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Obesity-linked genes' regulation in *Drosophila melanogaster*

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Abstract:

There are several serious diseases such as cancer, diabetes and obesity in this modern world. Insects are mostly used to investigate the functions of neuropeptides in regulation of feeding behavior and starvation. In *D. melanogaster* the regulation of *Twz, TfAp2, Hmgcr* and *G9a* were significantly affected by different periods of starvation. The expression of *Twz* and *TfAP-2* in adult flies controls the secretion and production of octopamine that regulates the function of *Drosulfakinin (DSK)* and *Drosophila cholecystokinin (CCK)*. In *D. melanogaster Hmgcr* is produced in the pars intercerbralis (PI) region of the brain and was supposed to regulate feeding behavior but the regulation of feeding was not affected either by *Hmgcr* nor *G9a* on different nutrient condition. *Hmgcr* catalyzes the production of mevalonate; the precursors of juvenile hormone *(JH)* component.

Key words: Obesity linked-genes, starvation in *Drosophila, TfAP-2, Twz*

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Abbreviation

Adipokinetic AKH

Central nervous system CNS

Cholecystokinina receptor CCKAR

Drosulfakinin DSK

Drosophila cholecystokinin CCK

Juvenile hormone JH

Insulin producing cells IPC

Pars intercerbralis PI

Transcription factor AP-2 TfAP-2

Acknowledgement

I express my gratitude and respect to my parents who always encourage and help me at every step of my education. I greatly appreciate the support and motivation of my supervisor Dr. Michael William whose office door was always open to me and Dr. Volkmar Passoth who guide and support me and give valuable comments on this manuscript. Thanks to Prof Helgi Schioth who gave me a chance to work in his lab. Thanks to all my friends and colleagues of Fly lab who always helped me whenever I needed. Finally I completed this manuscript with the help and support of my wife who has read every word of this manuscript and made numerous corrections.

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Zaid Khan

Introduction:

There are a number of severe diseases in this modern society like metabolic disorder, diabetes and obesity and huge attempts have been made to investigate the function of peptide hormones and neuropeptides in regulation of metabolism and feeding, particularly in mammals. Insects are largely used in research for the purpose to understand the mechanism of their feeding behavior and increase our knowledge of human biology.

The human genes *TFAP2B* (encoding AP-2β) and *KCTD15* have been recognized as novel loci linked with obesity. *TFAP2B* belongs to the *AP-2* family of transcription factors that function as main regulators in a different developmental mechanism, while in mice *TFAP2* ensures proper development of the CNS and peripheral noradrenergic neurons. *KCTD15* grouped into the family of potassium-channel tetramerization domain- including proteins. In zebra fish embryos, *KCTD15* limits neural crest formation due to inhibition of *AP-2α* function. In *Drosophila melanogaster TFAP2B* is expressed via the gene *TfAP-2*, that is highly conserved and vastly expressed in the central nervous system while in mice the noradrenergic system is regulated by *TFAP2B* [1]. *TfAP-2* and *Twz* in adults are genetically linked in order to control the production and secretion of octopamine that regulates the function of *Drosophila cholecystokinin* (*CCK*) homologue and *Drosulfakinin* (*DSK*). When food enters into the lumen of mammals then the gut releases a mammalian gastrointestinal hormone called *CCK* that attaches to the *cholecystokinina receptor* (*CCKAR*) present on vagal sensory terminals; a satiation message is delivered to the nucleus of the solitary tract to restrain feeding. In analogy to mammalian *CCK*, *DSK* in adult *D. melanogaster* performs the function to restrain overeating after starvation [2]. *D. melanogaster Sulfakinin* gene (*DSK*) has two peptides (*DSK-1 and 2*), which have a function in locomotion and color preferences while a third peptide *DSK-0* is involved in gut muscle contraction [3, 4]. *D. melanogaster* expresses the *Dsk* and other peptide products into the brain along with the insulin producing

cells IPCs. However IPCs appear to be only *DSK*- generating neurons into neurosecretory cells through axons terminating in neurohemal area of corpora cardiaca, anterior intestine and aorta [5].

***Drosophila melanogaster* insulin like peptides *Dilps*:**

Insulin producing cells IPCs, functionally similar with the mammalian pancreatic islet β cells, are situated in the central nervous brain. In *D. melanogaster* the main function of IPCs is to regulate metabolism and growth. Eight insulin like-peptides are present in *D. melanogaster* that regulate metabolism, growth, longevity and maturation. Four of these *dilps*, called *dilps*1-3 and *dilp*5 are mainly produced in IPCs [2, 5].

