The transcription factor dFOXO controls the expression of insulin pathway genes and lipids content under heat stress in Drosophila melanogaster

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Abstract. The insulin/insulin-like growth factor signaling (IIS) pathway is one of the key elements in an organism’s response to unfavourable conditions. The deep homology of this pathway and its evolutionary conservative role in controlling the carbohydrate and lipid metabolism make it possible to use Drosophila melanogaster for studying its functioning. To identify the properties of interaction of two key IIS pathway components under heat stress in D. melanogaster (the forkhead box O transcription factor (dFOXO) and insulin-like peptide 6 (DILP6), which intermediates the dFOXO signal sent from the fat body to the insulin-producing cells of the brain where DILPs1–5 are synthesized), we analysed the expression of the genes dilp6, dfoxo and insulin-like receptor gene (dInR) in females of strains carrying the hypomorphic mutation dilp641 and hypofunctional mutation foxoBG01018. We found that neither mutation influenced dfoxo expression and its uprise under short-term heat stress, but both of them disrupted the stress response of the dilp6 and dInR genes. To reveal the role of identified disruptions in metabolism control and feeding behaviour, we analysed the effect of the dilp641 and foxoBG00124 mutations on total lipids content and capillary feeding intensity in imago under normal conditions and under short-term heat stress. Both mutations caused an increase in these parameters under normal conditions and prevented decrease in total lipids content following heat stress observed in the control strain. Thus, we may conclude that dFOXO takes part in regulating the IIS pathway response to heat stress as well as the changes in lipids content caused by heat stress, and this regulation is mediated by DILP6. At the same time, the feeding behaviour of imago might be controlled by dFOXO and DILP6 under normal conditions, but not under heat stress.

Key words: Drosophila melanogaster; insulin/insulin-like growth factors signaling pathway; dInR; dilp6; dfoxo; gene expression; feeding behaviour; total lipids.

For citation: Eremina M.A., Menshanov P.N., Shishkina O.D., Gruntenko N.E. The transcription factor dFOXO controls the expression of insulin pathway genes and lipids content under heat stress in Drosophila melanogaster. Vavilovskii Zhurnal Genetiki i Selektcii = Vavilov Journal of Genetics and Breeding. 2021;25(5):465-471. DOI 10.18699/VJ21.053
Introduction
Nowadays, as living beings often encounter unfavourable environmental conditions such as pollution and global warming, the study of deeply conservative mechanisms that contribute to adaptation is of current interest. It is known that such influences launch the development of nonspecific adaptive defensive responses on molecular (Garbuz, Evgen’ev, 2017), behavioral (Kaluev, 1999), biochemical and physiological (Gruntenko, 2008; Even et al., 2012; Miyashita, Adamo, 2020) levels. The ability to respond to stress in an integrated manner, which comprises behavioral, metabolic and molecular reactions, is key for survival and adaptation of animals including insects (Koyama et al., 2020). It was proven that besides its role as crucial modulator of growth and metabolism, in insects, the IIS pathway is an essential component of the neuroendocrine stress reaction (Gruntenko, Rauschenbach, 2018; Lubawy et al., 2020). Due to the deep homology of this pathway in animals of different taxa including humans and flies, it is possible to use the latter as an object for investigating evolutionary-conservative mechanisms underlying molecular-genetic regulation of the IIS pathway, and carbohydrate and lipid metabolism it controls. As in most animals, in insects, carbohydrates and lipids serve as the main energy supply (Arrese, Soulages, 2010). The processes of producing and storing energy undergo complex modulation by many inner factors including heritage, lifestyle, hormones, metabolites, as well as various outside influences (Mattila, Hietakangas, 2017).

Drosophila’s applicability to the research of metabolism is defined by the simplicity of its IIS pathway regulation (Fig. 1), which involves homologues of insulin (DILPs1–5) and insulin-like growth factors (DILP6) of mammals connecting to a single insulin-like receptor (dInR), which activates the pathway (Gruntenko, Rauschenbach, 2018), and two homologues of relaxin (DILPs7,8) (Gontijo, Garelli, 2018). The dInR signal being transduced directly or via its substrate CHICO (the homologue of insulin receptor substrates of mammals, IRS1–4) causes Akt/PKB (protein kinase B homologue) to activate, which in turn modulates the activity of a number of proteins, in particular, it phosphorylates transcriptional factor of Forkhead box class O family, dFOXO (homologue of mammalian FOXO), which is synthesized in the fat body and controls the transcription of more than a thousand genes (Bai et al., 2012), and inhibits its translocation into the nucleus (Puig et al., 2003; Slack et al., 2011; Álvarez-Rendón et al., 2018). Under stress, dFOXO is translocated to the nucleus (Jünger et al., 2003; Hwangbo et al., 2004; Gruntenko et al., 2016) activating the expression of a number of genes including dInR via a feedback loop (Gruntenko, Rauschenbach, 2018). It was also previously shown that the expression of dlip6 in the fat body inhibits the expression of dlip2 and dlip5 in imago’s brain as well as the secretion of DILP2 into the hemolymph, and that dFOXO influence on the expression of DILPs produced in median neurosecretory cells is mediated by DILP6 synthesized in the fat body (Slaidina et al., 2009; Bai et al., 2012). Thus, DILPs seems to connect dFOXO, adipose tissue and endocrine function of the brain, creating a feedback loop back to dInR.

