ВІСНИК
УКРАЇНСЬКОГО ТОВАРИСТВА
ГЕНЕТИКІВ І СЕЛЕКЦІОНЕРІВ

УДК 575.21

THE RELATIONSHIP OF *WOLBACHIA* INFECTION AND DIFFERENT PHENOTYPES IN THE *DROSOPHILA MELANOGASTER* NATURAL POPULATIONS FROM RADIOACTIVELY POLLUTED AND CLEAR AREAS IN UKRAINE

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Aim. *The study was performed to investigate the relationship between Wolbachia infection and phenotypes that distinct from wild-type of Drosophila melanogaster from different localities in Ukraine including those from Chornobyl Exclusion Zone during 2013–2014.* ***Methods.*** *We have established isofemale lines from populations: Uman', Inkerman, Odesa, Varva, Kyiv, Drogobych, Yaniv, Poliske, Chornobyl, and Chornobyl Nuclear Power Plant (NPP). The ambient radiation (μSv/h) was measured in the sample sites. The flies were reared in the laboratory through two generations. We carried out the observation of F2 flies for visibly detectable phenotypes. According to whether the trait was inherited, observations were separated into three categories: with deviations of posterior cross-vein (C2) (incomplete penetrance), visible phenotypic changes (non-inherited) and mutations (inherited). Polymerase chain reaction (PCR) with primers specific to the 16S rRNA and Wolbachia surface protein (wsp) genes were used to determine infection presence in isofemale lines of the flies established for each population.* ***Results.*** *Examination of different phenotypes indicates that the highest mutation rate (but not C2 and not inherited changes) is in populations from Chornobyl Exclusion Zone and, therefore, connection with ambient radiation was detected (p = 0.0241). Generalized mixed linear regression has shown evidence that the presence of phenotypes with defects of C2 vein varies with endosymbiont infection presence (p = 0.03473) in the populations from radioactively polluted areas.* ***Conclusion****. Wolbachia is not related to occurring phenotypes neither with phenotypic changes nor with mutations, at least in surveyed populations. However, C2 defected phenotypes relates to the bacterial presence in populations from the contaminated area. Nonetheless, the origin of this relationship is unknown and the mechanisms of such a connection require further research.*

Keywords: *Drosophila melanogaster, Wolbachia, endosymbiont, ambient radiation, mutation, phenotypic change, posterior cross-vein.*

Introduction. *Wolbachia* is an endosymbiont which infects a variety of invertebrates around the world. These members of the alpha-proteobacteria group are known for reproductive parasitism that has a wide range of manifestations in their hosts. *Wolbachia* is maternally transmitted similarly to mitochondria, and, their interactions with host cell depend on host genetic background (Zug, Hammerstein, 2015). It is little known about influences of host-parasite interaction on penetrance and expressivity of mutations and induction of pheno-copies, especially, in the natural environment. However, there is some evidence which indicates that endosymbionts can modulate phenotypes of mutations in the model organism *Drosophila melanogaster* in which infection has rather a beneficial impact than deleterious (Zakharov et al., 2008).

The most prominent example of *Wolbachia* interruption into the fruit fly genetic program is a fertility rescue in *sxl* mutants (Starr, Cline, 2002). Clark et al. have shown that *chi*^{2} *(chico*^{2}*)* homozygotes *D. melanogaster* showed lethal effect when *Wolbachia* was removed (Clark et al., 2015).

However, bacteria interaction with *chi* product, an insulin-receptor substrate involved in growth regulation, is not direct as with SXL product. *Wolbachia*-infected flies have reduced activity of TEs (transposable elements), which are a major force of mutation rate acceleration in *D. melanogaster* (Teresa et al., 2015). All that indicates omnidirectional interactions between host-bacteria association in the context of hologenome (Rosenberg et al., 2009). In these examples, *Wolbachia* has positive effects on host and can be considered as one of the possible ways of bacteria to influence adaptation of host. In several instances, *Wolbachia* had deleterious effects on the fitness of the host. Presence of *Wolbachia* reduced activity superoxide dismutase what led to the increase of ROS that can level up mutation rate in the host (Wang et al., 2012). Increased level of ROS is associated with an increase in DNA damages in spermatocyte of sibling species *D. simulans* and the increase of *Wolbachia* density (Brennan et al., 2012).

