International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Seasonal Adaptations of Body Color Morphs of Tropical *Drosophila punjabiensis* involve Plastic Changes in Stress Resistance Traits and Energy Metabolites

Meenu Yadav[1]

[1]Department of Biochemistry and Genetics, Barkatullah University Bhopal - 462026
[1]Corresponding Author Email: *meenudu88[at]gmail.com*

Abstract: ***Despite numerous studies on body color polymorphism in diverse insect taxa, assessment of plastic responses to climatic stressors has received lesser attention. Drosophila punjabiensis is a sibling species of D. jambulina but its adaptive strategy is different to cope with environmental stressors. Field data on D. punjabiensis have shown simultaneous occurrence of dark and light morphs in colder as well as warmer seasons in the subtropical regions which seems inconsistent with thermal budget hypothesis. We investigated plastic changes in two cuticular traits (body melanisation and cuticular lipid mass); resistance to cold, heat and drought; and three energy metabolites (carbohydrates, total body lipids and proline) in laboratory reared dark and light body color morphs of D. punjabiensis. Developmental and adult acclimation (cold or drought acclimation and heat hardening) plastic responses were significantly higher for dark morph than light morphs. In the field, greater abundance of dark morph flies under colder climatic conditions seems consistent with its plasticity levels. In D. punjabiensis, occurrence of light morph under colder or drier environments seems compatible with its acclimation potential capacity. Dark and light body color morphs revealed compensatory plastic changes in the levels of three energy metabolites. Thus, in D. punjabiensis divergence of plastic responses of body color morphs to climatic stressors seem to have played a major role attaining its temperate as well as tropical locality.***

Keywords: Plastic changes, seasonal, Body color, *Drosophila punabiensis*, Energy metabolites

1. Introduction

Diverse insect taxa from temperate regions are able to cope with seasonally varying climatic stressors through developmental as well as adult acclimation during their life time (Whitman and Ananthakishnan, 2009; Denlinger and Lee, 2010). Effects of thermal acclimation on physiological as well as life history traits have been investigated in several ectothermic organisms (Angilletta 2009). In contrast, plastic changes induced by wet – dry conditions are less well understood (Tauber et al., 1998; Chown et al., 2011). Several tropical drosophilids encounter seasonally varying low vs high relative humidity conditions while thermal changes are quite limited i. e.24 to 30 °C (www.tropmet. res. in). In southeast Asia, changes in relative humidity associated with altered patterns of rainfall due to El nino and climate warming are likely to increase drier conditions which can affect survival of different tropical insect taxa (www.skymetweather. com; www.imd. gov. in). Assessment of acclimatization potential of stenothermal tropical drosophilids reared under wet – dry conditions can help in understanding species specific stress resistance potential to multiple stressors.

Ectothermic organisms found in temperate locations experience a wider variety of colder settings, ranging from freezing to mild cold. Additionally, they face changes in other abiotic actors that impact their ability to adapt and enhance their survival under extreme weather conditions (Sinclair, 2015). In tropical regions, seasonal changes in humidity conditions are likely to play a major role in affecting morphological, physiological and life history traits (Tauber et al., 1986; Tauber et al., 1998). The role of humidity for some tropical insects has been demonstrated for diapause (Pires et al., 2004; Seymour and Jones, 2000); as well as on the basis of increased level of desiccation resistance due to developmental acclimation in *D. leontia* (Parkash and Ranga, 2014); and adult acclimation in *D. simulans* (Bubliy et al., 2013). However, a single study has shown effect of low vs high humidity adult acclimation on mating related traits of darker and lighter morphs of tropical *D. jambulina* consistent with melanism - desiccation hypothesis (Parkash et al., 2009). This study has shown that the frequencies of melanic and non - melanic morphs of *D. jambulina* are driven by humidity changes and not due to thermal conditions (Parkash et al., 2009). However, previous studies have not investigated possible adaptive effects of humidity acclimation on energy metabolites of seasonally varying diverse tropical insect taxa (Tauber et al., 1998; Bubliy et al., 2013). Further, in context of climate warming effects, tropical insect taxa remain unassessed despite greater risk due to increased aridity conditions (Hoffmann, 2010). Thus, there is need to assess developmental as well as adult acclimation effects of ecologically relevant low vs high humidity conditions on stress resistance traits, cross - tolerance and physiological changes in metabolic fuels to cope with seasonally varying climatic stressors.

In insect taxa from temperate regions, adaptations to multiple stressors involve plastic changes to improve stress resistance level through direct and cross - tolerance effects as well as maintenance of energetic homeostasis in metabolic fuels after

possible perturbations due to climatic stressors (Benoit et al., 2009; Sinclair et al., 2013; Williams et al., 2014; Sinclair, 2015). Analysis of plastic changes in metabolites of Antarctic midge *Belgica antarctica* has shown some overlap in the accumulation of glycogen and erythritol due to cold or drought acclimation while reduction in the level of serine was evident in response to cold, drought and heat stress (Michaud et al., 2008). Previous studies have emphasized the role of different cryoprotective low molecular mass solutes such as sugars, polyols and free amino acids. However, for tropical insects, the role of possible heat induced thermoprotective and osmoprotective solutes has received less attention. Some studies have shown the role of exogenous trehalose to increase resistance to warmer temperature in *Belgica antarctica* from temperate region (Benoit et al., 2009). However, association between warmer temperature and proline has been shown in the beetle - *Alphitobius diaperinus* (Renault et al., 2006); and in a tropical drosophilid *Zaprionus indianus* (Kalra et al., 2017). However, previous studies have not considered plastic changes in energy metabolites induced by low vs high humidity rearing and / or adult acclimation condition for insect taxa from temperate as well as tropical habitats.

Proline has been considered as a multifunctional amino acid due to its role as cryoprotectant and / or osmoprotectant and in mitigating oxidative stress in plants (Liang et al., 2013). In insects, changes in some free amino acids accumulated in response to different climatic stressors has been less investigated (Fields et al., 1998; Misener et al., 200); and in a arthropod (Issartel et al., 2005) as compared to many studies on sugars and polyols (Overgaard et al., 2007; Kostal et al., 2011b; Colinet et al., 2012). Accumulation of proline in response to cold stress has been demonstrated in the larvae of *D. melanogaster* (Kostal et al., 2011b); in *Chymomyza costata* larvae (Kostal et al., 2011a); and due to drought stress in *D. immigrans* (Tamang et al., 2017). Laboratory flies of *D. melanogaster* subjected to cold shock at - 7 °C for 2 - 3 h; as well as strains resisting chilling injury at 0 °C for 30 to 60 h, revealed 3 to 6 fold increase in the level of proline (Misener et al., 2001). In contrast, the role of proline as an osmolyte to mitigate water stress was first reported in wilting perennial rye grass - *Lolium perenne* (Kemble and MacPherson, 1954) and subsequently in bacteria (Csonka and Hanson, 1991). Higher levels of proline have been observed in *Arabidopsis thaliana* (Liang et al., 2013) and in drought - tolerant rice varieties (Choudhary et al., 2005) but not in case of barley (Chen et al., 2007). Thus, associations between high proline levels and drought tolerance have shown mixed results in plants. Since heat and drought stress co - occur in natural habitats; it may be argued that proline might play a role in thermoprotection in insects. Therefore, despite the abundance of proline in insects, the physiological role (s) of stress induced changes in proline level in diverse insect taxa need further studies.

Temperature is considered as a major abiotic factor affecting geographical distribution and abundance levels of various insect taxa (Andrewartha and Birch, 1954**;** Angilletta, 2009). Narrow distribution patterns of tropical *Drosophila* species are limited by their low genetic potential to adapt to colder and drier habitats (Kellermann et al., 2009). Further, in context of climate warming, it has been argued that tropical drosophilids might be vulnerable due to expected higher aridity conditions (Hoffmann, 2010). Despite the fact that plastic changes in stress resistance traits in the generalist species, *D. melanogaster* are higher as compared with genetic differences, similar studies have not been carried out for tropical *Drosophila* species (Hoffmann et al., 2005). On the Indian subcontinent, there are contrasting seasonal patterns of ambient relative humidity (80 ± 5% RH during monsoon but 40 ± 6 % RH during autumn). Therefore, seasonal humidity conditions are likely to impact plastic changes to multiple climatic stressors. Thus, assessment of developmental acclimation under high or low humidity on basal and induced stress resistance to multiple stressors as well as their cross - tolerance effects can help in understanding adaptive potential of tropical *Drosophila* species to harsh climatic conditions.

D. punjabiensis is a member of the melanogaster group's montium species subgroup and is reportedly widely distributed throughout south - east Asia and India (Parshad and Paika, 1964). For the final two abdominal segments in females of this species, there is color dimorphism; light morph predominates over dark morph (Ohnishi and Watanabe, 1985; Parkash et al., 2009). Geographic populations of Drosophila species belonging to the subgenus Sophophora and mesic have been studied for water conservation on the Indian subcontinent (Parkash et al., 2008 a; b; 2010; 2012). However, despite the abundance of montium species of Drosophila on the Indian subcontinent, no research has been done on them. In the present work, we assessed season - specific plastic changes in stress related traits as well as changes in the levels of energy metabolites in the tropical *D. punjabiensis*. Wild – caught flies of *D. punjabiensis* from wet (monsoon or rainy) and dry (autumn) seasons were reared under season specific simulated growth conditions and G_1 & G_2 generations flies were tested for heat, desiccation and starvation resistance. For every stressor, we evaluated cross resistance for the remaining two stressors. Additionally, we looked into how plastic changes affected the patterns of accumulation and/or use for each of the three metabolic fuels. Energy budget adjustments were also assessed for control and acclimated flies. Three replicates of several groups of flies that were exposed to varying times of heat hardening, desiccation, or famine acclimation were used to measure the rates of change in the levels of three energy metabolites.