Stress reaction causes the mobilization of organism’s energy reserves along with a variety of metabolic changes. In a changing environment, feeding behaviour plays an important role in adaptation (Rabasa, Dickson, 2016). It is known that in mammals, acute stress is usually accompanied by feeding suppression and a decrease in weight gain; chronic stress can result in excessive food intake, weight gain and obesity (Rabasa, Dickson, 2016).

This study aimed to analyse the expression of dlnR, dlip6 and dfoxo genes of three key components of the IIS pathway, which is involved in neuroendocrine stress reaction, in D. melanogaster strains carrying dlip641 and foxoBG01018 mutations under heat stress, and to evaluate the latter’s influence on feeding behaviour and total lipids content in these strains.

Materials and methods
Drosophila melanogaster strains and stress conditions. Three D. melanogaster strains were used in this study: strain dlip641 with the deletion covering the 3’ region of phiI gene and 5’ upstream region of dlip6 including the first exon and part of the first intron (Rauschenbach et al., 2017); strain
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**Fig. 1. The scheme of insulin/insulin-like growth factors signaling pathway in Drosophila.**

DILPs are *Drosophila* insulin-like growth peptides, dInR – *Drosophila* insulin-like receptor, CHICO – homologue of mammalian insulin receptor substrate, PI3K – phosphoinositide 3 kinase, dPDK1 – *Drosophila* phosphoinositide-dependent kinase-1, dAkt/PKB – a homolog of mammalian protein kinase B, dFOXO – *Drosophila* forkhead box O transcription factor.

**Primers used in RT-PCR**

| Gene | Amplicon, bp | Forward/Reverse | Sequence (5’→3’) | Tm, °C | Reference |
|------|--------------|----------------|------------------|--------|-----------|
| dfoxo | 196          | F              | GCCTAGATCACTTTCCGAG | 53     | Gruntenko et al., 2016 |
|      |              | R              | GTACGCTCCGCTCATTGT | 55     |           |
| dilp6 | 149          | F              | CACGGAATACGAAACGACAG | 55     | Eremina et al., 2019 |
|      |              | R              | TCACGTTACATACTGCAG | 55     |           |
| dinR | 123          | F              | TGAACGTGAGACACATCAAGAC | 59     | Okamoto et al., 2013 |
|      |              | R              | CGAGGAGATTTCTGTCTGCTG | 58     |           |
| ActSC | 90           | F              | GCAGCTTTACTCTCTACCA | 58     | Guio et al., 2014 |
|      |              | R              | ATGTACCGAGACATTTCAG | 55     |           |

**foxoBG01018**, which carries a P[GT1] element transposon in the 5’ upstream region of the *dfoxo* gene, resulting in a mild loss of function (Dionne et al., 2006); and their progenitor strain w^118^ as a control. The stocks were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN, USA).

The cultures were raised on standard medium (agar-agar, 7 g/l; corn grits, 50 g/l; dry yeast, 18 g/l; sugar, 40 g/l) and kept at 25 °C, 12:12 h photoperiod, relative humidity 50 %.

Imagoes were synchronised at eclosion (flies were collected every 3–4 hours). Females were exposed to heat stress by transferring vials with flies from a 25 °C incubator to a 38 °C incubator for 60 or 90 min. After 60 min of stressing flies were returned to 25 °C, after 90 min they were subsequently frozen in liquid nitrogen and stored at –80 °C.

**Quantitative real-time polymerase chain reaction (qRT-PCR).** mRNA quantity of *dilp6*, *dfoxo* and *dinR* genes was evaluated in whole body homogenates of Canton-S females (15 flies/sample) using TRI reagent Lot #BCBT8883 (Sigma-Aldrich, USA) for total RNA extraction, Revert Aid First Strand cDNA Synthesis Kit #K1621 (Thermo Fisher Scientific, USA) with oligo (dT)18 primer for synthesis of cDNA, M-427 Kit with SYBR-Green I (Syntol, Russia) and CFX96 Touch qPCR System (Bio-Rad, USA) for performing qRT-PCR. Each reaction was performed in triplicates with three biological replicates. Data were normalized against *Act5C*. High stability of *Act5C* expression under heat stress was shown by Ponton et al. (2011). The primers used in the study are shown in the Table.

