1 **Energy metabolites mediated cross-protection to heat, drought and starvation induced**

2 **plastic responses in tropical *D.ananassae* of wet-dry seasons**

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4 **Chanderkala Lambhod, Ankita Pathak, Ashok K Munjal and Ravi Parkash***

5 Department of Genetics, Maharshi Dayanand University, Rohtak – 124001; Department of

6 Biochemistry and Genetics, Barkatullah University, Bhopal – 462026, India

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9 **Running title**: Seasonal changes in energy metabolites (34)

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14 *Author for correspondence: (rpgenetics@gmail.com)

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16 **Summary statement**

17 In the tropical *Drosophila ananassae*, low or high humidity induced plastic changes in energy

18 metabolites provide cross-protection to seasonally varying climatic stressors.

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24   **ABSTRACT**

25   Cross-tolerance effects for cold and drought stressors are well known for temperate and polar

26   ectothermic organisms. However, less attention has been paid to plastic changes induced by wet-

27   dry conditions for the tropical insect taxa. *Drosophila ananassae* is abundant in wet habitats but

28   its desiccation sensitivity is likely to make it vulnerable under expected drought conditions due

29   to climate change. We tested plastic effects of heat hardening, acclimation to drought or

30   starvation; and changes in trehalose, proline and body lipids in *D. ananassae* flies reared under

31   wet or dry season specific conditions. Wet season flies showed significant increase in heat

32   knockdown, starvation resistance and body lipids after heat hardening. However, accumulation

33   of proline was observed only after desiccation acclimation of dry season flies while wet season

34   flies elicited no proline but trehalose only. Thus, drought induced proline can be a marker

35   metabolite for dry season flies. Further, partial utilization of proline and trehalose under heat

36   hardening reflects their possible thermoprotective effects. Heat hardening elicited cross-

37   protection to starvation stress. Stressor specific accumulation or utilization as well as rates of

38   metabolic change for each energy metabolite were significantly higher in wet season flies than

39   dry season flies. Energy budget changes due to inter-related stressors (heat vs desiccation or

40   starvation) resulted in possible maintenance of energetic homeostasis in wet or dry season flies.

41   Thus, low or high humidity induced plastic changes in energy metabolites can provide cross-

42   protection to seasonally varying climatic stressors.

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INTRODUCTION

In temperate regions, ectothermic organisms encounter a greater range of colder environments (freezing to mild warm), as well as changes in other abiotic factors which affect their acclimatization to improve survival under harsh climatic conditions (Angilletta , 2009; Sinclair, 2015). Ectothermic organisms from temperate or polar regions are able to cope with seasonally varying climatic stressors through developmental as well as adult acclimation during their life time (Denlinger and Lee, 2010). However, lesser attention has been paid to acclimatization of tropical ectothermic organisms (Whitman and Ananthakrishnan, 2009). Tropical drosophilids encounter significant 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 tropical regions, seasonal changes in humidity conditions play a major role in affecting morphological, physiological and life history traits (Tauber et al., 1986; Tauber et al., 1998). For example, role of humidity has been demonstrated for dry season induced diapause in some tropical insects (Pires et al., 2000; Seymour and Jones, 2000); increased desiccation resistance due to low humidity developmental acclimation in *D. leontia* (Parkash and Ranga, 2014); and due to effect of humidity acclimation on heat resistance in adult flies of *D. simulans* (Bubliy et al., 2013). Further, another study on tropical *D. jambulina* has shown effect of low vs high humidity acclimation on mating related traits of darker and lighter morphs 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, plastic changes induced by multiple stressors for tropical insect taxa of wet – dry seasons have received less attention (Tauber et al., 1998; Chown et al., 2011).

In Southeast Asia, changes in relative humidity associated with reduced patterns of rainfall due to El nino and climate warming are likely to increase drier conditions affecting survival of tropical insect taxa (www.skymetweather.com; www.imd.gov.in). Therefore, 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 and likely consequences on their fitness and survival (Hoffmann, 2010).

During their life time, ectothermic organisms (freeze tolerant, freeze avoiding or chill susceptible) undergo stressor induced plastic changes in stress resistance traits as well as

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82   metabolic fuels for survival under harsh climatic conditions (Denlinger and Lee, 2010; Sinclair et

83   al., 2013). Analyses of cold induced plastic changes in metabolites have emphasized the role of

84   different cryoprotective colligative solutes such as sugars, polyols and free amino acids

85   (Overgaard et al., 2007; Michaud et al., 2008; Kostal et al., 2011b; Colinet et al., 2012). First, the

86   role of exogenous trehalose to increase resistance to cold has been shown in *Belgica antarctica*

87   (Benoit et al., 2009). Second, laboratory flies of *D. melanogaster* subjected to cold shock at -7

88   °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

89   increase in the level of proline (Misener et al., 2001). Accumulation of proline in response to

90   cold stress has been demonstrated in the larvae of *D. melanogaster* (Kostal et al., 2011b); in

91   *Chymomyza costata* larvae (Kostal et al., 2011a).Third, In the gall fly *Eurosta solidaginis,* cold

92   induced energy metabolites include glycerol and sorbitol (Lee, 2010). Fourth, as compared to

93   many studies on sugars and polyols, changes in free amino acids accumulated in response to

94   different climatic stressors have been investigated in few insect taxa (Fields et al., 1998; Misener

95   et al., 2001); and in a arthropod (Issartel et al., 2005) .Thus, plastic responses to cold involve

96   different physiological mechanisms based on different energy metabolites.

