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FINAL REPORT ON THE

SUMMER STUDENT PROGRAM

Gene expression analyses in two strains of D.melanogaster using RT qPCR

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Introduction

Drosophila melanogaster is a species of fly (the taxonomic order Diptera) in the family Drosophilidae. The species is known generally as the common fruit fly (though inaccurately) or vinegar fly. Starting with Charles W. Woodworth's proposal of the use of this species as a model organism, D. melanogaster continues to be widely used for biological research in genetics, physiology, microbial pathogenesis, and life history evolution. As of 2017, eight Nobel prizes had been awarded for research using Drosophila. D. melanogaster is typically used in research because it can be readily reared in the laboratory, has only four pairs of chromosomes, breeds quickly, and lays many eggs. Its geographic range includes all continents, including islands. D. melanogaster is a common pest in homes, restaurants, and other places where food is served. Flies belonging to the family Tephritidae are also called "fruit flies". This can cause confusion, especially in the Mediterranean, Australia, and South Africa, where the Mediterranean fruit fly Ceratitis capitata is an economic pest.

History of use in genetic analysis

Thomas Hunt Morgan's Drosophila melanogaster genetic linkage map: This was the first successful gene mapping work and provides important evidence for the chromosome theory of inheritance. The map shows the relative positions of allelic characteristics on the second Drosophila chromosome. The distance between the genes (map units) are equal to the percentage of crossing-over events that occurs between different alleles.

D. melanogaster was among the first organisms used for genetic analysis, and today it is one of the most widely used and genetically best-known of all eukaryotic organisms. All organisms use common genetic systems; therefore, comprehending processes such as transcription and replication in fruit flies helps in understanding these processes in other eukaryotes, including humans.

Thomas Hunt Morgan began using fruit flies in experimental studies of heredity at Columbia University in 1910 in a laboratory known as the Fly Room. The Fly Room was cramped with eight desks, each occupied by students and their experiments. They started off experiments using milk bottles to rear the fruit flies and handheld lenses for observing their traits. The lenses were later replaced by microscopes, which enhanced their

observations. Morgan and his students eventually elucidated many basic principles of heredity, including sex-linked inheritance, epistasis, multiple alleles, and gene mapping.

Reasons for use in laboratories

D. melanogaster types (clockwise): Brown eyes with black body, cinnabar eyes, sepia eyes with ebony body, vermilion eyes, white eyes, and wildtype eyes with yellow body

There are many reasons the fruit fly is a popular choice as a model organism:

- Its care and culture require little equipment, space, and expense even when using large cultures.
- It can be safely and readily anesthetized (usually with ether, carbon dioxide gas, by cooling, or with products such as FlyNap).
- Its morphology is easy to identify once anesthetized.
- It has a short generation time (about 10 days at room temperature), so several generations can be studied within a few weeks.
- It has a high fecundity (females lay up to 100 eggs per day, and perhaps 2000 in a lifetime).
- Males and females are readily distinguished, and virgin females are easily isolated, facilitating genetic crossing.
- The mature larva has giant chromosomes in the salivary glands called polytene chromosomes, "puffs", which indicate regions of transcription, hence gene activity.
- It has only four pairs of chromosomes – three autosomes, and one pair of sex chromosomes.
- Males do not show meiotic recombination, facilitating genetic studies.
- Recessive lethal "balancer chromosomes" carrying visible genetic markers can be used to keep stocks of lethal alleles in a heterozygous state without recombination due to multiple inversions in the balancer.
- The development of this organism—from fertilized egg to mature adult—is well understood.
- Genetic transformation techniques have been available since 1987.
- Its complete genome was sequenced and first published in 2000.

- Sexual mosaics can be readily produced, providing an additional tool for studying the development and behavior of these flies.

Genetic markers

Genetic markers are commonly used in Drosophila research, for example within balancer chromosomes or P-element inserts, and most phenotypes are easily identifiable either with the naked eye or under a microscope. In the list of a few common markers below, the allele symbol is followed by the name of the gene affected and a description of its phenotype. (Note: Recessive alleles are in lower case, while dominant alleles are capitalised.)

