RESEARCH ARTICLE

Effect of lead pollution on fitness and its dependence on heterozygosity in *Drosophila subobscura*

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Abstract

Lead is one of the most present contaminants in the environment, and different species respond differently to this type of pollution. If combined with genomic stress, lead may act synergistically, causing significant decrease of fitness components. We used two genetically diverse *Drosophila subobscura* populations (regarding both putatively adaptive inversion and microsatellite loci polymorphisms) originating from two ecologically distinct habitats. To establish different levels of genome heterozygosity, series of intraline, intrapopulation and interpopulation crosses were made. The progeny were reared on a standard medium and a medium with 200 μg/mL of lead acetate. Development time was significantly extended to all groups reared on lead. The progeny of intraline crosses showed significantly extended development time compared to all other groups. The obtained results suggest that genome heterozygosity reduces the effect of lead pollution.

Introduction

Human impacts on climate warming, pollution, habitat loss and fragmentation are likely to increase over 21st century (Smith *et al.* 2009). Habitat fragmentation can increase the risk of stochastic fluctuation in population size, the distribution pattern of populations and even metapopulation dynamics which consequently influence population genetic structure (Frankham 2005; Ewers and Didham 2006). Important issues arise regarding the limits and the ability of populations and species to adapt to different anthropogenic selective pressures, simultaneously. Habitat fragmentation could potentially lead to *de novo* population subdivision and a population with already challenged genetic structure could be more vulnerable to other environmental stressors, i.e. heavy metal pollution. Pollution increasingly affects variability of natural populations, which may be a direct consequence of pollutant impact on organism fitness, migration rate, or indirect by altering predation pressure or competition (Klerks and Levinton 1993). The direct effect may be obviated by increased tolerance caused by physiological acclimation after exposure to sublethal level of pollutants earlier in life (Klerks and Weis 1987), acclimatization or genetic adaptation in which a population has evolved towards increased tolerance.

Populations of various species have different ways of coping with environmental stress, either through adaptations including evolutionary change or phenotypic plasticity, or they can leave the affected habitats, establishing new communities (Williams *et al.* 2007). Further, genetically diverse and previously isolated populations may come into secondary contact with other populations, and hybridization may occur (Wellenreuther *et al.* 2010). The results of hybridization in terms of fitness depend largely on the genetic differences between populations. If they are genetically similar, effects of hybridization may be beneficial; but with increased genetic divergence, hybridization may lead to outbreeding depression, as a result of disruption of local adaptation under-dominance or breakdown of coadapted gene complexes (Edmands 2007; Kurbalija *et al*. 2010).

Numerous studies have shown depletion of genetic variability in populations exposed to heavy metal pollution (Xiao *et al.* 2000; Ross *et al.* 2002; Kim *et al.* 2003; Coors *et al.*

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2009; Lopes *et al.* 2009). Marine gastropods with higher levels of genetic diversity, have increased tolerance to heavy metal pollution (Nevo *et al.* 1986). In *Drosophila*, lead pollution affects various fitness components such as development (Cohn *et al.* 1992; Stark and Banks 2003; Kenig *et al.* 2013), longevity, fecundity and even complex behavioural traits such as courtship (Cohn *et al.* 1992; Massie *et al.* 1992; Hirsch *et al.* 2003). On the other hand, studies on the effects of different levels of population heterozygosity on fitness components in many species showed both benefits and outbreeding depression (Tallmon *et al.* 2004; Kurbalija *et al.* 2010).

