The Stockholm populations of *Adalia bipunctata* (L) (Coleoptera: Coccinellidae)—a case of extreme female-biased population sex ratio

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The genetic composition and sex ratio in the Stockholm populations of *Adalia bipunctata* have been studied. The overall frequency of melanics is 3.2 %, which is significantly lower than in the populations of St. Petersburg and other large cities along the Baltic Sea. The secondary sex ratio in the Stockholm populations is female-biased 82:18. More than half of *A. bipunctata* females are infected with the male-killing *Spiroplasma* bacterium. Beetles of the co-existing species *Adalia decempunctata* are infected with a different bacterium belonging to the genus *Rickettsia*.

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Adalia bipunctata is a classical subject of population and ecological genetics (see the reviews of MAJERUS 1994; ZAKHAROV 1995). Of special interest is melanism, i.e. the occurrence of black morphs in populations, and the distribution in populations of male-killing bacteria that change the sex ratio. We have previously studied in detail the populations of *A. bipunctata* from St. Petersburg and Leningrad region (SERGIEVSKII and ZAKHAROV 1983; ZAKHAROV and SERGIEVSKII 1983). These studies have demonstrated that among all examined populations of Eastern Europe the highest frequency of melanic morphs is observed in the population from the center of St. Petersburg (80–85 % of melanics) (ZAKHAROV 1990). We have also shown (ZAKHAROV et al. 1996; 1998) that *A. bipunctata* females in St. Petersburg are often infected with the *Spiroplasma* bacterium that kills male embryos due to which the sex ratio in the population is female-biased and makes up approximately 70:30.

The cause of mass infection is unknown. As for the melanism in the St. Petersburg population, we explain it, following LUSIS (1961), by an interaction of natural and anthropogenic factors, namely, by the marine climate and industrial pollution of the environment (ZAKHAROV 1990, 1995). This makes a very good argument for studying the *A. bipunctata* populations in Stockholm—the second largest city in the Baltic region located in the same latitude as St. Petersburg. The results of our study are presented in this communication.

Another species of the genus *Adalia*, *A. decempunctata*, inhabits some regions along with *A. bipunctata*. This species is not found in St. Petersburg. Little is known about the distribution of male-killing bacteria in the populations of *A. decempunctata*.

MATERIALS AND METHODS

Adalia beetles were collected in July 1998 in two regions of Stockholm—in the center, near the Royal Palace, and on the island of Djurgarden, as well at a distance of about 12 km from the center in the south-west direction in the Skarholmen-Kungens kurva region. The beetles were collected from different trees and bushes, mainly linden, willow, elder.

From the pupae collected in the region of Skarholmen-Kungens kurva, in addition to beetles of the more abundant species *A. bipunctata*, a small number of ladybirds of another species, *A. decempunctata*, emerged. They were also used in searching for male-killing bacteria.

Upon visual examination, the *A. bipunctata* beetles were divided by the coloration and the pattern on elytra in the following morphs: typica, anulata (red), 6-pustulata, 4-maculata (melanics). The sex of the beetles was determined by means of dissection using toothpicks that were changed after dissection of each female to avoid an artificial transfer of bacteria from infected beetles to noninfected ones.

Bacterial infection of the beetles by bacteria was established by the method of polymerase chain reaction.

For polymerase chain reaction (PCR) DNA was isolated by chloroform extraction followed by ethanol precipitation. Prior to DNA isolation, the beetles were kept frozen at −20°C overnight. Then, wings and elytra were cut off, and the bodies were homogenized in Eppendorf tubes in a lysing solution containing 0.1 M EDTA, 5 mM Tris-HCl (pH 8.0), and 0.75 M NaCl. After addition of SDS to a final concentration of 1 %, the suspension was incubated for 1 h at 65°C, and 10 M potassium acetate (1/7 of volume)

was then added. The mixture was agitated, incubated for 30 min at 0°C and centrifuged for 10 min at 8000g. An equal volume of chloroform was added to the supernatant, and the mixture was centrifuged at 8000g for 10 min. Ethanol (2.5 volumes) was added to the supernatant, and the DNA precipitate was pelleted at 10,000g for 10 min. DNA was dissolved in sterile water and directly used for amplification (0,1 μg).

PCR was performed in a volume of 25 μl in a thermocycler (Tula, Russia) using thermophilic Taq-DNA polymerase (Bion, Moscow) by the Hot start method (ZAKHAROV et al. 1998).

PCR was carried out with primers specific for the 16S rRNA genes of bacteria of the class *Mollicutes*, which includes the genus *Spiroplasma*. The following primers were used: MGSO-(5′-TGCACCATCTGT-CACTCTGTTAACCTC-3′) and FP-(5′-GCTCAAC-CCCTAACCGCC-3′) (VAN KUPPEVELD et al. 1992). PCR was used to attempt amplification of a 429 bp product under conditions: 35 cycles of denaturation at 95°C for 35″, annealing at 55°C for 1′, synthesis at 75°C for 1′, with the last synthesis at 75°C for 10′.

