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# Fertility differences between two wild-type *Drosophila melanogaster* lines correlate with differences in the expression of the *Jheh1* gene, which codes for an enzyme degrading juvenile hormone

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**Abstract.** Juvenile hormone plays a "status quo" role in *Drosophila melanogaster* larvae, preventing the untimely metamorphosis, and performs a gonadotropic function in imagoes, ensuring the ovaries' preparedness for vitellogenesis. The decreased level of juvenile hormone results in reproductive disorders in *D. melanogaster* females including a delay in the oviposition onset and a fertility decrease. Another factor that can affect the insect reproduction is an infection with the maternally inherited symbiotic α-proteobacterium *Wolbachia*. The present study is devoted to the analysis of the expression of two juvenile hormone metabolism genes encoding enzymes of its synthesis and degradation, juvenile hormone acid O-methyltransferase (*jhamt*) and juvenile hormone epoxide hydrase (*Jheh1*), respectively, in four wild-type *D. melanogaster* lines, two of them being infected with *Wolbachia*. Lines *w153* and *Bi90* were both derived from an individual wild-caught females infected with *Wolbachia*, while lines $w153^T$ and $Bi90^T$ were derived from them by tetracycline treatment and are free of infection. Line *Bi90* is known to be infected with the *Wolbachia* strain *wMel*, and line *w153*, with the *Wolbachia* strain *wMelPlus* belonging to the *wMelCS* genotype. It was found that infection with either *Wolbachia* strain does not affect the expression of the studied genes. At the same time, it was shown that the *w153* and $w153^T$ lines differ from the *Bi90* and $Bi90^T$ lines by an increased level of the *Jheh1* gene expression and do not differ in the *jhamt* gene expression level. Analysis of the fertility of these four lines showed that it does not depend on *Wolbachia* infection either, but differs between lines with different nuclear genotypes: in *w153* and $w153^T$, it is significantly lower than in lines *Bi90* and $Bi90^T$. The data obtained allow us to reasonably propose that the inter-line *D. melanogaster* polymorphism in the metabolism of the juvenile hormone is determined by its degradation (not by its synthesis) and correlates with the fertility level.

Key words: *Drosophila melanogaster*; *Wolbachia*; *jhamt*; *Jheh1*; gene expression; fertility; juvenile hormone metabolism.

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# Различия в плодовитости между двумя линиями *Drosophila melanogaster* дикого типа коррелируют с различиями в экспрессии гена *Jheh1*, кодирующего фермент деградации ювенильного гормона

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**Аннотация.** Ювенильный гормон играет у личинок *Drosophila melanogaster* роль "status quo" гормона, препятствуя преждевременному наступлению метаморфоза, а у имаго выполняет гонадотропную функцию, обеспечивая подготовку яичников к вителлогенезу. При снижении уровня ювенильного гормона у самок *D. melanogaster* наблюдаются нарушения репродукции, выражающиеся в задержке начала откладки яиц и снижении плодовитости. Еще одним фактором, способным повлиять на репродуктивную функцию насекомых, является инфицирование матерински наследуемой симбиотической α-протеобактерией *Wolbachia*. Настоящее исследование посвящено анализу экспрессии двух генов метаболизма ювенильного гормона, кодирующих ферменты его синтеза и деградации – кислую О-метилтрансферазу ювенильного гормона (*jhamt*) и эпоксидгидразу ювенильного гормона

(*Jheh1*) соответственно, у четырех линий *D. melanogaster* дикого типа, две из которых инфицированы *Wolbachia*. Линии *w153* и *Bi90* происходят от отдельных самок, отловленных в дикой природе, и инфицированы *Wolbachia*, а линии $w153^T$ и $Bi90^T$ получены на их основе посредством тетрациклиновой обработки и характеризуются отсутствием инфекции. Известно, что линия *Bi90* инфицирована штаммом *Wolbachia* генотипа *wMel*, а линия *w153* – штаммом *Wolbachia wMelPlus*, относящимся к генотипу *wMelCS*. Обнаружено, что инфицирование как одним, так и другим штаммом *Wolbachia* не влияет на экспрессию исследованных генов. В то же время показано, что обе линии с ядерным генотипом *w153* отличаются от линий с генотипом *Bi90* повышенным уровнем экспрессии гена *Jheh1* и не различаются по уровню экспрессии гена *jhamt*. Уровень плодовитости не зависел от инфицирования *Wolbachia*, но у линий с ядерным генотипом *w153* он оказался существенно ниже, чем у линий с ядерным генотипом *Bi90*. Полученные данные позволяют сделать обоснованное предположение о том, что межлинейный полиморфизм *D. melanogaster* по метаболизму ювенильного гормона коррелирует с уровнем плодовитости и определяется деградацией гормона, а не его синтезом.
Ключевые слова: *Drosophila melanogaster*; *Wolbachia*; *jhamt*; *Jheh1*; экспрессия генов; плодовитость; метаболизм ювенильного гормона.