Cy1: Curly; the wings curve away from the body, flight may be somewhat impaired

e1: Ebony; black body and wings (heterozygotes are also visibly darker than wild type)

Sb1: Stubble; bristles are shorter and thicker than wild type

w1: White; eyes lack pigmentation and appear white

bw: Brown; eye color determined by various pigments combined.

y1: Yellow; body pigmentation and wings appear yellow, the fly analog of albinism

Classic genetic mutations

Drosophila genes are traditionally named after the phenotype they cause when mutated. For example, the absence of a particular gene in Drosophila will result in a mutant embryo that does not develop a heart. Scientists have thus called this gene tinman, named after the Oz character of the same name. Likewise changes in the Shavenbaby gene cause the loss of dorsal cuticular hairs in Drosophila sechellia larvae. This system of nomenclature results in a wider range of gene names than in other organisms.

Adh: Alcohol dehydrogenase- Drosophila melanogaster can express the alcohol dehydrogenase (ADH) mutation, thereby preventing the breakdown of toxic levels of alcohols into aldehydes and ketones. While ethanol produced by decaying fruit is a natural food source and location for oviposit for Drosophila at low concentrations (<4%), high concentrations of ethanol can induce oxidative stress and alcohol intoxication. Drosophila's fitness is elevated by consuming the low concentration of ethanol. Initial exposure to ethanol causes hyperactivity, followed by incoordination and sedation. Further research has shown that the antioxidant alpha-ketoglutarate may be beneficial in

reducing the oxidative stress produced by alcohol consumption. A 2016 study concluded that food supplementation with 10-mM alpha-ketoglutarate decreased Drosophila alcohol sensitivity over time. For the gene that codes for ADH, there are 194 known classic and insertion alleles. Two alleles that are commonly used for experimentation involving ethanol toxicity and response are ADHs (slow) and ADHF (fast). Numerous experiments have concluded that the two alleles account for the differences in enzymatic activity for each. In comparing Adh-F homozygotes (wild-type) and Adh- nulls (homozygous null), research has shown that Adh- nulls have a lower level of tolerance for ethanol, starting the process of intoxication earlier than its counter partner. Drosophila show many of the same ethanol responses as humans do. Low doses of ethanol produce hyperactivity, moderate doses incoordination, and high doses sedation."

b: black- The black mutation was discovered in 1910 by Thomas Hunt Morgan. The black mutation results in a darker colored body, wings, veins, and segments of the fruit fly's leg. This occurs due to the fly's inability to create beta-alanine, a beta amino acid. The phenotypic expression of this mutation varies based on the genotype of the individual; for example, whether the specimen is homozygotic or heterozygotic results in a darker or less dark appearance. This genetic mutation is x-linked recessive.

bw: brown- The brown eye mutation results from pteridine (red) pigments inability to be produced or synthesized, due to a point mutation on chromosome II. When the mutation is homozygous, the pteridine pigments are unable to be synthesized because in the beginning of the pteridine pathway, a defective enzyme is being coded by homozygous recessive genes. In all, mutations in the pteridine pathway produces a darker eye color, hence the resulting color of the biochemical defect in the pteridine pathway being brown.

m: miniature- One of the first records of the miniature mutation of wings was also made by Thomas Hunt Morgan in 1911. He described the wings as having a similar shape as the wild-type phenotype. However, their miniature designation refers to the lengths of their wings, which do not stretch beyond their body and, thus, are notably shorter than the wild-type length. He also noted its inheritance is connected to the sex of the fly and could be paired with the inheritance of other sex-determined traits such as white eyes. The wings may also demonstrate other characteristics deviant from the wild-type wing, such as a duller and cloudier color. Miniature wings are 1.5x shorter than wild-type but are

believed to have the same number of cells. This is due to the lack of complete flattening by these cells, making the overall structure of the wing seem shorter in comparison. The pathway of wing expansion is regulated by a signal-receptor pathway, where the neurohormone bursicon interacts with its complementary G protein-coupled receptor; this receptor drives one of the G-protein subunits to signal further enzyme activity and results in development in the wing, such as apoptosis and growth.

se: sepia- The sepia eye color is brown. Ommochromes[brown] and drosopterins[red] are responsible for the typical eye color of Drosophila melanogaster. These mutations occur on the third chromosome. When mated with a wild type, flies with red eyes will be dominant over sepia color eyes. They are then classified as a recessive mutation, and can only result when both chromosomes contain the gene for sepia eyes. Sepia colored eyes are not dependent on the sex of the fly. The Sepia eye color decreases sexual activity in males and influences preference of females.

v: vermilion- Vermilion eye color compared to a wild type D. melanogaster is a radiant red. Vermilion eye color mutant is sex-linked recessive gene due to its absence of brown eye pigment. The red pigment is located on the X chromosome. The synthesis of brown pigment is due to the process of converting tryptophane to kynurenine, vermilion flies lack the ability to convert these amino acids blocking the production of brown pigment.

