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**THE IMMUNE SYSTEMS RESPONSE TO  
ENVIRONMENTAL STRESSORS IN *DRO-  
SOPHILA MELANOGASTER***

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Bachelor's thesis

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# TIIVISTELMÄ

Aino Malin: Banaanikärpäsen immuunijärjestelmän vaste ympäristön stressitekijöihin  
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*Drosophila melanogaster*, joka yleisesti tunnetaan banaanikärpäsenä, on laajalti käytetty koe-eläin biolääketieteellisessä tutkimuksessa. Monet immuunijärjestelmän geenit ovat konservoituneet kärpästen ja ihmisten välillä, eli näillä lajeilla on immuunijärjestelmissään samankaltaisia rakenteita ja mekanismeja. Banaanikärpänen on hyvä malliorganismi tutkimukseen myös nopean lisääntymisen ja suhteellisen halvan ylläpidon vuoksi. Banaanikärpäksillä ei ole lainkaan hankittua immuunipuolustusjärjestelmää, vaan niiden puolustusmekanismi perustuu synnynnäiseen immuni-teettiin. Näin ollen banaanikärpänen soveltuu hyvin luonnollisen immunitietin tutkimiseen. Yleisesti ajatellaan myös, että on eettisesti parempi käyttää tutkimuksessa hyönteisiä, joiden hermosto ei ole niin pitkälle kehittynyt, kuin esimerkiksi nisäkkäillä.

Tämän tutkimuksen tarkoituksena on selvittää banaanikärpäsen immuunijärjestelmän vastetta ympäristön stressitekijöihin. Aiemmin on ollut tiedossa, että ympäristötekijöillä on vaikutusta joidenkin immuunijärjestelmän geenien ilmentymiseen. Tutkimme *Drosophila melanogaster* lajia. Tutkitut stressitekijät ovat lämpötila, ruoan suolapitoisuus ja syömättömyys. Tutkitut geenit ovat nimeltään *IBIN* ja *IBIN-like*, joiden ilmentymistä infektiota on tutkittu indusoivan. Näiden geenien oletetaan kodaavan peptideitä. Tässä tutkimuksessa käytimme kahta eri *D. melanogaster* kärpäslinjaa.

Kun kärpäset oli altistettu tutkituille ympäristöolosuhteille, niiden RNA eristettiin ja geeniekspressio mitattiin kvantitatiivisella polymeerasiketjureaktiolla. Tulosten mukaan lämpötila lisää *IBIN* geenin ilmentymistä useimmissa tutkimissamme ryhmissä. Ruoan suola taas vaikuttaa *IBIN* geeniin sen ilmentymistä madaltavasti. Useimmat löydetyistä tuloksista eivät ole tilastollisesti merkitseviä, mutta niiden avulla voimme kuitenkin ymmärtää banaanikärpästen luonnolliseen immunitettiin vaikuttavia tekijöitä paremmin.

Avainsanat: Luonnollinen immunitetti, immunologia, *Drosophila melanogaster*, ympäristön stressi, lämpötila, geeniekspressio

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# ABSTRACT

Aino Malin: The immune systems response to environmental stressors in *Drosophila melanogaster*

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*Drosophila melanogaster*, more commonly known as fruit fly, is a widely used model organism in biomedical research. Many of the immunological genes are conserved between flies and humans. In other words, the immune systems of these species share some similar mechanisms and structures. The part of the reason why flies are so suitable in research is that they reproduce quite quickly and are easy to maintain. Fruit flies do not have an adaptive immune system and therefore rely only on the innate immunity. Therefore, fruit flies are suitable for the study of the innate immunity. For the ethical standpoint, it is commonly thought to be more ethical to use neurophysiologically less developed model animals such as insects compared to more complex animals such as mammals.

The purpose of this study is to find out how environmental stressors affect the expression of *Drosophila melanogaster* innate immunity. It is known that some environmental conditions have an impact on gene expression. We studied *D. melanogaster* in different temperature conditions. The effect of salt exposure and starvation was studied as well. The studied genes are *IBIN* and *IBIN-like*, which are shown to be highly induced by infection. These genes are expected to code peptides. In this study we use two different fly lines.