2. Materials and Methods

Collections and cultures

Collections of wild - living Drosophila flies were made during autumn (September - October) and winter (December - January) seasons in the years 2016 and 2017 of a midland locality Solan (1550m). *D. punjabiensis* flies were identified and sexes were separated from wild collected flies (Parshad and Paika, 1964). However, for both the morphs, about 30 isofemale lines were set up to check their progeny for five generations. True breeding strains of both the morphs were isolated and used for further laboratory cultures. Dark vs light morphs male flies of *D. punjabiensis* were not investigated in the present study. For each morph, pilot experiments were made to find possible morph specific differences in chill - coma recovery (CCR) and heat knockdown times. For each morph, differences between strains were separated and non -

significant were discarded. Mass cultures were made by pooling eight true breeding strains of one type morph (dark or light).

For each morph line, flies were allowed to lay eggs for 12 hours in each of four food vials kept at 21°C and after discarding parental flies, eggs were allowed to develop at 15 or 25°C to check changes in body melanization of female progeny. For mass population of each morph, laboratory cultures were maintained at low density in wide mouth large (250ml) culture bottles for two generations before reared on standard food medium comprising maize powder, yeast powder, agar - agar, jaggery water and live yeast was not included in the fly medium. For both the morph, flies were allowed to lay eggs at 21°C, 50% RH for 8 hours in ten bottles; and five bottles with eggs and covered with thin mesh gauze (and tied around the neck of each bottle) were kept in two different environmental chambers (providing 25°C and 40% RH; and other at 25°C and 80% RH) for egg to adult development and five to six days old female flies were tested for different stress related traits. Likewise, for each morph, egg laid at 21°C were transferred to either 15 or 25°C with 50% RH in separate incubators providing thermal and humidity conditions.

Setup for an Experiment

In wild - caught flies of *D. punjabiensis* from autumn and winter season, the assortment of body color morphs of female flies was based on careful scrutiny of melanisation of six abdominal segments. In pilot experiments, we confirmed the results of visual assortment with rearing effects of thermal (15 or 25°C) plasticity changes in abdominal melanisation patterns of body color morphs of *D. punjabiensis* Possible changes due to laboratories adaptation in true breeding strains of body color morph were checked on the basis of body melanisation, chill - coma recovery and heat knockdown. For each trait and morph, we did not find differences due to possible inbreeding and /or laboratory adaptation. However, laboratory experiments on body color morph strains were done with in 6^{th} to 7^{th} months of their isolation and confirmation. Wild - caught flies were kept in the laboratory at 21°C for one day before analysis of acclimation treatment in the laboratory. This was done to minimize the effects of field acclimatization of flies to ambient thermal or humidity conditions in the field.

Different sets of experiments were conducted with laboratory reared body color morph strains, first developmental acclimation effects of low vs high humidity (40 vs 80% RH and G. T. at 25°C) were assessed for physiological traits related to desiccation resistance; and for cold or heat survival so as to find effects of wet or dry conditions For these traits, effects of rearing thermal conditions (15 or 25°C) were also conducted. Second, in laboratory reared flies (15 or 25°C) body color morph strains, we analyzed changes in basal trait values (resistance to cold, heat or drought) after adult acclimation to one day thermal vs drought conditions. Third, for each morph strains, changes in the level of three energy metabolites (trehalose, proline or body lipids) were analyzed in control (unacclimated) and flies subjected to hardening / acclimation pretreatments related to either cold vs drought; or heat vs drought. For each treatment experiment, separate controls were run simultaneously.

Examine flies collected in the wild

We conducted pilot studies on three separate, randomly selected samples (n = ~80) of wild - caught female *D. punjabiensis* flies representing each of the two distinct morphs in order to study heat knockdown (winter) and chill - coma recovery. There was heterogeneity in the trait values for each morph among the samples, most likely as a result of age or diet in the field. Next, using a wet weight, we categorized flies for each morph - specific sample based on the body weight of each fly that was captured in the wild. Reduction of variation between samples for trait values (chill - coma recovery or heat knockdown) was achieved by varying ~10% of independent samples according to these criteria. Fly samples collected in the winter from the wild were deacclimated for 24 hours at 21°C in the laboratory before being assessed for stress resistance (cold or heat).

Analysis of cuticular traits

Among the female flies of *D. punjabiensis*, identification of dark and light morphs was easier to identified based on melanization patterns of last abdominal segments. Developmental plasticity induced changes in body melanization i. e.5^{th} + 6^{th} + 7^{th} abdominal segments were fully melanised in flies of dark morph but there was no melanization in these posterior abdominal segments in light morph. However, dark and light morph flies revealed no such plasticity for abdominal segments reared at any temperatures (reared at 15 or 21 to 25°C). Percent melanisation was analyzed in wild - caught individuals of each season and three replicates of 10 flies from each morph strain. Abominal melanisation was estimated from dorsal view of the female abdomen giving values ranging from 0 (no melanisation) to 10 (complete melanisation) for each of the six abdominal segments (2^{nd} to 7th). Since the abdominal segments differ in size (i. e.0.86, 0.94, 1.0, 0.88, 0.67 and 0.38 for 2nd to 7th segments respectively), these relative sizes were multiplied with segment - wise melanisation scores. Percent melanisation data were calculated as (Σ observed weighted melanisation scores of abdominal segments per fly/Σ relative size of each abdominal segment×10 per fly) × 100 (Parkash et al., 2008). Additionally, six - day - old flies (n = 3 replicates × 20 flies of each body color morph) were utilized to estimate the cuticular lipid mass. To get their dry mass—that is, without any body water—flies were dried for a whole night at 60°C. After being dried for five minutes in HPLC - grade hexane, each fly was taken out of the solvent, allowed to dry again at ambient temperature, and then weighed again on a Sartorius microbalance (model CPA26P, precision 0.001 mg; www.sartorious. com). The calculation of cuticular lipid mass per centimeter squared involved dividing the difference in mass after solute extraction by the surface area (µg / SA).

Analysis of desiccation related traits

Desiccation survival was calculated as the time to lethal dehydration (LT_{100}) in dry air for both adult acclimated flies (n = 3 replicates × 20 flies per body color morph) reared under temperature and humidity conditions specific to the season and laboratory - reared flies. Ten flies made up each duplicate. Water content of each fly was estimated from the difference between the fresh and dry mass at 60°C. To estimate total body water content, rate of water loss; and dehydration tolerance, individual flies were used. The Sartorius microbalance (model CPA26P; 0.001 mg precision) was used

to weigh individual flies for each morph strain. The flies were dried for 24 hours at 60 °C before being weighed again. The difference between the mass before and after drying at 60 °C was used to estimate the total body - water content. We used Wharton's (1985) method—modified by Benoit et al. (2005)—to calculate the rate of water loss. The formula for calculating total body - water content (m) was f - d, which is the difference between the fresh or wet (f) and dry (d) masses. Each fly was weighed, subjected to 10% relative humidity for a predetermined amount of time at 1 - hour intervals (from 1 to 8 hours), and then weighed again. Water loss rate was calculated using Wharton's (1985) exponential equation, mt = m0e - kt, where mt is the water mass at time t and m0 is the initial water content. Dehydration tolerance was calculated as [(wet body mass – body mass at death) / (wet body mass – dry body mass) × 100]. For different rearing conditions, such as temperatures (15 and 25°C) or humidities (40 and 80% RH), the percentage of total body water lost until death due to desiccation was used to estimate the dehydration tolerance.

Measures of cold resistance

To evaluate chill coma recovery, 60 adult females of each morph (20 female flies × 3 replicates each) were placed in 10 ml glass vials, which were submerged in a 10% glycol solution cooled to 0°C. The vials were taken out after 16h (or 12h in case of wild - caught flies) and recovery time was scored. The next step involved moving the flies to 9 cm diameter petriplates in a temperature - controlled chamber at 21°C, and marking how long it took for the flies to recover from a chill coma (in minutes). When the flies stood up on their legs, it seemed that they had recovered. Before evaluating the recovery of the chill coma, adult flies were subjected to 10°C for one day and allowed twelve hours to recover. The further increases or decreases in thermotolerance as a result of thermal acclimatization were noted. Adult females (n = 3 repetitions × 20 flies) were exposed to 0°C for several durations: 10, 20, 30, 40, 50, 60, 70h for flies reared at 25°C, and up to 100h for flies reared at 15°C. This was done to test their resistance to cold shock. Following each round of cold stress, control and treated flies were placed in food vials measuring 37 by 100 mm and allowed to recuperate for 24 hours. The percentage of survivors was then computed by dividing the total number of flies exposed to cold stress by the number of individuals recovered after 24 hours of recovery. The cold survival time (LT_{100}) was defined as the amount of time that flies would experience cold shock at a stressful temperature of 0°C. Even after recovering for 24 hours at that temperature, the percentage of flies that survived was zero.

