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Drosophila melanogaster inhabiting northern regions of European Russia are infected with *Wolbachia* which adversely affects their life span

N.V. Roshina[1, 2], A.V. Symonenko[2], A.V. Krementsova[2, 3], E.A. Tsybul'ko[2], V.E. Alatortsev[2], E.G. Pasyukova[2] ✉, D.V. Mukha[1] ✉

[1] Vavilov Institute of General Genetics, RAS, Moscow, Russia
[2] Institute of Molecular Genetics, RAS, Moscow, Russia
[3] Emanuel Institute of Biochemical Physics, RAS, Moscow, Russia

Wolbachia is a genus of bacteria causing intracellular infection in the natural populations of *Drosophila melanogaster* on all continents. In *D. melanogaster*, *Wolbachia* affects various life history traits, behaviour, sensitivity to stress and viral infection. The phenotypic effects of *Wolbachia* might evolve to promote its further spreading, increasing the interest in exploring the spread of *Wolbachia*, in particular, at the boundaries of the *D. melanogaster* habitat, in association with the effects on vital traits of host species. In this paper, we present data on the level of *Wolbachia* infection in two *D. melanogaster* populations from the northern regions of European Russia: Alexandrov (56.41° N, 38.72° E) and Valday (58.02° N, 33.24° E). The flies were collected in private apple gardens located in two small hamlets without supermarkets or fruit markets, from 2010 to 2015. The both populations demonstrated the same level of infection: in average, 69.7 % of the inbred lines (ILs) obtained from single females of the Alexandrov population and 68.4 % of ILs obtained from single females of the Valday population. The infection rate varied from year to year showing a tendency to reduction, its overall level being within the range previously observed in other habitats. Life spans were compared in sub-lines of the same IL, one infected with *Wolbachia* and the other treated with tetracycline healing this infection. In four out of five ILs, the lifespan of both males and females was severely affected by *Wolbachia*; in different ILs, the mean life spans reduced from 1.8 to 5.4 times and from 1.4 to 2.4 times, respectively. Our results confirm that, despite *D. melanogaster* widespread distribution, the *Wolbachia* effect on their life span has been mostly negative.

Key words: *Drosophila melanogaster*; *Wolbachia* sp.; PCR, natural populations; symbiont; life span.

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✉ e-mail: egpas@rambler.ru; dmitryVmukha@gmail.com

Drosophila melanogaster, обитающие на севере европейской части России, заражены *Wolbachia*, негативно влияющей на их продолжительность жизни

Н.В. Рощина[1, 2], А.В. Симоненко[2], А.В. Кременцова[2, 3], Е.А. Цыбулько[2], В.Е. Алаторцев[2], Е.Г. Пасюкова[2] ✉, Д.В. Муха[1] ✉

[1] Институт общей генетики им. Н.И. Вавилова Российской академии наук, Москва, Россия
[2] Институт молекулярной генетики Российской академии наук, Москва, Россия
[3] Институт биохимической физики им. Н.М. Эмануэля Российской академии наук, Москва, Россия

Wolbachia – род бактерий, вызывающих внутриклеточную инфекцию и широко распространенных в природных популяциях *Drosophila melanogaster* на всех континентах. Вольбахии оказывают влияние на ряд признаков *D. melanogaster*, определяющих ее приспособленность, а также на поведение мух, их чувствительность к стрессу и вирусной инфекции. Влияние вольбахии на фенотип вида-хозяина могло способствовать расселению бактерии, что увеличивает интерес к дальнейшему изучению ее географического распространения, в частности на границах среды обитания *D. melanogaster*, в сочетании с воздействием на жизненно важные признаки вида-хозяина. В настоящей работе приведены данные об уровне зараженности вольбахией двух популяций *D. melanogaster* из северных регионов Европейской России. Мух собирали с 2010 по 2015 г. в частных яблоневых садах, расположенных в двух небольших деревнях без супермаркетов или фруктовых рынков, недалеко от городов Александров (56.41° с. ш., 38.72° в. д.) и Валдай (58.02° с. ш., 33.24° в. д.). Обе популяции были заражены вольбахией на сходном уровне. В среднем было заражено 69.7 % инбредных линий (ИЛ), полученных от индивидуальных самок, выловленных близ Александрова, и 68.4 % ИЛ, полученных от индивидуальных самок валдайской популяции. Уровень заражения менялся из года в год, демонстрируя тенденцию к снижению, при этом в среднем находился в пределах, ранее наблюдавшихся в других местообитаниях. Сравнивалась продолжительность жизни в сублиниях одних и тех же ИЛ: одна сублиния оставалась зараженной вольбахией, а другая была вылечена тетрациклином. В четырех из пяти протестированных ИЛ продолжительность жизни как самцов, так и самок оказалась сильно сниженной в присутствии вольбахии; в разных ИЛ вольбахия сокращала продолжительность жизни в 1.8–5.4 раза у самцов и в 1.4–2.4 раза у самок. Полученные результаты подтверждают, что, несмотря на широкое распространение, вольбахия может негативно влиять на продолжительность жизни.