## Introduction

According to the current understanding of the genetic control of Diptera reproduction, a key role is played by 20-hydroxyecdysone (20E), while juvenile hormone (JH) only prepares ovaries for vitellogenesis, unlike in most other insect orders, where JH has the function that in Diptera is performed by 20E (Roy et al., 2018; Wu et al., 2021). The balance between these two hormones determines many events in the life of holometabolic insects from the larval period, where 20E initiates the start of moulting, and the JH level determines whether it will be larval moulting (if it is high), or the onset of metamorphosis (if it is low) (Truman, Riddiford, 2007), the neurohormonal stress response, which involves both hormones, and the regulation of changes in ovaries under heat stress or starvation (Gruntenko et al., 2003a; Terashima et al., 2005; Gruntenko, Rauschenbach, 2008).

Despite the secondary role of JH in oogenesis regulation and reproduction control in *Drosophila*, there are data indicating that in flies with a decreased level of JH, the reproduction process is disrupted, which is expressed as a delay in oviposition onset and a fertility decrease (Altaratz et al., 1991; Gruntenko et al., 2003b; Yamamoto et al., 2013; Meiselman et al., 2017), and endogenic JH treatment of females speeds up egg maturation (Richard et al., 2001). Thus, we can assume that through controlling vitellogenins uptake by oocytes (Berger, Dubrovsky, 2005), JH takes part in the determination of the fertility level in *Drosophila*.

The intracellular signaling of JH is well described in the literature (Jindra et al., 2015; Roy et al., 2018), including the JH receptor complex Methoprene-tolerant (Met) – Taiman – Germ cell-expressed (Gce), the heat shock protein HSP83 and nucleoporin Nup358, which interact with Met and ensure JH transfer into the nucleus and the activation of the transcriptional factor Kr-h1 by it. At the same time, the mechanisms of the JH level regulation are still underresearched.

To add to the knowledge regarding this subject, we have estimated the level of fertility and expression of the genes responsible for JH synthesis and degradation, *jhamt* and *Jheh1*, in four *Drosophila melanogaster* lines, two of which were earlier demonstrated to differ in the fertility level (Adonyeva et al., 2021). *jhamt* codes for juvenile hormone acid O-methyltransferase (JHAMT), transforming JH acid or inactive JH precursors into the active form of the hormone at the final stage of JH biosynthesis in insects (Niwa et al., 2008). *Jheh1* codes for one of the forms of JH epoxide hydrolase that inactivates the hormone *via* hydrolysis of the epoxide functional group producing JH diol (Flatt et al., 2005).

Notably, another factor capable of affecting fly fertility as well as JH metabolism is the infection with the maternally-inherited symbiotic α-proteobacterium *Wolbachia pipientis* (Werren et al., 2008; Burdina, Gruntenko, 2022). *Wolbachium* is a widely spread intracellular insect symbiont infecting more than 40 % of the studied species and greatly affecting host physiology (Werren et al., 2008; Burdina, Gruntenko, 2022). As lines $w153^T$ and $Bi90^T$, the differences in the fertility of which were shown earlier, were derived from lines *w153* and *Bi90*, which, in turn, were derived from single females caught in nature and were initially infected with *Wolbachia*, we decided to use for analysis lines *w153* and *Bi90*, carrying the infection, and lines $w153^T$ and $Bi90^T$, having undergone antibacterial therapy, to search for possible effects of *Wolbachia* on the fertility level and the expression of the JH metabolism genes.