97   In tropical habitats, heat as well as drought stress co-occur and proline has been shown to

98   play a role in thermoprotection of some plant taxa (Verbruggen and Hermans, 2008). The role of

99   proline as an osmolyte to mitigate water stress was first reported in wilting perennial rye grass -

100 *Lolium perenne* (Kemble and MacPherson, 1954) and subsequently in bacteria (Csonka and

101 Hanson, 1991). Higher levels of proline have been observed in *Arabidopsis thaliana* (Liang et

102 al., 2013) and in drought-tolerant rice varieties (Choudhary et al., 2005) but not in case of barley

103 (Chen et al., 2007). Therefore, associations between high proline levels and drought tolerance

104 have shown mixed results in plants and need further studies. Proline has been considered as a

105 multifunctional amino acid due to its role as cryoprotectant and / or osmoprotectant and in

106 mitigating oxidative stress in plants (Szabados and Savoure, 2009). However, a recent study has

107 shown accumulation of proline due to drought stress in *D. immigrans* (Tamang et al., 2017).

108 Thus, despite the abundant level of proline in insects, its physiological role(s) in diverse insect

109 taxa need further studies.

110   In ectothermic organisms, survival under harsh environments depends upon maintenance

111 of energetic homeostasis in metabolic fuels after possible perturbations due to climatic stressors

112 (Benoit et al., 2009; Sinclair et al., 2013; Williams et al., 2014; Sinclair, 2015). In this respect,

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113 some studies have evidenced cold induced changes in metabolites through targeted ^{1}H NMR
114 based metabolomics (Overgaard et al., 2007; Michaud et al., 2008; Colinet et al., 2012). Based
115 on metabolic profiling of temporal changes (during recovery), protective role of cold or heat
116 hardening is evident from faster attaining of homeostasis of metabolites to normal level as
117 compared to perturbations in metabolic pool in the control groups of *D. melanogaster*
118 (Malmendal et al., 2006; Overgaard et al., 2007; Kostal et al., 2011b; Williams et al., 2014).
119 However, efforts to find marker metabolites induced by cold or drought or heat stress have
120 shown similarities as well as differences. For example, Antarctic midge *Belgica antarctica* has
121 shown some overlap in the accumulation of glycogen and erythritol due to cold or drought
122 acclimation while reduction in the level of serine was evident in response to cold, drought and
123 heat stress (Michaud et al., 2008). In nature, acclimatization of ectothermic organisms involves
124 developmental as well as adult acclimation. Therefore, NMR metabolic profiling of field
125 acclimatized insects may further help in understanding stressor induced plastic changes in the
126 metabolome. Further, there is need to examine energy metabolites mediated cross-protection to
127 multiple stressors in insect taxa living under seasonally varying environments of tropical regions.

128 Temperature is considered as a major abiotic factor affecting geographical distribution
129 and abundance levels of various insect taxa (Andrewartha and Birch, 1970**;** Angilletta, 2009).
130 Narrow distribution patterns of tropical *Drosophila* species are limited by their low genetic
131 potential to adapt to colder and drier habitats (Kellermann et al., 2009). Further, in context of
132 climate warming, it has been argued that tropical drosophilids might be vulnerable due to
133 expected higher aridity conditions (Hoffmann, 2010; Chown et al., 2011). Despite the fact that
134 plastic changes in stress resistance traits in the generalist species, *D. melanogaster* are higher as
135 compared with genetic differences, similar studies have not been carried out for tropical
136 *Drosophila* species (Hoffmann et al., 2005). On the Indian subcontinent, there are contrasting
137 seasonal patterns of ambient relative humidity ($80 \pm 5\%$ RH during monsoon but $40 \pm 6\%$ RH
138 during autumn). Therefore, assessment of season specific humidity acclimation on induced stress
139 resistance as well as their cross-tolerance effects can help in understanding adaptive potential of
140 tropical *D. ananassae*.

141 In the present work, we assessed season-specific as well as sex- specific plastic changes
142 in stress related traits as well as changes in the levels of three energy metabolites in the tropical
143 *D. ananassae* which is characterized by low desiccation resistance. Wild – caught flies of *D.*

144   *ananassae* from wet (monsoon) and dry (autumn) seasons were reared under season specific

145   simulated growth conditions and flies were tested for basal as well as induced level of resistance

146   to heat, desiccation and starvation resistance. For each stressor, we tested cross resistance for

147   other two stressors. Further, we investigated patterns of changes (accumulation and / or

148   utilization) for each of the three metabolic fuels due to plastic changes. For control as well as

149   acclimated flies, changes in energy budget were also estimated. The rates of change in the levels

150   of three energy metabolites were assessed in three replicates of multiple groups of flies subjected

151   to different time durations of heat hardening, desiccation or starvation acclimation. Thus, we aim

152   to find stressor induced plastic changes for possible maintenance of energetic homeostasis in the

153   tropical *D. ananassae* from wet or dry seasons.