Ключевые слова: *Drosophila melanogaster*; *Wolbachia* sp.; ПЦР; природные популяции; симбионт; продолжительность жизни.

Wolbachia is a genus of bacteria causing intracellular infection and represents the most widespread maternally transmitted facultative arthropod endosymbionts (Werren et al., 1995; Werren, Windsor, 2000; Hingelbroeker et al., 2008). From 40 % (Zug, Hammerstain, 2012) to 76 % (Jeyaprakash, Hoy, 2000) of arthropod species are estimated to have been infected. *Wolbachia* is widespread in the natural populations of *D. melanogaster* on all continents (Hoffmann et al., 1994, 1998; Solignac et al., 1994; Riegler et al., 2005; Ilinsky, Zakharov, 2007; Verspoor, Haddrill, 2011; Richardson et al., 2012; Ventura et al., 2012). However, the *Wolbachia* infection has never been characterized for the northern European populations of *D. melanogaster*.

Wolbachia is able to affect the life history traits of their hosts, mainly their reproduction, by causing cytoplasmic incompatibility, parthenogenesis induction, male-killing, and feminization, or by altering fecundity, fertility, and progeny survival (reviewed in Maistrenko et al., 2015). In *D. melanogaster*, apart from various life history traits (Olsen et al., 2001; Fry et al., 2004; Markov et al., 2009; Sharon et al., 2010; Serga et al., 2014), *Wolbachia* also affects sensitivity to stress (Brownlie et al., 2009; Versace et al., 2014) and viral infection (Teixeira et al., 2008; Martinez et al., 2015; Lindsey et al., 2018), sleep and aggressive behaviour (Rohrscheib et al., 2015; Bi et al., 2018). Among other traits, lifespan was reported to be influenced by *Wolbachia* (Min, Benzer, 1997; Brummel et al., 2004; Alexandrov et al., 2007; Carrington et al., 2009). It is believed that some effects of *Wolbachia* on the traits of their hosts evolved to promote spreading of bacteria (Werren et al., 2008). This increases the interest in exploring *Wolbachia* spreading, in particular, near the boundaries of the *D. melanogaster* habitats, in association with effects on vital traits of the host species.

In this paper, we describe the effect *Wolbachia* had in *D. melanogaster* inhabiting two localities in the northern regions of European Russia, which can be regarded as the outskirts of this species' natural habitat. The level of infection declined in years, but remained relatively high in the both populations. Our study has demonstrated that the life spans of both the males and females were negatively affected by the presence of *Wolbachia* in four of the five cases tested. This is a confirmation that *Wolbachia* has an important impact on the vital traits of their host organisms.

Materials and methods

Fly strains. Flies were collected in private apple gardens located in two small hamlets without supermarkets or fruit markets, at a distance of several kilometers from Alexandrov, Russia (56.41° N, 38.72° E) and Valday, Russia (58.02° N, 33.24° E). The flies were collected manually from the surface of apple heaps (Alexandrov) or with baited traps (Valday). Fly collections were carried out from late August to early October of 2010, 2011, 2012, 2014 (Alexandrov) and in 2014, 2015 (Valday).

From 40 to 60 isofemale lines per population per year were started from females caught in nature. In their progeny, the male phenotype was checked to avoid contamination by *D. simulans*. Each line was maintained by brother × sister inbreeding during 20–22 generations. The inbred lines (IL) which survived inbreeding were further checked for the presence of *Wolbachia* and used for the lifespan measurements. Each IL was considered as genetically homogeneous (Falconer, Mackay, 1996) and represented one haploid genome of the population.

Tests for *Wolbachia*. DNA was extracted from 20–50 flies of the same genotype following the standard phenol-chloroform protocol. All ILs were checked for the presence of *Wolbachia* via quantitative PCR (MiniOpticon real-time PCR detection system, Bio-Rad) with primers to the *Wolbachia* 16S rRNA gene, 5′-CATACCTATTCGAAGGGATAG-3′ and 5′-AGCTTCGAGTGAAACCAATTC-3′ (Werren, Windsor, 2000). The lines showing positive results were treated with tetracycline (0.25 mg per 1 mL of fly food (Holden et al., 1993), with modifications) for three generations followed by three generations of recovery, before they were used in life span assays.

