Based on the LC$_{50}$ values, the adult flies were fed with PC with a sublethal concentration of 250 μg/ml in 0.5% DMSO for 7 days. The homogenate of these flies was used for studying the effect of PC on levels of oxidative stress markers and antioxidants in the oxidative stress induced flies treated with 15 mM paraquat. The PC fed flies showed significant diminution in the levels of oxidative stress markers like malondialdehyde hydroperoxide, reduced glutathione with respect to control flies, and modulatory effect was observed in the levels of Superoxide dismutase and Catalase that offer protection against paraquat induced oxidative stress in flies.

In the current study, paraquat caused high mortality (45–50%) among flies during exposure period. But the low incidences of mortality among test flies clearly indicate the protective nature of PC combination. The results obtained in "paraquat resistance assay" suggest that PC prophylaxis has the propensity to protect against neurotoxicant exposure largely due to its antioxidative potential. Further, paraquat induced neurotoxicity could be evidenced by high rate of locomotor deficits as measured in the negative geotaxis assay. Synergistic activity of PC has been shown with significant changes in the oxidative stress makers compared to curcumin alone. Based on biochemical evidences, we propose that dietary feeding of PC to *Drosophila* for a short duration has the propensity to attenuate paraquat induced oxidative stress owing to its antioxidative nature and its ability to modulate the activities of antioxidant defenses such as reduced GSH. Further work is essential to know the exact mechanism of action.

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Spiroplasma in natural populations of *Drosophila melanogaster* from Ukraine.

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Abstract

We have identified endosymbiotic bacteria of the genus *Spiroplasma* in wild populations of *Drosophila melanogaster* from Ukraine. These bacteria were identified from two different locations, and this is the first finding of the bacteria in Ukraine. Flies from populations infected with *Spiroplasma* spp. did not show any signs of male killing. The results are discussed in relation to previous findings concerning spatial distribution and male-killing behavior of *Spiroplasma*. Keywords: *Spiroplasma*, *Wolbachia*, *Drosophila melanogaster*, endosymbiotic, male killing, Ukraine.

Introduction

Endosymbiotic bacteria capable of modifying sexual reproduction, or "reproductive parasites", have been described from diverse invertebrate species (Duron, 2008; Mateos *et al.*, 2006). They are widely reported both in natural populations and in laboratory lineages of arthropods, causing cytoplasmic incompatibility, male killing, feminization, and parthenogenesis (O'Neill *et al.*, 1997). The mechanisms associated with their interactions with host organisms, however, are not well-understood, nor is the scale of their geographic distribution well documented.

Among the "reproductive parasites" of drosophilids, bacteria of the genus *Wolbachia* are the best-studied group, with most studies of sexual reproduction-modifying microorganisms being centered on this genus (Werren, 1997; Goryacheva, 2004). Bacteria of the genus *Spiroplasma* are no less interesting in this respect, although they are poorly studied compared to *Wolbachia* (Montenegro *et al.*, 2005; Haselkorn, 2010; Weinert *et al.*, 2007).

The genus *Spiroplasma* contains a group of very small (0.1-0.2 by 4-5 μm), motile, helical, wall-less prokaryotes that are associated primarily with insects, and occasionally with ticks and plants (Gasparich *et al.*, 2004). For instance, *S. citri*, *S. phoeniceum*, and *S. kunkelii* are known as plant pathogens transmitted by insect vectors; *S. apis* and *S. melliferum* as pathogens of the honeybee; and *S. poulsonii* is maternally inherited in *Drosophila* (Whitcomb and Tully, 1979; Kageyama *et al.*, 2006). *S. poulsonii* was first described as an agent causing sex ratio shifts in *Drosophila willistoni* offspring that spanned from minor changes in the proportion of females all the way up to complete disappearance of males (the phenomenon was termed *androcide* or malelessness; see ref. 12). Male killing *Spiroplasma* has also been found in Central and South American natural populations of other drosophilids of the group *willistoni*, namely *D. nebulosa*, *D. equinoxialis*, and *D. paulistorum* (Williamson and Poulson, 1979). Non-male-killing *Spiroplasma* has been detected in wild Japanese populations of *D. hydei* (Kageyama *et al.*, 2006; Ota *et al.*, 1979). More recently, the bacterium was identified in Brazilian (Montenegro *et al.*, 2000) and African (Pool *et al.*, 2006) populations of *D. melanogaster*, where it also caused male killing. Till now *Spiroplasma* was not detected in Drosophilidae populations from Eurasia.