The main tissues that express *dilps* are midgut, adult and larval brains, salivary glands and ovaries. The neurosecretory cells in the protocerebrum called pars intercerbralis (PI) express *dilps* 2, 3 and 5 [2, 6, 7]. *Dilps* 2, 3 and 5 are independently expressed in larval IPCs while *dilps* 5 and 3 are expressed when nutrients are available, because of starvation the quantity of *dilp5* and *dilp3* transcripts is decreased and accumulated in the axonal termini and IPCs. The removal or blocking of IPCs in *D. melanogaster* can cause late development and small body size [8, 9, 10].

***Drosophila melanogaster* as a model:**

T. H. Morgan has studied *D. melanogaster*, which is a model organism used in genetics, more than a century ago. He applied it to study hereditary. The reasons of selecting *D. melanogaster* are:

a) Compared to other model organisms like rats and mice its care is easy and economical.

b) *D. melanogaster* is a small fly and its life cycle is only about eight weeks.

c) Genetic and environmental factors alter its life cycle.

d) Male and female can clearly be distinguished from each other and virgins can easily be

isolated.

D. melanogaster is a small fly, showing similar behavioral activities compared to *Homo sapiens* such as courtship and male-male aggression. Recently scientists have been applied *D. melanogaster* in research on molecular developmental genetics [6, 11]. *D. melanogaster* genome size is about 180 Megabases (MB) in which 120 MB is euchromatin region whereas the remaining 60 MB genome consists of two blocks of ribosomal RNA genes, simple sequence satellites and transposons [12]. Lipids and carbohydrates in *D. melanogaster* are stored by Fat body and oenocytes while in vertebrates they are stored by adipose tissues and liver. Fat body in *D. melanogaster* is involved in metabolism, it is a region of glycogen storage, and it is also concerned with the synthesis of hemo lymph proteins. Adipokinetic *(AKHs)* and octopamine hormones are concerned to provoke mobilization of lipids. *AKHs* constituted of the vast family comprised of 8 to 10 amino acids peptides released into hemo lymph through neurosecretory cells of the corpora cardiac [13].

Aim of the Project:

The neuroendocrine system of human is complex and it is difficult to understand how the neuroendocrine system of a human regulates food intake and exposes the process causing eating disorder. *Drosophila melanogaster's* simple brain was used to study the eating behavior to answer to nutritional necessitates. Starvation has been also studied in order to obtain more information about these genes and help us to understand how these genes are regulated. This study investigates that how the *Hmgcr*, *G9A* and the two conserved obesity-linked genes *TfAP-2* and *Twz* are regulated.

Material and Methods:

Drosophila melanogaster stock:

Drosophila melanogaster CSORC flies were obtained from the stock existing in Uppsala University, Department of Functional Pharmacology. They were kept for 12:12 hour's period's light: dark cycles in a 25°C incubator and at 60% humidity. The flies were raised on the standard media containing fly food called jazz mix (Fischer Thermo Scientific).

Starvation Assay:

Drosophila melanogaster CSORC flies were raised on standard media having fly food called jazz mix (Fischer Thermo Scientific) in the 25°C incubator and at 60% humidity. The flies used for starvation assay were raised for 5-7 days on normal food called jazz mix (Fischer Thermo Scientific). Afterward, the flies were shifted on a 1% 6 ml agarose (10 g/l) in a vial that provided water and humidity to the flies enclosed in the vial, however, did not provide any food at all. The flies were starved in a 25°C incubator for 24 and 48 hours and then the flies were frozen in -80°C overnight. The control *CSORC* flies were also provided the normal food but these flies were not starved. After 5-7 days the control flies were frozen overnight. Heads of the starved and control *CSORC* flies were detached by banging tubes with a table for 20 seconds. Afterward, mRNA was extracted from the heads of the flies, cDNA was synthesized and a qPCR was performed on cDNA obtained from flies' heads.

Different concentration of diets used for flies:

We prepared 5 different types of foods. A standard diet consisted of (10:10 g dl^{-1} sugar: protein) named control. The rest of the diets were called the experimental diets having various concentrations, limited diet was, (2.5:2.5 g dl^{-1} sugar: protein), high protein diet (10:40 g dl^{-1} sugar: protein), high sugar diet (40:10 g dl^{-1} sugar: protein), and high sugar plus

high protein diet (40:40 g dl^{-1} sugar: protein). After aging the flies for 1-2 days, only male flies were collected under a microscope and raised for 5 days on the above-mentioned diets in a 25°C incubator. Afterward, to each Eppendorf tube 50 flies were added and frozen overnight in -80°C. The tubes were banged with a table and heads were detached from fly's body. Afterward, mRNA was extracted from 50 flies heads, cDNA was synthesized and qPCR was performed.

