Contrasting patterns of variations in body melanisation and desiccation resistance in two species of montium species subgroup.

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Introduction

In numerous animal species body melanisation is a highly variable trait, occurring either from genetic variability or phenotypic plasticity (Gibert *et al.,* 1996; Scheiner, 2004). Phenotypic plasticity is the ability of an organism to express different phenotypes depending upon the biotic and abiotic environments. Environmentally affected phenotypes were considered of lesser importance because of apparent lack of genetic basis. The modern view of plasticity rejects these findings, because phenotypic plasticity often has a genetic basis. This aspect has been explored in few cosmopolitan *drosophilids* (*D. melanogaster, D. simulans, and D. kikkawai*) whereas, there are no data on warm adapted and cold adapted species. Recent studies have reported lack of phenotypic plasticity for body melanisation in some warm adapted tropical *Drosophila* species, *e.g*., *D. polymorpha* (Brisson *et al.,* 2005), *D. jambulina* (Parkash *et al.,* 2009), and *D. ananassae*. The species patterns of plasticity that evolve not only depend upon local habitats but also on the nature of genetic variation for plasticity in relevant traits and population level patterns of dispersal. Some specific approaches to this field include descriptive surveys of reaction norms for given populations, population comparison of reaction norms, genetic studies of plasticity, and functional ecological studies of trait performance in alternative environments. There are no reports on simultaneous analysis of stress related traits in populations of sibling *Drosophila* species with contrasting distribution patterns across a given continent. Obviously, the complexity of this topic requires good model systems like *Drosophila* that are helpful in analyzing the shape of the reaction norms (David *et al.,* 1997). For present investigations, we have focused on a warm adapted tropical species *D. jambulina* and cold adapted temperate species *D. watanabei*.

D. jambulina belongs to montium species subgroup of *melanogaster* group and has been reported from India as well as south-east Asian regions (Parshad and Paika, 1964). This species exhibits color dimorphism for the last two abdominal segments in females; and light morph is dominant over dark morph (Ohnishi and Watanabe, 1985; Parkash *et al.,* 2009). Whereas, *D. watanabei* also belongs to the same species subgroup as the *melanogaster* group but lacks color dimorphism. Its distribution pattern is still not clear except that it was first reported from Cambodia. In the present study, we investigated the norm of reaction of abdominal pigmentation in these two sibling species. The aim was to compare two sibling species belonging to same subgroup in order to study their adaptive capacities. A comparison of stress related traits in population of a cold adapted – *D. watanabei* and a warm adapted species *D. jambulina* may help in explaining their differential evolutionary potential. Such species might encounter varying climatic stress conditions resulting in different shapes of reaction norms. I also compared the norms of reaction in two sibling species and analyzed their divergence. We found that both the species mainly diverged at extreme temperatures. I tried to know about the ecology of the two species on the basis of genetic and plastic changes in stress related traits? In this study, we have attempted to analyze traits that could be involved in discriminating as cold adapted and warm adapted species.

Present comparative data on *D. jambulina* and *D. watanabei* are quite interesting in several respects: (a) no plastic effects were observed on body melanisation and other ecophysiological traits in *D. jambulina*; (b) in contrast, *D. watanabei* showed quite high plasticity for all the traits; (c) our data are not in agreement with thermal-melanism hypothesis for *D. jambulina*, whereas *D. watanabei* supports this hypothesis. Laboratory controlled desiccating or high humidity conditions (which may correspond with seasonal variation in humidity levels) have altered the frequencies of dark and light morphs in *D. jambulina*. Thus, our past results support the role of varying humidity levels in maintaining body color polymorphism and its impact on water balance in warm adapted *D. jambulina* (Parkash *et al.,* 2009), whereas a high plastic response for all ecophysiological traits supports the role of temperature in *D. watanabei* (present study).

