significant changes in the expression of these ORs after HFD treatment were not shown and these gene expression patterns might not explain the functional changes in general. According to the previous research, olfactory sensory projections on the processing from antenna to brain indicated different regions of functional subdomains in the *Drosophila* antennal lobe of brain. Each OR showed responses to the specific regions corresponding to different types of odorants [34].

These findings demonstrate that olfactory behaviors of the flies are strongly regulated by nutritional status, with increased HFD consumption generally leading to decreased attraction to food related odorants except benzaldehyde and pentyl acetate. We reasoned that some regulators may be mediated to a benzaldehyde odorant, indicating either that they mediate the olfactory

responses or that they function in different ways under metabolic signaling and olfactory signaling [22,35,36].

### *4.2 The mechanisms of high-fat-induced olfactory modulation are conserved and mediated both metabolic and olfactory signaling*

We also found a direct link between high-fat diet treatment and olfactory modulation in Drosophila. Several high-fat-induced changes in the main olfactory receptor organs (the third antennal segments) of flies through our DEG analysis indicated conserved mechanisms common to mouse and human about olfactory dysfunction [6,18]. By showing decreased *DmOrco* gene expression in real-time PCR, we reconfirmed the similar expression patterns in our DEG results. We suggested that perhaps not only olfactory receptor genes and odorant binding proteins but also some mediators regulate expression of the *DmOrco* gene in flies.

The certain genes were observed in functional changes through the several different pathways. These genes may be associated with olfactory and nutrient-related pathways as well as novel pathways that modulate olfactory responses in the fly. Previous studies demonstrated microarray analysis after starvation [37] or heat treatment [28] that affected olfactory responses and feeding behaviors under nutritional status in adult fly. Both research groups in the analysis included the antennal part, which is the main olfactory receptor organ to make clear the importance of olfaction depending on internal state.

Specifically, some of genes were associated with neurological system process, cognition, sensory perception and nutrient metabolism in our GO results. Previous work demonstrated that neural activities of olfactory bulb in mammals is modulated by starved and fed states, and the similar result has been shown in the antennae of the fruit fly by increased food-searching behavior under starvation. Like mammals, it has been reported that insulin as a metabolic factor modulates olfactory responses in fly antennae by enhancing pre-synaptic facilitation and regulated food-searching behaviors according to internal state [19]. Furthermore, Drosophila insulin-like peptide 2 (DILP2) was shown to modulate short neuropeptide F receptor (sNPFR1), which regulating of feeding behaviors in antenna and also stimulated in starved flies [38-40]. Similarly, several studies demonstrate the importance of neuropeptides in regulation of feeding behavior and olfaction [41,42].

Interestingly, our DEG analysis has detected transcripts of DILP2 in significant changes of down-regulation compared to other insulin-related genes. This decrease pattern could be partially explained by the reduction of the olfactory sensitivity caused by HFD treatment. Probably this might cause the loss of controlling ability of attractive behaviors to food odors. Recent research has demonstrated that DILP2 was shown to different expression levels depending on the types of diets [43]. Moreover, diet treatment for oversupply of high-sugar made detrimental effects in homeostatic processing and caused obesity with insulin resistance by fat accumulation [35], indicating this internal state might prevent effective communication in each organ from blocking in the hemolymph. We concluded that such condition could not help functioning with other peripheral receptors, of course, including olfaction. These data revealed that olfactory responses to HFD treatment are affected not only by the overexpression of certain insulin signaling genes but also by the inhibition of others. Therefore, communication through

olfactory modulation could contribute to peripheral organs and subsequently linked to central nervous system [14,31,44].

Olfactory modulation was integrated by internal and external factors to provide the crucial evidence of olfactory responses to odors. Our study has demonstrated that the fluctuations in olfactory and metabolic cues regulate olfactory sensitivity at the receptor level and contributes to olfactory modulation in the applied HFD treatment. A better understanding of the complex mechanisms underlying olfactory modulation may help to characterize the olfactory systems affected by HFD treatment in the future. Identifying novel regulators of olfactory system and examining to find out specific odorant receptors to odors in intracellular level affected by HFD treatment, suggesting it might provide to understand the mechanisms and circuits from antenna to brain in the flies.

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# 고지방식이 스트레스로 유도된 노랑 초파리 (*Drosophila melanogaster*)에서 후각 민감성 및 행동 반응과 전사 프로파일 분석 연구

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## 국문초록

본 연구에서는 고지방식이 스트레스로 유도된 초파리 모델 (*Drosophila melanogaster*)을 이용하여 냄새 성분에 따른 후각 민감성과 행동 반응을 확인하였으며, 이에 따라 전사 프로파일 분석 연구를 통해 비만(obesity)과 후각(olfaction)의 상관관계를 연구하고자 하였다.

대부분 유기체는 생존을 위해서 에너지 항상성을 유지하며 체내의 영양 상태나 외부의 자극 요소들에 의해서 영향을 받게 된다. 이러한 요인들에 의해서 영양 불균형이 나타나 비만을 야기하게 되며, 또한 신진 대사에 중요한 역할을 담당하는 감각 기관에 해로운 영향을 미치게 된다. 특히, 후각 기능을 담당하는 기관들이 제대로 역할을

수행하지 못하게 되면, 냄새에 대한 제어가 불가능해져 지속적인 불균형 상태를 유지하게 되어 더 심각한 증상을 유발하게 된다.

비만과 스트레스를 야기시키는 요인 중 하나인 고지방식이는 수명을 감소시키고 움직임을 둔하게 하여 생체 내 에너지 항상성을 교란시키게 되며, 각종 대사증후군 및 만성질환을 일으키는 요인이 되기도 한다.

성충으로 태어난 수컷 초파리 모델을 이용하여 고지방식이 스트레스를 7 일과 14 일 기간으로 주었을 때, 스트레스 기간이 길수록 수명이 줄어 들었으며, 행동이 느려지는 것을 확인할 수 있었다. 또한, 냄새 성분에 따라 전반적으로 7 일과 14 일 섭취 기간 모두 후각 민감성이 감소하는 것을 확인하였으며, 후각 수용체와 관련된 유전자(Orco)의 발현 레벨이 낮아짐을 보임으로써 고지방식이 스트레스가 후각에 영향을 미친다는 사실을 확인하였다. 이러한 냄새 성분에 대하여 후각 행동 반응을 확인해 본 결과, 고지방식이 스트레스를 받은 후 기피하였던 벤즈알데하이드(benzaldehyde) 성분에 대하여 오히려 선호하는 경향을 보였다. 이에 따라, 고지방식이 스트레스에 의해 영향을 받는 유전자 그룹을 확인하기 위하여 전사 프로파일 분석을 진행하였다. 그 결과, 후각 신호 전달에 영향을 주는 후각 수용체와 후각 결합단백질에서 발현 차이가 나는 유전자들을 확보하였으며, 또한 영양 성분과 관련이 있는 인슐린 관련 유전자들을 확인할 수 있었다.

이러한 연구 결과들을 통해 고지방식이 스트레스에 의한 후각 기능과 대사 신호전달에 미치는 영향을 확인함으로써, 비만과 후각 기능의 상관관계에 대한 연구 기반을 마련하여 향후 후각 기능을 토대로 하여 비만을 예방하고 치료하는데 기여할 것이라 사료된다.

**주요어** : 노랑초파리, 고지방식이 스트레스, 후각 반응, 행동 변화, 전사 프로파일 분석

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