Isolation of RNA from flies' heads:

Drosophila melanogaster male flies were collected under a microscope anesthetized with CO_2 and shifted into 1.5 ml Eppendorf tubes. Flies' heads were detached from their bodies by hitting the tubes with a table. *Drosophila melanogaster* fifty flies heads were taken in each Eppendorf tube and RNA was extracted from flies' heads according to kit manual (miRNeasy Micro Kit Qiagen), with some modification. A maximum amount of the aqueous phase was collected from the RNA sample into a collection tube. After adding different buffers according to kit manual, the collection tubes that retained RNA were washed with 500 µl of 70% ethanol to eliminate impurities. The RNA tubes were dried for 10 minutes even to remove vapors of ethanol from the tubes. RNA column tubes that contained RNA were shifted into 1.5 ml Eppendorf tube. The RNA in the column tube was eluted with a 20 µl of autoclaved water added in the center of the column tubes. The RNA tubes were centrifuged for 1 minute at maximum speed to elute the RNA and the RNA was collected in 1.5 ml Eppendorf tubes and stored in a -20°C freezer.

RNA and cDNA Concentration:

Nanodrop 1000 spectrophotometer (Saveen Werner) was used to measure the concentrations of RNA and cDNA.

Complementary DNA (cDNA synthesis):

The cDNA was synthesized from 2 µg of total RNA. The cDNA reaction mix contained a total volume of 20 µl, including 10 µl reverse transcriptase buffer, 1 µl reverse transcriptase enzyme (AB applied biosystem, Qiagen), rest of the 9 µl reaction contained RNA plus water according to the calculation. The following RT-PCR program was used to make cDNA from RNA.

Initial denaturation at 95°C for 3 minutes,

Elongation (amplification) at 37°C for 1 hour,

Afterward, a PCR program having the following condition amplified the cDNA samples.

Initial denaturation at 94°C for 4 minutes,

30 cycles of denaturing at 94°C for 30 seconds,

Annealing temperature at 52.8°C for 30 seconds and extension at 72°C for 30 seconds.

The PCR mix contained Taq DNA polymerase enzyme (5 U/ µl), DMSO 3% 1 µl, primer tubulin forward and reverse 100 pmol/ µl, so 0.2 µl of primer was used for each reaction. Afterward, the cDNA samples were run on gel electrophoresis. A 1.5% agarose gel was used for the separation of cDNA samples i.e. 6 grams of agarose was dissolved in 400 ml of 1 X TAE (Tris-acetate-EDTA) buffer. Afterward, 5 µl of ethidium bromide was added to a 100 ml of agarose solution. Ethidium bromide is a fluorescent dye that inserts among the bases of nucleic acids and permits exposure of DNA fragments in the gels. Two µl of 6 X loading dye was mixed with a 10 µl of cDNA samples and loaded into the wells of the gel along with a 100 bp marker on one side of the gel. The 1 X TAE buffer was used for the separation of DNA. The buffer was used to ascertain pH, and to provide ions to maintain conductivity.

Once the process of electrophoresis was completed, the gel was put under the UV light box and a photo was taken from the gel in a dark room for a record [14].

Real time PCR (qPCR):

Relative mRNA expression levels of a housekeeping gene (Rp49) and of the genes of interest *Twz, TfAp-2*, *Hmgcr* and *G9A* were determined through quantitative RT-PCR (qPCR). A total volume of 20 µl per reaction was used in qPCR. The target genes were amplified using the instrument IQ5 (Bio-Rad). The concentration of each primer was 2 pmol/µl and the template concentration was 10 ng/µl. Beacon designer was used for primers' designing using SYBR Green as a setting. For each primer pair water was used as a negative control on a 96 wells plate of qPCR. All experiments of qPCR were run in duplicates. The desired genes were amplified under the following qPCR condition.

Initial denaturation at 95°C for 3 minutes,

50 cycles of denaturing at 95°C for 30 seconds,

Annealing temp at 52.8–63°C for 30 seconds different for each primer,

Extension at 72°C for 30 seconds and a final extension of 10 minutes.

My IQ5 software (Bio-Rad) was used to analyze data of qPCR [15]. Efficiencies of the primer were calculated using LinReg PCR program [16]. Subsequently, GeNorm protocol described by Vandesompele et al [17] was used to acquire normalization values from the expression of the housekeeping gene Rp49. Grubbs` test was used to eliminate outliers. Statistical analysis was performed through anova to find out gene expression differences between different groups. $P < 0.05$ value was used as significance level. All of the graphs

were made in Microsoft Excel 2007. The following primer pairs were used as shown in the following table (Table 1).

Table 1. List of primers and its annealing temperature.