154

155   **MATERIAL AND METHODS**

156   **Collection and Cultures**

157   Wild-caught *Drosophila* species were collected during two seasons wet or rainy (July and

158   August) and dry or autumn (Mid September to mid November) from five local sites but each one

159   separated by ~ 3 to 4 km in the university town Rohtak (Latitude 28.08 °N, Altitude 220m) in the

160   year 2015. The flies were collected during one week in the mid of rainy or autumn season. Based

161   on our past collection records, the relative abundance of *Drosophila ananassae* is ~30% in rainy

162   season and ~20% in the autumn. Therefore, we collected 730 flies in rainy season and 416 flies

163   in autumn season which included different *Drosophila* species. However in the laboratory,

164   assortment of wild-caught flies provided 192 flies of *D. ananassae* in rainy season and 136 in

165   autumn season. For each season, flies were used to set up three replicate populations in 300 ml

166   culture bottles, each with 40 pairs of *D. ananassae*. Further, adult flies of each bottle were

167   allowed to oviposit on cornmeal-yeast-agar medium in four culture bottles in order to maximize

168   the number of laboratory raised flies. The wet season wild-caught flies were reared under wet

169   season specific simulated condition ($26\pm1°C$ and $78\pm2\%$ RH). The resulting adult flies of G_1 and

170   G_2 were used for assessment of basal, acclimated, cross-tolerance effects of three stressors (heat,

171   drought and starvation) along with simultaneous analysis of control or unacclimated flies. For

172   wet season, one week old flies of G_1 and G_2 generations were analyzed for changes in three

173   stress related traits as well as energy metabolites (body lipids, proline and trehalose). Thus, all

174   experiments on wet season flies were completed before the onset of autumn season. Likewise,

175 for autumn or dry season, collection of wild flies, setting up of mass populations in triplicate and

176 rearing under dry condition (25 ± 1°C; $40\pm2\%$ RH) were conducted during the autumn season. For

177 dry season flies, 1stress resistance traits (heat knockdown, desiccation or starvation resistance) as

178 well as energy metabolites (trehalose, proline and total body lipids contents), were assessed in

179 three replicates of thirty flies. Control experiments were run simultaneously.

180

181 **Stress resistance assays**

182 **(a)** Heat knockdown time was measured in three replicates of thirty flies of both the seasons.

183 Individual males and females were placed into 5mL glass vials submerged into a water bath at a

184 constant temperature of 37 °C. Flies were scored for knockdown time (in minutes and seconds).

185

186 **(b)** Desiccation resistance was measured as the time to dehydration effect under dry air (~8%

187 RH) in flies of both the sexes of wet or dry season. Each vial contained 2 g of silica gel at the

188 bottom overlain with a foam disc to avoid contact with flies. We placed ten flies in such plastic

189 vials (40×100 mm) in which open end was covered with muslin cloth. Finally, such vials were

190 kept in the desiccator chamber (Secador electronic desiccator cabinet; www.tarsons.in) which

191 maintained ~8% relative humidity. Number of immobile flies was counted after hourly intervals;

192 and LT_{100} values were recorded.

193

194 **(c)** For three replicates of thirty flies, starvation resistance was measured as survival time till

195 death under humid conditions (~90% RH) but without food. Ten adult flies were placed in a dry

196 plastic vial which contained foam sponge impregnated with 8 ml of water + 2 mg sodium

197 benzoate (to prevent any bacterial growth). The mortality time was recorded twice a day till all

198 flies died from starvation.

199

200 **Assessment of direct and cross-tolerance effects**

201 Direct as well as cross-tolerance effects were assessed for each stressor, (heat or drought or

202 starvation) in *D. ananassae* flies of wet or dry seasons. For each stressor, different groups (three

203 replicates of thirty flies each) of acclimated flies were tested for changes in other two stress

204 related traits. Thus, for three stressors (heat or desiccation or starvation), we tested nine

205 acclimation-by-test combinations (i.e. three direct effects + six cross tolerance effects). For

206   analysis of cross-tolerance, acclimated/hardened fly groups were tested for changes
207   (increase/decrease/no effect) in the level of each stress resistance trait. Therefore, for testing
208   direct and cross tolerance due to (i) heat hardening, three groups of thirty flies of both the sexes
209   were subjected to 2 h heat stress followed by 2 h recovery period on nutrient agar medium; and
210   thereafter flies were analyzed for change in heat knockdown, desiccation or starvation
211   resistance. (ii) Desiccation acclimation was given to flies for 4 h followed by 4h recovery period;
212   and these flies were analyzed for heat knockdown, desiccation and starvation resistance. (iii)
213   Flies were exposed to 20 h for starvation acclimation followed by 15h recovery period; followed
214   by testing for changes in heat knockdown, starvation and desiccation resistance.

215

216   **Estimation of energy metabolites**
217   **(a)** Body lipid content was estimated on G_1 or G_2 flies (three replicates of thirty flies of each
218   season and sex) reared under wet or dry season specific conditions. For lipid content individual
219   fly was dried in 2 ml Eppendorf tubes (http://www.tarsons.in) at 60 °C for 48 h and then weighed
220   on Sartorius microbalance (Model-CPA26P; 0.001 mg precision; http://www.sartorious.com).
221   Thereafter, 1.5 ml di-ethyl ether was added in each Eppendorf tube and kept for 24 h under
222   continuous shaking (200 rpm) at 37 °C. Finally, the solvent was removed and individuals were
223   again dried at 60 °C for 24 h and reweighed. Lipid content was calculated per individual by
224   subtracting the lipid-free dry mass from initial dry mass per fly.