Materials and Methods

Cultures

Wild individuals of *D. jambulina* and *D. watanabei* (n = 100-120) were collected from a lowland locality, Rohtak of the Indian subcontinent. The collections were made in September – November months, 2007, with net sweeping and bait traps from fruit markets and godowns, as well as from nurseries. Based on the T_{ave} data of population, cultures were maintained at 21ºC. Density was kept low (30 – 40 eggs per vial) by limiting the egg laying period for 6 to 8 hours. In order to test the repeatability of trait values across two successive generations (G_1 and G_2), different isofemale lines (n = 20) were analyzed for various traits. For investigating plastic effects of body melanisation at different growth temperatures, 25 to 30 pairs of flies of each isofemale line were allowed to lay eggs at 21ºC in 8 replicate vials. Two such vials were then transferred to each of 17º, 21º, 25º, and 28ºC growth temperatures. Six day-old flies from these different developmental temperatures were analyzed for various ecophysiological and body size traits. Climatic data for the site of origin of population were obtained from Indian Institute of Tropical Meteorology (IITM; www.tropmet.res.in).

Analysis of body melanisation

For both the species, body size traits (thorax length, wing length, and wing width) and % body melanisation were simultaneously measured in laboratory reared female individuals. For each fly, wing length was measured from the thorax articulation to the tip of third longitudinal vein under Olympus SZ-11 microscope (www.olympus.com, Japan) fitted with a micrometer. Likewise thorax length was estimated as a distance from anterior margin of the thorax to the posterior tip of the scutellum from the dorsal view. As index of wing width, we used the distance between distal ends of the second and fifth longitudinal veins. Body melanisation patterns were visually inspected in six day-old adults. Melanisation was scored from dorsal view of the abdomen giving values ranging from 0 (no pigment) to 10 (complete darkness) for each of the six visible abdominal segments, *i.e.*, 2^{nd} to 7^{th} (David *et al.,* 1990) for both the species. Since the abdominal segments differ in size, relative sizes (*i.e.*, 0.87, 0.95, 1.0, 0.90, 0.62, 0.36 for 2^{nd} to 7^{th} segments, respectively, for both the species) were multiplied with segmentwise pigmentation scores. The flies were scored by two independent persons, and a high correlation ($r > 0.96 \pm 0.02$) between two sets of observations ensured repeatability. The data on % melanisation were calculated as Σ observed melanisation scores of six abdominal segments per fly/ Σ relative size of each segment × 10 per fly × 100. The abdomen of each fly minus viscera was mounted on a slide and total % body melanisation per fly was also estimated through Biowizard image analysis software - Dewinter Optical Inc. (www.dewinterindia.com).

Desiccation resistance

To measure desiccation resistance, ten individuals from each line were isolated in a dry plastic vial, which contained 2 g of silica gel at the bottom and were covered with a disc of foam piece. Such vials with foam plugs were placed in a desiccation chamber (Secador electronic desiccator cabinet), which maintains 4 - 5% relative humidity. The vials were inspected every hour and the number of dead flies (completely immobile) was recorded and LT_{100} values (lethal tolerance time at which all flies died) were calculated.

Thermoresistance assays

For thermoresistance traits, two assays (heat knockdown and cold recovery) were preferred, because these are ecologically relevant and measurements were found repeatable across replicates as well as generations. We controlled effects due to age, sex, anesthesia, ambient room temperature, and thermal conditions of assay vials. For all the assays, sexes were isolated in separate vials after mild anesthesia with ether (about 4 minutes) followed by one day recovery period. Six-day-old female flies were aspirated and introduced to assay vials, which were pretreated at experimental temperatures for 6 hr so as to minimize effects due to thermal variations. For all traits, measurements were made in a thermocontrolled room at 21°C.

Heat knockdown was measured on ten female individuals from each isofemale line (n = 20). Individual females were placed into 5 ml glass vials submerged into a water bath at a constant temperature (39ºC). Flies were scored for time (in minutes) taken to be knocked down.

For cold recovery, adult female flies were aspirated and placed individually in 5 ml glass vials. These vials were set in thermocol boxes (24 × 13 × 10 cm) containing ice flakes (made with an ice flaking machine; AICIL), which were kept at 0ºC in the refrigerator for 8 hours. This was followed by transfer of flies to petri-plates (9 cm diameter) in a temperature controlled room at 21°C, and cold recovery period (in minutes) was recorded.

Analysis of plastic effects in two species

To assess plastic effects for quantitative traits (body melanisation, desiccation resistance, heat knockdown, cold recovery, and body size) in both species, we compared slope values of laboratory reared individuals across temperatures. In *D. watanabei* changes in the slope values (regression coefficient slope = b) indicated plastic effects. The lack of changes in slope values for different ecophysiological traits across temperatures, however, suggested no plasticity in *D. jambulina*.