Rp49 F	5´-CACACCAAATCTTACAAAATGTGTGA-3´	Rp49 R	5´-AATCCGGCCTTGCACATG-3´	63 °C
TfAP-2 F	5´-CTAAGAGCAAGAACGGAG-3´	TfAP-2 R	5´-AACCAAGGATGTCAGTAG-3´	56.9 °C
Twz F	5´-GCCACATTCTGAACTTTATG-3´	Twz R	5´-CACCAAATAGTTGCCATT-3´	56.1°C
Hmgcr F	5´- CCTGAATGTGAGCAATAATC-3´	Hmgcr R	5´- AATAATCGCACGGAATCG-3´	56.9°C
G9A F	5´-TTATGCCAGTGATGCTGCTC-3´	G9A R	5´- TGAAGCATTCTGCAAGTGG-3´	58.3°C

Results:

Effect of starvation on the expression of *Twz, TfAp-2, Hmgcr and G9a* in fly brain:

Twz and *TfAP-2* genes activity could be regulated transcriptionally or post-translationally through the process of starvation. The qPCR analysis was performed on adult males (5 replicates) for 24 and 48 hours starvation. *TfAP-2* expression was significantly enhanced for 24 hours starving males ($p < 0.005$, SE±0.07, 1.66 fold), (Figure 2), however, starvation did not affect *Twz* expression after 24 hours. On the other hand adult males were starved for 48 hours and had significantly enhanced the expression of both *Twz* and *TfAP-2*, (Figure 1).

The expression of *Hmgcr* and *G9a* were also regulated transcriptionally when flies were starved for 24 hours or 48 hours. According to the qPCR data for 24 and 48 hours of starvation of adult males, *Hmgcr* and *G9a* genes expression in flies had significantly elevated after 24 hours of starvation (*Hmgcr*; $P<0.0038$: *G9A*; $P< 0.0001$), (Figure 3, 4), but there was no effect observed on *Hmgcr* and *G9a* expression in flies after 48 hours starvation.

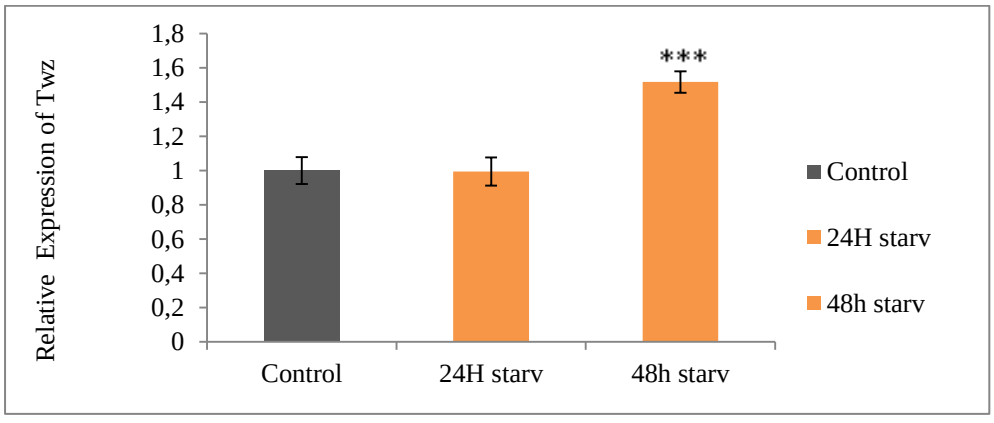

Figure. 1 Starvation elevates *Twz* transcription in the fly brain.

D. melanogaster CSORC flies were starved for 24 and 48 hours. The control *CSORC* flies were not starved. The mRNA expression of *Twz* was significantly elevated after 48 hours starvation compared with control. Six replicates were used per condition and each replicate consisted of 50 flies heads. Y-axis shows the mRNA expression level of the *Twz* gene. A triple asterisk (***) denotes significant difference to un-starved control.

One-way anova with bonferroni post hoc test was performed. P value of <0.05 was used as a significance level. The black bar indicates a control (un-starved) in the graph and its expression level is set to level 1 in the figure. The abbreviation denotes, 24 h starv, 24 hours starvation, 48 h starv, 48 hours starvation.