Nowadays, there is still a lack of knowledge about the purpose of *Wolbachia* success in spreading among natural populations of *D. melanogaster* (Serga, Kozeretska, 2013). Thus, genetic changes within infected populations are still far from understanding. Hunter et al. (2016) investigating inbreed wild *D. melanogaster* strains found that *Wolbachia* presence slightly affected recombination rate at X chromosome. However, another study has not shown any changes for different loci at X chromosome (Serga et al., 2010).

As known, spontaneous mutations are an essential process for natural selection as for evolution at all. Considering high levels of *Wolbachia* in the *D. melanogaster* natural populations and apparent links with predicted and possible unknown host phenotypes endosymbiont might be affected by dynamics of spontaneous mutation or some of them. It was found that *Wolbachia* can phenotypically as well as genetically adjust due to the novel genetic background of the host through three generations of *D. melanogaster* mutant (Newton, Sheehan, 2015).

To find out whether *Wolbachia* presence might have link with different fly phenotypes and phenotypically detectable mutations in nature we have surveyed populations of *D. melanogaster* from Ukrainian localities including those from Chornobyl Exclusion Zone that was chosen as an area for which the acceleration of mutation processes in fly's populations under influence of ionizing radiation can be considered.

Materials and methods

We collected flies in the summer-fall period (August through October) in 2013–2014 from different localities of Ukraine:

Uman' (48°45'45.26"N–30°14'38.97"E);
Inkerman (44°36'49"N–33°36'36"E);
Odesa (46°29'13.91"N–30°43'51.59"E);
Varva (50°29'33.30"N–32°42'50.93"E);
Kyiv (50°21'9.06"N–30°28'57.70"E);
Drogobych (49°21'0.00"N–23°30'0.00"E);
Chornobyl Exclusion Zone Chornobyl (51°16'21.5"N–30°13'16.9"E);
near the cooling pond of Chornobyl NPP (51°22'23.9"N–30°08'17.9"E);
Yaniv (51°23'12.9"N–30°04'23.9"E),
Poliske (51°16'44.0"N–29°23'39.8"E).

The measurement of ambient radiation level (μSv/h) was performed with Dosimeter-Radiometer MKS-05 «Terra-P». The isofemale lines were established for each population in the laboratory conditions. The flies were reared at 25 ± 1 °C and 70–80 % relative humidity on the standard nutritional media (Roberts, 1998). The flies of each isofemale line were carried out through two consequent generations (F1-F2).

The observation of visible phenotypes was performed for F2 flies. Regarding to inheritance of observed traits, all detected phenotypic manifestations have been grouped into three main categories: mutations (mut), phenotypic changes (phen_chang), and deviations of posterior cross-vein (C2). Mutations category (mut) consists of phenotypically detectable and inheritable: mutant phenotypes with bright-red eyes that composed of *scarlet*, *cinnabar* and other known mutations; *brown* mutants; mutants of L2 and L5 wing veins. Phenotypic changes (phen_chang) comprised of other phenotypic deviations that are rather caused by environmental effects or recognized as not inherited phenotypic changes (e.g. variations of wing veins, cut on the wing, balloon-like appearance of wing, white abdomen etc). Deviations of posterior cross-vein (C2) demonstrate incomplete penetrance that was investigated previously for Ukrainian populations of *D. melanogaster* (Kozeretska et al., 2016) and had a high level of presence in the current study, thus, the phenotypes with this trait were grouped separately. The frequencies of phenotypes have been calculated by dividing the number of flies with observed phenotype on the total flies' number per population.

We extracted total genomic DNA by the salting-out method from whole-bodies of 10–12 adult flies of each isofemale line (Aljanabi, 1997). To detect *Wolbachia*-presence, we performed PCR with the set of primers to bacterial *16S rRNA* (O'Neill et al., 1992) and *wsp* (Zhou, Neill, 1998) genes. The PCR products were visualized on 2 % agarose gel.