Statistical analyses

For all the traits (abdominal melanisation, desiccation resistance, heat knockdown, cold recovery, and body size traits) population means (n = 20 lines * 10 individuals each) along with SD were used for graphical illustrations and tabular data. Correlations between different traits were based on data from isofemale lines. Within-population correlations for melanisation and other traits were significantly high ($r > 0.88$) in both the species. The Statistica package (Statsoft Inc., Release 5.0, and Tulsa, OK, USA) was used for statistical calculations as well as illustrations.

Results

Segment-wise variability for melanisation across temperatures

Mean ± S.D. values for segment-wise melanisation at different temperatures for both the species are given in Table 1. *D. watanabei* showed a very high plasticity for all the segments (2^{nd} to 7^{th}), whereas in *D. jambulina* both the morphs showed same values throughout all temperatures

(Table 1). Melanisation of abdominal segments at 17 versus 28°C revealed significant differences, *i.e*., lower melanisation of all segments at 28°C indicates plastic effects (Table 1) in *D. watanabei*. For the last three posterior abdominal segments (5^{th}, 6^{th}, and 7^{th}), we observed a very much higher phenotypic variability as compared with anterior abdominal segments in *D. watanabei*.

In *D. jambulina*, the population means for three anterior segments (2^{nd} + 3^{rd} + 4^{th}) do not differ significantly between dark and light morphs. But significant differences characterize body color morphs with respect to the last three abdominal segments (5^{th} + 6^{th}+ 7^{th}). Body melanisation for dark as well as light morphs showed no effect due to different growth temperatures (17– 28°C), *i.e*., there is lack of phenotypic plasticity as compared to *D. watanabei.*

Table 1. Data on percent melanization (m ± S.D.) showing segment-wise variability across two temperatures in sibling species – *D. jambulina* and *D. watanabei* of montium subgroup.

Abdominal Segments	*D. watanabei*			*D. jambulina*		
	17°C	28°C	t-test	Dark morph (17 & 28°C)	Light morph (17 & 28°C)	t-test
2^{nd}	11.85 ± 1.00	5.00 ± 1.01	**	7.06 ± 1.20	7.10 ± 1.09	ns
3^{rd}	13.22 ± 1.36	6.00 ± 1.12	**	6.20 ± 1.17	6.34 ± 1.48	ns
4^{th}	13.78 ± 1.74	6.23 ± 0.98	**	4.94 ± 1.60	4.50 ± 2.10	ns
5^{th}	12.96 ± 2.85	4.15 ± 1.53	***	2.30 ± 1.78	2.56 ± 1.24	ns
6^{th}	18.84 ± 1.62	3.10 ± 1.10	***	20.00 ± 0.00	0.00	***
7^{th}	7.20 ± 0.00	2.00 ± 0.00	***	7.20 ± 0.00	0.00	***
2^{nd} + 3^{rd} + 4^{th}	38.85 ± 2.88	17.23 ± 2.40	**	18.20 ± 3.39	18.34 ± 3.53	ns
5^{th} + 6^{th} + 7^{th}	39.00 ± 2.24	9.25 ± 1.33	***	29.50 ± 1.15	2.56 ± 1.47	***
Sum (%)	77.85 ± 3.30	26.48 ± 3.10	***	47.70 ± 3.16	20.90 ± 3.05	***

** $p < 0.01$; *** $p < 0.001$, ns = non-significant.

Plasticity for ecophysiological traits

Reaction norms according to growth temperatures for various ecophysiological traits (Melanisation, desiccation resistance, Heat knockdown, and cold recovery) are illustrated in Figure 1. All the traits showed ~ 3-4 folds decrease in values at 28ºC for *D. watanabei*, whereas for *D. jambulina* a straight line was evident across temperatures (17 to 28ºC). *D. watanabei* showed higher body melanisation and desiccation resistance at 17 and 21ºC, but at 25 and 28ºC both the traits showed reduced trait values as compared to *D. jambulina* (dark morph).

Plasticity for body size traits

Data for body size traits (Wing length, Wing width, and Thorax length) showed a change of 12 – 14% for *D. watanabei* (Figure 2). By contrast, no change in body size traits was observed in either dark or light morphs for *D. jambulina*.