**Total lipid quantification.** Quantification of total lipids was performed using Van Handel’s method (1985) modified for *D. melanogaster* (Eremina, Gruntenko, 2020) under normal conditions or in 24 h after 60 min under 38 °C. Flies (1 fly per sample, 10–20 samples per each studied group) were decapitated to avoid the influence of eye pigment on the measurement results, homogenised on ice in 100 μl of chloroform-methanol (1:1) and shaken for 10 min. 50 μl of supernatant were transferred to new tubes and placed in microthermostat M-208 (Bis-N, Russia) at 90 °C till the solvent completely evaporated. Then 10 μl of 95% H2SO4 were added to each sample and they were again kept at 90 °C for 2 min. After
that the samples were cooled on ice and the phosphovanillin 
color reagent (85 % H₃PO₄ + 6 % vanillin solution (4:1)) was 
added up to 1 ml of volume. The samples were incubated for 
15 min at room temperature till pink colouration appeared and 
was stable for 1 h. Then the samples were measured by Smart 
Spec Plus spectrophotometer (Bio-Rad, USA) at 525 nm.

Feeding behaviour analysis (CAFE). Ingestion was mea-
sured using the Capillary Feeder (CAFÉ) method of Ja et 
al. (2007), modified by Williams et al. (2014). To provide 
flies with a humid environment, flat-bottomed glass vials 
(20 × 100 mm) with 1 % agarose (5 cm high) were placed into 
microcentrifuged 50 ml tubes filled with 7 ml of water. Each 
glass capillary (10 × 90 mm, Narishige, Japan) was filled with 
20 μl of liquid food containing 5 % sugar and 5 % east extract 
(Biospringer, France). Five females were placed into each vial 
(4–9 vials per group), which was plugged with a foam 
plug. A capillary was inserted into it through 10 μl and 200 μl 
pipette tips and was held in place by them. The vials with 
flies were kept in an incubator (Sanyo, Japan) at 25 °C, 50 % 
relative humidity, 12:12 h photoperiod for 24 or 48 h. Before 
that the experimental group was subjected to short-term heat 
stress (38 °C, 60 min). Initial and final food levels in capil-
laries were marked to determine total food consumption per 
day. To minimize food evaporation, capillaries were topped 
with a 0.1 μl oil layer. To adjust for food evaporation, a vial 
without flies was used.

Statistical analysis. Data on gene expression were analyzed 
by the 2^{-∆∆CT} method (Livak, Schmittgen, 2001). All data are 
presented as means ± SEM and analysed by ANOVA. The 
results were considered significant at p < 0.05.

Results and discussion

To discover whether disruption of the feedback loop of the 
IIS pathway regulation affects its stress response, we studied 
the expression of three key genes of the pathway, dilp6, foxo 
and dlnR, in D. melanogaster females carrying hypomorphic 
mutation dilp6BG01018 and hypofunctional mutation foxoBG01018 
under normal conditions or heat stress (38 °C, 90 min). There 
were no quantitative changes in mRNA expression level of 
dilp6 and dlnR genes in dilp6BG01018 and foxoBG01018 strains under 
heat stress, whereas in their progenitor strain w1118 the expres-
sion of dilp6 decreased, and the expression of dlnR increased 
under heat stress (Fig. 2, p < 0.05 for both genes). At the 
same, foxo expression level increased or had a tendency to 
increase under heat stress in all strains under study (see Fig. 2, 
STRAIN – F(2,12) = 3.14, p < 0.081; STRESS – F(1,12) = 12.80, 
p < 0.0038). Notably, dilp6BG018 mutants are characterised by 
a lower dilp6 expression (p < 0.001); however, foxo expres-
sion in foxoBG01018 mutants does not differ from the control 
strain w1118 (see Fig. 2). This allows us to assume that the 
previously described loss of dFOXO function in foxoBG01018 
strain (Dionne et al., 2006) is connected not with a lowered 
expression level of the corresponding gene but with a defect 
in its structure.

The results of qualitative measurement of total lipids in 
D. melanogaster females with dilp6BG018 and foxoBG01018 mutations 
under normal conditions or following heat stress (38 °C, 
60 min) signify that both mutations cause an increase in 
lipid content in comparison with the control strain w1118, and 
lipid content in dilp6BG018 and foxoBG01018 strains, unlike in their 
progenitor strain, does not decrease in 24 h after heat stress.
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These results correspond well with our data on the increased content of total lipids in females of dilp641 and foxoBG01018 strains (see Fig. 3, a), as well as with increased glucose and trehalose levels in dilp641 and foxoBG01018 mutants we previously demonstrated (Eremina et al., 2019).

Regarding regulation of feeding behaviour under heat stress it seems to occur independently from dilp6 and dfxoxo genes as their mutations do not inhibit loss of appetite following stress (see Fig. 3, b).

Conclusion

Thus, we have shown that the disruption of dilp6 and dfxoxo gene functions in Drosophila melanogaster (1) results in the feedback loop of the IIS pathway being disrupted under heat stress, (2) leads to an increase in total lipids content under normal conditions and impedes their decrease following heat stress, and (3) causes an increase in feeding intensity under normal conditions but does not impede its decrease following heat stress.

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dFOXO регулирует экспрессию генов инсулинового сигнального каскада и содержание липидов при стрессе

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