However, literature data regarding combined effects of environmental stress and interpopulation hybridization as genomic stress are scarce, and the results obtained are specific for particular stress and differ among taxa. Temperature stress reduces F_1 hybrid vigour but also reduces F_2 fitness breakdown in interpopulation *Drosophila* hybrids (Vetukhiv and Beardmore 1959) but crowding stress simply increases the benefits of interpopulation hybridization in *Wyeomyia smithii* (Armbruster *et al.* 1997). Groenendijk *et al.* (2002) found rapid loss of heavy metal tolerance in *Chironomus riparius* hybrids showing population-specific acclimation to cadmium pollution. Edmands and Deimler (2004) suggested that fitness response was environment dependent in interpopulation hybrids of tidepool copepod *Tigriopus californicus* exposed to high temperature and low salinity. Kjaersgaard *et al.* (2012) showed that differences in locomotor performances of inbred and crossbred *D. melanogaster* are temperature-specific, hybrid individuals performed better when exposed to higher temperatures, whereas inbred individuals were better in standard laboratory conditions, suggesting that heterosis may be stronger in stressful environments where the masking of conditionally expressed recessive deleterious alleles is of strong fitness benefit (Reed *et al.* 2002, 2012; Joubert and Bijlsma 2010; Bijlsma and Loeschcke 2012).

D. subobscura represents a useful model species in evolutionary biology research. Numerous studies have focussed on the analysis of genetic structure of different natural *D. subobscura* populations, using several adaptive and neutral genetic markers: inversion polymorphism, microsatellites and mtDNA variability (Krimbas 1993; Pascual *et al.* 2001; Stamenkovic-Radak *et al.* 2008; Kurbalija Novicic *et al.* 2011; Jelic *et al.* 2009; Stamenkovic-Radak *et al.* 2012). The results obtained with inversion polymorphism as an adaptive molecular marker suggest that populations in different habitats have been subjected to habitat-specific selection regimes (Stamenkovic-Radak *et al.* 2012). Literature data indicate that *D. subobscura* populations are genetically diverse (according to putatively adaptive polymorphism), even on a small geographic scale, with frequent strong and unpredictable consequences of interpopulation hybridization (Kurbalija *et al.* 2010). Results for chromosome markers and mtDNA to some extent suggest adaptive population divergence and local adaptation to specific microhabitat

characteristics (terrain topology, temperature, humidity, insolation and vegetation coverage). Gene arrangements are differently favoured in diverse microhabitats despite high gene flow and large effective population sizes (Kurbalija Novicic *et al.* 2013). Also, Kenig *et al.* (2013) showed difference in tolerance to lead pollution between two populations of *D. subobscura* from different habitats indicating local genetic adaptation to heavy metal pollution. Under such conditions, selection is effective in driving the evolution of a population.

The objectives of the present study are: (i) to investigate the heterozygote performance under stressful conditions (lead contamination), (ii) to estimate if the amount of heterozygosity (genetic background) is able to moderate a stressful environment (lead pollution), and (iii) to investigate whether populations from ecologically different habitats differ in fitness performance under lead pollution.

Materials and methods

Population sample

For the present study, *D. subobscura* individuals were sampled from two ecologically distinct habitats; Sicevo Gorge, SG and Botanical Garden, BG, both located in Serbia. SG, located in Eastern part of Serbia (43°19′N, 22°08′E), known as a glacial refugium of the central Balkans, has polydominant forests, endemic flora and represents a stable ecosystem where fluctuations in temperature are minimal. The BG *Arboretum* is located in a central, urban part of Belgrade (44°49′N; 20°28′E) and chronically exposed to anthropogenic activity. Soil analysis showed 18-fold higher levels of zinc and nine-fold higher levels of lead in BG than in SG. Stamenkovic-Radak *et al.* (2012) showed significant differences between two particular populations (BG and SG) based on inversion polymorphism, variability of mtDNA haplotypes and microsatellite variability.

The flies were collected using conventional fruit traps. Approximately 50 isofemale lines (IF) per population were made, each from a single gravid wild-caught female. All lines were maintained and all experiments were performed under constant laboratory conditions, at 19±0.5°C, ~60% relative humidity, light of 300 lux and 12/12 h light/dark cycles. Experimental procedure started on four generations old flies after bringing them from the field to allow acclimatization to laboratory conditions and prevent false decrease in fitness components due to acclimatization.