The *Wolbachia* infection rate calculated by dividing the number of infected isofemale lines on their total number in each population (Clopper-Pearson confidence interval was used).

Statistical analysis was performed on two groups sampling sites the group of sites for which the ambient radiation level was measured (RadStat «Radiation Status», 2014) and another has not (NonRadStat «None Radiation Status», 2013). RadStat included populations form polluted area (Chornobyl Exclusion Zone) subset RadStat:Poll, and without radioactive contamination (rest of the populations) subset RadStat:Clear. We performed hierarchical clustering analysis of phenotype frequencies with Euclidean distance with average linkage and visualized results with heatmap building (Babicki et al., 2016).

Assuming that we have count data (Poisson distribution), for further analysis of relationship between *Wolbachia* and host phenotypes among natural populations, we chose the mixed generalized linear regression with sample site as a random factor. Additionally, to test whether radiation (Rad) has influence on the fly phenotype-*Wolbachia* relationship, ambient radiation was chosen as an additive factor (for RadStat data only). Taking into account the localities with different radiation levels as possible mutagen factors, we used generalized linear regression to test the effect of ambient radiation on phenotypes, followed by ANOVA with Chi-square test. All statistical operations were performed with R version 3.5.0 using *lme4* packages (2018).

Results and discussion

The flies were sampled from 14 localities in Ukraine. We have analyzed 12989 F2 flies from 404 isofemale lines for the presence of different phenotypes. For populations that were sampled in 2014, *Wolbachia* status has been investigated in our previous paper (Gora et al., 2016). However, for this work, isofemale lines that were examined for phenotype presence have been taken and infection status was recalculated (fig. 1).

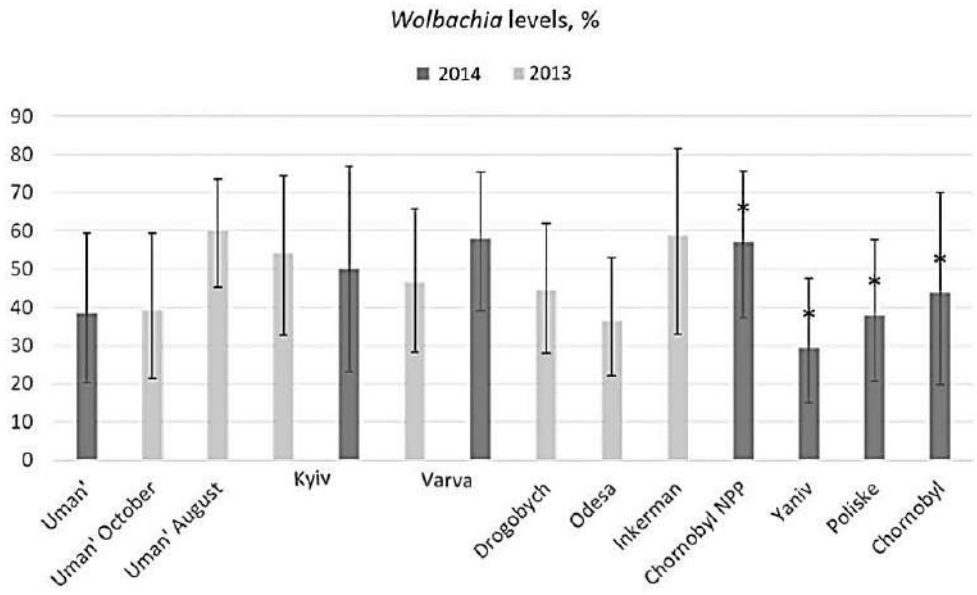

Fig. 1. *Wolbachia* infection levels in Ukrainian *Drosophila melanogaster* populations 2013–2014. 95 % confidence Clopper-Pearson intervals were used. * populations from Chornobyl Exclusion Zone.

Among examined flies, phenotypes with deviations of C2 and different phenotypic changes were the most frequently observed (fig. 2). The rarest phenotypes were the mutation of L2 and L5 veins (0.222 % and 0.075 % respectively). Kyiv 2014 population is not included because of the absence of any phenotype.