The male-killing *Spiroplasma* infection rate in natural populations is typically low: 0.1-3% in the group *willistoni* and 2-3% in populations of *D. melanogaster* (Kageyama *et al.*, 2006). A non-male-killing form has been described from natural populations of *D. hydei* from Japan, in which the infection rate reached up to 46% in the late 1970s (Ota *et al.*, 1979) and up to 66% in the 2000s (Kageyama *et al.*, 2006). Non-male-killing spiroplasmas are also known to infect *D. aldrichi, D. mojavensis* (Mateos *et al.*, 2006), *D. wheeleri, D. tenebrosa, D. simulans, D. atripex, and D. ananassae* (Haselkorn *et al.*, 2009). In a laboratory experiment, transmission of a non-male-killing *Spiroplasma* from *D. hydei* to *D. melanogaster* has been documented (*Oregon R* strain) (Kageyama *et al.*, 2006). The infection persisted for 2-3 generations with no male killing effect. Until recently, *Spiroplasma* in drosophilids had only spread in South America (Montenegro *et al.*, 2000) and Japan (Ota *et al.*, 1979). In African drosophilid populations (Uganda), male-killing *Spiroplasma* was detected in a wild-caught *D. melanogaster* female in 2005 (Pool *et al.*, 2006). Recent studies show an increase in *Spiroplasma* infection in *D. neotestacea* from around Rochester, NY, from ≈10% in the 1980s to ≈80% today. This increase has been attributed to the adaptive value of the infection in that the infected females are more resistant against nematodes (Jaenike *et al.*, 2010). The evolutionary relationships, however, of other newly discovered spiroplasmas remain poorly understood, as does the relatedness of the male-killing and non-male-killing *Drosophila* spiroplasmas. One explanation for the expression of male killing caused by spiroplasmas is proposed in the "threshold density hypothesis" (Anbutsu and Fukatsu, 2003) whereby the male-killing phenotype requires that the bacterium be at some relatively high titre within the host before the male-

killing effect is observed. In addition, there is evidence that the expression of spiroplasma-induced male killing in *D. melanogaster* may depend on host genotype with some genetic backgrounds being more likely to express male-killing when infected than others (Kageyama *et al.*, 2009). In spite of these studies, however, the mechanism of male killing still remains unknown.

The objectives of this study were to use DNA sequence-based phylogenetic tools to: 1) document the presence or absence of *Spiroplasma* infection in Ukrainian populations of *D. melanogaster*, and 2) determine whether or not *Spiroplasma* found in Ukrainian populations were capable of male-killing.

Materials and Methods

Fly collection sites

Natural populations of *D. melanogaster* were sampled during the summer (August-September) seasons of 2006, 2007, and 2008 in different regions of Ukraine, including near Kyiv, Odesa, Uman', Varva, Lubny, Pyryatyn, Magarach (Yalta), as well as from three locations within the Chornobyl nuclear exclusion zone (Poliske, Chornobyl, and near the cooling pond of the Chornobyl Nuclear Power Plant).

DNA extraction

DNA was extracted from 10 wild-caught females from each of the studied natural populations. DNA extraction was performed using QIAamp DNA Micro Kit (Qiagen, USA) following manufacturer's protocol.

Spiroplasma detection

Detection of the bacteria in the fly DNA preparations was performed as described in Montenegro *et al.* (2005). We used PCR with primers specific to a highly conserved 360 bp long region of *Spiroplasma* 16S rRNA (5'-GCT TAA CTC GAG TTC GCC; 5'-CCT GTC TCA ATG TTA ACC TC). The PCR profile was as follows: denaturation 94ºC/3 min; 30 cycles –denaturation 94ºC/30 s, annealing 51ºC/45 s, elongation 72ºC/60 s; final elongation 72ºC/4 min. The reaction mix volume was 20 μl (2 μl DNA, 2 μl 10× buffer, 1 μl 25 mM MgCl₂, 2 μl 2 mM dNTP, 2 μl 10 μM primers, 0.25 μl 5U/μl *Taq*, 10.75 μl H₂O).

Sequencing

The 16S rRNA fragment was amplified and purified using a Gel Extraction Kit (Qiagen, USA). Sequencing was performed in the Engencore sequencing lab of the University of South Carolina, USA, using the 3130 Genetic Analyzer (Applied Biosystems, USA). Sequences were analyzed using BLAST (Altschul *et al.*, 1990).