After the flies were exposed to the studied environmental conditions their RNA was extracted and gene expression was measured by using quantitative polymerase chain reaction. Results show that the temperature upregulates the expression of the *IBIN* gene in most of the studied groups. Salt in the food of the flies on the other hand downregulates the *IBIN* gene expression. Findings are not statistically significant in most cases but still help us understand more about *D. melanogaster* innate immunity.

Keywords: Innate immunity, immunology, *Drosophila melanogaster*, environmental stress, temperature, gene expression

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# 1. INTRODUCTION

*Drosophila melanogaster*, more commonly known as a fruit fly, is a popular model animal in research especially when studying immune system. Their immune system has similar structures and molecules as human, and many groundbreaking medical discoveries are done by using them. For example, Noble prize for discovering Toll like receptors (TLRs) was first researched in *D. melanogaster* (Lemaitre *et al.*, 1996; Weiss and O'Neill, 2022). Fruit flies reproduce quickly enabling the usage of several dozens of flies in research settings. *D. melanogaster* has a low genome redundancy that makes even single mutations visible in the phenotype (Rämet, 2012). Innate immunity has been studied in *Drosophila melanogaster* because the species has homologous structures and mechanisms in their immunity system as humans (Valanne *et al.*, 2019). It is also considered that usage of the *D. melanogaster* as model animal is more ethical than the usage of other neurophysiologically more developed animals such as mammals (Rämet, 2012).

Immunity can be divided into innate and adaptive ones. In this research we focused on the innate immunity of the *Drosophila melanogaster*. It has been used to study innate immunity since the species lacks an adaptive one (Ulvila, Vanha-Aho and Rämet, 2011; Arch *et al.*, 2022). *Drosophila* innate immunity consists of humoral and cell-mediated ones. Humoral branch of the immunological system involves the production and release of antimicrobial peptides (AMP), which are mainly activated by Immune deficiency (IMD) or Toll pathways (Salminen and Vale, 2020). Cell mediated part consists of blood cells called hemocytes, which can be divided into three major categories: plasmatocytes, crystal cells and lamellocytes (Salminen and Vale, 2020).

Gene CG44404 known as *IBIN* (Induced By Infection) is a peptide coding gene that was shown to be highly induced by infection on which it has gotten its name (Valanne *et al.*, 2019). In this research the aim was to study if environmental stressors had an effect on the gene expression levels in *Drosophila melanogaster*. *IBIN* and *IBIN-like* genes are expressed via Toll pathway and NFkB signalling mediates the pathway (Myllymäki, Valanne and Rämet, 2014; Valanne *et al.*, 2022). Research shows that overexpression of *IBIN* gene enhances sugar metabolism in *D. melanogaster* (Valanne *et al.*, 2019, 2020). *IBIN* and other infection induced genes are expressed very little in normal conditions By studying more this *IBIN*-gene and discovering more about the effect of outside stress, it is possible to understand more about *D. melanogaster* innate immunity and through that also human's immunity. This research and the results shown in this work are part of the larger *IBIN*-gene related research done by the Experimental immunology group in Tampere University.

In this study the goal was to learn whether different environmental stressors affect the expression of *IBIN* and *IBIN-like* genes. The studied environmental conditions were temperature, salt concentration of food and starvation. Temperatures were room temperature as control, 4°C and 36°C. The temperatures and salt concentration were chosen so that the flies would survive them because it is important when studying gene expression (Belyi *et al.*, 2020). *D. melanogaster*, as insects, are ectothermic species, so the temperature of their surrounding environment affects greatly their body temperature (Ito and Awasaki, 2022). It is shown that some *D. melanogaster* genes are expressed differently in different temperatures, such as for example the heat shock proteins that are found in insects. (Wojda, 2017) The temperature affects the flies metabolism and mitochondrial functions (Jørgensen *et al.*, 2023). Chosen salt concentration for the study was 4%; food with no salt was used as control. The salt in the sustenance of the flies has been shown to affect the expression of some innate immune system related genes in *D. melanogaster* (Overend *et al.*, 2012). Flies do not normally eat salty food, so the goal of this experiment was to study how it affects the immunity of the flies.