Experimental setup

In this experiment, we randomly chose 32 IF lines from both populations, SG and BG. To create a series of lines with different levels of genome heterozygosity, three types of crosses were performed as three experimental groups: (i) random crosses within IF line (intraline), (ii) random crosses between IF lines within each of populations (intrapopulation hybrids), and (iii) random crosses between two subsets

of IF lines originated from two natural populations (inter-population hybrids). For interpopulation and intrapopulation hybrids both direct and reciprocal crosses were made to detect any possible maternal effect. In this way, eight experimental groups according to the type of cross were established. All crosses were made with 3–5 d old virgin flies of both sexes, collected every 24 h and kept separately. Three males and three females were used for each type of cross (intraline, intrapopulation and interpopulation). The crossing scheme is provided in table 1.

After allowing flies to mate for 48 h, females were transferred to vials containing fresh standard cornmeal *Drosophila* medium to lay eggs and females were transferred everyday to a fresh medium, until enough eggs were collected. From each cross, 15 eggs were collected and transferred to the standard medium and 15 to a medium containing 200 μg/mL lead acetate (Pb(CH$_3$COO)$_2 \cdot$ 3H$_2$O). In this way, 16 experimental groups were established (eight with and eight without lead in medium). This concentration of lead acetate was chosen after a pilot experiment on the SG population showed LC20 for this concentration (results not shown). Three replicates in three subsequent days were made on each medium.

To assess the fitness components under stressful (lead contamination) and nonstressful conditions, development time and egg-to-adult viability in all experimental groups were monitored.

Development time (*DT*) was calculated in days, once all the adults were emerged:

$$DT = \frac{\sum n_d * d}{\sum n_d},$$

where n_d is the number of flies emerging in d days after the eggs were laid.

Egg-to-adult viability (*V*) was calculated as the ratio of the emerged adults to the number of transferred eggs:

$$V = \frac{n}{N},$$

where N is the number of transferred eggs and n is the number of adults emerged from them.

Viability data were analysed as arcsine square root transformed proportions. No transformation was needed for development time. Tests for normality and homogeneity of data variances were confirmed by Jarque-Bera, Bartlett's and Levene's tests incorporated in PAST software (Hammer *et al.* 2001). Post-hoc Fisher LSD test was performed within

two-way ANOVA to resolve the variability between populations, type of cross and treatment for egg-to-adult viability and development time. All analyses were performed with Statistica for Windows 6.0 (StatSoft, Aurora, USA).

Results

Development time

The mean values and variances of development time for all types of crosses (intraline, intrapopulation and interpopulation, direct and reciprocal) reared on the standard and lead media are shown in table 2.

Two-way ANOVA for development time with the type of cross and treatment as factors (table 3) showed that lead pollution has a highly significant effect on development time ($F = 1066$, $P < 0.001$). Also, crosses significantly differ in development time ($F = 5$, $P < 0.001$). However, there was no significant interaction between the type of cross and treatment regarding development time.

Lead pollution significantly increased the development time in all types of crosses (Post-hoc Fisher LSD $P < 0.01$). Intraline crosses from both populations (BG and SG) reared on standard medium showed significantly longer development time compared to reciprocal intrapopulation hybrids (SG) reared on the same medium. Also, intraline crosses from both populations (BG and SG) reared on the standard medium showed significantly longer development time compared to interpopulation hybrids reared on the same medium. However, there was no significant difference in development time between intrapopulation and interpopulation hybrids reared on the standard medium.

Intraline BG crosses reared on lead showed significantly longer development time than all types of hybrid crosses reared on lead. Intraline SG crosses reared on lead showed significantly longer development when compared to reciprocal SG and reciprocal interpopulation hybrids reared on lead. There was no significant difference in development time between intrapopulation and interpopulation hybrids reared on lead. Also, there was no significant difference in development time between direct and reciprocal crosses of any type.