225

226   **(b)** For trehalose content estimation, each of the three replicates of thirty flies of each season and
227   sex were homogenized in a homogenizer (Labsonic@ M; http://www.sartorious. com) with 300
228   μl Na2CO3 and incubated at 95 °C for 2 h to denature proteins. An aqueous solution of 150 μl
229   acetic acid (1 M) and 600 μl sodium acetate (0.2 M) was mixed with the homogenate. Thereafter,
230   the homogenate was centrifuged (Fresco 21, Thermo-Fisher Scientific, Pittsburgh, USA) at
231   12,000 rpm (9660 ×g) for 10 min. For trehalose estimation, aliquots (200 μl) were placed in two
232   different tubes; one was taken as a blank whereas the other was digested with trehalase at 37 °C
233   using the Megazyme trehalose assay kit (K-Treh 10/10, http:// www.megazyme.com). In this
234   assay, released D-glucose was phosphorylated by hexokinase and ATP to glucose-6-phosphate
235   and ADP, which was further coupled with glucose-6-phosphate dehydrogenase and resulted in
236   the reduction of nicotinamide adenine dinucleotide (NAD). The absorbance by NADH was

237   measured at 630 nm (UV-2450-VIS, Shimadzu Scientific Instruments, Columbia, USA). The

238   pre-existing glucose level in the sample was determined in a control reaction lacking trehalase

239   and subtracted from total glucose concentration.

240

241   **(c)** Proline content was estimated in each of the three replicates of thirty flies of each season and

242   sex**.** Proline concentrations in fly homogenates were determined by the modified method after

243   Bergman and Loxley (1970). In this assay, interference from primary amino acids gets

244   eliminated by nitrous acid treatment and the excess nitrous acid is removed by heating with

245   ammonium chloride followed by hydrochloric acid. Interfering materials are also removed by

246   absorption to the protein-sulphosalicylic acid complex.

247   Thirty adult flies were homogenized in 3 ml of sulphosalicylic acid. Following

248   centrifugation, 50 μl of the homogenate was added to 15 μl of freshly prepared 1.25 M sodium

249   nitrite solution and content were mixed and kept at room temperature for 20min. Further, 15 μl

250   of 1.25Mammoniumchloride solution added and content were mixed followed by addition of 60

251   μl of concentrated hydrochloric acid. The content were mixed and heated in a boiling water bath

252   for 20 min. The tubes were cooled and 60 μl of 10 N sodium hydroxide was added. To the

253   resulting solution, we added 200 μl glacial acetic acid and 200 μl of ninhydrin solution in each

254   capped tube. The solutions were mixed and incubated for 60 min. at 100 °C. Following

255   incubation, the samples were extracted with toluene, and absorbance of the aqueous phase was

256   quantified spectro-photometrically at 520 nm and the amount of proline was estimated in

257   reference to a standard curve.

258

259   **Analysis of rate of change in energy metabolites after hardening/acclimation pre-**

260   **treatments**

261   We conducted independent experiments for each stressor to find change in the rate of

262   accumulation or utilization of three energy metabolites (trehalose, proline and body lipids). Such

263   changes were assessed in three replicates of thirty flies of each season as well as sex as a

264   function of different time durations of hardening/ acclimation by a stressor. Independent groups

265   of flies were (a) heat hardened for 1h, 2h, 3h, 4h and 5h at 34°C with 2h recovery ;(b)

266   desiccation acclimation for1h, 2h, 3h, 4h and 5h at 8% relative humidity with 4h recovery ; (c)

267 starvation acclimation for 10h,15h, 20h, 25h and 30h followed by 15h recovery; and these

268 respective flies were tested for changes in the level of each of three energy metabolites.

269

Assessment of energy metabolites mediated cross-protection

271 For acclimated as well as non-acclimated (control flies), changes in three energy metabolites

272 (trehalose, body lipids and proline content) due to each stressor were measured in three replicates

273 each of thirty flies. This was done for flies reared under season-specific wet or dry conditions.

274 For such analysis, we used data on flies after heat hardening (2 h heat stress followed by 2 h

275 recovery); (ii) desiccation (5 h acclimation followed by 4 h recovery); (iii) starvation (25 h

276 starvation acclimation followed by 15h recovery). Finally, percent change in accumulation or

277 utilization of three energy metabolites due each stressor were calculated to find possible cross-

278 protection between multiple stressors.

279

Treatment and analysis of data

281 Data for heat knockdown time at 37°C (in minutes and seconds) were recorded on individual

282 male and female flies of *D. ananassae* reared under wet or dry season specific simulated

283 condition. For other two stressors (desiccation or starvation), we recorded survival mortality of

284 10 flies per vial as a function of shorter (at hourly) for desiccation; and twice daily (8am and

285 8pm) for starvation resistance till all the flies died. For each season and sex, (three replicates of

286 thirty flies), data on basal level (or control) and hardened/acclimated flies were represented as

287 mean ± s.e.m. (Table 1) while effects of treatments and sex were calculated on the basis of

288 ANOVA (supplementary tables). Seasonal differences in stress related traits were compared with

289 't'test as well as in terms of fold differences (Table 1).

290 The data on assays for three stressors (heat, drought or starvation) were used for

291 calculating absolute acclimation/hardening capacity -AAC (i.e. difference in trait values between

292 acclimated flies – control flies) following Kellett et al (2005). Further, we also calculated relative

293 acclimation capacity – RAC (i.e. absolute acclimation capacity divided by control value of

294 unacclimated flies). For all the three stress related traits, we represented AAC in the form of bars

295 while RAC values were depicted on the top of each bar. Illustrations depicted simultaneous

296 comparison of direct acclimation effect as well as cross tolerance effects for male and female

297 flies of wet or dry season (Fig 1 to 3). For heat resistance, correlation between direct heat

10

298   hardening effect and cross-tolerance effects due to desiccation acclimation was represented (with

299   Box-whisker) for flies of two seasons as well as sexes. Further, we represented relationship

300   between heat hardening effect on starvation as well as on heat knockdown (Fig. 4).

