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Thermal Adaptation and Stress Resistance in *D. subobscura* Populations from Two Altitudes at Stara Planina Mountain (Serbia) †

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Abstract: Climate change and global warming are affecting many insect species in different ways. Organisms develop diverse mechanisms responding to variable environmental conditions. Due to global warming and the rise in mean and extreme temperatures, the importance of an individual's ability to adapt to temperature stress will further increase. Our focus in this study is the thermal response, which is considered to be one of the crucial elements of individual fitness and survival in a fast changing environment. We investigated stress resistance traits: desiccation and heat knock-down time in two *Drospohila subobscura* populations from two different altitudes (1080m and 1580m a.s.l.) along Stara planina mountain slopes in Serbia. The F1 progeny of the mass populations from both localities was used to establish six experimental groups at three different temperature regimes: 25^{0}C, 19^{0}C and 16^{0}C. Our aim was to determine whether there is a correlation between altitude/origin of populations and/or laboratory thermal evolution within these resistance traits. Flies from all groups were tested (scored every hour) for desiccation mortality at those temperatures. To score heat knock-down time all groups were placed into vials with moistened cotton plugs to prevent desiccation, kept at 37^{0}C for seven hours, and knock down flies were scored every 30 minutes. Our results indicate that the population origin, as well as laboratory thermal evolution have a significant influence on the analysed traits. Individuals originating from the higher altitudes reared at higher temperatures show better resistance to thermal shock.

Keywords: *D. subobscura*; desiccation tolerance; heat knock-down tolerance; global warming; life history; adaptation

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1. Introduction

Temperature is one of the most important factors shaping the distribution and evolution of different species populations and is considered to be a particularly important stressor, because variable thermal environments are common and may represent substantial challenges for survival and reproduction, especially in the light of global warming [1,2]. It is estimated that the average global temperature has risen rapidly since the 1880s, and temperature extremes are going to be 1.5^{0}C to 4.5^{0}C higher by the end of this century [3]. These are affecting all the known species on Earth. To better understand thermal adaptation, a geographic gradient along which climate strongly varies is of particular interest. Studies of altitudinal changes in phenotype and genotype can complement studies of latitudinal patterns and provide evidence of natural selection in response to climatic factors, especially temperature which is thought to be one of the most important selective agents [4].

To study the adaptation of insects to climate change, especially to extreme temperatures on different biological levels, many Drosophila species have been used. They are a great model organisms in the research of biology, ecology, evolution and genetics due to their small body size, short life cycle, ease to manipulate and rear, limited genetic redundancy, etc. *D. subobscura* Collin (Diptera: Drosophilidae), a Palearctic species widespread throughout Europe which already showed population-level genetic responses to various stressful conditions [5], can be treated as an ideal model system for thermal adaptations research. Latitudinal variation in phenotype and genotype has been well studied in *Drosophila sp*, but altitudinal patterns have rarely been investigated [6] although high altitudes and latitudes share similarly extreme environmental conditions [6,7]. For example, selection at low latitudes/altitudes (or at warm temperatures in the lab) may lead to decreased body size in these species; but the decreased size might lead to reduced stress tolerance [8]. Our aim was to determine if there is a correlation between altitude, precisely origin of populations, and laboratory thermal adaptation.

In studies of experimental thermal adaptation, the choice of ecologically relevant traits is of special importance. Desiccation and heat knock-down resistance (HKDR) are both considered reliable indicators of thermal adaptation, both being related to heat stress tolerance [7, 9]. For heat adaptation, knock-down resistance has been suggested to be important and to correlate with natural adaptation to high-temperature environments [9,10]. In nature, high temperature is also correlated with desiccation and there are examples of adaptive patterns in desiccation resistance [10]. The present study analyzes the dynamics and variation of these two stress resistance traits in *D. subobscura* flies originating from two natural populations sampled from different altitudes. The two populations were maintained in laboratory conditions at three different temperatures: 25^{0}C, 19^{0}C and 16^{0}C, for more generations before we started this study. In the light of climate change and global warming, results obtained from this study will contribute to a better understanding of the mechanisms underlying thermal adaptation and the impacts of global warming in biological systems [11].