Within population analysis and correlations

For all the traits, correlation values are significantly higher for both the species. For assorted dark and light morphs of *D. jambulina*, correlations of body melanisation with desiccation resistance, Heat knockdown, Cold recovery are highly significant (r = 0.8 – 0.9). The correlations based on within population trait variability suggest correlated changes in these traits. Results of ANCOVA,

with body size as a covariate have shown significant trait variability due to temperature for *D. watanabei* and due to morphs (dark and light) for *D. jambulina* (data not shown).

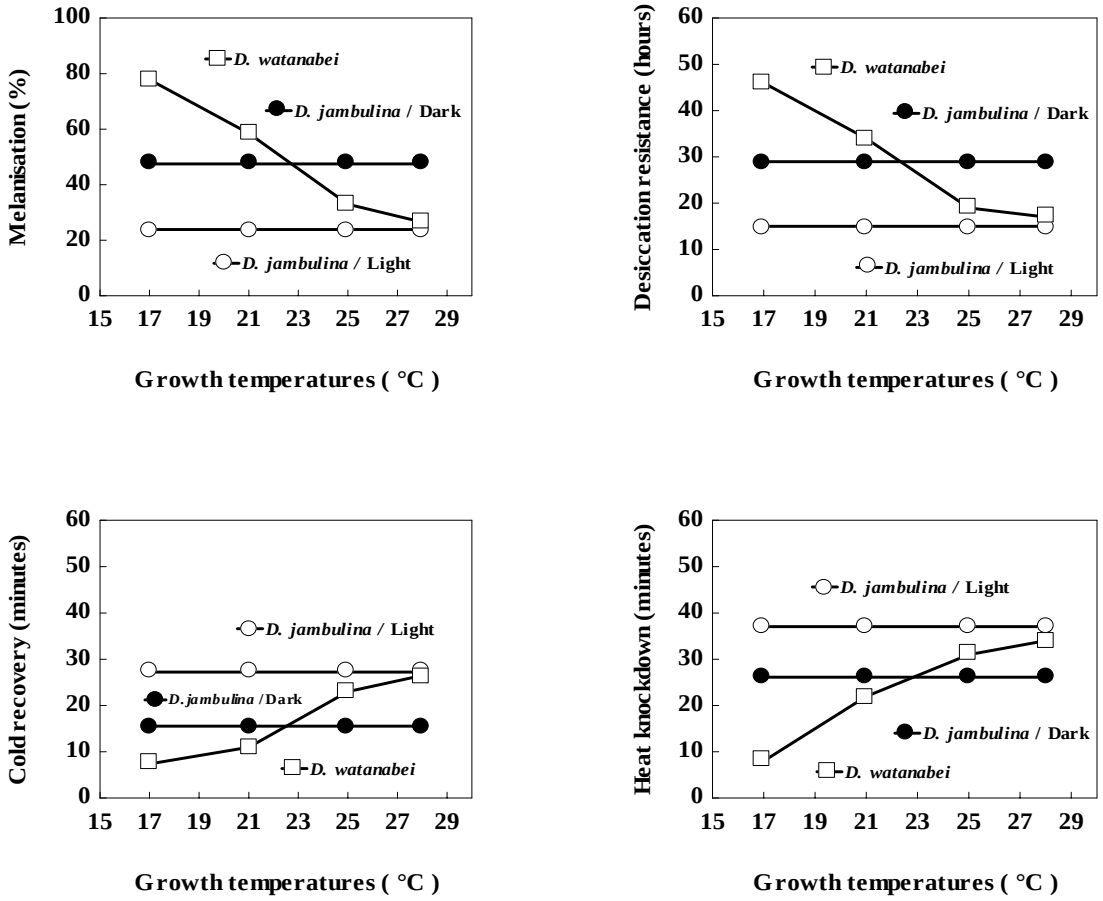

Figure 1. Reaction norms for four traits: % melanisation, desiccation resistance, cold recovery, and heat knockdown in a warm adapted species, *D. jambulina*, and a cold adapted species, *D. watanabei*.