Lifespan assays. The lifespan was measured according to (Roshina et al., 2014). Five virgin flies of the same genotype and sex, all collected on the same day from cultures with moderate density, were placed in replicate vials. Flies were transferred weekly to vials with fresh food containing approximately 5 mL of standard medium without live yeast on the surface. Dead flies were recorded daily. Experiments comparing fly life spans were conducted simultaneously. Sample sizes were 50 flies/sex/line. Lifespan was estimated for each fly as the number of days alive from the day of eclosion to the day of death. Mean and median lifespan and survival curves were primarily used to characterize lifespan.

Statistical analyses. Fisher's exact test was used to compare the proportions of infected ILs. Standard descriptive statistic analysis of lifespan (Wilmoth, Horiuchi, 1999; Carey, 2003) was performed to determine mean lifespan and accompanying variances, standard deviations and standard errors; median, minimum and maximum lifespans; lifespans of the lower and upper quartiles, 10 and 90 percentiles. Survival curves were estimated using the Kaplan–Meier procedure. The nonparametric, distribution-free Mann–Whitney test and Kolmogorov–Smirnov test were used to evaluate the statistical significance of the difference between the survival curves.

Results

The presence of *Wolbachia* was checked in inbred lines (ILs), each obtained from a single female collected in the private apple gardens located near Alexandrov, Russia (56.41° N, 38.72° E) and Valday, Russia (58.02° N, 33.24° E). From 20 to 75 lines per population per year were studied (Table 1). The level of infection was high in Alexandrov in 2010 and 2011, moderate in 2012 and became significantly lower in 2014 ($p < 0.01$, compared to 2010 or to 2011, Table 1). In Valday 2014, the level of infection was significantly higher than in Alexandrov 2014 ($p < 0.05$, see Table 1) and didn't change in 2015, although showed a tendency to reduction. When averaged over years, the level of infection was similar in the both populations: 69.7 % in Alexandrov and 68.4 % in Valday.

To assess the effects of *Wolbachia* on the lifespan, five infected ILs from Alexandrov 2010 were divided into two sub-lines each and, while one sub-line was maintained in the same way as earlier, the other was treated with tetracycline healing the *Wolbachia* infection. After treatment and subse-

Table 1. The level of *Wolbachia* infection in the natural populations of *D. melanogaster* inhabiting northers regions of European Russia

Population	Year	Number of ILs	Number of infected ILs	% of infected ILs
Alexandrov	2010	20	16	80.0
	2011	45	37	82.2
	2012	28	19	67.9
	2014	75	38	50.7
Valday	2014	60	42	70.0
	2015	48	32	66.7

Table 2. Parameters of male and female life spans in the Alexandrov ILs ($N = 50$)

Line	Sex	*Wolbachia*	Mean	Median	Lower quartile	Upper quartile	*p*-values for comparisons with the infected sub-line	
							Mann–Whitney test	Kolmogorov–Smirnov test
1	♂	+	7 ± 0.5	7	4	27		
		–	38 ± 1.9	39	10	47	**0.0000001**	**< 0.001**
	♀	+	17 ± 0.9	19	11	23		
		–	41 ± 1.5	42	39	49	**0.0000001**	**< 0.001**
11	♂	+	25 ± 1.0	24	19	30		
		–	57 ± 1.6	57	52	64	**0.0000001**	**< 0.001**
	♀	+	20 ± 1.1	19	15	26		
		–	53 ± 2.1	51	45	68	**0.0000001**	**< 0.001**
19	♂	+	27 ± 1.3	31	18	35		
		–	50 ± 1.1	49	44	54	**0.0000001**	**< 0.001**
	♀	+	27 ± 1.2	27	21	33		
		–	46 ± 1.3	47	43	52	**0.0000001**	**< 0.001**
30	♂	+	31 ± 1.1	35	27	38		
		–	56 ± 2.3	55	45	68	**0.0000001**	**< 0.001**
	♀	+	31 ± 1.6	32	22	43		
		–	44 ± 1.4	44	31	56	**0.0000001**	**< 0.001**
33	♂	+	40 ± 1.7	39	34	47		
		–	44 ± 1.7	41	38	48	0.067205	> 0.10
	♀	+	45 ± 1.7	48	40	54		
		–	49 ± 1.9	51	44	59	0.111280	> 0.10