301   In accordance with the objectives of this study, data on sum of three energy metabolites

302   (ug mg^{-1} fly^{-1}) in control flies were compared with heat hardened or flies acclimated to

303   desiccation and starvation; and percent changes (+ or -) were calculated to compare acclimation

304   effects across seasons and sexes (Table 2).

305   Data obtained from independent sets of experiments on rate of metabolite change as a

306   consequence of different durations (1h, 2h, 3h, 4h or 5h) for heat hardening or desiccation

307   acclimation; were subjected to regression analysis for calculation of regression slope values

308   (Table 3); and seasonal differences in slope values were compared with 't' test. Finally, season

309   specific differences in the accumulation and utilization (calculated in terms of percent increase or

310   decrease) of three energy metabolites (body lipids, trehalose and proline) due to either heat

311   hardening or acclimation to desiccation or starvation were schematically represented to highlight

312   possible energy metabolite mediated cross-protection in wet or dry season female flies (Fig. 5).

313   For stressor acclimated/hardened flies, the energy content (trehalose, proline and body lipids)

314   was calculated using standard conversion factors (Schmidt-Nielsen, 1990). The amount of each

315   energy metabolite was multiplied by conversion factor i.e. for trehalose (17.6 Jmg^{-1}); for body

316   lipid (39.3 Jmg^{-1}); and proline (17.8 Jmg^{-1}). Statistical calculations and illustrations were made

317   with the help of Statistica 5.0 as well as Statistica 7.

318

319   **RESULTS**

320   **Seasonal differences in stress resistance and energy metabolites**

321   Data on seasonal differences (wet vs dry) for six traits of *D. ananassae* flies reared under season

322   specific simulated conditions are shown in Table 1. For heat knockdown, starvation resistance

323   and body lipids, wet season flies revealed significantly higher trait values as compared with dry

324   season flies (Table 1). For dry season flies, desiccation resistance and the amount of trehalose

325   and proline were significantly higher. For all the traits, season specific differences were

326   significant for both the sexes ($p < 0.001$; Table 1).

327   **Plastic changes in heat knockdown of wet vs dry season flies**

328   For heat knockdown, data on absolute hardening capacity (acclimated – control values) due to

329   direct hardening as well as cross tolerance effects due to desiccation or starvation acclimation are

330   illustrated in Fig. 1. The wet season flies (both sexes) showed significant increase in heat

331   knockdown due to heat hardening as well as cross resistance effect of desiccation (Fig. 1A,B).

332   However, starvation acclimated flies of wet season showed lesser increase in heat knockdown

333   (Fig. 1A,B). In contrast, in dry season flies, heat knockdown duration decreased as a

334   consequences of cross tolerance effect due to starvation acclimation (Fig. 1C). However, plastic

335   changes in heat knockdown of dry season flies due to direct heat hardening and cross tolerance

336   effect after desiccation acclimation were 50 to 60% lower (Fig. 1C,D). Thus, direct effect due to

337   heat hardening as well as cross tolerance effects significantly improved heat resistance of wet

338   season flies as compared with dry season flies (Fig. 1).

Season specific plastic changes in desiccation resistance

340   Wet season flies exhibited lower acclimation effects due to desiccation acclimation as well as

341   cross tolerance effect of heat hardened flies (Fig. 2A,B) as compared with dry season flies (Fig.

342   2C,D). There was a trade-off between desiccation and starvation resistance. We observed two

343   fold reduction in desiccation resistance of starvation acclimated flies of dry season as compared

344   with wet season.

Seasonal plastic changes in starvation resistance

346   For starvation resistance, wet season flies exhibited significantly higher effect of direct starvation

347   acclimation as well as cross-tolerance due to heat hardening as compared with dry season flies

348   (Fig. 3). In case of desiccation acclimated flies, there was greater reduction in starvation

349   resistance of dry season flies as compared with wet season flies.

Relationship between heat hardening effects with other traits

351   *D. ananassae* flies are acclimatized to multiple stressors in its natural habitats across season. For

352   seasonal changes in heat knockdown, we found positive relationship between direct heat

353   hardening and cross tolerance due to desiccation acclimation (Fig. 4A). Thus, significant

354   increase in heat resistance of wet season flies results due to plastic effects of heat as well as

355   drought (Fig. 4A). Inter-related plastic changes between resistance to heat and starvation as a

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356 consequence of heat hardening are shown in Fig. 4B. Therefore, heat hardening of wet season

357 flies resulted in higher resistance to both heat and starvation while such effects were lower for

358 dry season flies.

Assessment of relative hardening / acclimation capacity

360 Results of relative acclimation capacity (RAC) for direct as well as cross-tolerance plastic effects

361 for three stressors are shown in Fig. 1-3. In wet season flies, direct acclimation effects were

362 maximum for starvation acclimation followed by heat hardening. However, direct effect of

363 desiccation acclimation was higher for dry season flies as compared with wet season flies. RAC

364 values for cross-tolerance effects (SA on HK); (DA on HK) and (DA on HH) were quite similar

365 i.e. RAC = ~0.20 (Fig. 1-3). These observations suggest that RAC values for direct acclimation /

366 hardening are stressor specific while cross tolerance effects could be conserved for a species.