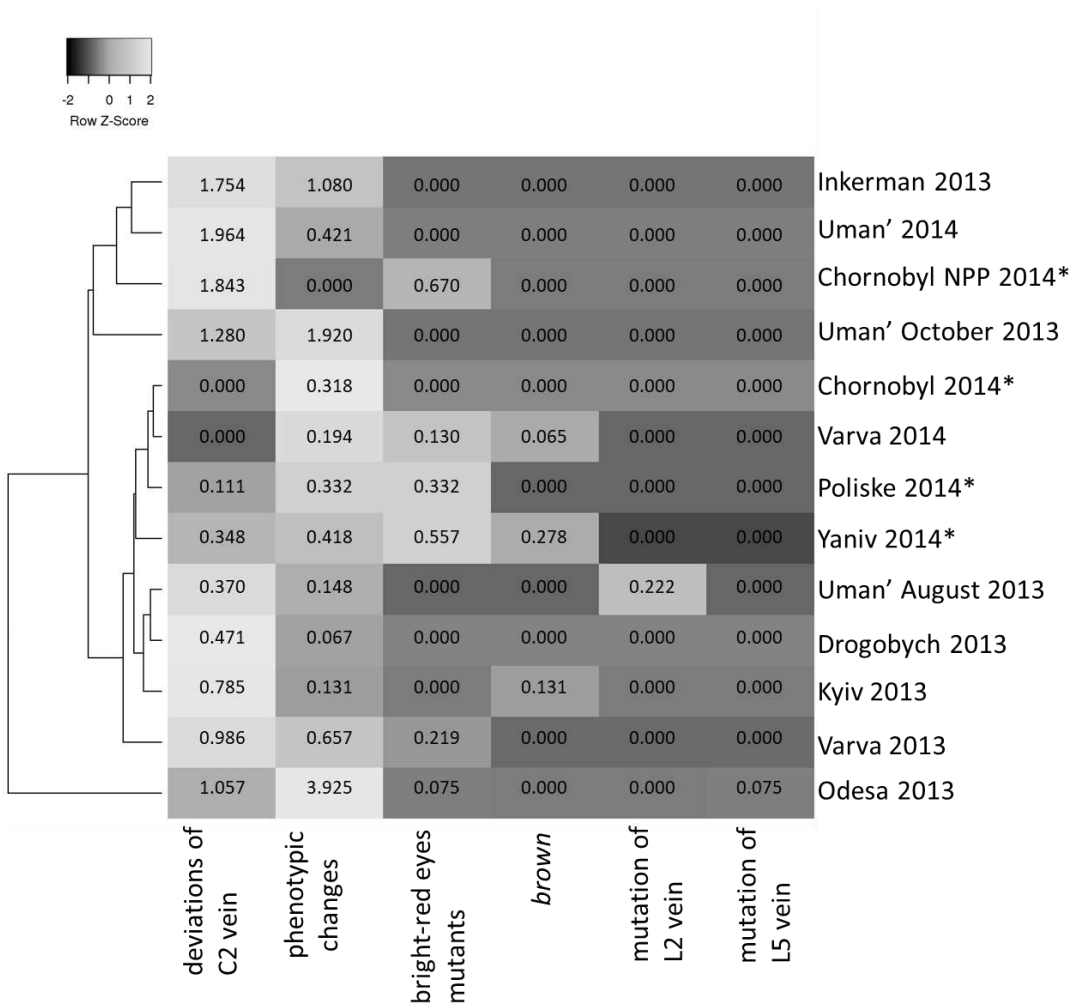

Fig. 2. The clustering of populations based on phenotype frequencies (presented in percentages within heatmap). * populations from Chornobyl Exclusion Zone. Z-score based on Euclidean distance with average linkage.

Interesting distribution of phenotypes has a population from Odesa 2013 presented as separate branch (fig. 2). We have observed the highest frequency of different phenotypic changes comparing to other populations. Also, L5 vein mutant phenotype has been detected only in this population.