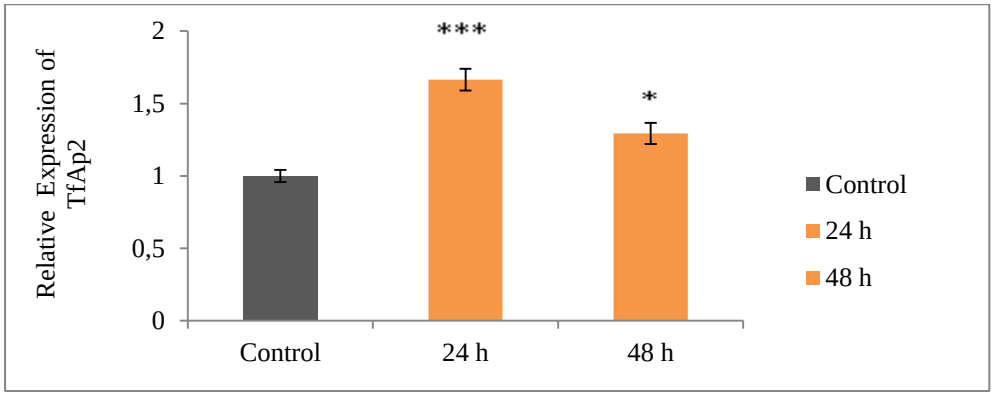

Figure 2. Starvation elevates *TfAp2* transcription in the fly brain. *D. melanogaster CSORC* flies were starved for 24 and 48 hours. Un-starved *CSORC* flies were used as a control. *TfAp2* mRNA expression level was significantly increased in 24 and 48 hours starvation after comparing with control. Six replicates were applied per condition and each replicate comprised of 50 flies heads. All values are given as average ± SE. One-way anova with bonferroni post hoc test was performed. The asterisk sign denotes significant difference to control ($p<0.0001$). P value of <0.05 was used as a significant level. Y-axis shows the expression level of the *TfAp-2* gene. *CSORC* Control is indicated with a black bar and the expression level was set as 100% indicated as 1 on the graph. The abbreviation denotes, control, *CSORC* flies, 24 h, 24 hour starvation, 48 h, 48 hours starvation.

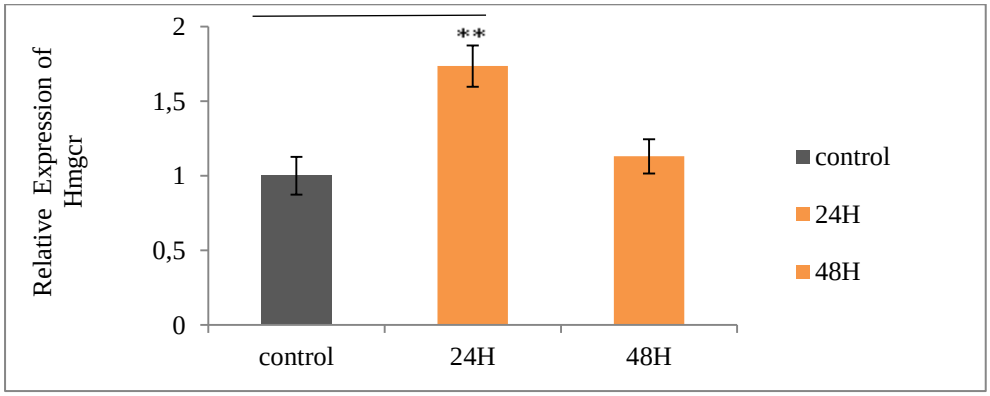

Figure 3. Starvation elevates *Hmgcr* transcription in fly brain.

D. melanogaster CSORC flies were starved for 24 and 48 hours. *CSORC* un-starved flies were used as a control in the experiment. In 24 hour starvation mRNA expression level of *Hmgcr* was significantly increased compared to control (un-starved). The control is indicated as a black bar in the graph and the expression level of control was set to level 1 on the graph. All values are given as average ± SE (P<0.0038). One-way anova with bonferroni post hoc test was applied. A double asterisk (**) shows significant difference to control. n=6 replicates were used for every condition and each replicate consisted of 50 heads. The abbreviations stand for, 24h, 24 hour starvation, 48 h, 48 hours starvation.

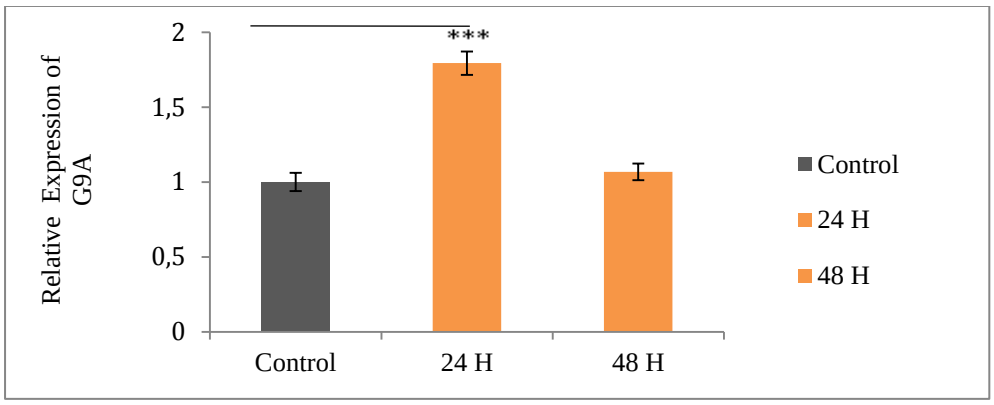

Figure 4. Starvation elevates *G9A* transcription in the fly brain.