Measures of heat resistance

For heat resistance assays, individual adult females of *D. punjabiensis* grown at 15 and 25°C (20 female flies × 3 replicates each) were placed in 10 ml glass vials submerged in a glass tank with water held at a constant temperature of 38°C. Resistance was scored as the time taken for flies to be knocked down. Before determining their heat knockdown resistance, the flies were heat acclimated for one day at 32°C and then recovered for twelve hours at that temperature. Heat survival time is the duration of heat shock at stressful temperature after which percent survival of flies becomes zero (LT_{100}) even after recovery of 24 h at the rearing temperature. For heat - shock survival, four - day old adult females flies were subjected to heat stresses at 38°C for different time durations. Thereafter, flies were transferred to separate fresh food vials and percent survival was calculated for all of them after 24 h. To evaluate the heat acclimation (at 32°C for 1 day) response, we compared the percent survival after heat shock of each duration and LT_{100} in control and heat acclimated flies.

Energy metabolites estimation

Body lipids

Individual flies of each body color (dark and light) and three replicates of ten flies each of the control and hardened or acclimated conditions had their body lipid content measured. After 48 hours of drying at 60°C, each fly dry mass was measured using a Sartorius microbalance (model CPA26P, precision 0.001 mg). Individual flies were placed in 2 ml centrifuge tubes (http: //www.tarsons. in) with 1.5 ml of diethyl ether to remove body lipids. These tubes were shaken for four hours at 200 rpm and 37 °C. After that, the solvent was changed, and the procedure was repeated. After the solvent had been eliminated, individual flies were once again dried for 48 hours at 60°C and weighed.

Trehalose

In order to estimate the trehalose concentration, thirty female of *D. punjabiensis* flies of each body color morph were homogenized using 300 µl Na2CO3 in a homogenizer (Labsonic[at]M; http: //www.sartorious. com) and then incubated at 95°C for two hours in order to denature the proteins. The homogenate was combined with an aqueous solution of 600 µl sodium acetate (0.2 M) and 150 µl acetic acid (1 M). After that, the homogenate was centrifuged for 10 minutes at 12, 000 rpm (9660 ×g) using a Fresco 21 centrifuge (Thermo - Fisher Scientific, Pittsburgh, USA). Aliquots (200 µl) were used for trehalose measurement. One tube served as a blank and the other, which was treated with trehalose at 37 °C using the Megazyme trehalose assay kit (K - Treh 10/10, http: //www.megazyme. com), was taken. In this experiment, hexokinase and ATP phosphorylated released D - glucose to produce glucose - 6 - phosphate and ADP. This was then combined with glucose - 6 - phosphate dehydrogenase, leading to a decrease in nicotinamide adenine dinucleotide (NAD). At 340 nm, the absorbance by NADH was measured using a UV - 2450 - VIS instrument from Shimadzu Scientific Instruments located in Columbia, USA.

Proline

Thirty adult flies were homogenized in 3ml of sulphosalicylic acid to estimate the proline concentration of both the morphs. After centrifugation, 15µl of freshly made 1.25 Msodium nitrite solution was mixed with 50µl of the homogenate. The mixture was then left to stand at room temperature for 20 minutes. After mixing the components, 15µl of a 1.25M ammonium chloride solution was added. Next, 60µl of concentrated hydrochloric acid was added. After mixing, the mixture was cooked for 20 minutes in a bath of boiling water. After cooling the tubes, 60µl of 10N sodium hydroxide was added. In each capped tube, we added 200 µl of glacial acetic acid and 200 µl of ninhydrin solution to the resulting mixture. After mixing the solutions, they were incubated at 100°C for 60 minutes. After the samples were incubated, they were extracted using toluene. The aqueous phase's absorbance was

measured spectrophotometrically at 520 nm, and the amount of proline was determined using a standard curve.

Statistical analysis

For computations and graphics, we applied Statistica (version 7.0; statsoft, Tulsa, ok, USA). Student t - tests were used to evaluate cold or heat resistance data for wild flies in order to determine statistical differences between basal and hardened/acclimated flies of two body color morphs. Relative acclimation capacity (RAC), which is the trait value of hardened or acclimated flies minus basal trait value, was used to describe the hardening or acclimatization responses in wild - caught flies. These differences were then divided by basal trait value (see Kellett et al., 2005). Welch's test was utilized to assess changes in trait values for every trait. The data are displayed as mean values (±s. e.) based on three replicates of twenty flies per trait, body color morph strains raised under different humidity conditions (40 vs.80% RH). ANOVA was used to determine the variation caused by body color morphs, rearing temperatures, and their interactions when analyzing data on the impacts of developmental plasticity for six stress - related characteristics and three energy metabolites in morph strains raised under two growth temperatures (15 vs.25°C). We assessed basal amounts of three energy metabolites (proline, trehalose, and total body lipids) and used conventional conversion factors (J/mg) based on Schmidt - Nielson (1990) to compute the energy content per fly (each body color morph).

3. Results

Seasonal changes in body color

Wild collected female flies of *D. punjabiensis* out of selected body color morphs (across two seasons) revealed abundance of dark morph (40%) in winter while only 15% in autumn. Likewise, percent light morph flies were about (15 - 20 %) in the winter but about 30% in the autumn (Fig.1A). These seasonal changes across seasons revealed differences in the percent abundance. Thus, we observed seasonal fluctuations in the absolute number of flies of dark and light morph ($p<0.001$)).

Developmental plasticity of body melanisation and cuticular lipid mass.

As a proxy for winter or autumn ambient temperature, plastic changes resulting from raising *D. punjabiensis* flies at 15 or 25°C are displayed in Figure 1 B and C. In response to rearing body color morph developed at 15 or 25°C, we have demonstrated alterations in cuticular lipid mass and body melanization. At both rearing temperatures, the melanization of dark - morph flies was higher than that of light - morph flies (Fig.1 B & C).

Assessment of thermo - resistance in light and dark body color morphs of wild - caught flies.

Table 1 displays information on the basal and post - acclimation cold or heat resistance in the separate samples of *D. punjabiensis* wild - caught flies (dark and light morphs). Relative acclimation capacity (RAC) with cold acclimation was higher, while chill - coma recovery (CCR) of morph flies (subjected to 0°C for 24h) in control groups revealed significant variations between light versus dark morph. The acclimation capacity (RAC = - 0.28) of the light morph was found to be equal to dark morph flies (Table 1) in the CCR of flies accustomed to low humidity. Thus, a trade - off between basal and acclimated capacity was seen in field - captured flies, wherein the light morph's cold tolerance was shown to be greatly boosted upon cold or low humidity acclimatization, despite it having a lower basal value of cold resistance. A faster recovery and therefore a higher tolerance to cold are indicated by negative values of RAC for CCR (chill coma recovery).

Table 1 provides data on relative acclimation capacity (RAC) in response to rapid heat hardening or acclimation to heat or low humidity for wild - caught and varied body color morph samples with respect to heat resistance. The basal level of heat resistance in winter - collected flies was high in the light morph than in the dark morph (Table 1). When compared to the dark morph, the light morphs demonstrated a considerable improvement in heat tolerance (high RAC values) during heat hardening, heat acclimation, and following low humidity acclimation (Table 1). Therefore, drought acclimation and heat hardening, could increase the stress tolerance of winter - collected flies.

Plastic changes due to rearing conditions

Table 2 presents information on traits associated to desiccation, as well as heat and cold survival at 0°C and 38°C for both dark and light body color morphs raised at growth temperatures (25°C) and of 40% and 80% relative humidity, respectively. Cuticular lipid mass increase in flies grows at low humidity (40%) and also in lower rearing condition (15°C & 50% RH). Table 1 shows that raising morph at 15°C as opposed to 25°C greatly enhanced their ability to Table 2 displays statistical differences using Welch's test and ratio differences for each trait, growth temperatures, and body color morph. Table 3 displays the effects of developmental plasticity and body color morphs on various physiological variables, as well as the interaction between these effects and the ANOVA test results. In Table 3, morphs effect was more in traits like percent melanisation, dehydration tolerance, cuticular lipid mass and cold shock survival caused by thermal rearing conditions was more than 70%. Therefore, for various physiological characteristics of *D. punjabiensis*, plasticity changes differ considerably.

Plastic changes in cold resistance

A comparison of basal and plastic changes in cold resistance (chill - coma recovery or cold shock survival) in dark and light body color morph (due to growth temperature 15 vs 25°C) and their respective adult acclimation to cold (10°C, 1d) and drought (35% RH, 1d) are shown in Figure 2 A & B. Flies reared at 25°C took longer time duration of chill - coma recovery (min.) than 15°C reared flies. Further, an increase in cold tolerance was higher after cold or desiccation (low RH) acclimation. In addition, cold shock survival ability was also increased after acclimation treatment in both the morph flies (Fig.2 C & D).

Developmental and adult plasticity changes in heat or drought resistance

The basel and acclimated levels (after heat or drought acclimatization) of adult flies of two body color morphs raised at 15 or 25°C are depicted in Figure 3. Two measures (A & B: heat knockdown time at 38°C; and C & D: heat shock

survival at 38°C) were used to quantify heat resistance. The dark morph clearly had less heat survival and less heat resistance. However, following adult acclimatization (to heat or drought), the heat resistance of the light morph was stronger. Thus, there was a greater change in heat resistance after drought acclimation was given to flies. However, heat acclimation is also beneficial to raise the basal level tolerance.

Developmental and adult plasticity changes in desiccation stress and rate of water loss

As adults get used to the cold or a drought, they exhibit different levels of desiccation resistance (A & B) and altered rates of water loss (C & D), as shown in the bar diagram in Figure 4. As the rate of water loss and desiccation resistance are inversely correlated, we observed that morph raised at 15°C exhibited greater levels of resistance to desiccation when exposed to cold or drought, whereas the rate of water loss was decreased in both morphs when pre - treatments were added. If flies were raised at 15°C, the modifications brought about by drought acclimation were greater than those caused by cold acclimation (Fig 4). However, in terms of both features, drought acclimation responses were more significant for morph raised at 25°C than cold acclimation (Fig 4).