Quantitative real-time polymerase chain reaction (qRT-PCR) is a method that was used in this research to analyse gene expression after the RNA was extracted from the flies. PCR is widely used tool in research. qRT-PCR can detect and count RNA from the samples. RNA is first transcribed to complementary DNA (cDNA), which acts as a template in qPCR. The amount of amplified gene product is measured after every PCR cycle by using fluorescence. (Kralik and Ricchi, 2017; Artika *et al.*, 2022)

## 2. MATERIALS AND METHODS

### 2.1 *Drosophila melanogaster*

The study was conducted by using *Drosophila melanogaster*, a model animal known more commonly as a fruit fly. Two different fly lines were used in this research, wild type wRHP and Canton S. Both of these fly lines are commonly used in research (Qiu, Xiao and Meldrum Robertson, 2017). As can be seen from the picture of the flies (Figure 1) they are somewhat different looking. Most notably wRHP flies have white eyes and Canton S have red eyes. The difference between the eye color of the flies is caused by a deletion in wRHP line's X-chromosome, which results to white eyes as well as other neurodegenerative traits (Qiu, Xiao and Meldrum Robertson, 2017). The used fly individuals were all male. 15 flies of both lineages were used in each experiment conditions and they were divided into three tubes to extract RNA.



**Figure 1.** wRHP (A) and Canton S (B) *Drosophila melanogaster* pictured under a light microscope (Nikon DeltaPix).

## 2.2 Studied conditions

The effect of environmental stressors on gene expression was studied by exposing the flies on different temperatures, food's salt concentrations and starvation. Four different temperature conditions were used to study the gene expression: room temperature (22°C), 4°C, 36°C. The fly vials were kept in 36°C water bath for 1 hours or 4 hours.

For the salt exposure experiment, the food with 4% of salt concentration was prepared. Food with salt and blue dye (Brilliant blue, Carbosynth) was made as well. Flies were kept in the salty blue food for 8 and 24 hours and photographed by microscope after that. The effect of starvation of the flies was studied as well. The flies were not given food, but only 3 cm thick cotton infiltrated with water was put into the vial. The flies were kept in these no food conditions for 24 hours.

## 2.3 Laboratory techniques

### 2.3.1 Fly laboratory

Inside the fly laboratory the fruit flies were handled under a light microscope. The flies were sedated by using carbon dioxide. The flies were flipped onto new food weekly. The bottles were emptied and the flies that were going to be used in experiments were collected to vials two days after the bottles were emptied. We collected only male flies. After the flies had been exposed to environmental stress, they were decapitated by using a small scalpel and forceps. Heads and bodies were then studied separately.

### 2.3.2 RNA-extraction

Three parallel samples with five flies each were used in every experiment conditions. RNA was extracted by using the RNA isolation procedure with TRI reagent protocol (Thermo Fischer Scientific). Firstly, the fly tissue samples were homogenized in eppendorf tubes with TRI reagent by using micropestles. Then the homogenate was stored at room temperature for five minutes. Chloroform was added to the samples and the tubes were shaken by vortex for 15 seconds and then incubated at room temperature for 15 minutes. The tubes were centrifuged at 12,000 xg at 4°C for 15 minutes. After that the top aqueous phase was collected. Isopropanol was added to tubes, and then they were vortexed briefly and incubated at room temperature for ten minutes. The tubes were centrifuged for 8 minutes in same conditions as before. The supernatant was discarded after that. Then 500 µl of 75% ethanol was pipetted into the tubes. Tubes were centrifuged at 7500xg at 25°C for

five minutes, then the ethanol was removed completely. Then the pellet with RNA from the fly bodies was dissolved in 50 µl of milliQ-water and heads 20 µl of milliQ-water. The next step was to measure the extracted RNA concentration by using the nanodrop 2000.