367 This assumption needs further verification for its generality among *Drosophila* species. Further,

368 we observed a trade-off between RAC values of cross-tolerance effects of DA vs SA but such

369 effects were significantly higher for dry season flies (Fig. 2 & 3). Finally, cross-tolerance effect

370 of SA on heat knockdown was positive in wet season flies but there was a trade off (SA on HK)

371 in dry season flies (Fig. 1). We also found sex-specific minor differences in RAC for stress

372 related traits of wet or dry season flies (Fig. 1-3).

Season specific plastic changes in energy budget

374 Energy budget based on three energy metabolites (trehalose, proline and body lipids per fly) in

375 control and heat hardened or flies acclimated to desiccation or starvation are shown in Table 2.

376 There is significant increase (~ 73% in females and ~ 62% in male) in the energy budget of wet

377 season flies due to heat hardening (Table 2) while such changes in the energy budget of dry

378 season flies are sixty percent lower. In contrast, there is ~ 12% gain in energy budget due to

379 desiccation acclimation of dry season flies as compared with ~ 4% in the wet season flies.

380 Further, starvation acclimated flies of wet season consumed about 40 percent of energy budget

381 per fly as compared with ~ 14% in dry season flies. These observations show plastic changes in

382 energy budget are stressor as well as season specific while both the sexes reveal similar trends.

Cross-protection between accumulation and utilization of energy metabolites

384   In *D. ananassae* flies reared under wet or dry conditions, we found significant differences in the

385   stressor specific levels of accumulation and utilization of trehalose, proline and body lipids (Fig.

386   5). This schematic diagram shows that body lipids increase due to heat hardening i.e. ~ 79% in

387   wet season flies as compared with 48% for dry season flies. There is cross-protection between

388   accumulation of body lipids due to heat hardening and utilization under starvation. In contrast,

389   proline was elicited by desiccation acclimated flies of dry season only. However, desiccation

390   acclimated wet season flies accumulated 115% more trehalose than control flies. Further, heat

391   hardening of wet season flies utilized 50% of the accumulated trehalose. For dry season flies,

392   desiccation induced plastic changes accumulated only 30% more trehalose but 60% more amount

393   of proline which were partially utilized under heat hardening. Thus, plastic changes in three

394   energy metabolites (trehalose, proline and body lipids) are consistent with cross-protection

395   between three stressors (heat, drought and starvation).

396   **Stressor specific rates of change in energy metabolites**

397   We found significant seasonal differences in stressor specific (heat, drought or starvation)

398   changes (accumulation or utilization) in the levels of each of the three energy metabolites as a

399   function of different durations of hardening / acclimation of wet or dry season flies (Table 3).

400   For body lipids and trehalose, the rates of change were significantly higher for wet season flies

401   as compared with dry season flies. Sex-specific differences in the rates of metabolite change

402   were observed only in the wet season flies (Table 3). Further, changes in the level of proline

403   were found only in dry season flies.

404   **Results of ANOVA on stress related traits**

405   In supplementary Table S1, we have shown results of ANOVA for three stress related traits

406   (heat, drought or starvation) with respect to three variables (control vs acclimated; sexes and

407   seasons) in *D. ananassae* flies reared under wet or dry season specific conditions. For all the

408   three stress related traits, we found highly significant differences (p <0.001) across sexes,

409   seasons and due to acclimation as well as due to their respective interactions (Table S1).

410   Similarly the results of ANOVA on three energy metabolites are shown in supplementary table

411   S2 which also showed significant differences ($p < 0.001$) for all the variables.

412

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DISCUSSION

We observed humidity driven significant plastic changes in the stress related traits (heat knockdown, and resistance to desiccation or starvation); and three energy metabolites (proline, trehalose and body lipids) in *D. ananassae* flies reared under wet or dry season specific simulated conditions. Basal levels of stress resistance and energy metabolites differ significantly due to developmental acclimation. The effects of heat hardening as well as desiccation acclimation significantly improved heat knockdown in wet season flies. Further, heat hardening also increased starvation resistance in wet season flies. However, dry season flies showed higher levels of proline as well as desiccation resistance but a lower amount of trehalose. Therefore, proline can be considered as a marker metabolite because accumulation of proline was evident only in drought acclimated flies of dry season. Stressor specific changes in energy budget per fly support cross-protection between heat hardening and desiccation or starvation resistance. Thus, seasonal differences in relative humidity (wet or dry) induced cross-protective plastic changes in three energy metabolites are consistent with energetic homeostasis and for coping season specific stressful environments.

Seasonal differences in cross-tolerance effects

Previous studies on cross-tolerance to multiple stressors did not consider impact of both developmental as well as adult acclimation effects of wet or dry conditions (Angilletta, 2009; Sinclair et al., 2013). In the present work, we examined such acclimation effects on tropical *D. ananassae*. In heat hardened flies (of wet or dry seasons), cross-tolerance effects on desiccation was higher for dry than wet season flies. However, such cross-tolerance effect on starvation tolerance was more in wet than dry season flies (Fig. 2,3). In contrast, in starvation acclimated flies cross-tolerance effects on heat resistance showed season specific contrasting differences i.e. a positive effect in wet season flies (Fig. 1A,B) but a negative effect in dry season flies (Fig. 1C,D). Therefore, cross-tolerance effects of starvation acclimated flies on heat knockdown differ across seasons. Further based on genetic effects, a previous study has shown lack of trade-off between resistance to heat and other stress related traits in *D. melanogaster* (Williams et. al., 2012). Thus, genetic and plastic effects of multiple stressors seem to differ in impacting heat tolerance but this assumption needs further analysis. In *D. ananassae*, cross-tolerance effects in two cases (a) plastic response of starvation acclimation on desiccation resistance (Fig. 2); (b) effect of desiccation acclimation on starvation resistance (Fig. 3) showed trade-off with greater