Effects of acclimation treatments on energy metabolites

Table 4 provides information on how stress (cold, heat, or drought acclimatoin) affected the levels of three energy metabolites (trehalose, proline, and body lipids) and their relative levels of acclimation capacity in body color morph strains raised at 15°C. When comparing the dark morph to the light morph, the basal level of an energy metabolite was higher for trehalose and proline (Table 4). Cold acclimation raised the trehalose content but decreased the proline content in both morphs. On the other hand, heat acclimation increased the level of trehalose only but decrease the level of body lipids (Table 4). Therefore, in both the morphs, the modifications brought about by different acclimation condition (Table 4). While proline was used when acclimated to cold, and also raised the body lipids levels. Additionally, there were increase in the content of trehalose and proline after low humidity acclimation, but no such change was observed for body (Table 4). Comparing low humidity acclimation to cold or heat acclimation, these observations support complementing adjustments. As a result, we discovered that three energy metabolites changed in different ways when stressed in different pre - treatments. Lastly, with regard to the quantity or level of energy metabolite and the energy budget (determined using conventional conversion factors). Our findings suggested complimentary changes for drought versus cold to potentially preserve energy balance.

4. Discussion

To understand the possible physiological basis of widespread ability of tropical *Drosophila punjabiensis*, we tested resistance to cold, heat and desiccation in body color morphs of female flies reared at 15°C. Our results in morph strains reared at 25°C are consistent with lower resistance to cold, heat and drought. However, morph strains reared at 15°C gave unexpected results i. e. significantly higher level of resistance to cold and drought in both the morphs (including light morph). Adult acclimation responses to cold, drought and low humidity stress further increased tolerance levels of morph strains reared at 15°C. Besides light morph strain showed lack of trade - off for heat versus cold tolerance. Analysis of basal and acclimated responses (chill - coma recovery and heat knockdown duration) in samples of wild - caught flies (i. e. assorted morphs) of *D. punjabiensis* also showed similar patterns. For morph strains reared at 18°C, we found compensatory changes in the levels of three energy metabolites (trehalose, proline and total body lipids) induced by adult acclimation to cold vs drought (for cold tolerance) and to heat vs drought (for heat resistance).

Based on field observations on seasonal changes (autumn vs rainy) in the absolute abundance of body color morphs of *D. punjabiensis*, we predicted divergence in the physiological tolerance levels of morphs to cope with seasonal environmental heterogeneity. Analysis of wild - caught (autumn) samples of body color morphs of *D. punjabiensis* revealed cold resistance in both the morphs consistent with co - existence of light morph flies under colder environments during rainy. As expected, heat resistance (heat knockdown time) of wild - caught morph flies was lower for dark but higher for light. In the laboratory, body color morph strains reared at 15°C or 25°C with 50% RH revealed significant differences in resistance to cold, heat and drought consistent with developmental acclimation effects which seem adaptive under seasonally varying environments.

The basal and acclimation responses (heat or low humidity) of body color morph strains reared at 25°C are in agreement with thermal budget hypothesis i. e. lighter morph to be heat resistance as compared to heat sensitive dark morph. These observations are consistent with earlier findings in the ladybird beetle (*Adalia bipunctata*) showing higher cuticular reflectance of solar radiation in light body color individuals so as to avoid detrimental effects of heat (Brakefield and Willmer, 1985). Therefore, decline in the abundance of dark body color morph of *D. punjabiensis* in rainy seems adaptive. In contrast, in several insect taxa, species with darker body color inhabit cooler areas due to their lower cuticular reflectance (of solar radiation), and darker individual can heat up faster to thermoregulate their bodies for flight, for aging, and mating etc (True, 2003; Clusella - Trullas et al., 2001). Therefore, in cooler environments melanic species show greater fitness than light body color species (True, 2003). In *D. punjabiensis* both fields captured light morph flies (~25%) during autumn season and also higher cold resistance of laboratory reared flies of light morph strain at 15°C, are not in agreement with thermal - melanism hypothesis. Thus, we predicted possible role of acclimation potential (to cold or drought) and in the storage level of energy metabolites (proline and trehalose) which confer ability to resist colder and drought conditions. Our data have shown that light morph flies recover later than dark flies i. e. basal level of cold tolerance is lower in light morph flies. In contrast, adult acclimation (to cold or drought) of light morph resulted in significant increase in cold resistance. Further, light morph flies reared at 15°C store higher amount (~50%) of trehalose, proline and total body lipids as compared to dark morph.

Previous studies have shown cryoprotective role of proline and trehalose (Benoit et al., 2009; Colinet et al., 2012; Kostal et al., 2011). For example, hyperprolinemic larvae of drosophilid *Chymomyza costata* are able to tolerate

cryopreservation in liquid nitrogen (Kostal et al., 2011). In *Belgica Antarctica*, resistance to heat and cold is associated with dehydration induced increase in the level of trehalose (Benoit et al., 2009). In the present work also, we found significant increase in the level of proline and trehalose in response to cold or drought acclimation of the light morph as compared with dark. There are no data on reflectance level (of solar radiation) in light body color morph (and other morphs) of *D. punjabiensis* and light morph flies could be at a disadvantage in the ability to warm up (under solar radiation) in the colder season. However, higher adult acclimation responses as well as adaptive changes in the storage and acclimated levels of three energy metabolites could possible account for adaptability of dark morph of *D. punjabiensis* to cooler environments during winter season (15) in the temperate locality of origin of flies.

For heat resistance of Drosophila species, flies reared under warmer conditions (25 or 28°C) show higher basal tolerance level as compared to growth under colder conditions (Hoffmann et al., 2003; Angilletta, 2009). Such expectations were evident in two body color morphs (light and dark) reared at 25°C. Similar levels of plastic responses were evident after adult acclimation to heat or low humidity although acclimation effects were higher with draught than heat. In contrast, for body color morph reared at 15°C exhibited about 30% reduction in the basal level of heat knockdown time but heat acclimation responses were similar to those observed for morph reared at 25°C. However, increase in heat resistance after drought acclimation was equal for morph flies reared at 15 and 25°C. Further, higher basal as well as induced levels of heat resistance of light body color morph strain reared at 15°C is quite unexpected. A similar lack of trade - off between cold and heat resistance has been observed in field flies of *D. obscura* (Sorensen et al., 2016). Thus, as compared to lower plasticity for cold and heat resistance in tropical Drosophila species with restricted distribution (Mitchell et al., 2011; Overgaard et al., 2011). The higher plasticity level for cold and heat resistance in morph flies reared at 15°C supports its higher adaptive potential expected under invasion.

Tropical drosophilid, with restricted distribution patterns are limited by their survival under drier habitats (Kellermann et al., 2009). Widespread and narrowly distributed tropical Drosophila species are able to increase their desiccation resistance after drought hardening but level differ across species (Kellermann et al., 2018). In the present work, body color morph strains of *D. punjabiensis* reared at 15°C, basal as well as plasticity changes in desiccation resistance due to adult acclimation (cold or drought) are significantly higher as compared with morph strains reared at 25°C, showing lack of trade - off between basal and induced levels of desiccation resistance. For 15°C reared flies, morph specific differences in desiccation resistance, plasticity effects due to drought acclimation were higher than cold acclimation. Plasticity (developmental acclimation under low vs high 40% vs 80% RH humidity or at 15°C vs 25°C) showed changes in desiccation resistance and cuticular lipid mass. The plastic changes in body melanization and cuticular lipid mass could affect rate of water loss. Thus, for two physiological mechanism of desiccation resistance (rate of water loss and dehydration tolerance) in morph strains of *D. punjabiensis*, cold or drought acclimation (developmental or adult) vary in their effects on desiccation resistance plasticity. Thus, body color morphs of *D. punjabiensis* reared at 15°C are able to increase their drought resistance through developmental and acclimation to cold or drought. Further, studies are needed to find generality of plasticity for physiological mechanisms of desiccation resistance in other tropical Drosophila species.

In the subgenus sophophored, montium species subgroup (of melanogaster species group) is represented by more than two dozen species endemic to south - east Asia but very few species (e. g. *D. kikkawai*) have invaded other continents e. g. South America and Africa (Ashburner, 1989; Markow and O'Grady, 2006). Despite the fact that several species of montium subgroup show genetic polymorphism for body color, these species show narrow distribution patterns only in the oriental region (Ohnishi and Watanabe, 1977; Ashburner 1989; Markow and O'Grady, 2006). Field data on geographical (latitudinal) differences in allele frequencies for dark and light body color morphs (in *D. jambulina*, *D. montium*, *D auraria* and *D. kikkawai*) support adaptive changes on different continents due to (India, Brazil) climatic selection (Freire - Maia & Freire - Maia, 1954; Mechado et al 2001; da costa et al 2003; Parkash et al 2000). However, plasticity changes in body color morphs and other ecological stress related traits have received less attention so far. For example, in *Drosophila polymorpha* (Cardini species group) and *D. falleni* (quinaria species group), three body color morphs lack plastic responses when flies were reared at 18, 21 and 25°C (Dombeck and Jaenike, 2004; Brisson et al 2005) but there had been no such data on *D. punjabiensis* It may be questioned that laboratory analysis of plasticity changes in resistance to cold, drought and heat for body color morphs reared at 15°C may not represent field conditions encountered during winter. Although direct analysis of field - caught samples of *D. punjabiensis* is likely to be biased due to sampling and age of flies in the wild during in winter. We assumed that relative acclimation capacity analysis of plastic changes in chill - coma recovery as well as for heat knockdown duration could help in comparing the plastic changes due to acclimation of adult flies of body color morphs of *D. punjabiensis (*Table 1). We observed that body color morphs vary in their basal as well as induced level of plasticity for cold or heat and there was broad similarity in such patterns of plasticity between wild - caught flies and laboratory analyzed morphs of *D. punjabiensis*. However, further studies on *D. punjabiensis* are needed to check survival of control and hardened / or acclimated body morphs in field cages as has been already attempted in Australian populations of *D. serrata* which also belongs to montium species subgroup (Jenkins and Hoffmann, 1999). In *D. serrata*, there is evidence of seasonal selection (pre - winter vs post - winter) of evolved genetic changes in cold tolerance (Magiafoglou and Hoffmann). There is need to conduct similar studies for assessing genetic and plastic potential of body color morph strains.