Egg-to-adult viability

The mean values and variances of egg-to-adult viability for intraline crosses, intrapopulation and interpopulation hybrids

Table 1. Crossing scheme. N = number of crosses and number of successful crosses is shown in brackets.

| | | | Intrapopulation | | | | Interpopulation | |
| | Intraline | | BG | | SG | | BG $\times$ SG | |
	BG	SG	Direct	Reciprocal	Direct	Reciprocal	Direct	Reciprocal
N	Bi♀ $\times$ Bi♂ 32(30)	Si♀ $\times$ Si♂ 32(31)	Bi♀ $\times$ Bj♂ 32(23)	Bj♀ $\times$ Bi♂ 32(30)	Si♀ $\times$ Sj♂ 32(26)	Sj♀ $\times$ Si♂ 32(29)	Bi♀ $\times$ Si♂ 32(24)	Si♀ $\times$ Bi♂ 32(27)

Table 2. Mean development time (in days) $\pm$ standard error and variance for all types of crosses reared on standard medium (control) and medium with lead.

		Control		Lead	
		Mean $\pm$ SE	Variance	Mean $\pm$ SE	Variance
Intraline	BG	21.61 $\pm$ 0.19	1.11	25.14 $\pm$ 0.30	2.72
	SG	21.49 $\pm$ 0.16	0.83	24.66 $\pm$ 0.18	0.97
Intrapopulation	BG direct	21.21 $\pm$ 0.21	0.99	24.36 $\pm$ 0.19	0.81
	BG reciprocal	20.95 $\pm$ 0.15	0.65	24.17 $\pm$ 0.22	1.46
	SG direct	21.10 $\pm$ 0.19	0.91	24.55 $\pm$ 0.19	0.98
	SG reciprocal	21.25 $\pm$ 0.15	0.70	24.12 $\pm$ 0.18	0.95
Interpopulation	BG $\times$ SG direct	21.32 $\pm$ 0.15	0.52	24.14 $\pm$ 0.20	1.00
	SG $\times$ BG reciprocal	20.96 $\pm$ 0.17	0.85	24.10 $\pm$ 0.17	0.82

for both, direct and reciprocal crosses reared on standard and lead medium are shown in table 4.

Two-way ANOVA with the type of cross (intraline, intrapopulation and interpopulation) and treatment (lead pollution) as factors (table 5) showed that the type of cross significantly influences egg-to-adult viability ($F = 9.846$, $P < 0.001$), but lead treatment showed no significant effects on this component of fitness.

Intraline crosses from both populations showed significantly lower viability when compared to intrapopulation and interpopulation hybrids on both lead-polluted and control media (post-hoc Fisher LSD in all comparisons, $P < 0.05$). Significantly, higher viability was obtained between intraline crosses reared on the control medium and both intrapopulation and interpopulation hybrids reared on lead polluted medium (post-hoc Fisher LSD in all comparisons, $P < 0.01$). There was no significant difference between intraline crosses reared on the lead or standard medium (table 4).

No significant differences in viability were observed between intrapopulation hybrids from two populations (SG and BG) reared on the standard or lead-polluted medium for both direct and reciprocal crosses. There was also no significant difference between intrapopulation hybrids reared on the standard medium and the ones reared on the lead-polluted medium. Further, no significant difference in viability was observed between interpopulation hybrids reared on different types of medium. No significant difference was detected

Table 3. Two way ANOVA for development time for individuals from all type of crosses reared on standard medium (control) and medium with lead.

Source	df	MS	F
Treatment	1	1099	1066***
Type of cross	7	5	5***
Treatment * type of cross	7	1	1
Error	427	1	

***Statistically significant.

when interpopulation hybrids were compared with intrapopulation hybrids from both populations (SG and BG) in direct and reciprocal crosses.

Discussion

The present study represents a rare empirical dataset using model organism *D. subobscura* with a well-known genetic background and ecology, and is aimed at investigating fitness performances of two distinct natural populations under stressful conditions such as lead pollution. These populations have different genetic backgrounds, defined by different evolutionary history of each population and microclimatic conditions. The populations were sampled from localities characterized by different levels of lead contamination of soil (concentrations of lead in soil were 68.5 mg/kg in BG and 7.41 mg/kg in SG localities). The experimental design was made so as to detect a difference in fitness performance under stressful environmental conditions among hybrids with different population origin and to estimate if genome hybridization at different levels (inbred, outbred) is able to moderate the effects of stressful environments. Contrary to expectations, the obtained results showed no population-specific response to lead pollution under laboratory conditions. The results rather go in favour of the hypothesis that positive heterosis could be the prevalent mechanism that stabilizes the fitness performance in a population exposed to lead in a stressful environment.