The highest frequency of mutant category was in populations from Chornobyl Exclusion Zone. On the Fig. 2, they are mostly appeared in one cluster along with Varva 2014. However, for Chornobyl 2014 any mutations have not been detected. The most frequently observed mutant phenotypes were bright-red eyes in Chornobyl NPP 2014 (0.67 %) and *brown* in Yaniv 2014 (0.278 %). On the contrary, phenotypic changes were rarer than in other populations. Additionally, these populations had the lowest presence of C2 phenotypes, except Chornobyl NPP 2014 which vice versa had a fairly high frequency of C2 deviated phenotypes. According to the clusters on the fig. 2, the crucial role in the clustering has played distribution of C2 phenotypes. Populations of Inkerman 2013, Uman' 2014, Chornobyl NPP 2014 and Uman' October 2013 are gathered in one cluster as those that have the highest C2 frequencies. Despite that Uman' October 2013 is in this cluster, Uman' August 2013 is included into the other cluster together with Drogobych and Kyiv which had the lowest incidences of phenotypic changes in contrast to other populations.

Using generalized mixed Poisson regression, we have conducted analysis whether *Wolbachia* had an influence on phenotypes for populations from all localities with/without ambient radiation as an additive factor (table 1). Also, we investigated whether ambient radiation influences phenotypes in contamination free and polluted area. All dataset was separated on subsets according to definite phenotype presence in each population (for instance, NonRadStat subset included those populations that have phen_chang > 0)

For some subsets that included only one population, the analysis was not performed (e.g. mut ~ Wol*Rad analysis for RadStat:Clear is absent in table 1).

Table 1. The result of generalized regressions for phenotype-*Wolbachia* and phenotype-ambient radiation relationships performed according to different dataset

Model	*Dataset*	*Subset*	*Df*	*Chisq*	*P-value*
phen_chang ~ Wol	All data	-	1	0.0371	0.8473
	phen_chang > 0*	-	1	0.0448	0.8324
		NonRadStat	1	0.9648	0.326
		RadStat	1	3.6777	0.05515
		RadStat:Poll	1	1.1429	0.285
		RadStat:Clear	1	2.7725	0.0959
phen_chang ~ Wol×Rad	All data	-	2	4.3441	0.1139
	phen_chang > 0	RadStat	2	3.2946	0.1926
		RadStat:Poll	2	0.922	0.6306
		RadStat:Clear	2	3.6573	0.1606
phen_chang ~ Rad	phen_chang > 0	RadStat	1	1.687	0.194
		RadStat:Poll	1	0.626	0.4289
		RadStat:Clear	1	1.127	0.2884
mut ~ Wol	All data	-	1	0.1836	0.6683
	mut > 0	-	1	0.0757	0.7832
		NonRadStat	1	4e-04	0.9844
		RadStat	1	0.1049	0.746
		RadStat:Poll	1	0.6317	0.4267
		RadStat:Clear	1	0.7443	0.3883
mut ~ Wol×Rad	All data	-	2	2.5489	0.2796
	mut > 0	RadStat	2	2.7567	0.252
		RadStat:Poll	2	2.1151	0.3473
mut ~ Rad	mut > 0	RadStat	1	6.8999	0.00862**
		RadStat:Poll	1	5.0875	0.0241**
C2 ~ Wol	All data	-	1	1.1373	0.2862
	C2 > 0	-	1	0.9635	0.3263
		NonRadStat	1	2.0344	0.1538
		RadStat	1	0.064	0.8002
		RadStat:Poll	1	4.4586	0.03473**
C2 ~ Wol×Rad	All data	-	2	0.7957	0.6718
	C2 > 0	RadStat	2	0.7609	0.6835
		RadStat:Poll	2	3.9111	0.04797**
C2 ~ Rad	C2 > 0	RadStat	1	0.93476	0.3336
		RadStat:Poll	1	0.85518	0.3551

***** «one of three phenotypic categories» > 0 a subset of data to have been chosen due to the presence of defined categorical phenotype more than zero per population.