PCR with primers for a 16S rRNA gene fragment of bacteria of the genus *Wolbachia*: 16S Bf-(5′-TTCG-GCCGGATTTTACACAA-3′) and 16S Br-(5′-TAGGGATTAGCTTAGGCTTG-3′) (WERREN et al. 1995) was performed under conditions: 35 cycles of denaturation at 95°C for 20″, annealing at 55°C for 30″, synthesis at 72°C for 30″, with the last synthesis at 72°C for 10′. A product of 260 bp was expected in the presence of *Wolbachia*.

PCR was used to attempt amplification of 434 bases of the 17 kDa antigen gene of bacteria of the genus *Rickettsia* using the primers Rr17.61p-(5′-GCTCTTG-CAACTTCTATGTT-3′) and Rr17.492n-(5′-CATT-GTTCGTCAGGTTGGCG-3′) (WILLIAMS et al. 1992) under cycle conditions:30 cycles of denaturation at 95°C for 40″, annealing at 57°C for 1′, syntesis at 72°C for 1′, and the last syntesis at 72°C for 10′. PCR cocktail (reaction mix without DNA) was used as a negative control in all PCR reactions.

The amplified products were electrophoretically separated in 1.5 % agarose gel (type I agarose with a low electroendosmosis was obtained from Dia-M, Moscow), at 5 V/cm.

RESULTS

The frequencies of differently colored morphs in our samples are given in Table 1. The compositions of the populations from the center of the city and its neighborhood do not differ significantly. The overall frequency of melanic morphs in the Stockholm populations is 3.2 %.

The beetles produced from pupae under laboratory conditions were studied for the sex ratio. Thus, we registered the secondary sex ratio undistorted by a possible differential mortality at the imago stage. The results are presented in Table 2. The proportion of males in the *A. bipunctata* population is 0.18. The deviation from the 1:1 ratio is highly significant. Part of collected females served as a material for determining the bacterial infection.

14 females and 1 male were discovered among the *A. decempunctata* ladybirds emerged from pupae.

To find bacteria living in the cytoplasm of Stockholm's *A. bipunctata* cells, PCR was run with primers specific for a region of the 16S small subunit rRNA gene of bacteria of the class *Mollicutes*. The primers directed amplification of a 429-bp fragment from bacterial DNA. A positive control in the experiments was DNA isolated from insects of the line I-12 from St. Petersburg containing DNA of *Spiroplasma*, earlier analyzed and sequenced by us (ZAKHAROV et al. 1998). The PCR analysis resulted in the amplification of the expected fragment of approximately 429 bp only in 26 of 48 beetles (all females) collected in the vicinities of Stockholm (Fig. 1, Table 2). The percentage of infection is 54.2 %.

There are some other known symbiotic bacteria that are inherited cytoplasmically (i.e., maternally transmitted through egg cytoplasm) and affect the sex ratio in host Eurasian populations of *A. bipunctata* L. (WERREN et al. 1994; HURST et al. 1999a,b; MAJERUS et al. 2000; ZAKHAROV et al. 2000). So, 22 females non-infected with bacteria of the class *Mollicutes* were studied by PCR with primers specific for rickettsial 17 kDa-protein gene and primers specific for a 16S rRNA gene fragment of bacteria of genus *Wolbachia*. But specific PCR fragments were absent from DNA of these ladybirds.

Table 1. *Polymorphism for elytrum coloration in the Stockholm populations of A. bipunctata*

Place of collection	Number of beetles	typica	anulata	6-pustulata	4-maculata	% melanics
Center of the city	103	96	2	5	–	4.9
Kungens kurva	86	84	1	–	1	1.2
Total	189	180	3	5	1	3.2

Table 2. *Secondary sex ratio and infection of A. bipunctata females by Spiroplasma in the Stockholm populations*

Sex	Number of beetles	χ^2; p	Infected	Noninfected
Females	82	$\chi^2 = 40.96$	26	22
Males	18	$p < 0.001$	n.s	n.s

n.s.—not studied.

We also tested the beetles of the *Adalia decempunctata* population collected in Stockholm (Kungens kurva). DNA isolated from 18 *A. decempunctata* females failed to amplify with both the *Mollicute* and the *Wolbachia* specific primers, whereas four templates did amplify in the *Rickettsia*-specific PCR. The presence of rickettsial genus-specific DNA sequences was examined by PCR amplification of a 434-bp sequence of the gene for a 17-kDa antigen, specific for rickettsiae of the *R. typhi* group (Fig. 2).

DISCUSSION

We expected the *A. bipunctata* populations from Stockholm to be similar to the population from St. Petersburg taking into account similar climatic and anthropogenic conditions in these cities. This was, however, not quite the case. The frequency of melanics in Stockholm has turned out to be much lower than in St. Petersburg. This frequency is lower than in Helsinki (10.5 % of melanics) (MIKKOLA and ALBRECHT 1988), in Tallinn (25.1 %) (ZAKHAROV 1990), in Riga (40.6 %) (LUSIS 1961) and in Kaliningrad (31.5 %) (ZAKHAROV 1990). It is likely that the degree of atmospheric pollution in Stockholm has always been lower than in St. Petersburg where a large number of industrial enterprises are concentrated. To elucidate the role of pollution in the accumulation of melanics, it seems to be of interest to study the populations of *A. bipunctata* in other cities of Sweden.