Flies were starved for 24 and 48 hours, which increased mRNA expression level for *G9A* after 24 hours starvation compared to the un-starved control. Fifty flies' heads were used for each replicate and 6 replicates were applied per condition. All values are given as average ± SE (P<0.0001). One-way anova with bonferroni post hoc test was used. The expression level of *CSORC* un-starved control was set as 100% indicated as 1 in the figure and is shown with a black bar on the graph. Y-axis shows the expression level of *G9a* gene. A triple asterisk(***) indicates significant difference to control. The abbreviations stand for, control, *CSORC* flies, 24h, 24 hours starvation, 48 h, 48 hours starvation.

Effect of different diets on the transcription of *Hmgcr* and *G9A*:

D. melanogaster CSORC flies were raised for five days on various nutrient diets. Afterward, mRNA was isolated from the heads of the flies, cDNA was formed and qPCR was performed on cDNA of flies' heads. There were no significant changes observed for the mRNA expression of *Hmgcr* and *G9a* (P<0.6832) on all dietary regimes compared with the standard diets (sugar10: 10 yeast) figure 5A-B. The expression level of the standard diet (control) was set as 100%, represented as 1 on the graph.

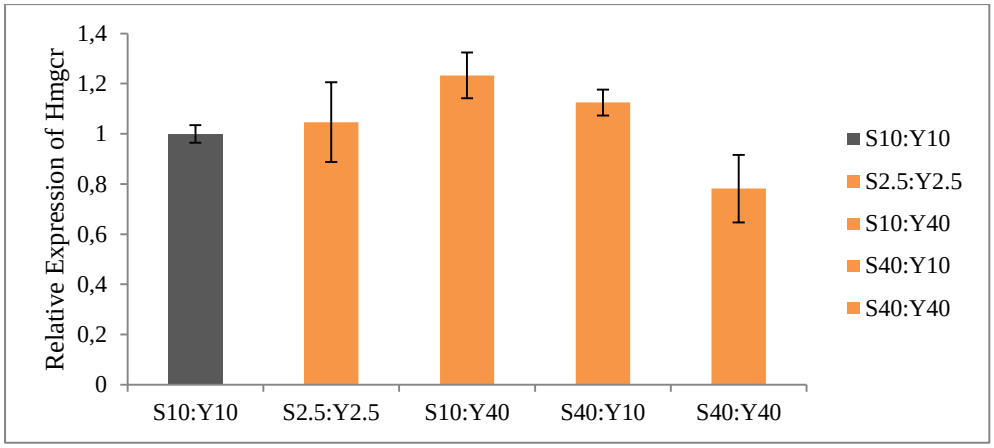

Figure. 5A Impact of dietary regimes on *Hmgcr* expression. *D. melanogaster* flies were raised for five days on various dietary regimes, a restricted diet (2.5:2.5 g dl^{-1} protein:sugar), a standard diet (10:10 g dl^{-1} protein:sugar), high protein diet (10:40 g dl^{-1}), high sugar diet (40:10 g dl^{-1}) and a protein plus high sugar diet (40:40 g dl^{-1} protein:sugar). After 5 days on different diets flies were headed off and mRNA was extracted and a quantitive PCR was carried out on cDNA of flies heads. Fifty flies heads were used for each replicate. All values are given as mean± SE. No significant difference was found to control. The Expression level of control was set as 100% indicated as 1 and is shown with a black bar in the graph. One-way anova with bonferroni post hoc test was run.