****** significant value $p < 0.05$.

The statistically significant result of ANOVA ($p = 0.00862$) for the relationship between ambient radiation and presence of mutations (mut ~ Rad) indicates that for populations that we collected from sites where ambient radiation was measured and mutation frequency more than zero, ionizing radiation has influences on the phenotypes manifestation of which is related to mutant genomes. Only mutant phenotypes of the eye were related to ambient radiation in these localities ($p = 0.0241$).

Intriguingly, a weak relationship was detected between deviations of C2 and *Wolbachia* in RadStat:Poll dataset (p = 0.03473). However, we did not observe significant effect in the model with ambient radiation as an additive factor (C2 ~ Wol × Rad, RadStat:Poll dataset, p = 0.04797) and due to insignificant influence of radiation on deviations of posterior cross-vein (C2 ~ Rad, RadStat:Poll dataset, p = 0.3551).

Previously, Kozeretska et al. (2016) have been reported that the aberrant C2 vein trait in Ukrainian populations was heritable and characterized by elevated frequencies. According to study of Waddington in the context of genetic assimilation, deviations of C2 can be fixed as heritable trait through generations of flies during influence of higher temperatures (Waddington, 1953). According to this, Kozeretska et al. (2016) have suggested that recent elevation of summer temperature in Ukraine can be connected to elevation of C2 deviations caused by miRs (Schertel et al., 2012). It is known that *Wolbachia* can alter aae-miR-2940 in *Aedes aegypti* and therefore lead to upregulation of arginine methyltransferases (AaArgM1-8), silencing of which decreases *Wolbachia* replication (Hussain et al., 2011). Nonetheless, miR and *Wolbachia* mechanisms of interactions remain to be investigated in the future.

Rutherford and Lindquist have shown that heat shock protein HSP90 affects development of wing veins under environmental perturbations, including oscillations of temperature (Rutherford, Lindquist, 1998). Recently association between fly temperature prevalence and *Wolbachia* infection was shown indicating that infected flies prefer lower temperature (Truitt et al., 2018). It is known that during host-symbiont interactions *Wolbachia* can affect defensive system of host and have impact on down-regulation of some HSP genes, e.g. *HSP22* (Zheng et al., 2011).

Accounting possible indirect relationships between aberrant C2 phenotype and *Wolbachia* throughout signaling pathway in which Hsp proteins and miRs are involved, our results, on the one hand, can be explained in this way. We hypothesize that phenotype changes of C2 could be explained by interactions between *Wolbachia* and some HSP proteins and/or miRs. Nonetheless, we did not find a *Wolbachia* relationship with phenotypes of other mutant genotypes.

Conclusion

For analyzed populations, we did not find the relationship of *Wolbachia* presence with non-heritable phenotypic changes and mutations. We found that C2 defected phenotypes relates to the bacterial presence in populations from the contaminated area (p = 0.03473). However, the origin of this relationship is unknown and can depend on indirect interactions in the symbiont-host association.

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Presented by Ya. I. Chernyk
Received 05.12.2018

ЗВ'ЯЗОК МІЖ ЧАСТОТОЮ ІНФІКУВАННЯ *WOLBACHIA* ТА РІЗНИМИ ФЕНОТИПАМИ В ПРИРОДНИХ ПОПУЛЯЦІЯХ *DROSOPHILA MELANOGASTER* З РАДІОАКТИВНО ЗАБРУДНЕНИХ ТА ЧИСТИХ ТЕРИТОРІЙ УКРАЇНИ

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Мета. Дослідити взаємозв'язок між частотами інфікування *Wolbachia* та змінених фенотипів *Drosophila melanogaster* з різних локалітетів України, включаючи Чорнобильську зону відчуження, протягом 2013–2014. **Методи.** Були започатковані ізосамкові лінії для популяцій Умані, Інкерману, Одеси, Варви, Києва, Дрогобичу, Янова та Чорнобильської зони відчуження (Поліське, Чорнобиль та Чорнобильська атомна електростанція). Також було проведено заміри фонового рівня радіації (мкЗв/год) в сайтах збору. Мухи утримувались в лабораторних умовах протягом двох поколінь. Для мух покоління F2 кожної ізосамкової лінії був проведений аналіз візуально помітних фенотипових відмінностей від дикого типу. Виявлені фенотипові зміни було розділено на три категорії: порушеннями поперечної жилки крила C2, помітні фенотипові зміни (які не успадковуються) та мутації (успадковуються). Для визначення присутності