Development time showed significant increase and the same pattern of response in all groups reared under lead contamination. The obtained result is not in agreement with some previously reported data (Shirley and Sibly 1999; Kenig *et al.* 2013) which suggests that individuals exposed to lead pollution during several generations showed faster development compared to those reared on the standard medium, explaining that shorter development time is an advantage, at least, for *Drosophila* species, because the larval stage is less exposed to the stressful environment. Wu *et al.* (2006) detected that the total duration of the development from larva to adult of *Boettcherisca peregrine* was

Table 4. Mean egg-to-adult viability (%) ± standard error and variance for all types of crosses reared on standard and medium with lead.

		Control		Lead	
		Mean±SE	Variance	Mean±SE	Variance
Intraline	BG	0.66 ± 0.04	0.04	0.64 ± 0.04	0.05
	SG	0.66 ± 0.03	0.02	0.64 ± 0.03	0.03
Intrapopulation	BG direct	0.82 ± 0.02	0.01	0.81 ± 0.02	0.01
	BG reciprocal	0.78 ± 0.03	0.03	0.79 ± 0.04	0.04
	SG direct	0.73 ± 0.04	0.05	0.76 ± 0.04	0.05
	SG reciprocal	0.75 ± 0.04	0.04	0.74 ± 0.04	0.04
Interpopulation	BG × SG direct	0.79 ± 0.03	0.03	0.84 ± 0.02	0.01
	SG × BG reciprocal	0.84 ± 0.02	0.01	0.84 ± 0.02	0.01

prolonged by cadmium exposure at a higher Cd concentration. Similar developmental alterations have been described for other insects such as *Aiolopus thalassinus* (Schmidt *et al.* 1991), *Lymantria dispar* (Gintenreiter *et al.* 1993), *Chironmus riparius* (McCadhon and Pascoe 1991) and *Oncopeltus fasciatus* (Cervera *et al.* 2004). Our results obtained for the duration of development in lead exposed groups shows the ability of flies to cope with the lead concentration used in this particular experiment, which needs increased time to complete their development under such stressful conditions. It is possible that some compensating physiological mechanisms such as elevated expression of heat shock proteins (Feder and Hofmann 1999; Sørensen *et al.* 2003) increased the time needed for completion of their development under such stressful conditions, but this needs further studies.

Development time showed significant difference between different types of crosses within each of the populations tested. The slowest development time was found in the progeny of intraline hybrids (for both populations), followed by a decrease in intrapopulation hybrids (for both direct and reciprocal crosses). Finally, the fastest development time under the lead contamination was found for interpopulation hybrids (hybrids between BG and SG populations). The results showed that a higher level of genome heterozygosity significantly influences fitness under lead pollution, decreasing development time, enabling flies to emerge faster. This may be due to the higher metabolic efficiency of heterozygotes because of the increased level of genetic variability.

The results of egg-to-adult viability showed a significant difference between the types of crosses and no significance was observed in presence of lead. It shows that for all groups, the presence of lead was an environment with a low level of stress (Lopes *et al*. 2004, 2006). These results are in agreement with Kenig *et al.* (2013) where authors found no intergenerational differences in viability between the groups from analysed populations of *D. subobscura* reared on different lead concentrations, which indicates that a low concentration is not sufficient to be a stress agent. Despite the fact that lead has no significant influence on viability in *D. subobscura*, our results of egg-to-adult viability showed significant differences between the types of crosses, such that the viability of intraline crosses was reduced on both media. These results confirm the hypotheses that hybridization shows positive fitness effects under lead pollution.

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Table 5. Two-way ANOVA for egg-to-adult viability of individuals from all types of crosses reared on standard medium (control) and medium with lead.

Source	df	MS	F
Treatment	1	0.005	0.124
Type of cross	7	0.422	9.846***
Treatment * type of cross	7	0.013	0.299
Error	421	0.0429	

***Statistically significant.

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