References

[1] Andrewartha, H. G., Birch, L. C. (1954). The distribution and abundance of animal. *The University of Chicago Press, Chicago. Illinois.*

[2] Angilletta, M. J. (2009). *Thermal adaptation: a theoretical and empirical synthesis. Oxford University Press, Oxford*.
[3] Ashburner M (1989). *Drosophila* A laboratory manual. *University of Cambridge, Cambridge, UK Cold Spring Harbor Laboratory Press.*
[4] Benoit, J. B., Lopez - Martinez, G., Elnitsky, M. A., Lee, R. E. and Denlinger, D. L. (2009). Dehydration induced cross tolerance of *Belgica antarctica* larvae to cold and heat is facilitated by trehalose accumulation. *Comp. Biochem. Physiol. A*.152, 518 - 523.
[5] Benoit, J. B., Yoder, J. A., Rellinger, E. J., Ark J. T., and Keeney, G. D. (2005). Prolonged maintenance of water balance by adult females of the American spider beetle, *Mezium affine* Boieldieu, in the absence of food and water resources. *J. Insect Physiol.*51: 565–573.
[6] Brakefield, P. M. and Willmer, P. G. (1985). The basis of thermal melanism in the ladybird *Adalia bipunctata*: differences in reflectance and thermal properties between morphs. *Heredity*.54, 9 - 14.
[7] Brisson, J. A., Toni, D. C. D., Duncan, I. and Templeton, A. R. (2005). Abdominal pigmentation variation in *Drosophila polymorpha*: geographic variation in the trait and underlying phylogeography. *Evolution*.59, 1046 - 1059.
[8] Bubliy, O. A., Kristensen, T. N. and Loeschcke, V. (2013). Stress - induced plastic responses in *Drosophila simulans* following exposure to combinations of temperature and humidity levels. *J. Exp. Biol*.216, 4601 - 4607.
[9] Chen Z, Zhou M, Newman I, Mendham N, Zhang G, Shabala S (2007) Potassium and sodium relations in salinised barley tissues as a basis of differential salt tolerance. *Funct Plant Biol* 34 150 - 162
[10] Choudhary, A. K., Thakur, R. C. and Kumar, N. (2005) Effect of integrated nutrient management on protein content and protein yield in wheat - rice crop sequence in north - western Himalayas. *Himachal Journal of Agricultural research,* 31 (2): 6 - 14.
[11] Chown, S. L., Sørensen, J. G. and Terblanche, J. S. (2011). Water loss in insects: an environmental change perspective. *J. Insect Physiol*.57, 1070 - 1084.
[12] Colinet, H., Larvor, V., Laparie, M. and Renault, D. (2012). Exploring the plastic response to cold acclimation through metabolomics. *Fun Ecol*.26, 711 - 722.
[13] Csonka L. N. and Hanson A. D. (1991). Prokaryotic osmoregulation: genetics and physiology. *Annu. Rev. Microbiol.*45: 569 - 606
[14] Da Costa BEP, Rohde C, da Silva Valente VL (2003). Temperature, urbanization and body color polymorphism in south Brazilian populations of *Drosophila kikkawai* (diptera, Drosophilidae) *Iheringia Série Zoologia*.93: 381–393.
[15] Denlinger, D. L. and Lee, R - E. (2010). Low temperature biology of insects. *Cambridge University Press, Cambridge.*
[16] Dombeck, I. and Jaenike, J. (2004). Ecological genetics of abdominal pigmentation in *Drosophila falleni*: a pleiotropic link to nematode parasitism. *Evolution*.58, 587 - 596.
[17] Fields, P. G., Fleurat - Lessard, F., Lavenseau, L., Febvay, G., Peypelut, L. and Bonnot, G, (1998). The effect of cold acclimation and deacclimation on cold tolerance, trehalose and free amino acid levels in *Sitophilus granaries* and *Cryptolestesferrugineus* (Coleoptera). *J. Insect Physiol.*44, 955 - 965.
[18] FREIRE - MAIA, N. (1954). The HARDY - WEINBERG law in Brazilian natural populations of *D. kikkawai*. *Drosophila Information Service* 28: 119–120.
[19] Hoffmann, A. A. (2010). Physiological climatic limits in Drosophila: patterns and implications. *J. Expt. Biol*.213, 870 - 880.
[20] Hoffmann, A. A., Hallas, R. J., Dean, J. A. and Schiffer, D. M. (2003). Low potential for climatic stress adaptations in a rainforest *Drosophila* species. *Science*.301, 100 - 102.
[21] Hoffmann, A. A., Shirriffs, J. and Scott, M. (2005). Relative importance of plastic vs genetic factors in adaptive differentiation: geographical variation for stress resistance in *Drosophila melanogaster* from eastern Australia. *Fu. Ecol*.19, 222 - 227.
[22] Issartel J., Renault D., Voituron Y., Bouchereau A., Vernon P. and Hervant F. (2005). Metabolic responses to cold in subterranean crustaceans. *J. Exp. Biol.*208, 2923 - 2929.10.1242/jeb.01737
[23] Jenkins, N. L. and Hoffmann, A. A. (1999). Limits to Southern border of *Drosophila serrata:* cold resistance, heritable variation, and trade - off. *Evolution* 53, 1823 - 1834.
[24] Kalra, B., Tamang, A. M. and Parkash, R. (2017). Cross - tolerance effects due to heat hardening, desiccation and starvation acclimation of tropical drosophilid - *Zaprionus indianus*. *Comp. Biochem. Physiol*. A 209, 65 - 73.
[25] Kellermann, V., Heerwaarden, B. V., Sgro, C. M. and Hoffmann, A. A. (2009). Fundamental evolutionary limits in ecological traits drive Drosophila species distributions. *Science*.325, 1244 - 1246.
[26] Kellermann, V., Hoffmann, A. A., Overgaard, J., Loeschcke, V. L. and Sgra, C. M. (2018). Plasticity for desiccation tolerance across Drosophila species is affected by phylogeny and climate in complex ways. *Proc. Royal Society B*.285 (1874): 20180048
[27] Kemble A. R. and MacPherson H. T. (1954) Liberation of amino acids in perennial ray grass during wilting. *Biochem. J.;* 58: 46 - 59
[28] Kostal, V., Korbelova, J., Rozsypal, J., Zahradnickova, H., Cimlova, J., Tomcala, A. and Simek, P. (2011a). Long - term cold acclimation extends survival time at 0°C and modifies the metabolomic profiles of the larvae of the fruit fly. *Drosophila melanogaster*. *PLOS One* 6, 1 - 10.
[29] Kostal, V., Zahradnickova, H. and Simek, P. (2011b). Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata* survive cryopreservation in liquid nitrogen. *Proc. Nat. Acad.* USA.108, 13041 - 13046.
[30] Liang X., Zhang L., Natarajan S. K., Becker D. F. (2013) Proline mechanisms of stress survival. *Antioxidants & Redox Signaling*, 19, 998–1011.
[31] Machado, M. X., D. C. De Toni and P. R. P. Hofmann, (2001). Abdominal pigmentation polymorphism of *Drosophila polymorpha* (Dobzhansky and Pavan, 1943)

collected on Ilha de Santa Catarina and neighboring islands. *Biotemas* 14: 87–107.

[32] Magiafoglou, A., Carew, M. E. and Hoffmann, A. A. (2002). Shifting clinal patterns and microsatellite variation in *Drosophila serrata* populations: a comparison of populations near the southern border of the species range. *J. Evol. Biol*.15, 763 - 774.

[33] Majerus, M. E. N. (1994). Ladybirds: *The new Naturalist library, Harper Collins, London*

[34] Majerus, M. E. N. (1998). *Melanism: Evolution in action. Oxford University Press. Oxford, U. K.*

[35] Markow, T. A. and O'Grady, P. (2006). *Drosophila: a guide to species identification and use.* Academic Press, San Diego, California. management on protein content and protein yield in wheat - rice crop sequence in north - western Himalayas. *Himachal Journal of Agricultural research*, 31 (2): 6 - 14.

[36] Michaud, M. R., Benoit, J. B., Lopez - Martinez, G., Elnitsky, M. A., Lee, R. E. Jr. and Denlinger, D. L. (2008). Metabolomics reveals unique and shared metabolic changes in response to heat shock, freezing and desiccation in the Antarctic midge, *Belgica Antarctica*. *J. Insect physiol*.54, 645 - 655.