2. Materials and Methods

Flies were collected in mid-August 2018, from two different elevations, 1080m (N 43.395255; E 22.603995) and 1580m (N 43.374145; E 22.618110), along Stara planina mountain slope in Serbia. Wild caught females were placed into the vials to establish isofemale lines (IF). After one generation, three to five pairs of males and females from each IF line were mixed to found two mass populations (V – high altitude and S – lower altitude) in order to preserve the original genetic variability. The F1 progeny of the mass populations from both localities were used to establish six experimental groups at three different temperature regimes: 25^{0}C, 19^{0}C and 16^{0}C. All groups were reared on the standard Drosophila medium (water/cornmeal/yeast/sugar/agar/nipagine). For all experiments, five to six days old virgin flies were used.

Desiccation

Five to six days old single flies were transferred into empty 3ml tubes. Approximately 100 flies (50♀ and 50♂) were used for each group. All groups were tested for desiccation mortality at three different temperatures: 25°C, 19°C and 16°C. Mortality was scored every hour. Flies that were not able to move were considered as dead.

Heat knock-down resistance

To score heat knock-down resistance, five flies (five to six days old) were placed into empty falcon tubes (50ml volume), and 50 flies for each sex per group were observed. Falcon tubes were closed with moistened plugs to prevent desiccation. All groups were placed into an incubator set to 37^{0}C, and knock-down flies were scored every 30 minutes.

Statistics

The results for both traits were analyzed using the full factorial general linear model (GLM) procedure and Bonferroni *post hoc* test in the STATISTICA ver 12. Desiccation resistance was analyzed with fixed factors: Population, Rearing temperature, Treatment and Sex and for heat knock-down time fixed factors were: Population, Rearing temperature and Sex**.**

3. Results

Desiccation

Mean values, variance and standard error for desiccation resistance are shown in Table 1.

Table 1. Mean values with standard error and variance for all groups for time of death (measured in hours) under desiccation stress.

			Population S		**Population V**	
Rearing temperature	**Treatment**	**Sex**	**Time of death**	**Variance**	**Time of death**	**Variance**
16°C	**16°C**	**F**	70.86± 2.06	214.1229	79.85±2.84	394.25
		M	63.28±1.83	167.3486	66.98±2.76	373.8954
	19°C	**F**	63.6±2.35	134.8163	73.63±2.35	269.6539
		M	58.67±1.91	178.4328	59.54±1.42	67.84354
	25°C	**F**	33.48±1.44	103.6016	32.90±1.18	100.2943
		M	28.2±0.91	41.71429	28.04±0.93	43.26367
19°C	**16°C**	**F**	82.23± 3.16	510.4235	86.64±3.10	481.8678
		M	67.76±2.22	246.9616	52.98±2.33	249.5302
	19°C	**F**	70.86± 2.37	280.6535	74.28±1.66	138.5322
		M	62.94±2.17	234.5065	46.14±1.42	100.7759
	25°C	**F**	44.68±1.19	159.5282	39.44±1.35	90.45551
		M	30.28±1.02	52.36898	26.1±1.03	53.43878
25°C	**16°C**	**F**	98.14±5.00	1250.735	64.44± 4.06	823.1902
		M	72.84±4.21	886.7086	34.46±2.18	237.2739
	19°C	**F**	72.64±5.01	1256.235	78.4±4.18	872.0816
		M	63.82±2.67	356.6404	49.62±2.22	245.5873
	25°C	**F**	30.56±1.42	100.8229	39.16±1.91	181.6065
		M	28.82±1.58	125.0486	29.14±1.17	68.57184

As expected, flies died faster from desiccation at high temperature treatment in all scored groups. Also, females showed higher overall desiccation resistance, especially females from lower altitude reared at 25°C.

Results of full factorial GLM analysis with fixed factors Population, Rearing temperature, Treatment and Sex are shown in Figure 1. Population, Treatment and Sex showed statistically significant influence on desiccation resistance (F=30.76, p=0.00; F=788.14, p=0.00; F=315.50, p=0.00 respectively). There is also statistically significant interaction between almost all combinations of factors, excluding interaction between all four factors and Population, Rearing temperature and Sex combined.