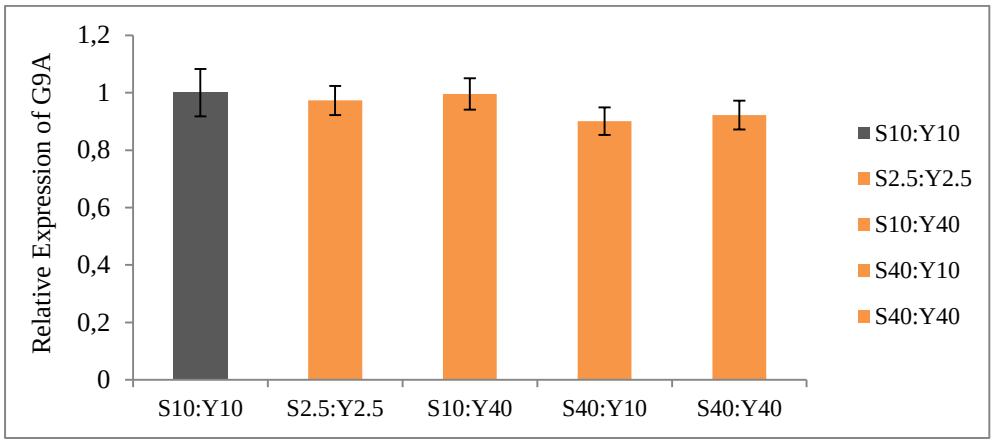

Figure. 5B Impact of dietary regimes on *G9A* expression. *D. melanogaster CSORC* flies were feed for five days on various diets called restricted diet (2.5:2.5 g dl^{-1} protein:sugar), control diet (10:10 g dl^{-1} protein:sugar), high protein diet (10:40 g dl^{-1}), high sugar diet (40:10 g dl^{-1}) and nutrient thick diet (40:40 g dl^{-1} protein:sugar). A quantitive RT-PCR was carried out on cDNA of flies heads. Fifty flies heads were used for each replicate and 5 replicates were applied for each diet. Control is shown with a black bar in the graph and the expression level of the standard diet was set to level 1 on the graph. All values are given as mean ± SE. No significant difference was found to control ($P<0.6832$). One-way anova with bonferroni post hoc test analysis was carried out.

Discussion:

Drosophila melanogaster flies were starved for various time frames or in a condition of using low macronutrients diets. Afterward, their CNS (heads) were used in order to find out the expression of *TfAP-2, Twz, Hmgcr* and *G9a* genes. In *D. melanogaster Hmgcr* is produced in the par intrecerabralis (PI) region of the brain and more correctly in insulin producing cells (IPCs). *Hmgcr* is a transmembrane glycoprotein attached to the endoplasmic reticulum and chiefly produced in liver tissues. *Hmgcr* also contributed to the mechanism of embryogenesis and cancer. Cholesterol is not synthesized in insects; however, *Hmgcr* catalyzes the production of mevalonate, the precursor of Juvenile hormone (*JH*) family components [18, 19]. It was proposed that *Hmgcr* regulates feeding behavior in *D. melanogaster* but however there was no significant effect of *Hmgcr* observed when flies were tested on different diets. The expression of *G9a* in adults is limited to gonads in males and females. The *G9a* protein can be found throughout the embryogenesis, oogenesis and larval development. All of these cells were metabolically active and consisted of numerous copies of *G9a* gene, however these cells have shown an abundant transcription and translation of the gene product. *D. melanogaster G9a* protein is mostly expressed in gonads of adult flies [20]. In *D. melanogaster* the expression of *Hmgcr* and *G9a* was not significantly affected when the flies were tested on different diets compared with the control flies, (Figure 5 A-B).

Our qPCR data shows that during starvation condition *TfAP-2* was activated, which could be a result of the interaction of *TfAP-2* and *Twz*, which stimulates *TfAP-2* expression. Moreover, under severe starvation conditions more *Twz* is required in order to activate *TfAP-2*, after 48 hours of starvation. Furthermore, the expression of *Twz* in flies does not need *TfAP-2,* while for the expression of *TfAP-2 Twz* is required [2].

The *CSORC* flies were starved for 24 and 48 hours and then the flies' mRNA were also tested for *Hmgcr* and *G9a* expression. The expression levels of *Hmgcr* and *G9a* in flies were significantly enhanced after 24 hours (unpublished data). Furthermore under severe starvation condition the expression levels of *Hmgcr* and *G9a* in flies were not significantly affected after 48 hours because the store nutrients in the flies were depleted upon starvation [18, 19]. An elevated level of expression of *G9a* after starvation indicates the high level of expression of hormonal activity [20].