## Materials and methods

***Drosophila* lines.** In the work, we used four *D. melanogaster* lines: the *w153* and *Bi90* lines, derived from single females and carrying *Wolbachia* strains of the *wMelCS* and *wMel* genotypes, respectively (Ilinsky, 2013), and their derivatives, $w153^T$ and $Bi90^T$, which underwent antibacterial therapy prior to the start of experiments. The lines were received from the collection of the Institute of Cytology and Genetics SB RAS. Notably, the *Wolbachia wMelPlus* strain, infecting the *w153* line, differs from other published strains of *wMelCS* by a large chromosomal inversion (Korenskaia et al., 2022).

Flies were kept on a standard medium (agar-agar, 7 g/l; corn flour 50 g/l; dry yeast 18 g/l; sugar 40 g/l) in an incubator (Sanyo, Japan) at a temperature of 25 °C, relative humidity of 50 %, and 12:12 h light cycle. For the experiments, flies were synchronized at eclosion (they were collected 3–4 h afterwards). To analyze fertility and gene expression levels, 10-days-old females were taken.

**Total RNA isolation and real-time RT-PCR.** To assess the number of mRNA of the *jhamt* and *Jheh1* genes, 15 females per biological replicate per line were frozen in liquid nitrogen in 1.5 ml Eppendorf tubes. In total, three biological

replicates of all four *Drosophila* lines were performed. After removing the tubes from liquid nitrogen, 150 µl of TRI reagent No. BCBT8883 (Sigma, USA) was added to each tube and flies were homogenized. To remove large tissue fragments from the homogenate, the tubes were centrifuged for 5 min at 10,000 rpm in an Eppendorf centrifuge at a temperature of 7 °C and then the homogenate was transferred to clean 0.5 µl tubes. 30 µl of cold chloroform was added, and after shaking the tubes were left for 15 min at room temperature. Afterwards, the homogenate was centrifuged for 15 min at 12,000 rpm and a temperature of 7 °C. 75 µl of cold isopropanol was added to the supernatant, and after shaking the tubes were left for 10 min at room temperature. After centrifugation (12,000 rpm; 10 min), pellets were washed with 150 µl of 75° ethanol twice with centrifugation in-between, dried and dissolved in 100 µl of deionized water. RNA concentration was measured with the use of Nanodrop OneC (Thermo Scientific, USA) and adjusted to 200 ng/µl by the addition of deionized water. cDNA synthesis was performed with the use of ABScript III RT Master Mix for qPCR with gDNA Remover No. RK20429 (ABclonal Technology, China) in accordance with the manufacturer's protocol.

*jhamt* and *Jheh1* expression were analyzed with CFX96 Touch amplificator (Bio-Rad, USA) using real-time RT-PCR with the M-427 set with SYBR-Green I (Syntol, Russia). Data were normalized on *Act5C*. For every sample, three technical replicates were performed. Primer sequences used in the study are presented in Table 1.

**Fertility.** To assess fertility, three male-female pairs aged 0–5 h were placed in cultivation vials (10 vials per experimental group), where they were left to lay eggs under standard conditions; flies were transferred to new vials every 24 h for 10 days. Fertility was calculated as the number of offspring (imagoes) eclosed from the eggs laid by experimental flies during the 10th day per one parent female.

**Statistical analysis.** Statistical significance of the differences in fertility (number of eggs per day per female) in the experimental groups was assessed using Student's *t*-test. Pairwise comparisons were performed using the Benjamini–Hochberg correction. In all cases, $p<0.05$ was considered statistically significant. Histogram data are presented as average means ± SEM.

Data on gene expression were analyzed by $2^{-\Delta\Delta CT}$ method (Livak, Schmittgen, 2001) using three biological replicates, each of which was obtained from three technical ones. Since Real-Time CFX96 Touch amplificator (Bio-Rad) provides only the average mean of three technical replicates and standard mean error, it is impossible to check normality, use non-parametric criteria or bootstrap. However, these data are sufficient to calculate sum of squares of the three replicates.

The general formula for the squared error of the mean:

$$SEM^2(\bar{x}) = \left(\sum x_i^2 - N\bar{x}^2\right)/\left(N\times(N-1)\right).$$

For each biological replicate, $N = 3$, and

$$\sum x_i^2 = 6SEM^2(\bar{x}) + 3\bar{x}^2.$$

This is sufficient to calculate both the average mean and it error:

$$\bar{\bar{x}} = \left(\sum \bar{x}_k\right)/3;$$

$$SEM^2(\bar{\bar{x}}) = \left(6\sum SEM^2(\bar{x}_k) + 3\sum \bar{x}_k^2 - 9\bar{\bar{x}}^2\right)/72.$$

The total sum of technical values for each average mean equals 9. When calculating Student's criterion of the significance of the difference between two average means, $2\times9 - 2 = 16$ degrees of freedom are obtained. It is known that significance criteria like Student's criterion are resistant to deviations from normality (Kendall, Stewart, 1961) due to the distribution of means approaching normality with increasing sample size.