A bias in favor of females in the secondary sex ratio in Stockholm is record-breaking. In populations of France studied by us previously, the sex ratio is 1:1 (ZAKHAROV and GORYACHEVA 1998), in England the proportion of females is 0.47 (HURST et al. 1993), in Moscow 0.46, in St. Petersburg 0.30 (ZAKHAROV and GORYACHEVA 1998). Up to the present, the bias in St. Petersburg has been the largest one following what we or other authors observed in populations of *Adalia*. It should be noted that regarding another species of ladybirds, *Harmonia axyridis*, it was found that in the population of Sapporo City (Japan) 49 % of females were attacked by the male-killing bacterium (MAJERUS et al. 1998). The propor-

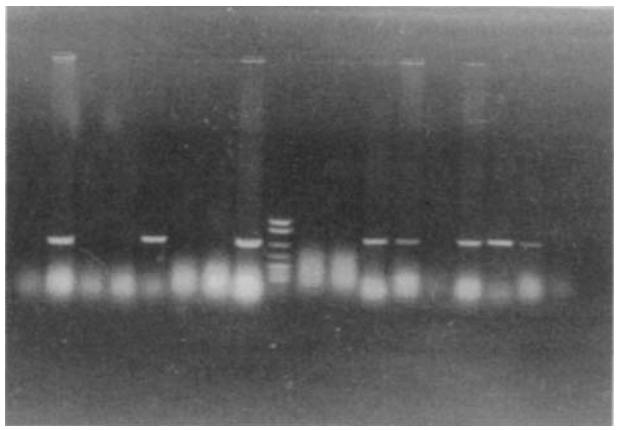

Fig. 1. Detection of bacterial DNA in total DNA isolated from *A. bipunctata* adults. A fragment of 429 bp of the bacterial gene for 16S small subunit rRNA was amplified with primers common for bacteria of the class *Mollicutes*. Lines: (1–16) *A. bipunctata* females from the Stockholm population; (10) molecular weight marker (pBR322 digested with MspI); (17) female *A. bipunctata* line I-12, containing *Spiroplasma*—positive control; (18) negative control (lanes are numbered left to right).

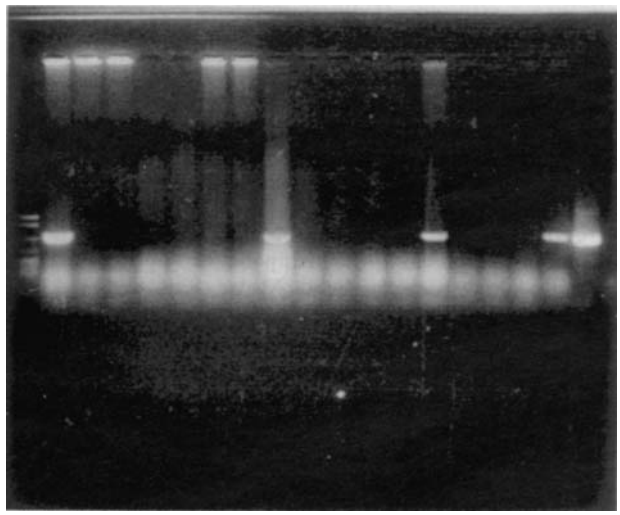

Fig. 2. Detection of bacterial DNA in total DNA isolated from *A. decempunctata* adults. A fragment of 434 bp was amplified with primers specific for the DNA fragment of the 17-kDa-protein gene of bacteria of genus *Rickettsia*. Lines: (1) molecular weight marker (pBR 322 digested with MspI); (2–18) *A. decempunctata* females; (19) positive control—*Rickettsia* typhi DNA; (20) negative control (lanes are numbered left to right).

tion of females turned out to be 0.39 (taking into account the tertiary sex ratio among beetles in nature at the stage of imago). In Stockholm a factor causing a deviation from the normal sex ratio is, as in St. Petersburg, the bacterium of the genus *Spiroplasma* which infects more than half of females. It is not clear, however, what determines such a high level of infection in this population. Since *Spiroplasma* is found with a high frequency (46.7 %) in the population of St. Petersburg too (ZAKHAROV et al. 1998), it would be very interesting to elucidate whether it is characteristic of all Baltic populations of *A. bipunctata* or only of some of them, maybe in large cities. We suppose to clarify this question later on.

The presence of *Rickettsia* in the *A. decempunctata* populations from Germany has already been reported (GRAF VON DER SCHULENBURG et al. 2001). It is, therefore, of interest that we collected *A. bipunctata* and *A. decempunctata* pupae on leaves of the same tree, i.e. ladybirds of both species co-exist together and are in close contact. Despite this fact, they are infected with different bacteria. This suggests the absence of a regular horizontal transfer of male-killing bacteria in ladybird populations.

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