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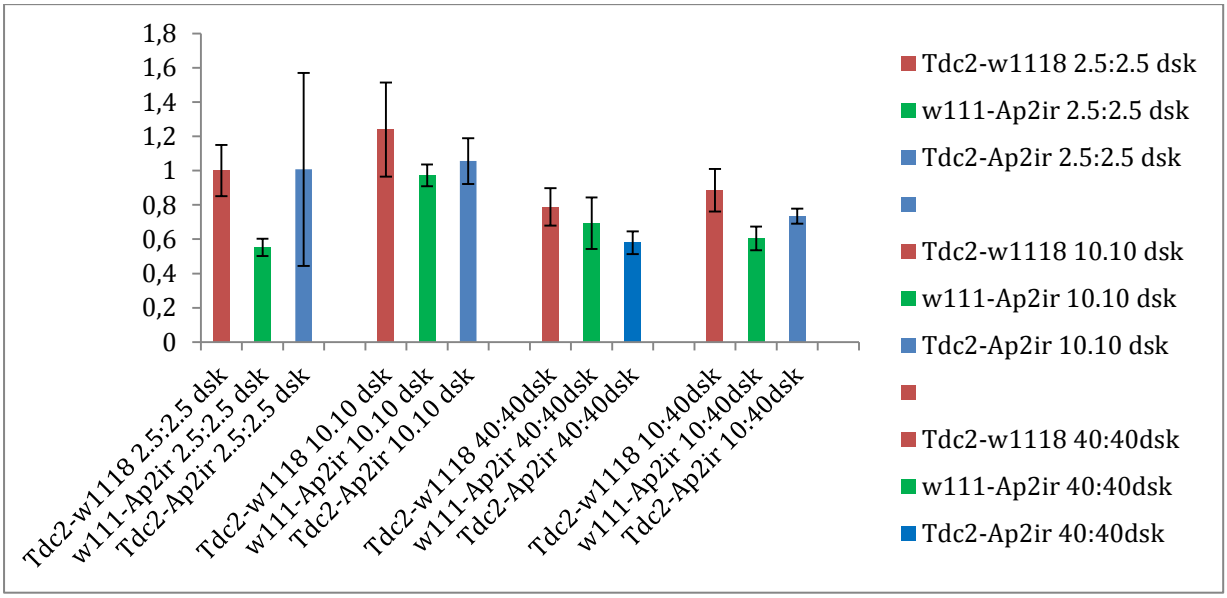
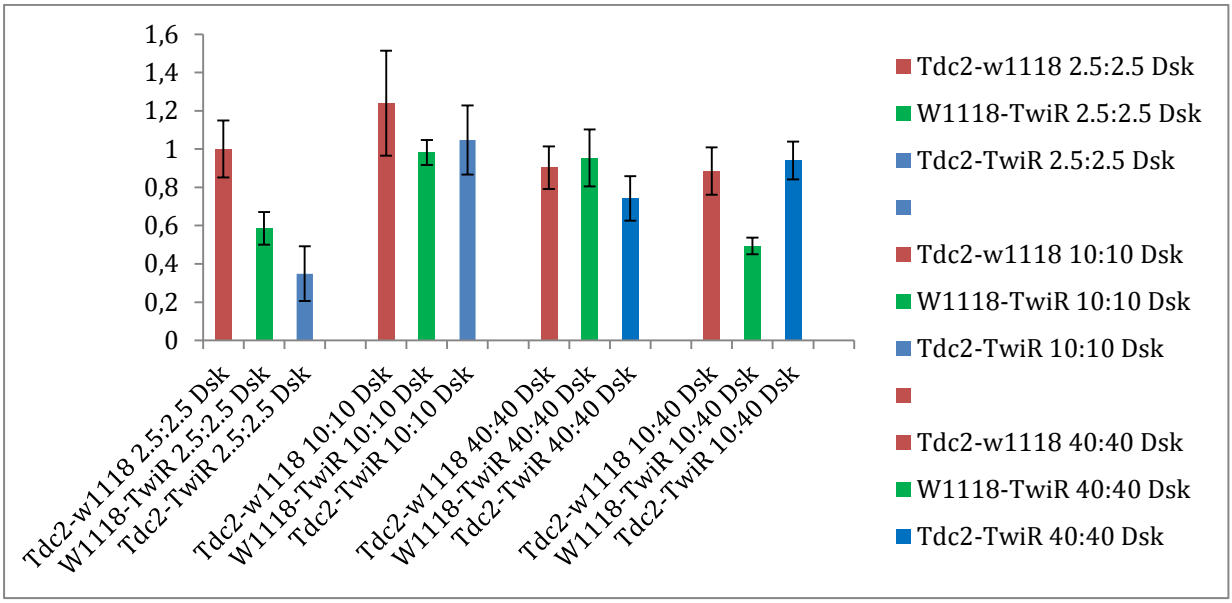
Appendix:



Different diets of *Tfap2* with *Dsk* gene: *D. melanogaster CSORC* flies were feed on various dietary regimes, a restricted diet (2.5:2.5 g dl^{-1} protein:sugar), standard diet (10:10 g dl^{-1} protein:sugar), high protein diet (10:40 g dl^{-1}), high sugar diet (40:10 g dl^{-1}), and nutrient thick diet (40:40 g dl^{-1} protein:sugar). After 5 days on the different diets flies were headed off and a quantitive RT-PCR was carried out on cDNA of flies head. Five replicates were used per condition and each replicate consisted of 50 individuals (heads). All values are given as mean± SE. No significant difference was found to control. One-way anova with bonferroni post hoc test was run.



Different diets of *Twz* and *Drosulfakinin* (*Dsk*). *D. melanogaster* wild-type flies were raised for five days on various dietary regimes called restricted diet (2.5:2.5 g dl^{-1} protein:sugar), standard diet (10:10 g dl^{-1} protein:sugar), high protein diet (10:40 g dl^{-1}), high sugar diet (40:10 g dl^{-1}) and nutrient thick diet (40:40 g dl^{-1} protein:sugar). After 5 days on the different diets flies were headed off and a quantitive RT-PCR was performed on cDNA. Five replicates were applied per condition and every replicate consisted of 50 individuals (heads). All values are given as mean± SE. No significant difference was found when compared to the control ($p<0.0375$). P value of < 0.05 was used as a significant difference. One-way anova with bonferroni post hoc test analysis was performed.