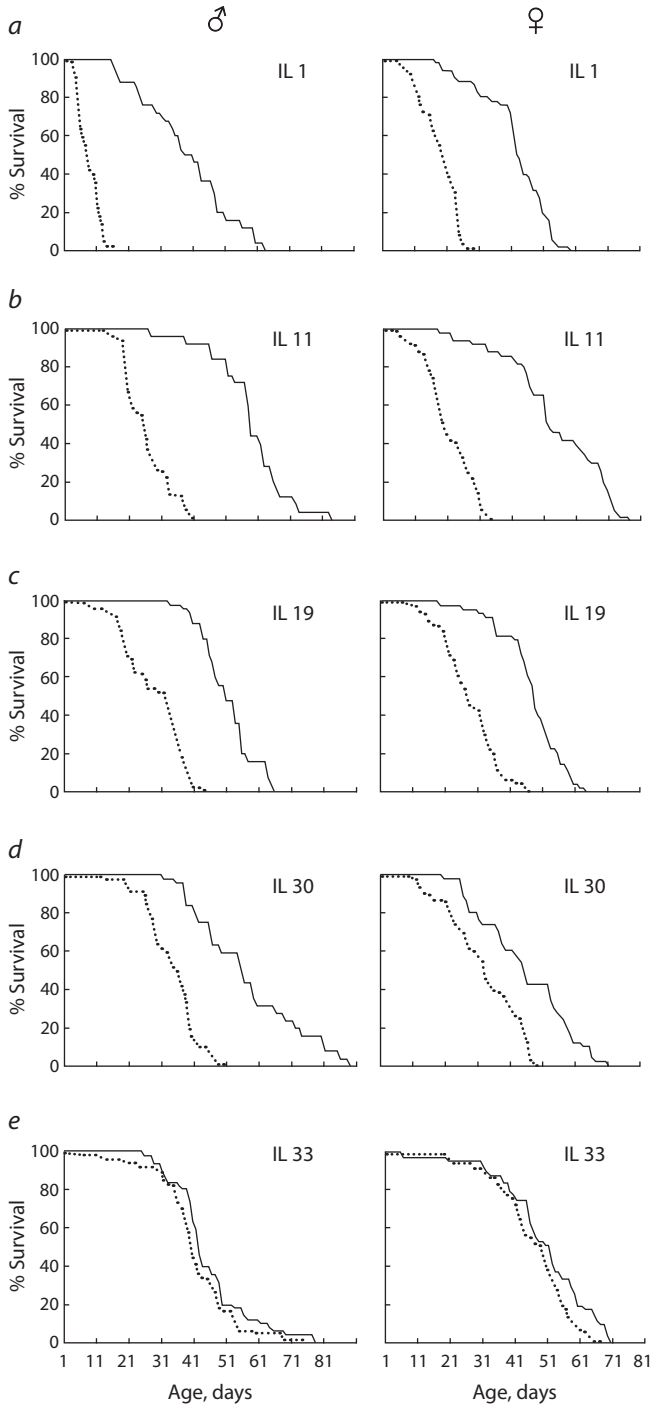
Note: Significant *p*-values are in bold case.

quent recovery, male and female life spans were measured in the both sub-lines of each of five ILs.

The mean life spans of infected males and females were relatively low (Table 2, Figure), the lowest being only seven days. In four ILs, the life spans of cured males and females were significantly longer than those of infected males and females (see Table 2, Figure): depending on the line, the mean life spans were increased from 1.8 to 5.4 times in males and from 1.4 to 2.4 times in females. The positive effect was thus smaller in females than in males. In one of the ILs, the life spans of infected males and females were not different from the life spans of cured males and females. In this line, the life spans of infected males and females were the highest, compared to the other four ILs.

Discussion

Though the level of *Wolbachia* infection was analyzed in many natural populations of *D. melanogaster* all over the world, the number of populations studied is limited and they are distributed unevenly across the Earth's territory. The level of infection can be as high as 100 %, which was registered in Australian (Hoffmann et al., 1998) and Brazilian (Ventura et al., 2012) populations, and as low as 5 % (in Australian populations, Hoffmann et al., 1998) and 1 % (in Sub-Saharan African populations, Verspoor, Haddrill, 2011). In the large-scale work of Ilinsky and Zakharov (2007) populations from Ukraine, Belarus, Moldova, Caucasus, Central Asia, Ural, Udmurtia, Altai, West and East Siberia, and the Far East were studied in different years, from 1974 to 2005, and it was shown that the



Life spans of *Wolbachia*-infected males and females (dotted line) compared to the life spans of tetracycline-cured males (solid line).