[37] Misener, S. R., Chen, C. P. and Walker, V. K. (2001). Cold tolerance and proline metabolic gene expression in *Drosophila melanogaster*. *J. Insect Physiol.47*, 393 - 400.

[38] Ohnishi, S. and Watanabe (1985). Genetic analysis of color dimorphism in the Drosophila montium subgroup. *Japanese J. Genetics*.60, 355 - 358.

[39] Overgaard, J., Malmendal, A., Sorensen, J. G., Bundy, J. G., Loeschcke, V., Nielsen, N. C. and Holmstrup, M. (2007). Metabolomic profiling of rapid cold hardening and cold shock in *Drosophila melanogaster*. *J Insect Physiol*.53, 1218 - 1232.

[40] Parkash R, Kalra B and Sharma V (2010). Impact of body melanisation on contrasting levels of desiccation resistance in a circumtropical and a generalist *Drosophila* species. Evolutionary Ecology, Netherland, Springer publications. DOI 10.1007/s 10682 - 009 - 9301 - 9.

[41] Parkash R, Rajpurohit S and Ramniwas S (2008a) Parallel changes in body melanisation and desiccation resistance in highland vs. lowland populations of *D. melanogaster. Journal of Insect Physiology*, 54: 1050 - 1056.

[42] Parkash R, Ramniwas S, Rajpurohit S and Sharma V (2008b) Variations in body melanization impact desiccation resistance in *Drosophila immigrans* from western Himalayas. *Journal of Zoology* 276: 219 - 227.

[43] Parkash, R. and Ranga, P. (2014). Seasonal changes in humidity impact drought resistance in tropical *Drosophila leontia*: testing developmental effects of thermal versus humidity changes. *Comp. Biochem. Physiol. A*.169, 33 - 43.

[44] Parkash, R., Rajpurohit, S. and Ramniwas, S. (2008c). Changes in body melanization and desiccation resistance in highland vs lowland populations of *Drosophila melanogaster*. *J. Insect Physiol*.54, 1050 - 1056.

[45] Parkash, R., Singh, S. and Ramniwas, S. (2009). Seasonal changes in the humidity level in the tropics impact body color polymorphism and desiccation resistance in *Drosophila jambulina* - evidence for melanism – desiccation hypothesis. *J. Insect Physiol*.55, 358 - 368.

[46] Parkash, R. Aggarwal, D. D., (2012). Trade - off of energy metabolites as well as body color phenotypes for starvation and desiccation resistance in montane populations of *Drosophila melanogaster*. *Comp. Biochem. Physiol.* A 161, 102–113.

[47] Parshad, R. & Paika, I. J. (1964). Drosophilid survey of India. II. Taxonomy and cytology of the subgenus *Sophophora* (*Drosophila*). *Res. Bull. Panjab Univ*., 15: 225 - 255.

[48] Pires JC, Zhao J, Schranz ME et al (2004) Flowering time divergence and genomic rearrangements in resynthesized Brassica polyploids (Brassicaceae). *Biol J Linn Soc* 82: 675–688

[49] Renault, D., Bouchereau, A., Delettre, Y. R., Hervant, F. and Vernon, P. (2006). Changes in free amino acids in *Alphitobiusdiaperinus* (Coleoptera: Tenebrionidae) during thermal and food stress. *Comp. Biochem. Physiol. A* 143, 279 - 285.

[50] S Clusella - Trullas, TM Blackburn, SL Chown (2011) Climatic predictors of temperature performance curve parameters in ectotherms imply complex responses to climate change, *The American Naturalist* 177 (6), 738 - 751

[51] Schmidt - Nielsen, K. (1990. Animal Physiology: Adaptation and Environment, *4th edn. Cambridge University Press, Cambridge.*

[52] Seymour, J. E. & Jones, R. E. (2000) Humidity - terminated diapause in the tropical braconid parasitoid *Microplitis demolitor*. *Ecological Entomology*, 25, 481–485.

[53] B. J. Sinclair, L. V. Ferguson, G. Salehipour - shirazi, H. A. MacMillan. (2013). Cross - tolerance and cross - talk in the cold: relating low temperatures to desiccation and immune stress in insects. *Integr. Comp. Biol.*, 53, pp.545 - 556

[54] Sinclair, B. J., L. E. Coello Alvarado, and L. V. Ferguson. (2015). An invitation to measure insect cold tolerance: methods, approaches, and workflow. *J. Therm. Biol*.53: 180–197

[55] Sørensen, J. G., Kristensen, T. N. and Overgaard, J. (2016). Evolutionary and ecological patterns of thermal acclimation capacity in *Drosophila:* is it important for keeping up with climate change. *Curr. Opin. Insect Sci*.17, 98 - 104. doi: 10.1016/j. cois.2016.08.003

[56] Tamang, A. M., Kalra, B. and Parkash, R. (2017). Cold and desiccation stress induced changes in the accumulation and utilization of proline and trehalose in seasonal populations of *Drosophila immigrans*. *Comp. Biochem. Physiol. A* 203, 304 - 313.

[57] Tauber MJ, Tauber CA, Nyrop JP and Villani MG (1998) Moisture, a vital but neglected factor in the seasonal ecology of insects: hypothesis and tests of mechanisms. *Environmental Entomology* 27: 523 - 530.

[58] Tauber, M. J., Tauber, C. A. and Masaki, S. (1986). Seasonal Adaptations of Insects. *New York: Oxford University Press. tolerance. Funct Plant Biol* 34 150–162.

[59] True, J. R. (2003). Insect melanism: the molecules matter. *Trends in Ecology and Evolution*.18, 640 - 647.

[60] Wharton, G. W., (1985). Water balance of insects. In: Kerkut, G. A., Gilbert, L. I. (Eds.), *Comprehensive Insect Physiol., Biochem. Pharmacol., vol.4. Pergamon Press, Oxford,* pp.565–603.

[61] Whitman, D. W. and Ananthakrishnan, T. N. (2009). Phenotypic plasticity of insects: mechanisms and consequences. *Science Publishers, Enfield, USA.*

[62] Williams, C. M., Watanabe, M., Guarracino, M. R., Ferraro, M. B., Edison, A. S., Morgan, T. J., Boroujerdi, A. F. B. and Hahn, D. A. (2014). Cold adaptation shapes the robustness of metabolic networks in *Drosophila melanogaster*. *Evolution* 68, 3505 - 3523.

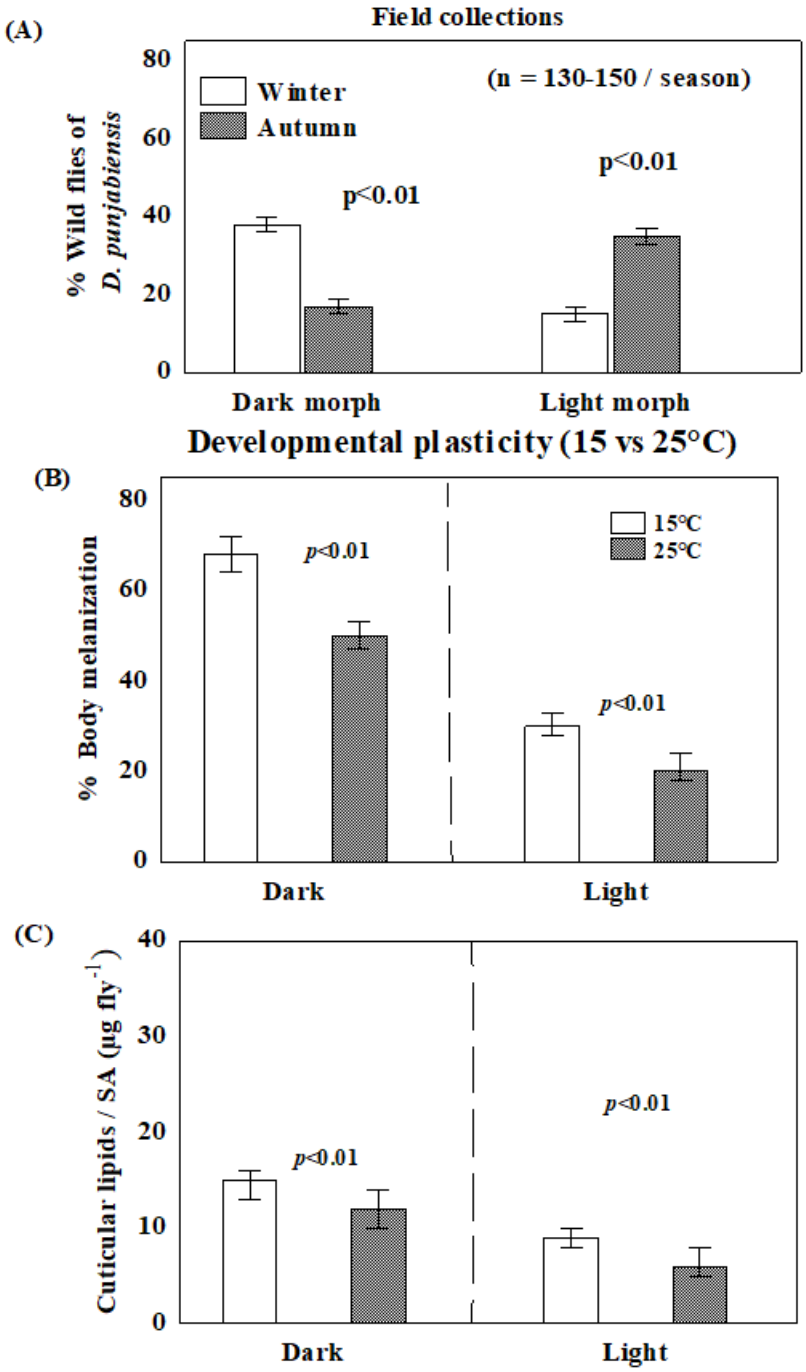

Figure 1: (A) Variations in the percentage abundance of the Dark and Light body color morphs of *D. punjabiensis* females collected over a two - year period (2016–2017) according to the season (winter vs. autumn). Due to developmental acclimatization at 15°C and 25°C, changes in (B) body melanization percentage and (C) cuticular lipid mass / surface area (µg fly - 1)