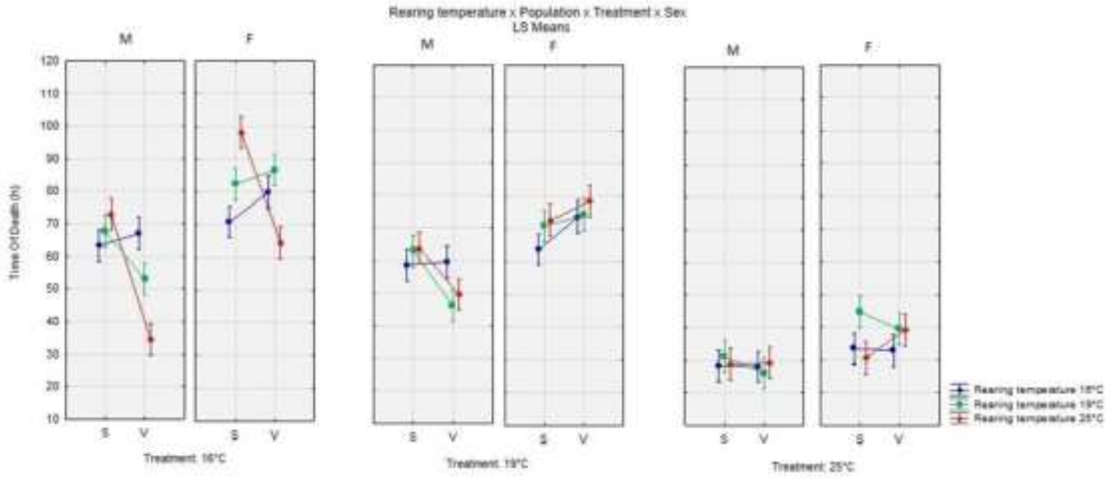

Figure 1. Full factorial GLM analysis with fixed factors Population, Rearing temperature, Treatment temperature and Sex.

Under cold temperature conditions (treatment 16°C) both sexes from S reared at 25°C have higher desiccation resistance than individuals from V (Bonferroni p=0.00). In the same conditions, S males reared at 19°C have higher desiccation resistance than V males (Bonferroni p=0.012). For females different trend is observed since, although not statistically significant, females from V reared at 19°C and 16°C showed higher desiccation resistance than S. Under optimal temperature for *D. subobscura* species (treatment 19°C) S males reared at 19°C and 25°C have higher desiccation resistance than V (Bonferroni p=0.017, p=0.035, respectively). Situation is different for females under the same conditions, since V females have better, but not statistically significant, desiccation response. Under high temperature (treatment 25°C) there is no significant difference between groups, but V females reared at 25°C have higher desiccation resistance than S females.

Heat knock-down resistance

Mean values, variance and standard errors for heat knock-down time are shown in Table 3; Individuals reared at 25°C showed the longest heat knock-down time.

Table 3. Mean values with standard error and variance for all groups for heat knock-down time (minutes).

		Population S		**Population V**	
Rearing temperature	**Sex**	**Heat knock-down time**	**Variance**	**Heat knock-down time**	**Variance**
16°C	**F**	115.8 ± 3.93	771.7959	126 ± 3.53	624.4898
	M	105 ± 2.74	376.5306	116.4 ± 3.16	501.0612
19°C	**F**	163.8 ± 3.66	668.9388	182.4 ± 5.06	1279.837
	M	162 ± 3.64	661.2245	183.6 ± 4.67	1088.816
25°C	**F**	310.2 ± 12.99	8434.653	357.5 ± 14.06	9491.489
	M	349.17 ± 17.77	11373.57	282.6 ± 14.32	10252.29

Results of full factorial GLM analysis with fixed factors Population, Rearing temperature and Sex are shown in Figure 2. Population, Rearing temperature, Sex and Rearing temperature x Sex interaction showed a statistically significant influence on heat knock-down time (F=10.707, p=0.001; F=20.389, p=0.00, F=610.236, p=0.00; F=11.981, p=0.00 respectively).