## 2.4 Quantitative real-time PCR

The qRT-PCR was done with iTaq Universal SYBR green One step kit (Bio-Rad, Hercules, CA, USA). The used amount of RNA was approximately 40 ng/sample. The kit turns the extracted RNA into cDNA using reverse transcriptase enzyme. The primers that were used in quantitative PCR reactions are shown in table 1. *IBIN* gene was measured in temperature experiment and *IBIN* and *IBIN* like in salt and starvation experiment. The expression of the housekeeping gene *ND-39* was measured in all the different stress conditions as well. That expression was used as a comparison when normalizing the differences in the RNA amounts between measured samples, because it is known that that gene is expressed similarly regardless of outside stress (Valanne *et al.*, 2019).

**Table 1.** Used primers in qRT-PCR

Gene	forward 5'→3'	reverse 5'→3'
IBIN	CAACTGCTGCCAATCCTCG	GCCTGGGATCGTAGTCACTT
IBIN-like	GCTCTTGTTATTTGTGCTGCC	GGCATAGGGATTTCGAAGGC
ND-39	ACCGACAAGGTTCTGACTGG	CTCCOCTTAGGCAAACAGAC

## 2.5 Statistical analysis

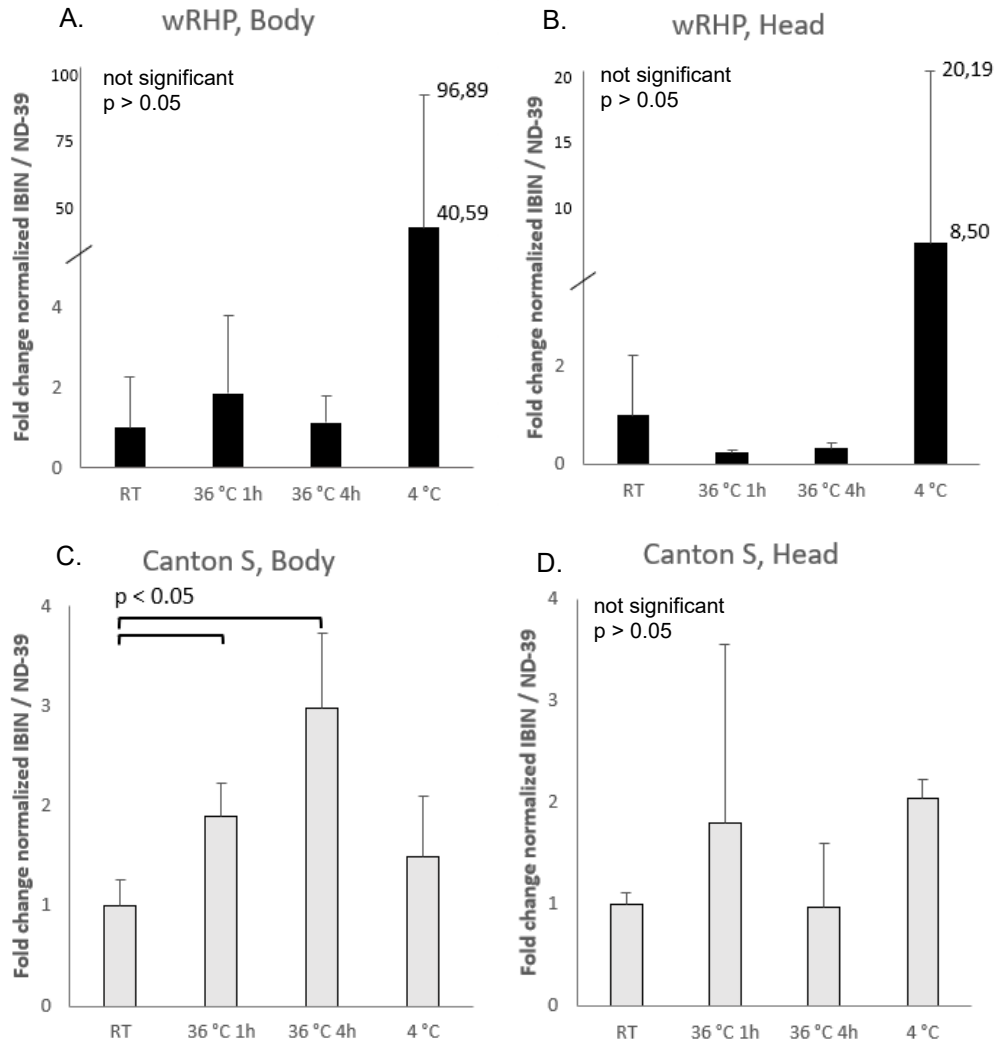
Bar graphs of the results were made by using Microsoft Excel. Statistical analysis was used when studying the statistical significance of the qRT-PCR gene expression results. Studied fly groups were compared to the room temperature flies in the temperature stress experiment. In salt and starvation experiment the groups were compared to the control flies which were given normal food. The used test was two-tailed t-test for two samples. Tests were conducted using R version 4.3.3. The level of significance was set to  $p < 0.05$ .