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444   effect in dry than wet season flies (Fig. 2,3).These observations are consistent with a trade-off

445   between resistance to desiccation or starvation in geographical populations of some tropical

446   drosophilids on the Indian subcontinents (Parkash and Munjal, 1999). Thus, we find similarity

447   between plastic and genetic effects for desiccation vs starvation resistance in geographical as

448   well as seasonal populations of tropical *Drosophil*a species.

Stressor specific relative acclimation capacity differ across wet-dry seasonal flies

450   Relative acclimation capacity (RAC) is a quantitative measure to compare stressor induced

451   plastic changes across species as well as within species i.e. seasonal or geographical populations

452   of ectothermic organisms (Kellett et al., 2005). The magnitude of RAC for heat knockdown has

453   been found to be similar (RAC = 0.25) in interspecific comparisons of four species each of

454   *melanogaster* species group and montium species group (Kellett et al., 2005). In contrast, in *D.*

455   *melanogaster* flies reared at 18, 25 and 28 °C showed variable effects of thermal developmental

456   acclimation on heat knockdown i.e. RAC ~ 0.35 for 18 or 25 °C reared flies but no RAC effect

457   for 28 °C flies (Cavicchi et al., 1995). Further, geographical populations of *D. melanogaster*

458   from east coast of Australia revealed RAC values in the range of 0.13 to 0.58 for heat

459   knockdown (Sgro et al., 2010). However, we are not aware of studies related to assessment of

460   quantitative differences in stressor specific relative acclimation capacity in seasonally varying

461   populations of diverse insect taxa. In the present work, we found significant season specific (wet

462   or dry) as well as stressor specific differences in relative acclimation capacity (Fig. 1-3). For

463   direct acclimation effects, RAC values were in the order of SA = 0.53 > HH = 0.36 > DA = 0.25.

464   In contrast, cross-tolerance RAC values were quite similar (RAC = ~ 0.20) for HH on SR, DA on

465   HK (wet season flies) and HH on DR (dry season flies). The generality of such observations

466   need further studies. Another interesting observation was cross-tolerance effect of starvation

467   acclimation on heat knockdown i.e. a positive effect in wet season flies but a negative

468   relationship (trade-off) in dry season flies. Thus, we found season specific and stressor specific

469   differences in RAC effects in low vs high humidity reared flies of *D. ananassae*.

470

Wet-dry seasonal flies differ in dehydration induced accumulation of osmoprotectants

472   Cold induced plastic changes in energy metabolites have revealed accumulation of trehalose as

473   osmoprotectant (Shimada and Riihima, 1990; Benoit et al., 2009; Colinet et al., 2012) but not in

474   *Sarcophaga crassipalpis* (Michaud and Denlinger, 2007). In the present work, drought induced

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475   accumulation of proline is associated with acclimatization of *D. ananassae* to drier conditions.

476   *D. ananassae* flies (reared under dry season condition) showed significant accumulation of

477   proline but a lower level of trehalose. However, *D. ananassae* flies reared under wet condition

478   did not elicit proline at all despite accumulation of a significant level of trehalose (Fig. 5).

479   Further, drought induced plastic changes in proline have been observed both for a cold adapted

480   *D. immigrans* (Tamang et al., 2017) as well as warm adapted *Z. indianus* (Kalra et al., 2017).

481   These studies suggest a possible role of proline as osmoprotectant in drosophilids reared under

482   either colder and drier; or warmer and drier conditions. However, further studies are needed to

483   support such observations.

484   **Cross-protection through inter-related plastic changes in metabolic fuels**

485   In wild habitats, colder or warmer environments are coupled with wet or dry or possible

486   starvation conditions; and insects are likely to evolve cross-protection mechanisms to multiple

487   stressors. It is known that cold stress with coupled drought level is able to elicit multifunctional

488   colligative solutes (sugars, polyols, proline as free amino acids). Possible inter-related

489   modulatory changes (cross-protection) in the accumulation and utilization of metabolic fuels

490   could favor energetic homeostasis. For example, a previous study has shown drought stress

491   induced higher level of proline but exihibited its utilization under cold stress in winter population

492   of *D. immigrans* (Tamang et al., 2017). In present work on tropical *D. ananassae*, we observed

493   cross-protection between heat hardening and two coupled stressors i.e. drought or starvation. We

494   found inter-related changes in three metabolic fuels both for wet or dry season flies of *D.*

495   *ananassae*. Inter-relationship between heat hardening induced accumulation of body lipids and

496   its consumption in starvation acclimated flies was evident for both the seasons but body lipid

497   changes were 60 % higher for wet season flies as compared with dry season. Such observations

498   are consistent with season specific differences in starvation resistance levels due to

499   developmental and adult acclimation. However, inter-related metabolic changes due to heat

500   hardening or desiccation acclimation involved accumulation and utilization of proline only in dry

501   season flies. In contrast, we observed four fold higher (~ 115 %) accumulation of trehalose in

502   wet season flies as compared to dry season flies. Trehalose as osmoprotectant is known to

503   counter the detrimental effects of drought stress on cellular membranes and cellular proteins.