Male fruit fly (Drosophila melanogaster) exhibiting the black body mutation, as well as being vestigial winged and brown eyed.

vg: vestigial- A spontaneous mutation, discovered in 1919 by Thomas Morgan and Calvin Bridges. Vestigial wings are those not fully developed and that have lost function. Since the discovery of the vestigial gene in Drosophila melanogaster, there have been many discoveries of the vestigial gene in other vertebrates and their functions within the vertebrates. The vestigial gene is considered to be one of the most important genes for wing formation, but when it becomes over expressed the issue of ectopic wings begin to form. The vestigial gene acts to regulate the expression of the wing imaginal discs in the embryo and acts with other genes to regulate the development of the wings. A mutated vestigial allele removes an essential sequence of the DNA required for correct development of the wings.

w: white- Drosophila melanogaster wild type typically expresses a brick red eye color. In January 1910, Thomas Hunt Morgan first discovered the white gene and denoted it as w. The discovery of the white-eye mutation by Morgan brought about the beginnings of genetic experimentation and analysis of Drosophila melanogaster. Hunt eventually discovered that the gene followed a similar pattern of inheritance related to the meiotic segregation of the X chromosome. He discovered that the gene was located on the X chromosome with this information. This led to the discovery of sex-linked genes and also to the discovery of other mutations in Drosophila melanogaster. The white-eye mutation leads to several disadvantages in the flies that possess it such as a reduced climbing ability, shortened life span, and lowered resistance to stress than those with Wild Type eye color. Drosophila melanogaster has a series of mating behaviors that enable them to copulate within a given environment and therefore contribute to their fitness. After Morgan's discovery of the white-eye mutation being sex-linked, a study lead by Sturtevant (1915) concluded that white-eyed males were less successful then wild-type males in terms of mating with females. It was found that the greater the density in eye pigmentation, the greater the success in mating for the males of Drosophila melanogaster.
y: yellow- The yellow gene is a genetic mutation known as Dmel\y within the widely used data base called flybase. This mutation can be easily identified by the atypical yellow pigment observed in the cuticle of the adult flies and the mouth pieces of the larva. The y mutation comprises the following phenotypic classes: the mutants that show a complete loss of pigmentation from the cuticle (y-type) and other mutants that show a mosaic pigment pattern with some regions of the cuticle (wild type, y2-type). The role of the yellow gene is diverse and is responsible for changes in behaviour, sex-specific reproductive maturation and, epigenetic reprogramming. The y gene is an ideal gene to study as it is visibly clear when an organisim has this gene, making it easier to understand the passage of DNA to offspring.

Similarity to humans

A March 2000 study by National Human Genome Research Institute comparing the fruit fly and human genome estimated that about 60% of genes are conserved between the two species. About 75% of known human disease genes have a recognizable match in the

genome of fruit flies, and 50% of fly protein sequences have mammalian homologs. An online database called Homophila is available to search for human disease gene homologues in flies and vice versa. Drosophila is being used as a genetic model for several human diseases including the neurodegenerative disorders Parkinson's, Huntington's, spinocerebellar ataxia and Alzheimer's disease. The fly is also being used to study mechanisms underlying aging and oxidative stress, immunity, diabetes, and cancer, as well as drug abuse.

Drosophila melanogaster is an important non-mammalian model system that has had a critical role in basic biological discoveries, such as identifying chromosomes as the carriers of genetic information (Morgan, 1910) and uncovering the role of genes in development (Lewis, 2007),(Nüsslein-volhard & Wieschaus, 1980). Because it shares a substantial genic content with humans (Rubin et al., 2000) , Drosophila is increasingly used as a translational model for human development, homeostasis and disease (Spradling, 2006).