## 3. RESULTS

Temperature was chosen as a study subject because previous research shows that some of the immune system's genes, such as heat-shock protein coding genes, of the *Drosophila melanogaster* are expressed differently in different temperatures (Wojda, 2017). The salt in the sustenance of the flies has been shown to affect the expression of some innate immune system related genes in *D. melanogaster* (Overend *et al.*, 2012). The starvation of the flies had been studied earlier and the starvation time and the choice to keep the flies hydrated was chosen according to that (Valtonen *et al.*, 2010).

### 3.1 Temperature

The expression of the *IBIN* gene was measured and the fold change was calculated by comparing the value to flies room temperature gene expression value. The results can be seen in figure 2. wRHP flies in 4°C expressed the *IBIN* gene. However, the results show that the change was there only in one of the three parallel samples. Because of that the standard error is large and the result is not statistically significant. It was found that in Canton S flies the expression was statistically significantly higher in hot conditions than in room temperature ( $p < 0,05$ ). Other groups were not significantly differentially expressed compared to the room temperature values. All other samples the *IBIN* gene was expressed more compared to the control room temperature groups but in the wRHP heads 36°C the expression was smaller.

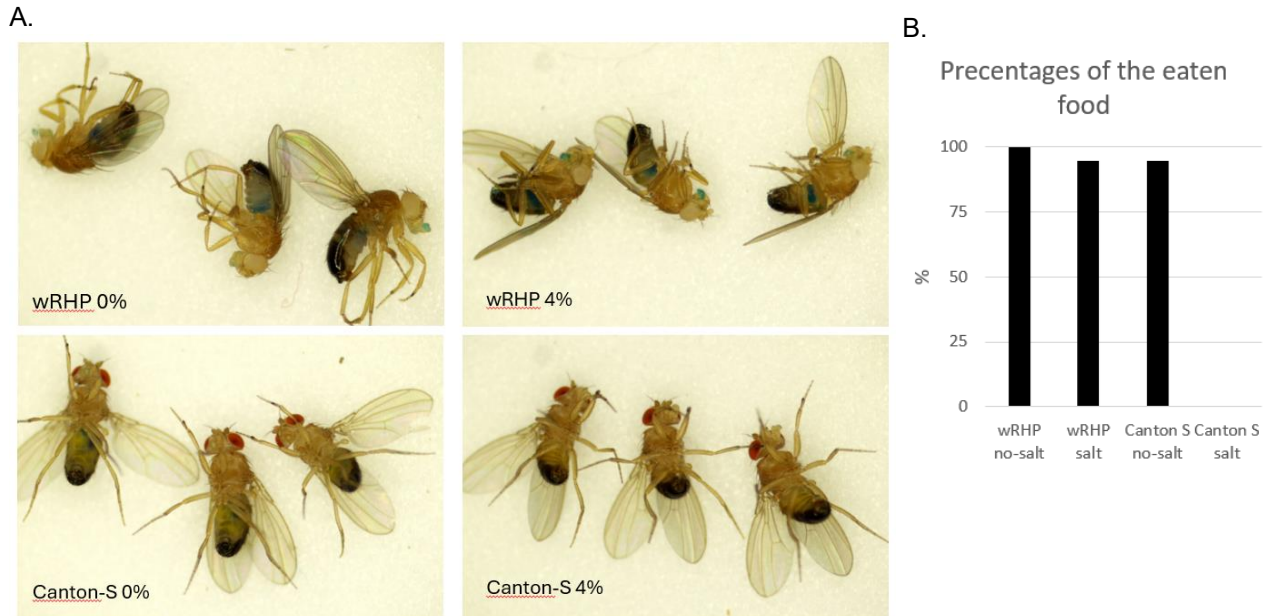


**Figure 2.** The expression of the IBIN gene in different conditions. wRHP body (A) and head (B), and Canton S body (C) and head (D)