Wolbachia infection rate varied from 70 % in Middle Asia to 40 % in Eastern Europe (Ukraine, Belarus, Moldova). Populations of the southern regions of European Russia (Caucasian) were analyzed by Bykov and co-authors (2014) who reported that the levels of *Wolbachia* infection were moderate in this region and higher than in the populations of Eastern Europe described in (Ilinsky, Zakharov, 2007). However, the published data are related only to the populations inhabiting the southern latitudes of the northern hemisphere and the northern latitudes of the southern hemisphere. Nothing has been previously known about the presence of *Wolbachia* in the northern populations of the northern hemisphere, including European Russia. Our study has demonstrated that *Wolbachia* presents in *D. melanogaster* inhabiting latitudes as high as 58° N. Infection frequencies appear to be moderate and vary over years.

While analyzing the frequency distribution of *Wolbachia* infection in various geographic regions, it should be taken into account that the studies were conducted in different years and seasons, and, overall, were nonsystematic. Monitoring of infection frequencies in Australian natural populations indicated that they could be both stable and fluctuate over time (Hoffmann et al., 1998). In Caucasian population, minor variations in the infection level were observed (Bykov et al., 2014). In our study, we observed a decline in infection frequencies over years. Laboratory cage studies showed that low larval density contributed to the stability of infection frequencies (Hoffmann et al., 1998). A plausible explanation would be that either the presence of *Wolbachia* is beneficial for the host or contamination occurs easier at high population density. However, there is no sufficient evidence to state that natural fluctuations in the level of infection are not random. Whether the observed fluctuations are explained by the fitness benefits, which *Wolbachia* provides for the host so that to persist in populations or, alternatively, by its unfavorable effects on fitness, remains to be elucidated.

Life span is a vital trait affected by *Wolbachia*. Interestingly, opposite effects were described by different authors: Min and Benzer (1997) and Alexandrov and co-authors (2007) demonstrated that, in infected flies, the life span decreased, while Brummel and co-authors (2004) reported the increased life span in infected flies. In the flies from Alexandrov population, healing from *Wolbachia* increased the life span and substantially slowed down aging in four out of five cases. Why both male and female life spans did not improve due to tetracycline treatment in one of the lines remains an enigma. One possible explanation is that the life span of infected flies in this line was the highest, compared to other ILs. Generally, it remains largely unclear why the *Wolbachia* infection in some cases leads to life extension, while in other cases shortens flies' life span. Previously, it was suggested that the effect on the life span depends on the strain of *Wolbachia* and ambient temperature (Brummel et al., 2004; Rohrscheib et al., 2016). At high temperatures of 25 to 29 °C, the deleterious effects of virulent *Wolbachia* strains on the host lifespan manifest themselves much more clearly, whereas at lower temperatures of 16 to 19 °C, the lifespan impact may be negligent or even slightly positive (Rohrscheib et al., 2016). During the late summer and early autumn, the outdoor temperature in Alexandrov varies from approximately 15 to 20 °C (https://ru.climate-data.org/location/929946/); accordingly, detrimental lifespan effects of *Wolbachia* may be compensated and pathogenic virulent strains could spread in the natural population of flies. At the laboratory conditions of 25 °C, the progeny of flies caught in late summer and early autumn would suffer evident lifespan shortening from the infection, as we have reported in this paper. In addition and in part as opposed to what has been said above, the divergence in longevity between different *D. melanogaster* lines was demonstrated to be mostly associated with the host genetic background rather than the type of *Wolbachia* infection (Carrington et al., 2009). Eventually, we

have to recognize that in spite of some available interesting data, it is not yet exactly clear how the life spans measured in the laboratory are related to the life spans under natural conditions. Further research is needed to describe the processes underlying the actual population dynamics of *Wolbachia* to the full extent.

What molecular mechanisms can determine the effect of *Wolbachia* endosymbiont on the *D. melanogaster* life span? In recent years, it has been demonstrated that *Wolbachia* can affect expression of the several genes related to aging. For example, it has been suggested that *Wolbachia* is able to upregulate insulin signaling downstream of InR (Insulin Receptor), thus negatively affecting the life span of flies (Ikeya et al., 2009). Specific interactions between insulin signaling and *Wolbachia* in lifespan regulation were confirmed in (Grönke et al., 2010). Effects of mutations in *Indy* also depended on the presence of *Wolbachia* (Toivenen et al., 2007). Further studies are needed to shed light on the molecular basis of *Wolbachia* effects on lifespan.

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Conflict of interest
The authors declare they have no conflict of interest.

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