The Drosophila species originated in sub-Saharan Africa, and only recently expanded its range to inhabit diverse habitats around the commensal ((David & Capy, 1988) Lachaise et al. 1988). Its recent global expansion and well-documented large population sizes have implied a capacity to quickly adapt to local ecological conditions. These insights, together with powerful functional genomic and genetic tools available for *D.melanogaster*, position it as a compelling model species with which to study the molecular mechanisms and evolutionary processes of range expansion and local adaptation. Foundational to understanding recent adaptive differences between populations of *D. melanogaster* is an understanding of its recent demographic history.
Past surveys of genetic diversity have placed the origin of *D. melanogaster* in sub-Saharan Africa (Begun and Aquadro 1993; Lachaise and Silvain 2004), and more recent African sampling has begun to illuminate an increasingly fine-scale understanding of its genetic variation over the continent (Pool et al. 2012). The common understanding is globe. At some point early in the species' history, *D. melanogaster* evolved to be a human that *D. melanogaster* began to expand north in concert with the recession of the

last ice age (David and Capy 1988; Li and Stephan 2006), resulting in a single “out-of-Africa” population bottleneck, possibly in concert with human dispersal (Henn et al. 2012). Current estimates place this divergence between African and European lineages at 12–19,000 years ago (assuming ten generations per year), though its severity and timing have been topics of debate (Thornton and Andolfatto 2006; Stephan and Li 2007). There was an initial conjecture based on elevated phenotypic divergence that some Asian populations might pose an exception, possibly having an older independent colonization unrelated to human movement (referred to as an ancient “Far Eastern race”; David et al. 1976; Lemeunier et al. 1986; David and Capy 1988). However, subsequent modeling using Southeast Asian samples (Kuala Lumpur) was unable to identify genetic signatures of such a scenario (Laurent et al. 2011). As a result, the factors underlying the morphological characteristics of Asian subpopulations have remained enigmatic.

Although genome-wide approaches to identify and estimate admixture between populations of *D. melanogaster* have been expanding (Kao et al. 2015; Bergland et al. 2016), demographic models for non-African populations have been limited to three general locales: the aforementioned Southeast Asian sample (Laurent et al. 2011), a North American sample (Duchen et al. 2013), and a European sample (the Netherlands [Li and Stephan 2006]). Estimates from these analyses have supported a single out-of-Africa event for Eurasian populations (12,000–19,000 ya; Li and Stephan 2006; Laurent et al. 2011; Duchen et al. 2013) associated with a severe bottleneck. They have additionally provided extant and ancestral population size estimates, which indicate a recent population expansion.

According to these facts, it seems to be important to discover adaptational genetic based differences between strains of D.melanogaster, originating from different locations.

During summer students programme we explored gene expression differences between European D.melanogaster strain – D18 (Domodedovo 18) and American strain Canton S.

Methods

During the summer student practice, I learned to apply many modern molecular genetic

methods. These include DNA and RNA isolation, gel electrophoresis, primer design, sequencing and real-time PCR.

Object of analyses:

D. melanogaster

Fig.1. *D. melanogaster*

For analysis of gene expression, we isolated RNA from two *Drosophila Melanogaster* strains D18 and CS, checked the quality using gel electrophoresis, treated with DNases, set up cDNA synthesis, and then performed real-time PCR. As a reference, we took two housekeeping genes. In total, we analyzed 14 genes. for each of them there is a significant difference in expression between the strains.

RNA isolation protocol:

1. Lyse and homogenize samples in TRIzol Reagent according to your starting material.
2. For tissues add 1 ml of TRIzol Reagent per 50-100 mg of tissue to the sample and homogenize using a homogenizer.
3. If samples content a high fat content, centrifuge the lysate for 5 minutes at 12000g at 4-10 C.
4. Incubate for 5 minutes to permit complete dissociation of the nucleoproteins complex.
5. Add 0,2 ml of chloroform per 1 ml of Trizol Reagent used for lysis, then securely cap the tube