Discussion

Temperature is the most important component of the abiotic environment for understanding the distribution and abundance of ectotherm species (Andrewartha and Birch 1954; Precht *et al.,* 1973; Hoffman and Parsons, 1991). Numerous life history traits, such as rate of development, viability, progeny production, and longevity have been measured in many species in relation to ambient temperature. All these results contribute to our understanding of geographic distributions. At an intraspecific level, numerous *Drosophila* species exhibit latitudinal clines, *i.e*., regular genetic variations, which are assumed to reflect adaptive responses to local climatic conditions. Body pigmentation variability in *Drosophila* has been investigated in many species, and there is a general consensus to recognize that it is a fast evolving trait which is not strongly influenced by phylogenetic constraints (Gibert *et al.,* 1996; Hollocher *et al.,* 2000; Kopp *et al.,* 2000; Wittkopp *et al.,* 2003).

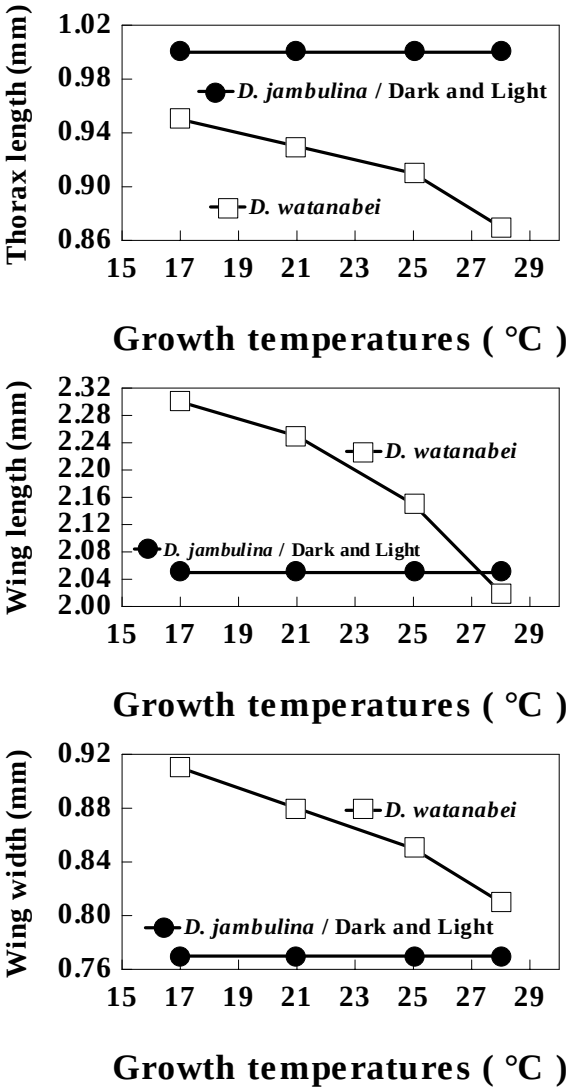

Figure 2. Comparison of reaction norms for morphometrical traits (Thorax length, Wing length and Wing width) in *D. jambulina* and *D. watanabei.*

Pigmentation varies within species and can respond rapidly to selection, and these variations provide an opportunity to study genetic changes in a population – genetic architecture. Analysis of allele genealogies and frequencies in populations from different geographic regions that experience different kinds and strengths of selection, together with understanding of basis of the functional differences between alleles, offers the prospect of a fully integrated picture of evolution – from the individual, to whole populations. Phenotypic plasticity is mainly related to growth temperature – at lower temperature flies become darker, in agreement with thermal budget hypothesis. Several studies have shown that thermal attributes of dark *vs*. light body color surfaces confer adaptive responses under spatially and temporally varying environments (Brakefield and Willmer, 1985; Holloway *et al.,* 1997; Kingsolver and Wiernasz, 1991). Seasonal changes in different species and genera of hoverflies have been explained on the basis of developmental phenotypic plasticity (Holloway *et al.,* 1997). Further, field populations of a tropical (north Australian) butterfly- *Hypolimnas bolina* exhibited phenotypic plasticity (*i.e*., dark individuals during winter dry season and light morph in the wet season). This study has also supported the adaptive hypothesis of crypsis and thermoregulation (Kemp and Jones, 2001). Thus, seasonal adaptive changes involve phenotypic plasticity in several insect taxa. In contrast, there is lack of phenotypic plasticity for body melanisation in some warm adapted tropical *Drosophila* species, *e.g*., *D. polymorpha* (Brisson *et al.,* 2005), *D. jambulina* (Parkash *et al.,* 2009), and *D. ananassae*. There is supporting evidence for the role of genetic polymorphism in seasonal adaptations, *e.g*., seasonal changes in the relative frequency of elytral patterns (morphs) in ladybird beetle - *Harmonia axyridis* (Tan, 1949; Osawa and Nishida, 1982) and in diverse insect taxa (Majerus, 1998; and references therein).