### 3.2 Salt eating

Before analysing the results for salt and starvation experiment, we studied if the flies would eat the salty food or not. Food with 4% salt and blue dye (Brilliant blue, Carbosynth) was made. If the flies eat the food, it shows in their stomachs when observed by a microscope.

The pictures and how many flies had eaten the food can be seen in figure 3. Blue colour is clearly visible in all but Canton S 4% picture. The graph (Figure 3B) shows that all or nearly all flies from both of the lines ate the blue food without salt, but when salt concentration was 4% none of the Canton S flies ate the food.

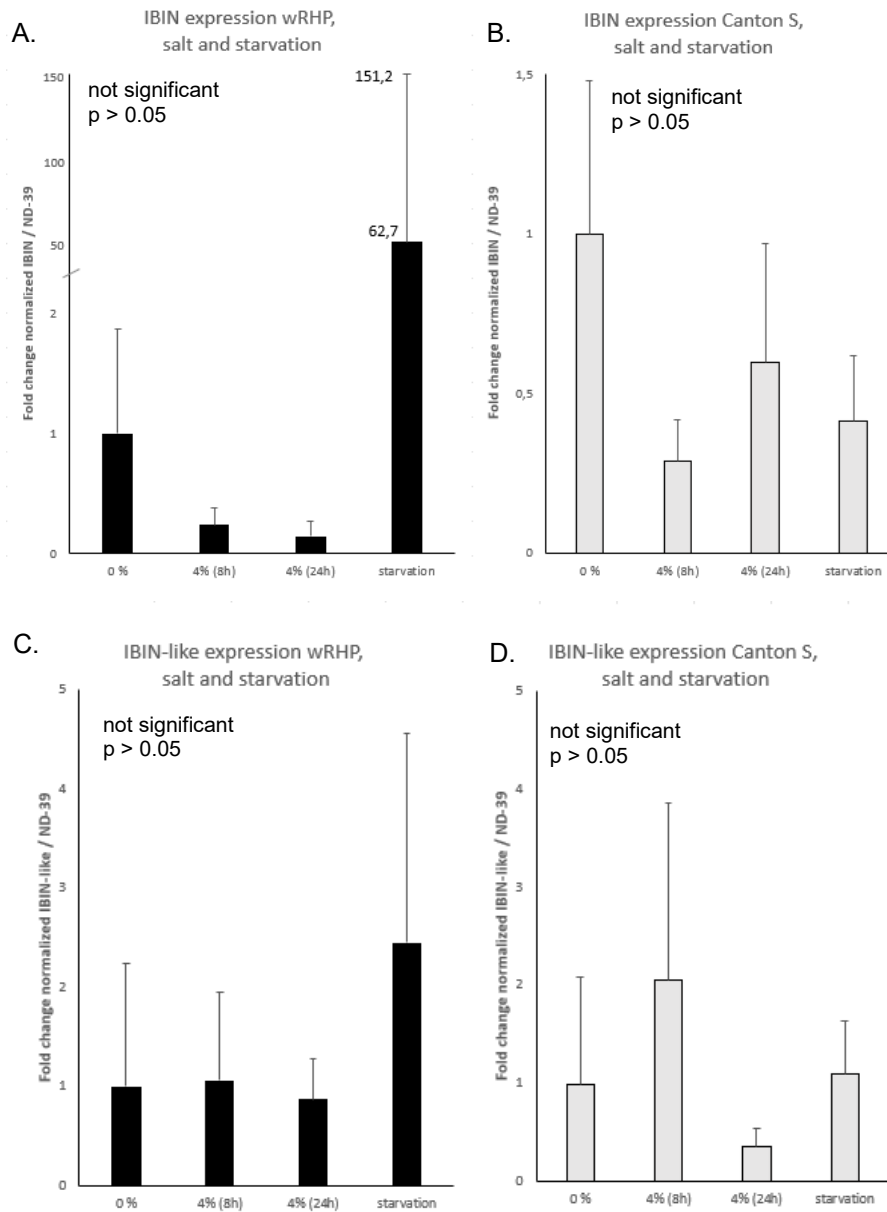


**Figure 3.** A. Pictures taken from microscope (Nikon DeltaPix) after flies were 24 hours prior given the blue food. B. Graph shows the percentages of how many flies had eaten blue food.

The Canton S flies did not eat the food that had 4% salt. Therefore, in the salt and starvation experiment part of the research only the starvation of the flies was in fact studied. wRHP flies ate the salty food, so it was possible to study salt exposure in them.

### 3.3 Salt exposure and starvation

*IBIN* and *IBIN-like* genes fold changes were calculated in a similar manner compared to the temperature flies. Only the bodies of the flies were studied in this experiment, because in the temperature experiment there was not a clear difference in the gene expression between bodies and heads. Gene expression was compared to the control groups that were given standard fly food with no salt in it. Results are shown in figure 4. It was found that none of the results were statistically significant. The *IBIN* gene was expressed less in wRHP flies in salty food than in the normal saltless food. Starvation of the wRHP flies affected in a way that the *IBIN* and *IBIN-like* genes fold changes are larger than in the control groups. However, the standard errors are quite big, and for that reason the result is not statistically significant. On the other hand, the wRHP flies which were given salty food seem to express the *IBIN* gene less than the control group. In the Canton S flies since they did not eat the blue food as shown in the materials and methods section, the results all show the effect of starvation to the flies. The *IBIN* gene is expressed less when the Canton S flies were starved than in control group. Salt exposure did not affect the *IBIN-like* gene expression significantly.