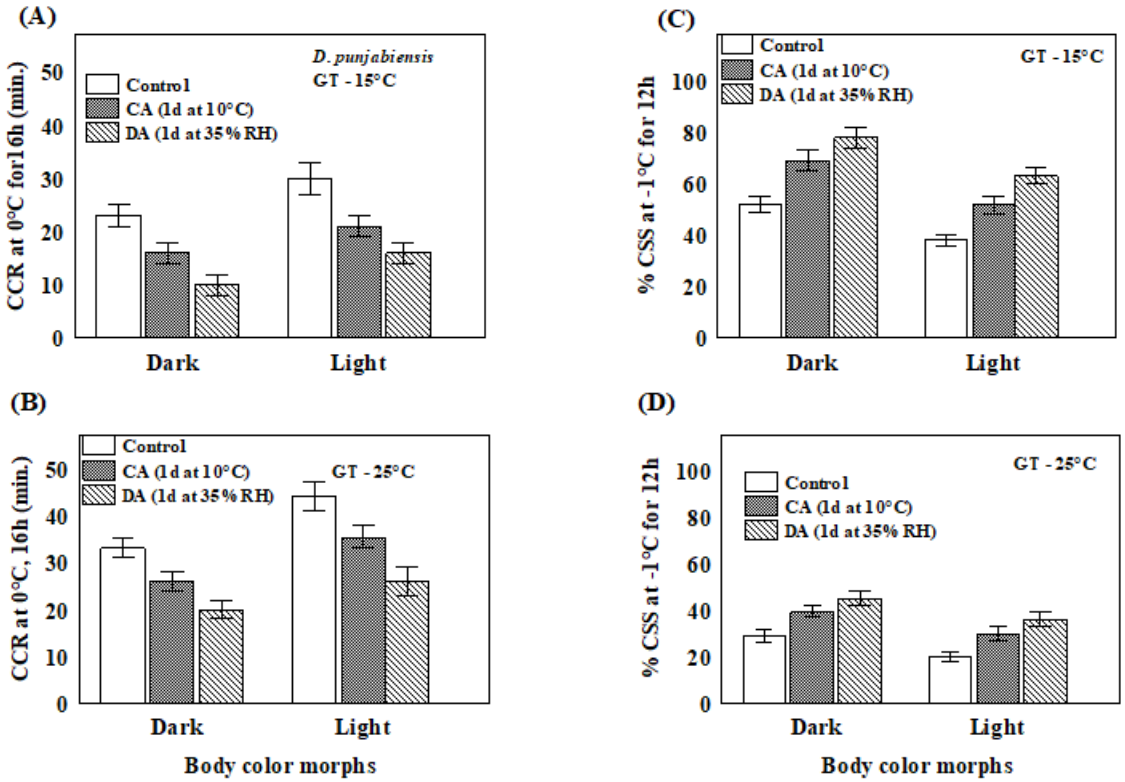

Figure 2: Changes in body color morphs of *D. punjabiensis* (A & B) for cold tolerance (CCR: chill - coma recovery); and cold shock survival (CSS; C & D) as a result of developmental acclimation (15°C Vs 25°C); and after adult acclimation (10°C for 1d; or 35% RH for 1d). Twenty female flies of each body color morph were used in three repetitions to generate the data (m ± s. e.) values for each trait.

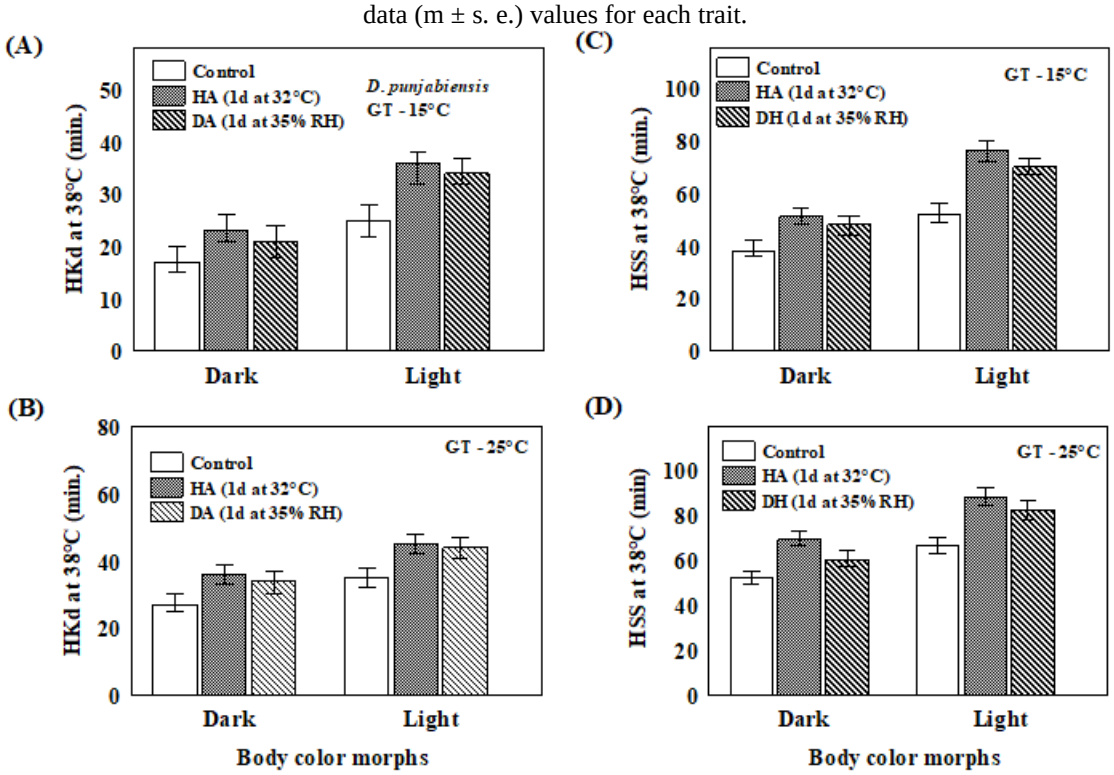

Figure 3: Due to developmental plasticity (15 vs.25°C) for heat resistance, *D. punjabiensis* body color morphs (A & B: HKd; heat knockdown; and C & D: HSS; heat shock survival at 38°C) exhibit notable changes. The data (m + s. e.) for basal and after heat acclimation (HA - 32°C for 1d) or drought acclimation (35% RH, 1d) shows that flies raised at 25°C had a higher heat tolerance than those raised at 15°C. Twenty female flies of each body color morph were used in three sets to generate the data (m ± s. e.) values for each trait.

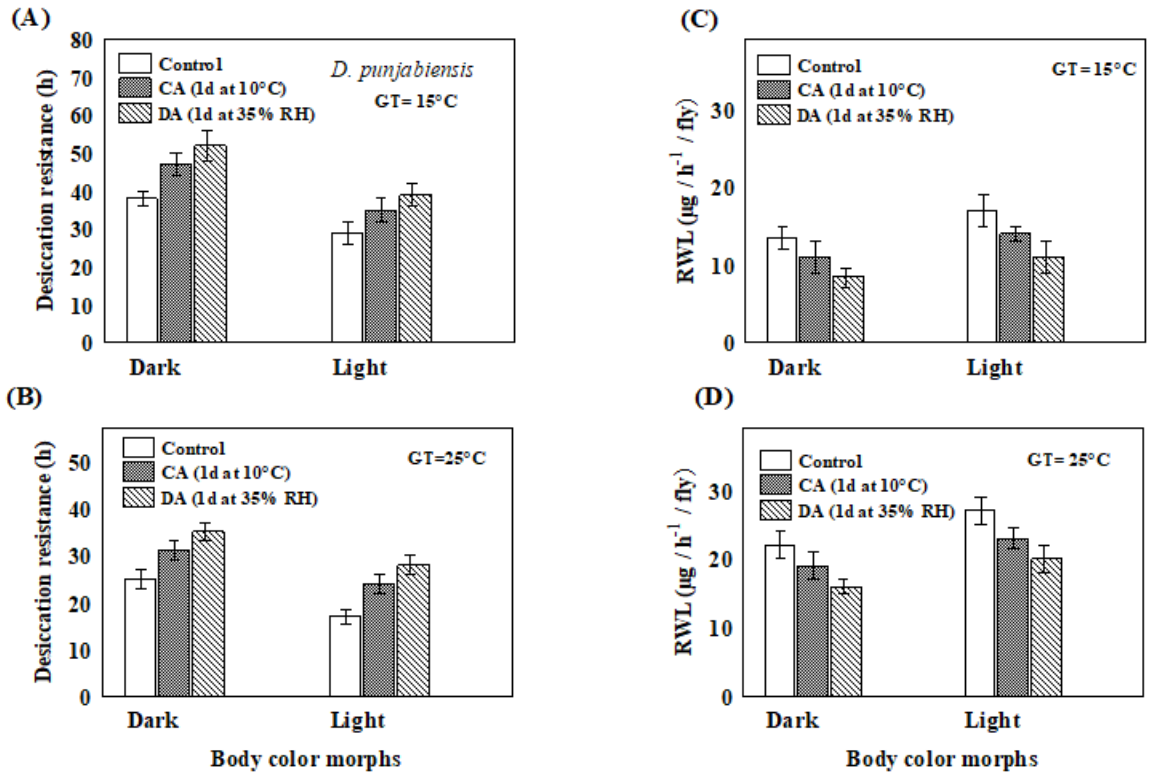

Figure 4: The desiccation - related traits (A & B: desiccation resistance; and C & D: rate of water loss) of the body color morphs (dark and light) of *D. punjabiensis* vary in response to developmental acclimation (15°C Vs.25°C); and after adult acclimation (cold or drought). The data (m ± s. e. values) for each trait are derived from three replicates of twenty female flies of each body color morph.