6. Incubate for 2-3- minutes.
7. Centrifuge the samples for 15 minutes at 12000 g at 4 C.
8. The mixture separets into a lower red phenol-chloroform, and interphase, and a colorless upper aqueous phase.
9. Transfer the aqueous phase containing RNA to a new tube.
10. Add 0,5 ml of isopropanol to the aqueous phase, per 1 ml of Tryzol Reagent used for lysis
11. Incubate for 10 minutes.
12. Centrifuge for 10 minutes at 12000g at 4 C. total RNA precipitate forms a white gel-like pellet at the bottom of the tube.
13. Discard the supernatant with a micropiperror
14. Resuspend the pellet in 1 ml of 75 EtOH per 1 ml of TRIzol Reagent used for lysis.
15. Vortex the sample briefly, then centrifuge for 5 minutes at 7500g at 4 C.
16. Discard the supernatant with a micropipettor.
17. Vacuum or dry the RNA pellet for 5-10 minutes.
18. Resuspend pellet in 20-50 mkl of RNAse free water, 0,1 mM EDTA solution by pipetting up and down.

After RNA isolation we checked the RNA quality and concentration using gel electrophoresis.

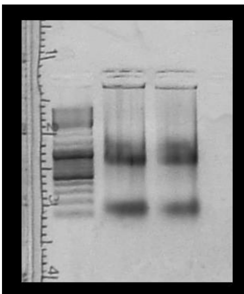

Fig. 2. Gel electrophoresis for RNA quality check

Then we eliminated DNA fro samples using this protocol:

Genomic DNA elimination

1. Add the following reagents into a sterile, RNase-free

tube on ice:

- 10X dsDNase Buffer 1 µL
- dsDNase 1 µL
- Template RNA 1 pg - 5 µg
- Water, nuclease-free To 10 µL

2.Mix gently and spin down.

3. Incubate for 2 min at 37°C in the preheated thermomixer

or water bath.

4.Optional. If RNA sample is to be used for RT-PCR

amplification of long fragments (≥ 3 kb), perform

dsDNase inactivation by incubating the sample at 55°C

for 5 min in the presence of 10 mM DTT.

5.Chill on ice, briefly centrifuge again and place on ice.

And then we made **cDNA reaction**.

1. After genomic DNA elimination, add the following reagents into the same tube in the indicated order:

Primers oligo (dT)18 primer 0.25 µL (25 pmol)

10 mM dNTP Mix 1 µL

Water, nuclease-free to 15 µL

2.Optional. If the RNA template is GC-rich or is known to contain secondary structures, mix gently, centrifuge

briefly and incubate at 65 °C for 5 min. Chill on ice, briefly centrifuge again and place on ice.

3. Add the following components to the reaction tube in the indicated order:

5X RT Buffer 4 μL

Maxima H Minus Enzyme Mix 1 μL

Total volume 20 μL

Mix gently and centrifuge.

4. Incubate for 10 min at 25°C followed by 15 min at 50 °C.

Then we made RT-qPCR reaction for 14 genes.

Primers for them was carefully designed.

Quantitative real-time PCR (qPCR) is a sensitive and robust technique directly evolved from the "end-point detection" polymerase chain reaction (PCR). Reverse transcription and subsequent quantitative real-time PCR (RT <qPCR) is a sensitive method that is currently widely used to quantify gene expression. However, the method requires normalization of the results with a suitable reference gene (RG), which is critical to minimizing differences in sample preparation.