**Figure 4.** The expression of IBIN (A,B) and IBIN-like (C,D) genes when the flies were given food with 4% salt or no food at all.

## 4. CONCLUSIONS

The results of this study were not very clear and easily interpreted. Some conditions affected the genes by upregulating and other by downregulating them. Regarding the temperature experiment, in Canton S flies the *IBIN* gene expression was statistically significantly higher in hot conditions than in room temperature. All other samples the *IBIN* gene was expressed more compared to the control room temperature groups but in the wRHP heads 36°C the expression was smaller. It seems that temperature might upregulate the expression of the *IBIN* gene.

The Canton S flies did not eat the food that had 4% salt. Therefore, in the salt and starvation experiment part of the research only the starvation of the flies was in fact studied. That was a notable finding in this study and could be an interesting research topic in the future. Discovering reasons why the different fly lines reacted differently to the salty food might have something to do with the mutation in the gene that controls the pigmentation of the eye. In wRHP flies the *IBIN* gene was expressed less in salty food, but more in starvation than in the normal saltless food. In other groups, it seems, that the expression fold change did not differ much from the fold change of the control groups.

In conclusion it can be said that environmental stressors might influence the expression of *IBIN* and *IBIN-like* genes in some cases downregulating and other cases upregulating the expression. There were not any conditions in this study that highly upregulated the expression. Perhaps there is some other conditions that could affect the expression which still need more research. The expression of the *IBIN* gene is close to zero in normal conditions. That is why there should be seen highly induced expression if the gene was regulated by environmental stressors. However, there was not that clear effect, and therefore it can be said that the studied stress factors did not affect the gene expression significantly in most of the conditions.

*Drosophila melanogaster* is an important model animal in many different biomedical research fields. Ethical aspects are important when considering what research model animal to use in study. Fruit flies are a good choice of model animal in several aspects including ethical reasons. They are insects and therefore have not as developed brain functions as more complex animals such as mammals. Although different tissue engineered alternatives are constantly being developed to replace animal models in research, *Drosophila melanogaster* remains an important organism to biomedical research.

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