Electrophoresis

PCR products were electrophoretically separated in a 1% agarose gel. The DNA fragments were determined by comparing the length of the PCR product with a sequenced fragment from the Varva population originally collected in 2006.

Fly rearing conditions

Thirty females collected from nature near Varva, Magarach, and Kyiv during three years (2006-08) were placed on a standard medium (6 g agar + 15 g yeasts + 50 g sugar + 55 g semolina per 1 L water) and reared for five generations at 25ºC as isofemale strains. Sex ratio was calculated

for offspring of each generation. Male killing usually manifests itself as the complete or nearly complete absence of males within a family making determination of infection unambiguous at the level of individual female (Anbutsu and Fukatsu, 2003).

Results and Discussion

PCR-based screening

PCR-based screening of 10 natural populations of *D. melanogaster* from Ukraine demonstrated the presence of the diagnostic 360 pb fragment in samples from only two populations (Varva and Magarach) collected in 2006, 2007, and 2008. This fragment was absent from samples collected from Kyiv, Odesa, Uman', Lubny, Pyryatyn, and all three populations from the Chornobyl zone (Figure 1).
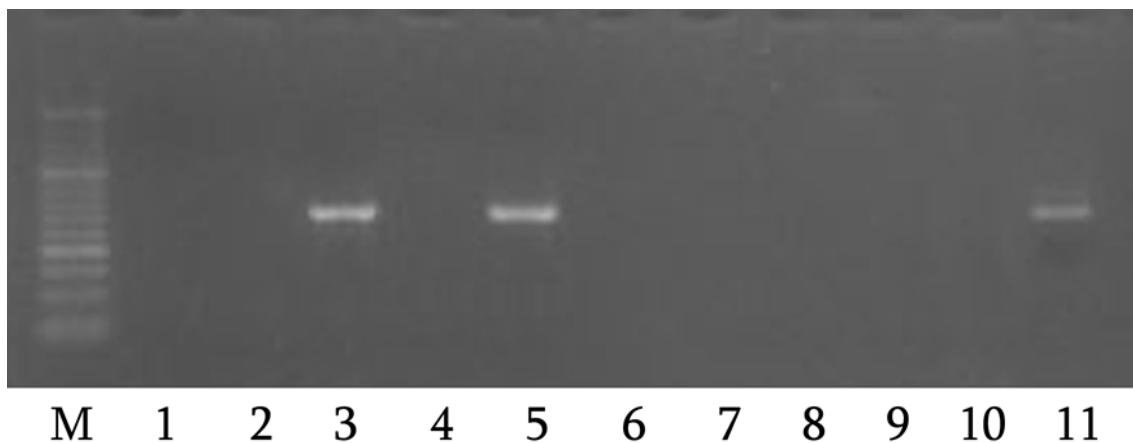

Figure 1. Electrophoregram of a PCR of a *Spiroplasma* 16S rDNA region. M – molecular weight marker; populations: 1 - Kyiv, 2 – Odesa, 3 – Varva, 4 – Uman', 5 – Magarach, 6 – Lubny, 7 – Pyryatyn, 8 – Apple Garden, 9 – Cooling Pond, 10 – Poliske, 11 – positive control (the PCR product which had been sequenced).

PCR products were sequenced to verify that our diagnostic DNA fragment was indeed *Spiroplasma* via comparison to sequences archived in GenBank (Seq1 HM775085). Our PCR product was 90% identical to the sequence of *Spiroplasma* 16S rRNA from GenBank (FJ657180).

This paper represents the first report of spiroplasma infection in *D. melanogaster* anywhere in Eurasia. *Spiroplasma* has, however, been reported from other hosts in Russia, *e.g.* the two-spotted ladybird beetle *Adalia bipunctata* (Sokolova *et al.*, 2002; Zakharov *et al.*, 2000). Also in Western Europe, *S. ixodetis* has been isolated from Arachnida and Insecta (but not Drosophilidae). The only event of infection with *S. poulsonii* in Europe (infected *Alopecosa pulverulenta*) was reported near Bern, Switzerland, in 2005 (Duron, 2008).