Table 1: Data on cold or heat resistance in wild - caught flies of assorted body color morphs (dark and light) of *D. punjabiensis*. Using a t - test, differences between morphs are displayed. Trait values are based on three replicates of ten wild - caught female flies of each body color morph (n=30 flies) for each hardening or acclimation treatment. Trait values of the control group (unacclimated) were assessed simultaneously for each treatment.

Acclimation effects	**Chill - coma recovery (min.) of wild - caught flies**		
(A). Cold resistance (0°C, 16h)	Dark	Light	t - test
1. Control (unacclimated)	24 ± 2.22	38 ± 3.04	78.25***
2. RCH (4°C, 2h)	18 ± 2.85	29 ± 4.22	44.41***
RAC	**- 0.25**	**- 0.23**	**ns**
3. CA (10°C, 1d)	16 ± 1.35	25 ± 2.00	22.16**
RAC	**- 0.33**	**- 0.34**	**ns**
4. DA (35% RH, 1d)	17 ± 1.78	27 ± 1.12	11.33*
RAC	**- 0.29**	**- 0.28**	**ns**
	Heat resistance (min.) of wild - caught flies		
(B). Heat knockdown at 38°C	Dark	Light	t - test
1. Control (untreated)	21 ± 2.20	34 ± 3.46	43.71***
2. RHH (32°C, 1h)	23 ± 1.54	38 ± 2.82	35.22***
RAC	**+ 0.09**	**+ 0.12**	**2.02***
3. HA (32°C, 1d)	26 ± 2.26	42 ± 3.44	85.42***
RAC	**+ 0.24**	**+ 0.24**	**ns**
4. DA (35% RH, 1d)	22 ± 1.57	37 ± 2.55	56.37***
RAC	**+ 0.045**	**+ 0.88**	**4.12****

*RCH = rapid cold hardening; CA = cold acclimation; DA= Desiccation acclimation; RHH = rapid heat hardening; HA = heat acclimation; RAC = relative acclimation capacity.

Table 2: Comparing the plastic changes in desiccation - related characteristics, cold survival at 0°C, and heat survival at 38°C resulting from morph (Dark and Light) of *D. punjabiensis* developmental plasticity under humidity (40 vs.80%) or rearing temperatures (15 vs.25°C). Welch's test is used for statistical analysis and data (m ± s. e., three replicates of 20 female flies per trait and treatment) is given.

Traits		Developmental humidity (%) GT - 25°C				Developmental temperature (°C) RH 50%			
	Morph	40%	80%	Fold ratio	Welch's test	15°C	25°C	Fold ratio	Welch's test
1) Desiccation Survival (h) LT_{100}	Dark	18 ± 2.02	14 ± 1.74	1.28	5.45*	39 ± 3.15	25 ± 2.05	1.56	15.23***
	Light	15 ± 1.85	10 ± 1.12	1.50	17.32***	30 ± 2.74	17 ± 1.23	1.76	26.12***
2) Dehydration tolerance	Dark	47 ± 2.88	43 ± 2.33	1.09	5.26*	64 ± 4.23	49 ± 2.74	1.30	35.23***
	Light	42 ± 3.41	37 ± 3.05	1.13	16.45**	55 ± 3.44	42 ± 2.36	1.31	42.48***
3) Cuticular Lipid mass (µg / SA)	Dark	13 ± 1.03	9 ± 1.42	1.44	9.23**	12 ± 1.20	10 ± 0.85	1.20	2.03*
	Light	12 ± 2.00	8 ± 1.05	1.50	5.47**	13 ± 1.00	10 ± 0.75	1.30	3.56*
4) Melanization (%)	Dark	43 ± 2.68	42 ± 2.45	1.02	ns	68 ± 3.75	45 ± 3.20	1.51	48.23***
	Light	28 ± 2.00	26 ± 1.44	1.07	ns	30 ± 2.89	20 ± 2.16	1.50	23.74***
5) Cold survival at 0°C (h)	Dark	29 ± 3.23	27 ± 2.77	1.07	ns	52 ± 3.88	29 ± 3.14	1.79	68.9***
	Light	24 ± 1.22	22 ± 1.16	1.09	ns	38 ± 3.56	21 ± 2.45	1.80	58.12***
6) Heat survival at 38°C (min.)	Dark	37 ± 3.66	33 ± 3.85	1.21	8.82**	38 ± 2.11	50 ± 2.47	1.31	33.26***
	Light	58 ± 3.56	53 ± 3.21	1.09	8.09**	52 ± 2.60	65 ± 2.35	1.30	25.41***

sults of Two - way ANOVA were used to explain the variation in six ecological factors and three energy metabolites of *D. punjabiensis* caused by morphs (dark and light) and developmental acclimation temperatures (15 vs.25°C). For six ecological features, sixty flies of each morph are utilized, and for three energy metabolites for both developmental temperatures, thirty flies of each morph are used.

		Melanization			**Cold shock survival**			**Heat shock survival**		
	df	**MS**	**F**	**% Var**	**MS**	**F**	**% Var**	**MS**	**F**	**% Var**
Dev. Acc. (1)	1	2937.37	18945.2	4.64	11288.8	2360.87	26.48	12760.4	4212.8	56.25
Morph (2)	1	58063.7	10264.06	90.66	31144.8	6513.42	73.07	9728.3	3211.8	42.88
1 × 2	1	2337.5	413.21	3.69	183.7	38.43	0.43	190.8	63.0	0.84
Error	236	5.7		0.22	4.8		0.011	3.0		0.01
		Cuticular lipids mass			**Dehydration tolerance**			**Desiccation survival**		
	df	**MS**	**F**	**% Var**	**MS**	**F**	**% Var**	**MS**	**F**	**% Var**
Dev. Acc. (1)	1	451.55	752.60	1.74	15073.4	3515.5	13.06	10600.1	2239.63	66.41
Morph (2)	1	2143.23	3572.13	82.55	5684.3	1325.7	78.12	5348.7	1130.09	33.51
1 × 2	1	0.79	1.32	0.03	20.4	4.8	2.83	7.0	1.48	0.043
Error	236	0.60		0.02	4.3		5.97	4.7		0.029
		Trehalose (µg / mg DM)			**Proline (µg / mg DM)**			**Body Lipids (µg / mg DM)**		
	df	**MS**	**F**	**% Var**	**MS**	**F**	**% Var**	**MS**	**F**	**% Var**
Dev. Acc. (1)	1	8508.5	2550.7	41.47	2527.86	1471.24	67.89	14758	3298.8	38.91
Morph (2)	1	11999.2	3597.1	58.48	1130.57	658.01	30.36	23050	5152.4	60.78
1 × 2	1	4.5	1.4	0.021	62.94	30.63	1.69	109	24.4	0.287
Error	116	3.3		0.016	1.72		0.05	4		0.01

Table 4: Plastic changes in the levels of three energy metabolites (trehalose, proline and body lipids: µg / mg DM / fly) in response to HA: heat acclimation, CA: cold acclimation, DA: Desiccation acclimation at 35% RH) of dark and light body color morph of *D. punjabiensis* female flies reared at lower temperature (15°C). For each treatment and morph, relative acclimation capacity (RAC) for changes in the amount of each energy metabolite is also given (+ sign indicates increase; and – sign indicates decrease).

	Trehalose (µg / mg DM)		**Proline (µg / mg DM**		**Body Lipids (µg / mg DM)**	
Treatments*	**Dark**	**light**	**Dark**	**light**	**Dark**	**Light**
1. Control	62 ± 2.44	48 ± 2.55	21 ± 1.71	17 ± 2.63	76 ± 3.00	98 ± 3.82
2. HA	75 ± 3.35	57 ± 2.50	22 ± 1.41	18 ± 2.55	56 ± 2.65	84 ± 2.88
(RAC)	**+ 0.21**	**+ 0.19**	**+ 0.047**	**+ 0.058**	**- 0.26**	**- 0.14**
3. CA	82 ± 3.77	69 ± 3.02	16 ± 1.32	14 ± 2.34	84 ± 3.20	120 ± 4.22
(RAC)	**+0.33**	**+ 0.44**	**- 0.23**	**- 0.18**	**+ 0.11**	**+ 0.22**
4. DA	56 ± 2.88	39 ± 2.82	32 ± 2.07	26 ± 2.73	72 ± 2.06	92 ± 2.42
(RAC)	**- 0.097**	**- 0.19**	**+ 0.52**	**+ 0.52**	**- 0.052**	**- 0.061**

HA – heat acclimation; CA – cold acclimation; DA – drought acclimation; RAC = relative acclimation capacity.