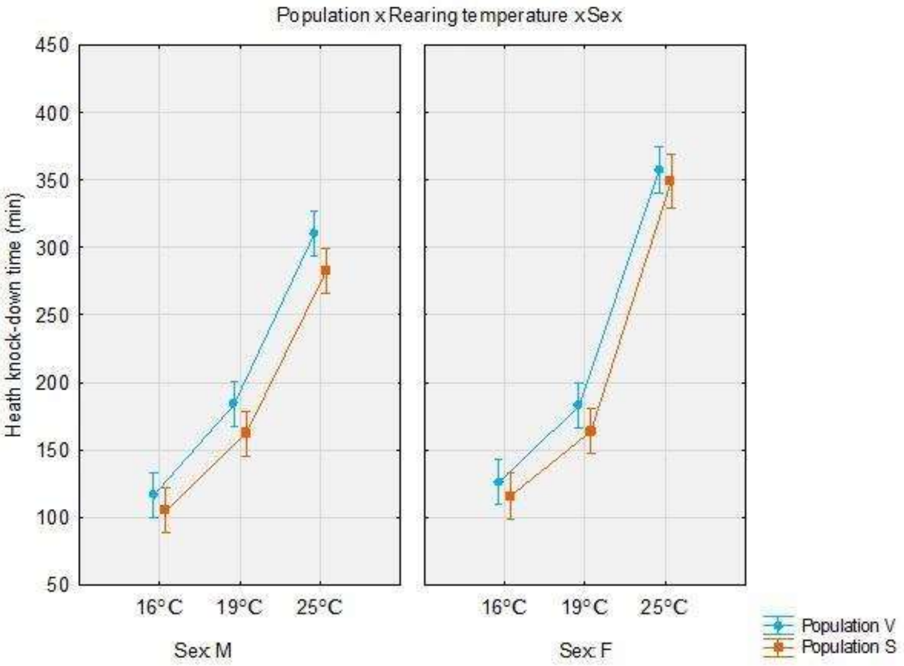

Figure 2. Results of full factorial GLM analysis with fixed factors Population, Rearing temperature and Sex.

Individuals from higher altitudes showed significantly longer heat knock-down time than individuals from lower altitude (Bonferroni p=0.00). Higher rearing temperature significantly prolongs heat knock-down time (Bonferroni p=0.00 for all combinations). For both populations females reared at 25°C have statistically significant longer heat knock-down time than males (Bonferroni p=0.006 and p=0.000 respectively).

4. Discussion

Environment and climate are changing rapidly, and to cope with these changes, organisms have to adapt. Adaptation can take many shapes and occur at different speeds, depending on the type of response, the trait, the population, and the environmental conditions [12]. Periods of environmental stress such as extreme temperatures, droughts, or food shortage may wipe out a population unless it is capable of coping with these conditions [13]. Studies of laboratory thermal evolution may provide evidence of natural selection in response to climatic factors, especially temperature which is thought to be one of the most important selective agents [14]. The present study analyzes the dynamics and variation of two stress resistance traits, desiccation and HKDR, in *D. subobscura* flies originating from two natural populations from two different altitudes kept under different temperature regimes in laboratory conditions.

Both analyzed traits showed a significant impact of population origin. For desiccation resistance, lower altitude population generally responds better to desiccation than the high altitude population. Given that high temperature is a cause of desiccation in the wild [7], and temperature generally decreases with increasing altitude, it is expected that the S population is better adapted, and has higher desiccation resistance than the V population from high altitude. For heat knock-down resistance we have a different population response, where the high-altitude population showed better resistance to heat knock-down. High altitude conditions are correlated with temperature extremes, and population V reared at 25^0C has shown the highest resistance to short-term extreme temperature.

Laboratory thermal evolution also showed effect on both analysed traits, but as expected is more prominent for heat knock-down resistance, which is positively correlated with high developmental temperature [9,10]. With increased rearing temperature, heat knock-down time prolongs, reflecting that preadaptation to specific thermal conditions has a strong influence on population ability to endure temperature extremes and that individuals accustomed to colder environments may face serious challenges in light of global warming. In this experiment, we have shown that laboratory thermal evolution has a significant influence on the population response to desiccation resistance in cold conditions, but doesn not have the same influence in the higher temperature conditions [15,16].

Not surprisingly, the traits studied here were strongly influenced by sex. Females, as previously described [17], have shown better heat resistance performances than males, indicating that males have more difficulties coping with global climate change and rising temperature. For instance, exposure to 25°C can induce male sterility in this species [18].

Drosophila suboscura is considered a cold-adapted species with a thermal range between 6 and 26°C [19] and a thermal optimum of 18°C [18]. Behavioral assays revealed that this species shows a thermal preference of 16.6°C when placed in a linear thermal gradient [20]. Desiccation and heat knock-down resistance are both considered reliable indicators of thermal adaptation, both being suggested to correlate with natural adaptation to high-temperature environments [14]. Although temperatures used in this experiment are not extreme, and are in the range experienced by Drosophila developing in nature, our results show that both traits are sensitive to rearing temperature and to the origin of the population. However, the patterns of the traits did not respond the same: they show different sensitivities to non-optimal temperatures, indicating different mechanisms responsible for thermal stress adaptation.

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