Insects can cope with seasonal environment through phenotypic plasticity, which allows a single genotype to produce different phenotypes. In order to test genetic as well as plastic effects, we compared slope values across temperatures for various ecophysiological traits in both the species. Changes in slope values for quantitative traits across temperatures suggest plastic responses. By contrast, lack of differences in slope values suggested the absence of plastic effects for quantitative traits in *D. jambulina*. Thus, in cold adapted temperate species *D. watanabei* phenotypic plasticity

for body melanisation led to darker flies at lower temperatures and lighter flies at higher temperatures; and such plastic effects corresponded with changes in other ecophysiological traits.

Body melanisation in D. jambulina – evidence for no plasticity

Body color polymorphism is a potentially adaptive trait in *D. jambulina*. Firstly, there was a lack of thermal effects on body melanisation in the field as well as in laboratory grown true breeding dark and light strains. A similar result on the lack of thermal plastic effects has also been reported for Brazilian populations of *D. polymorpha* (Brisson *et al.*, 2005). Thus, changes in body melanisation in this species are not in agreement with thermal melanism hypothesis. Secondly, in *D. jambulina* the dominance of light allele over dark allele may help this species in adaptation to warm and humid environments in the tropics (Parkash *et al.,* 2009). Thirdly, significant differences occur between dark *vs*. light morphs for desiccation stress related as well as life history traits, but not for body size traits. Thus, the observed trait variability can be explained on the basis of genetic polymorphism for body melanisation. In this respect, *D. jambulina* may adapt to variable environmental conditions through changes in allele frequency for dark and light morphs.

Body melanisation in D. watanabei - evidence for plasticity

Unlike *D. jambulina*, we obtained high thermal effects on melanisation for all the segments in *D. watanabei*. In *D. melanogaster*, homeotic genes control body segmentation, *i.e*., ultrabithorax regulates gene expression in the thoracic region while three anterior (2^{nd}, 3^{rd}, and 4^{th}) and three posterior abdominal segments (5^{th}, 6^{th}, and7^{th}) are controlled by *Abdominal-A* and *Abdominal-B*, respectively (Carroll, 1995). Lack of phenotypic correlations ($r = 0.14 \pm 0.12$, $p = 0.34$) between two groups of abdominal segments, *i.e*., (2^{nd}+3^{rd}+4^{th}) *versus* (5^{th}+6^{th}+7^{th}) for all populations, suggest their independent genetic control. For abdominal segments 2^{nd} to 4^{th}, we obtained non-significant differences in slope values at 17 and 25ºC as well as for the wild (Parkash *et al.,* 2008). By contrast, segments (5^{th}, 6^{th}, and 7^{th}) showed significant differences in slope values. We may infer that genetic changes might play a major role for abdominal segments (2^{nd} to 4^{th}) while thermal effects seem significant for 5^{th}, 6^{th}, and 7^{th} segments (Parkash *et al.,* 2008). Thus, *D. melanogaster* populations may involve genetic and plastic effects for body melanisation along an altitudinal gradient. In contrast, *D. watanabei* showed plasticity for all the segments 2^{nd} to 7^{th} suggesting adaptive strategies for survival under changing cold climatic conditions.