As we made six comparisons in total, the Benjamini–Hochberg correction was additionally calculated for the *p*-value to compare with three standard significance levels (Narkevich, Vinogradov, 2020).

## Results and discussion

Real-time RT-PCR did not reveal significant differences in the expression level of the gene of juvenile hormone acid O-methyltransferase, *jhamt*, both between *D. melanogaster* lines *Bi90/Bi90$^T$* and *w153/w153$^T$* (the same genetic background but infected/uninfected with *Wolbachia*), and between lines *Bi90/w153* and *Bi90$^T$/w153$^T$* (the same infection status but different genetic background) (Fig. 1, *a*). At the same time, the expression of the JH epoxide hydrolase gene, *Jheh1*, was significantly decreased ($p<0.001$) in lines *Bi90* and *Bi90$^T$* compared to lines *w153* and *w153$^T$* (see Fig. 1, *b*). This means that *Wolbachia* infection has no influence on the expression level of *jhamt* and *Jheh1*, which allows us to assume that synthesis and degradation of JH do not change under the bacteria effect. On the other hand, the existing differences in the gene expression level of *Jheh1*, which encodes an enzyme degrading JH, between *D. melanogaster* lines *Bi90/Bi90$^T$* and *w153/w153$^T$* allow us to assume the existence of differences in the enzyme activity level and, as a result, in JH content,

Primer sequences used in RT-PCR

| Gene name | | 5′→3′ | Length | Tm, °C | Reference |
|---|---|---|---|---|---|
| *jhamt* | F | GTCAGTTGTTGAACGATGTGGGT | 23 | 58 | Qu et al., 2017 |
| | R | CCTCATTATTTTCACCTTGCTGC | 24 | 55 | |
| *Jheh1* | F | AGTTTACCAGGTTATGGCTGGTC | 23 | 57 | Guio et al., 2014 |
| | R | CAGTCTCCACCCTGGATAAAGAA | 23 | 56 | |
| *Act5C* | F | GCGCCCTTACTCTTTCACCA | 20 | 58 | Guio et al., 2014 |
| | R | ATGTCACGGACGATTTCACG | 20 | 55 | |

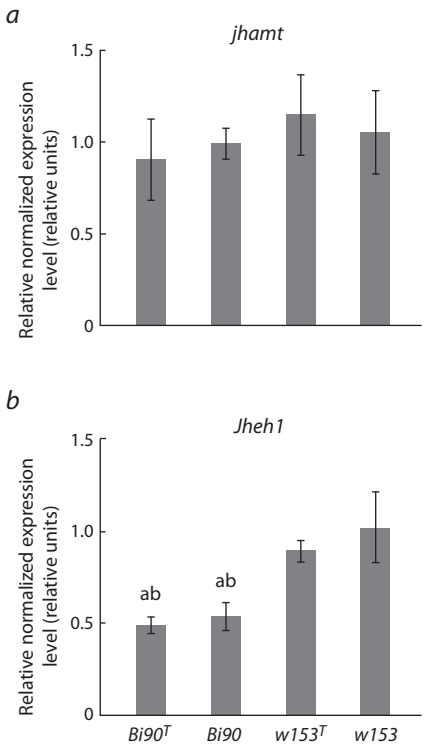


**Fig. 1.** Relative expression level of the *jhamt* (*a*) and *Jheh1* (*b*) genes in females of *D. melanogaster* lines *Bi90* (infected with the *wMel* strain of *Wolbachia*), *w153* (infected with the *wMelPlus* strain of *Wolbachia*), $Bi90^T$ (uninfected), $w153^T$ (uninfected).

Each value is the average mean of three biological replicates ± SEM. a – the significance of the differences from females of the w153 line ($p < 0.001$); b – the significance of the differences from females of the $w153^T$ line ($p < 0.001$).