RT qPCR protocol

1: 95,0°C for 5:00

2: 95,0°C for 0:10

3: 60,0°C for 0:30

Plate Read

4: GOTO 2, 39 more times

5: Melt Curve 65,0°C to 95,0°C: Increment 0,5°C 0:05

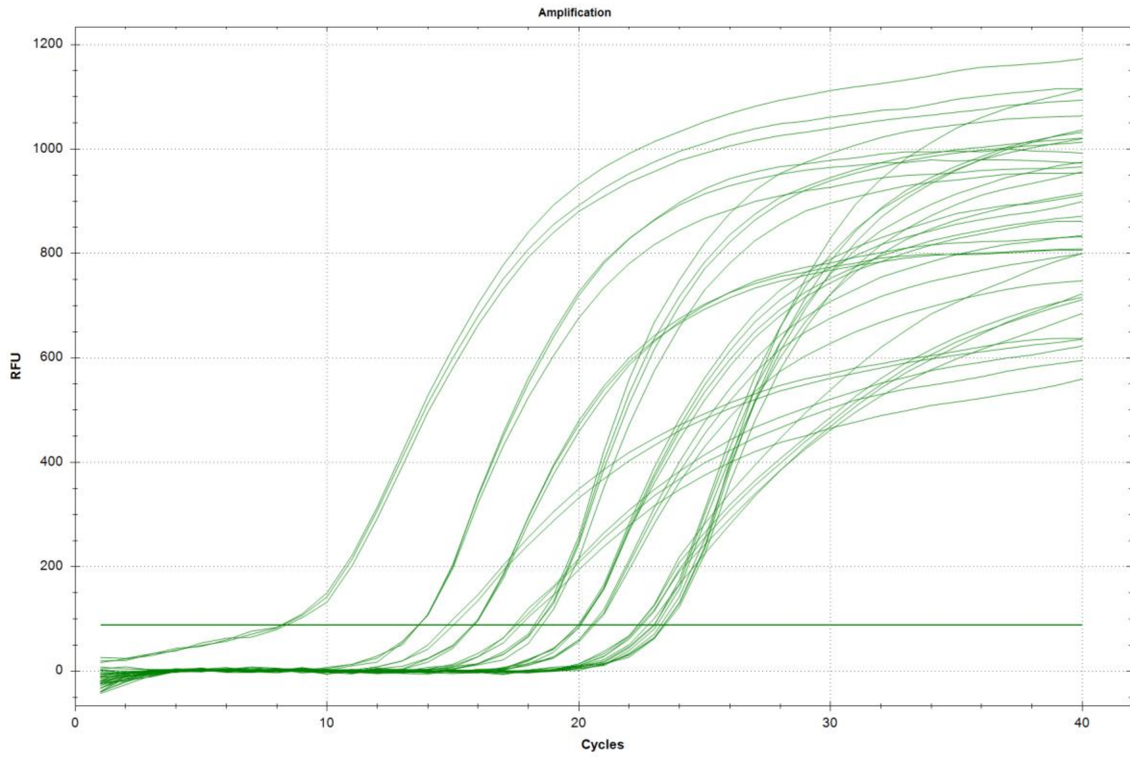

Fig. 3. RT qPCR result

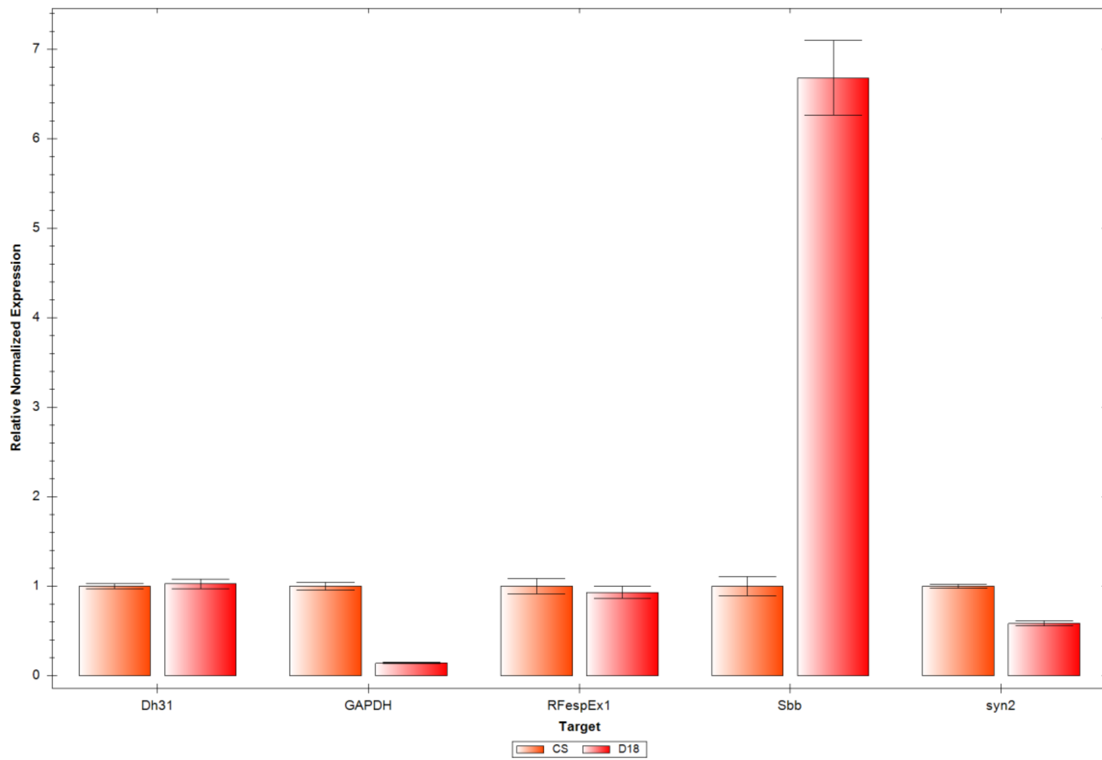

Fig. 4. Gene expression change according to reference genes.

Results

We discovered expression change for 14 genes responsible for different metabolic pathways, including iron metabolism and energy resources of the cell.

It may be caused by the fact that in place of origin for the fly strains average annual temperature differs for 5,5 °C. So it may be adaptation for different climate conditions.