The sequence we obtained for Ukrainian spiroplasma shows a high degree of similarity to *Spiroplasma* 16S rRNA sequences archived in GenBank. It has 87.8% identity with the 16S rRNA gene of *S. poulsonii* strain NSRO isolated from a Japanese population of *D. nebulosa* (GenBank #AB434486). The sequence is a little less similar to its correspondent in *S. citri* − 87.2% (GenBank #AM157769) and 79.3% identity with that of *S. ixodetis* (GenBank #GU585671). This suggests,

based on the 16S rRNA sequence similarity, that the *Spiroplasma* we detected is a close relative to the *S. poulsonii* type sequences. A more detailed classification with species identification of our samples was not possible due to the low variability of the 16S rRNA gene fragment within our collections, but it is interesting that our samples have a 90% identity with the 16S rRNA of *Spiroplasma* endosymbionts of *D melanogaster* isolated from Uganda in 2006 (GenBank #FJ657180.1), suggesting a possible African origin for the Ukrainian endosymbionts. Other isolates from non-Drosophilidae species (http://www.ncbi.nlm.nih.gov/sites/entrez?db+nucleotide) have a 90% identity with the *Spiroplasm*a from our Ukrainian sites suggesting the possibility of lateral transfer of the bacterium from another species.

We have shown that *Spiroplasma* infection does not occur in all Ukrainian drosophila populations, and thus we may be observing the early stages of *Spiroplasma* colonization of *D. melanogaster* in this region. This colonization could have resulted from horizontal transfer (Kageyama *et al.*, 2009) from another host species in Eurasia (Duron, 2008). It is also worth noting that environmental factors may also inhibit the spread of infection. For example, we have suggested that the expression of winter diapause in temperate zone and high latitude *D. melanogaster* populations (Schmidt *et al.*, 2005) may reduce the likelihood of infection through direct effects of temperature on *Spiroplasma* growth, hence resulting in its relative rarity in such regions.

Sex ratio and male-killing

Spiroplasma is known to be capable of inducing male killing in drosophilids (Montenegro *et al.*, 2005). In order to characterize the influence of the bacterium on sexual reproduction in *D. melanogaster* from Ukrainian natural populations, we analyzed sex ratios in the offspring of isofemale strains of populations from Varva and Magarach (both infected with the bacterium) and Kyiv (not infected) for 5 generations. We did not detect male killing in any of the isofemale lineages derived from 2006, 2007, and 2008 collections, either from populations infected with *Spiroplasma* or those that were uninfected.

The absence of a male killing effect in populations infected with the bacterium could be associated with several different factors. Explanations to account for the lack of male-killing include genetic changes in the bacterium, inhibition of the effect of *Spiroplasma* by other microorganisms, low titers of the bacterium in the host organism (Anbutsu and Fukatsu, 2003), or the genetic make-up of the host populations (Kageyama *et al.*, 2009). The study by Kageyama *et al.* (2006) suggests that there may be host genotypic effects on both the titre of bacteria and their ability to express the male-killing phenotype. It is worth noting that all past studies of *Spiroplasma* infecting *D. melanogaster* have only detected aggressive strain that causes the disruption of male embryo development that results in high rates of male killing. However, injection of the non-male-killing strain of *Spiroplasma* from *D. hydei* into *D. melanogaster* flies did not result in male killing (Kageyama *et al.*, 2006), suggesting genetic variation for this trait among *Spiroplasma* strains. In addition, the *Kageyama et al.* (2006) study found that the expression of male killing was dependant on the genotype of *Spiroplasma*, rather than host, in *D. hydei*. When *Spiroplasma* was transferred from *D. hydei* to *D. melanogaster*, it did not result in male killing. A low titer of the infection was, however, shown to coincide with the absence of male killing (Kageyama *et al.*, 2006), and it was suggested that *D. melanogaster* genotype regulated the expression of male killing specifically by means of titer down regulation. This suggests that although the male-killing phenotype may depend on the genotype of the bacterium, it is also likely influenced by the genotype of the host.

The influence of multiple co-infections with bacteria of different genera on the manifestation of *Spiroplasma* effects in *D. melanogaster* has not been well investigated, except for studies of its co-infection with *Wolbachia*. However, no signs of interaction effects related to co-infection with *Spiroplasma* have been observed in the presence of *Wolbachia* (Protsenko *et al.*, 2006). *Wolbachia*

infection has been reported for all investigated populations in Ukraine (Protsenko *et al.*, 2006), and it is possible that the presence of *Wolbachia* has a direct effect on the titre of *Spiroplasma* in these populations (Mouton *et al.,* 2004). If the threshold density hypothesis for the expression of male killing by *Spiroplasma* (Anbutsu and Fukatsu, 2003) is in effect, and co-infection with *Wobachia* reduces *Sprioplasma* titres, then it is possible that this interaction may be responsible for the lack of any observed male killing in these populations (Montenegro *et al.*, 2006). Further research is needed to confirm or rule out such interactions as a mechanisms underlying variation in the expression of male-killing.