Evidence for melanism-desiccation hypothesis

Possible role of body melanisation in conferring desiccation resistance was initially demonstrated for ebony mutant strains of *D. melanogaster* (Kalmus, 1941 a,b,c). Subsequently, darker and lighter laboratory strains of *D. polymorpha* were shown to differ in desiccation resistance (Brisson *et al.,* 2005). For altitudinal populations of *D. melanogaster*, assorted dark and light flies exhibited significant differences in desiccation stress resistance and cuticular water loss (Parkash *et al.,* 2008). We found a consensus between wild *vs*. laboratory data on the role of body melanisation in desiccation resistance in *D. jambulina* as well as *D. watanabei*. The mechanistic basis of desiccation resistance has been investigated in laboratory selected strains resistant and sensitive to desiccation (Gibbs *et al.,* 1997). Natural populations have, however, been mainly examined for clinal variation in desiccation resistance (Hoffmann and Harshman, 1999; Parkash and Munjal, 1999; Parkash *et al.,* 2005). Since desiccation resistance evolves through changes in cuticular permeability, the target of selection might be cuticular components (either cuticular lipids or cuticular melanisation).

Body size

Body size is a plastic trait, but there are no reports on the impact of seasonal changes in body size on desiccation related traits. A larger body size has a smaller surface area/volume ratio, which can minimize the cuticular water loss under desiccation stress (Zachariassen *et al.,* 1987; Gibbs and Matzkin, 2001). Thus, differences in body size across seasons might confer desiccation resistance, but our data are not in agreement with such expectations. We observed no plasticity for body size traits in *D. jambulina* whereas, quite high plasticity was observed in *D. watanabei.*

Conclusions

The present study in itself is a first attempt to know the ecological adaptations of two sibling species belonging to the same species subgroup. We analyzed traits that could be involved in discriminating as cold adapted and warm adapted species. *D. jambulina* has adapted to different seasons by modifying the frequencies of color morphs through assortative matings. Humidity rather than temperature is, therefore, the primary selection agent for body color polymorphism, whereas *D. watanabei* has established its niche in seasonally varying cold climatic conditions of temperate regions through a very high degree of phenotypic plasticity for all ecophysiological and body size related traits. Thus, we can infer that phenotypic plasticity somehow acts as a tool for survival among cold adapted temperate species (*D. watanabei, D. nepalensis*), whereas humidity acts as a major factor among warm adapted tropical species (*D. jambulina, D. ananassae*), which lack phenotypic plasticity for various stress related traits.

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The effects of dibutyl phthalate (DBP) on the development and fecundity of *Drosophila melanogaster.*

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Abstract

Dibutyl phthalate (DBP) is a plasticizer used in the manufacture of several industrial and household articles. They get easily released to the environment and may cause adverse effects to living organisms. In this study, effects of DBP on the development and fecundity of *Drosophila melanogaster* have been studied. 72 h larvae of *D. melanogaster* were exposed to 0.25 mL/L, 0.5 mL/L and 1 mL/L DBP. The percentages and times of transition from larvae to pupae and from pupae to adults were determined, and the daily mean egg number was examined using the female offspring, which had been exposed as larvae. No differences were found in the transition percentages from larvae to pupae and from pupae to adults ($p > 0.05$). It was found, however, that both the mean pupation and the mean maturation time were accelerated with 0.25 mL/L DBP exposures ($p < 0.05$). In addition, it was determined that 1 mL/L DBP exposure caused a delay in the mean pupation time ($p < 0.05$). Also in the 0.25 mL/L exposure group was found a statistically significant decrease in mean fecundity as compared to the control groups ($p < 0.05$). Keywords: Dibutyl phtalate, *Drosophila melanogaster*, developmental time, fecundity.

Introduction

In recent years there has been growing concern that certain chemical compounds released into the environment may have a harmful influence on the development and reproduction of several animal species. As widely used industrial chemicals, phthalate esters (PAEs) are mainly used in the polymer industry as external plasticizers in polyvinyl chlorides (PVCs). PAEs have also found application in the manufacture of children's toys, printing inks, nail polishes, varnishes, shampoos, sunscreens, skin emollients, garden hoses, building materials, lubricating oils, automobile parts, paints, glues, insect repellents, photographic films, perfumes, and food packaging (Heudorf *et al*., 2007; Liu *et al*., 2009). Their low melting point and high boiling point make them very useful as heat transfer fluids and carriers (Van Wezel *et al*., 2000). The world wide production of phthalates approximates 5.2 million metric tons a year (Liu *et al*., 2009). PAEs tend to migrate slowly out of the plastic, either into the air by volatilization or into water or other solvents by dissolution (Zhao *et al*.,