Wolbachia в ізосамкових лініях було проведено полімеразну ланцюгову реакцію (ПЛР) зі специфічними праймерами до генів *16S rRNA* та *wsp* (*Wolbachia surface protein)* бактерії. **Результати.** Найвищий рівень мутантних фенотипів виявлено в популяціях з Чорнобильської зони відчуження, відповідно, було показано їхній зв'язок з фоновим рівнем радіації (p = 0.0241). Результат змішаної генералізованої регресії показав, що фенотипи з порушеннями C2 корелюють з присутністю ендосимбіонту (p = 0.03473) в популяціях з радіоактивно забруднених територій. **Висновок**. Частота інфікування *Wolbachia* не впливає на частоту фенотипових змін та мутацій у досліджуваних популяціях. Тим не менше, показаний зв'язок частоти інфікування бактерією з частотою порушень жилки C2 для популяцій з забруднених територій, хоча механізми такого зв'язку потребують подальших досліджень.

Ключові слова: *Drosophila melanogaster*, *Wolbachia*, ендосимбіонт, фонова радіація, мутація, фенотипова зміна, дальня поперечна жилка.

СВЯЗЬ МЕЖДУ ЧАСТОТОЙ ИНФИЦИРОВАНИЯ *WOLBACHIA* И РАЗЛИЧНЫМИ ФЕНОТИПАМИ В ПРИРОДНЫХ ПОПУЛЯЦИЯХ *DROSOPHILA MELANOGASTER* ИЗ РАДИОАКТИВНО ЗАГРЯЗНЕННЫХ И ЧИСТЫХ ТЕРРИТОРИЙ УКРАИНЫ

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Цель. Исследовать взаимосвязь между частотами инфицирования *Wolbachia* и измененных фенотипов *Drosophila melanogaster* с разных локалитетов Украины, включая Чернобыльскую зону отчуждения, в течение 2013–2014. **Методы.** Были начаты изосамковые линии для популяций Умани, Инкермана, Одессы, Варвы, Киева, Дрогобыча, Янова и Чернобыльской зоны отчуждения (Полесское, Чернобыль и Чернобыльская атомная электростанция). Также было проведено замеры фонового уровня радиации (мкЗв/ч) в сайтах сбора. Мухи содержались в лабораторных условиях в течение двух поколений. Для мух поколения F2 каждой изосамковой линии был проведен анализ визуально заметных фенотипических отличий от дикого типа. Обнаруженные фенотипические изменения были разделены на три категории: нарушение поперечной жилки крыла C2, заметные фенотипические изменения (не наследуются) и мутации (наследуются). Для определения присутствия *Wolbachia* в изосамковых линиях было проведено полимеразную цепную реакцию (ПЦР) со специфическими праймерами к генам *16S rRNA* и *wsp* (*Wolbachia* surface protein) бактерии. **Результаты.** Самый высокий уровень мутантных фенотипов обнаружено в популяциях из Чернобыльской зоны отчуждения, соответственно, было показано их связь с фоновым уровнем радиации (p = 0.0241). Результат смешанной генерализованной регрессии показал, что фенотипи с нарушениями C2 коррелируют с присутствием ендосимбионта (p = 0.03473) в популяциях с радиоактивно загрязненных территорий. **Вывод.** Частота инфицирования *Wolbachia* не влияет на частоту фенотипических изменений и мутаций в исследуемых популяциях. Тем не менее, показана связь частоты инфицирования бактерией с частотой нарушений жилки C2 для популяций с загрязненных территорий, хотя механизмы такой связи требуют дальнейших исследований.

Ключевые слова: *Drosophila melanogaster*, *Wolbachia*, ендосимбионт, фоновая радиация, мутация, фенотипическая изменение, дальняя поперечная жилка.