For the first 30 years of studies on the dynamics of geographic range expansion of *Spiroplasma* in natural drosophilid populations, infection by this endosymbiotic bacterium had been detected primarily in two drosophila species – *D. hydei* (46 to 66%) and *D. neotestacea* (10 to 80%) (Jaenike *et al.*, 2010); only a few rare incidents of *Spiroplasma* in *D. melanogaster* have been recorded from Brasil (Montenegro *et al.*, 2005) and Uganda (Pool *et al.*, 2006). Our results suggest that this endosymbiont has now spread to Ukrainian drosophila populations.

Although it is clear that *Spiroplasma* is presently spreading throughout the world, in general, the reasons underlying this range expansion are not known. In the case of *D. neotestacea* from North America (Jaenike *et al.*, 2010), it seems likely that *Spiroplasma* range expansion is promoted by the adaptive effect of infection on the resistance to parasitic nematodes. It is possible that range expansion of endsymbionts is frequently associated with either beneficial or benign effects. It is perhaps worth noting that infection rates of natural populations of drosophilids with the male-killing *Spiroplasma* are typically very low (up to 3%) (Kageyama *et al.*, 2006) suggesting that the negative fitness consequences associated with the male-killing phenotype may at least in part be responsible to its relatively slow rates of range expansion and rarity in Drosophilids.

Overall, much remains to be learned from the spread of endosymbiotic organisms and that further studies of Ukrainian populations may be particularly useful in unraveling to mechanisms underlying both the spread of endosymbionts as well as variation in the expression of what are usually considered maladaptive traits like male-killing.

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Post-reproductive survival and the Faulkner Effect in *Drosophila melanogaster.*

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Survival and reproduction are the central components of life history. As noted by Novoseltsev *et al.* (2005), much is known about individual variation in survival among adult flies, but there exists relatively little information about individual variation in fecundity. Fecundity data are typically collected by counting the total number of eggs and live females in a cohort at each age. The cohort method obscures variation between individual flies, and provides no information about post-reproductive survival (PRS). If PRS were common it could cause substantial underestimation of fecundity rates in cohort studies by adding individuals, but not eggs, to the counts. High levels of PRS would suggest a lack of coordination between life history components. On the other hand, zero or brief post-reproductive survival would negate some concerns about the cohort method for estimating fecundity trajectories, and also suggest an intrinsic coordination of viability and reproduction.

Here we report on individual variation in life span and fecundity schedules among lab-reared female *D. melanogaster.* We used RI7, a long-lived recombinant inbred line derived from Luckinbill's artificial selection experiment for late age of reproduction (see Curtsinger and Khazaeli, 2002; Khazaeli and Curtsinger, in press). Flies were reared under controlled density of 100 larvae per vial. Mixed-sex pairs were placed in 8-dram shell vials on cornmeal-molasses medium within 12 hours of emergence. Vials were maintained at 24°C under constant illumination. Pairs were transferred daily without anesthesia to fresh vials. After transfer the daily egg production was counted under a dissecting microscope. To ensure that sperm availability did not limit fecundity, dead males were replaced with males of the same genotype as needed. Transfers and egg counts continued until the death of the last female. The finished data consist of complete life spans and daily fecundities for 126 females studied contemporaneously, with one case trimmed for sterility.

Female life spans averaged 50.8 days (*sd* = 14.9), while average lifetime fecundity was 764 eggs (*sd* = 321). PRS, defined as the number of days alive after the last observed egg, was highly variable, ranging from 0-43 days with an average of 6.6 days (*sd*=9 days). Total life spans and PRS for each fly are shown in Figure 1. The figure was produced by sorting individual life spans from shortest to longest and then plotting each as a horizontal bar; the resulting outline shows cohort survivorship. PRS is represented by the black segment of each bar.

Long periods of PRS are common, but not universal, in this genotype. Of the total 6400 fly-days lived by the cohort, 13% was PRS. Half of the flies exhibited PRS of three days or more. In