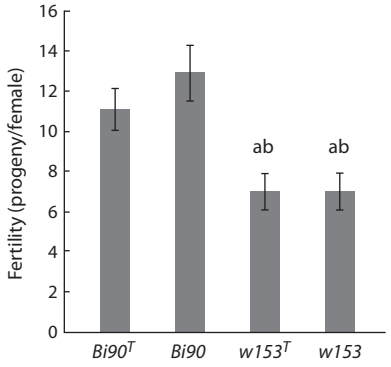


**Fig. 2.** Fertility level of *D. melanogaster* lines *Bi90* (infected with the *wMel* strain of *Wolbachia*), *w153* (infected with the *wMelPlus* strain of *Wolbachia*), $Bi90^T$ (uninfected), $w153^T$ (uninfected).

Each value is the average mean of 10 replicates (three females per replicate) ± SEM. a – the significance of the differences from females of the *Bi90* line ($p < 0.001$); b – the significance of the differences from females of the $Bi90^T$ line ($p < 0.001$).

the level of which should be elevated in the *Bi90* and $Bi90^T$ lines with a decreased expression level of the gene coding for an enzyme degrading the hormone.

Fertility analysis of the *Bi90*, $Bi90^T$, *w153* and $w153^T$ lines revealed that the line with a supposedly lower JH level (*w153* and $w153^T$) are characterized by significantly lower fertility ($p < 0.001$) compared to both the *Bi90* line and the $Bi90^T$ line (Fig. 2). This agrees well with earlier data on the correlation of low fertility level with low JH level (Altaratz et al., 1991; Gruntenko et al., 2003b; Yamamoto et al., 2013; Meiselman et al., 2017) or with the $Met^{27}$ mutation in the gene of the JH receptor (Gruntenko et al., 2000). Data regarding a decrease of the number of germline stem cells in the ovaries of *D. melanogaster*, carrying mutations in the *jhamt* and *Met* genes or with a knockdown of the latter, also indicate the important role JH plays in fertility regulation (Luo et al., 2020).

However, it is worth noting that most researchers attribute the demonstrated disruptions in reproduction and the decrease in fertility to disturbances in JH synthesis or the functioning of its receptor (Altaratz et al., 1991; Yamamoto et al., 2013; Meiselman et al., 2017; Luo et al., 2020), whereas our results indicate a correlation of interline differences in fertility in *D. melanogaster* with differences in the expression of gene encoding the enzyme that does not synthesize but degrades JH.

The lack of difference in fertility between the lines with the same genetic background infected and the uninfected with *Wolbachia* (*Bi90*/$Bi90^T$ and *w153*/$w153^T$) correlates with the lack of difference in the expression of genes responsible for JH synthesis and degradation and allows us to assume that *Wolbachia* does not influence this trait. There is a slight contradiction with our earlier data obtained on the $Bi90^{wMelPlus}$ line, where fertility and JH degradation differed from those of the *Bi90* line (Gruntenko et al., 2019).

However, it is necessary to note that the $Bi90^{wMelPlus}$ line was received by transferring cytoplasm carrying a *Wolbachia* strain from the *w153* line to the nuclear background of the *Bi90* line (*via* 20 generations of backcrossing females carrying the corresponding *Wolbachia* strain with males of the $Bi90^T$ line), and there is a nonzero probability that some aspects of *Wolbachia* influence on the physiology of the host can be attributed to the recent bacteria transfer and not merely to its presence in the cytoplasm. This hypothesis is indirectly confirmed by the lack of *Wolbachia* effect on fertility and JH degradation in the *Bi90* line, which was discovered in the same study (Gruntenko et al., 2019).

Additionally, transcriptomic analysis data obtained on infected *D. melanogaster* females revealed no changes in the differential expression of genes of the JH signaling pathway and the enzymes of its metabolism compared to uninfected females, which correlates with the lack of *Wolbachia* effect on the expression of the *jhamt* and *Jheh1* genes in *D. melanogaster* females in our work (Detcharoen et al., 2021; Lindsey et al., 2021).

## Conclusion

To sum up, three reasonable hypotheses could be made based on our data: 1) JH does play a certain part in the regulation *D. melanogaster* reproduction; 2) JH catabolism has no less, or perhaps more, of a role in providing interline polymorphism by the JH level; 3) *Wolbachia* does not affect the JH level and fertility in *D. melanogaster* given the long history of symbiosis between a certain bacterium strain and host line.

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