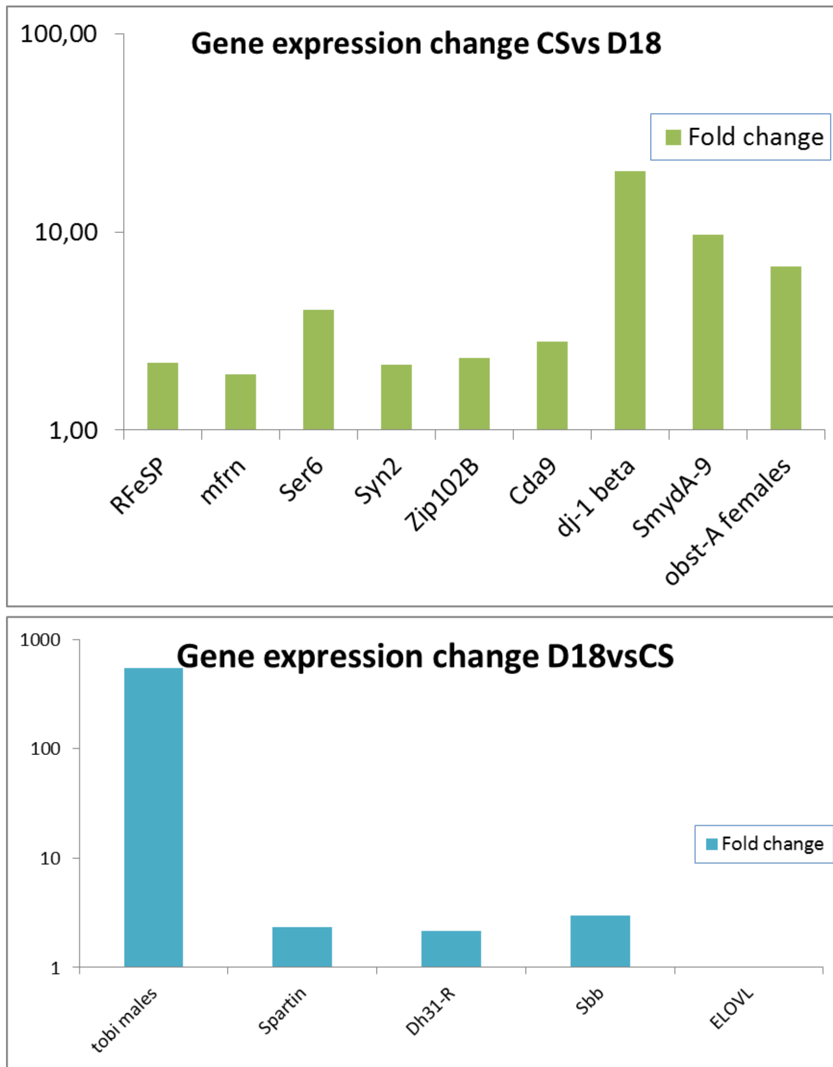

Fig.5. Fold change gene expression result

The results obtained in this study shed light on how organisms adapt to different conditions at the genetic level, since the two Drosophila lines were kept under identical

laboratory conditions for a year, and they also lived in laboratories for several decades. this suggests that the differences in expression that we observe are based solely on genetic differences between species.

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