504   Thus, plastic changes in metabolic fuels due to multiple stressors seem to involve inter-related

505   compensatory mechanisms to cope with the season specific wet or dry conditions.

17

506   Energetic changes in stressor induced and control groups of flies can also help in
507   understanding cross-protection. Energy budget changes per fly (based on three metabolites) were
508   significantly higher (~ 70%) in wet season flies subjected to heat hardening but only (~ 4%) due
509   to desiccation acclimation while starvation acclimation consumed energy budget (~ 40 %) as
510   compared to control or unacclimated flies (Fig.5). Thus, wet season flies facing multiple
511   stressors (heat or drought or starvation) are able to maintain its energy budget homeostasis. In
512   case of dry season flies, energy budget changes revealed ~ 30% increase due to heat hardening
513   and 12 % with desiccation acclimation while starvation acclimation consumed 15 %, thereby
514   showing a favorable energy balance under harsh dry conditions (Fig. 5). Thus, inter-related
515   stressor induced changes in three energy metabolite seem consistent with possible energetic
516   homeostasis.

517   **Thermoprotective effects of proline and trehalose**
518   Some studies have examined detailed mechanisms for thermal protection of various cellular
519   proteins and cell membranes by trehalose and / or proline supporting their ubiquitous role in
520   stabilizing cellular components against detrimental effects of cold or heat (Kaushik and Bhatt,
521   2003; Yancey et al., 1982; Reina-Bueno et al., 2012; Liang et al., 2013). Both proline and / or
522   trehalose provide cryoprotection in insects such as temperate *Chymomyza costata*, *Belgica*
523   *Antarctica* and *D. melanogaster* adults as well as larvae (Kostal et al., 2011b; Benoit et al., 2009;
524   Colinet et al., 2012; Kostal et al., 2011a) and also in different plant taxa (Szabados and Savoure,
525   2009). However, thermoprotective role of these two energy metabolites to heat stress have been
526   less documented. First, metabolic profiling of heat hardened *D. melanogaster* adults showed
527   elevated levels of alanine; and tyrosine (a precursor of stress hormones in insects) during
528   recovery period (Malmendal et al., 2006). Since, alanine results due to proline oxidation during
529   energy generation, utilization of proline can be argued during recovery of heat hardened *D.*
530   *melanogaster* as reported by Malmendal and colleagues (2006). Second, association between
531   heat and proline has been observed in beetle– *Alphitobius diaperinus* maintained at 28 °C
532   because proline amount constituted 50% out of a pool of 16 amino acids (Renault et al., 2006).
533   Third, thermoprotective role of proline as well as of trehalose has been suggested in some plant
534   taxa (Verbruggen and Hermans, 2008; Szabados and Savoure, 2009); and in soil bacterium
535   *Rhizobium etli* (Reina-Bueno et al., 2012). For *D. ananassae*, we observed partial utilization of
536   proline when dry season flies were subjected to heat hardening (Fig. 5) but we did not examine

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537 possible elevation of alanine due to proline oxidation. Thus, further studies are needed to analyse

538 osmo- as well as thermoprotective roles of proline in seasonally varying wild populations of

539 different warm adapted drosophilids.

540 We observed complementary metabolic changes in accumulation and utilization of

541 trehalose and proline in response to desiccation and heat stress in wet or dry season flies.

542 Accumulation of proline occurred only in dry season flies in response to desiccation acclimation.

543 We observed variable levels of trehalose in wet vs dry season flies. However, heat hardening

544 resulted in partial utilization of trehalose in wet season flies but both proline and trehalose in dry

545 season flies. Thus, proline could be a marker metabolite for dry season flies. Inter-related

546 changes due to heat hardening and starvation acclimation involved accumulation and utilization

547 of body lipids. Finally, we assessed energy budget changes per fly in control vs flies hardened /

548 acclimated to heat or drought or starvation. For both the seasons (wet or dry) and sexes, heat

549 hardening increased energy budget per fly due to build up of body lipids which were used during

550 starvation. Thus, energy budget changes due to stressors resulted in cross-protection as well as

551 maintenance of energetic homeostasis both under wet or dry climatic conditions. We may

552 suggest that plastic induced changes in stress resistance traits and energy metabolites in *D.*

553 *ananassae* are likely to counter future drier conditions expected due to climate change.

554

Competing interest

556 The authors declare no competing or financial interest

557

Author contributions

559 A.P. and C.L. did laboratory as well as data analysis; and R.P. wrote the MS.

560

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713 **Legends to figures**

714 **Figure 1. Plastic changes (acclimated trait value – control) in heat knockdown (minutes) as**
715 **a consequence of heat hardening (direct effect) and due to cross tolerance effects of**
716 **desiccation - DA or starvation – SA in *D. ananassae* of wet or dry season. Relative**
717 **acclimation capacity (RAC) value is shown on top of each bar.**

718

719 **Figure 2. Wet or dry season as well as sex specific changes in desiccation resistance (h) as a**
720 **consequence of desiccation acclimation (direct effect) and due to cross tolerance effects of**
721 **heat hardened - HH or starvation acclimated -SA in *D. ananassae* of wet or dry season.**

722

723 **Figure 3. Comparison of plastic changes in starvation resistance (h) due to starvation**
724 **acclimation (direct effect) and cross tolerance effects of heat hardened - HH or desiccation**
725 **acclimated – DA in *D. ananassae* of wet or dry season.**

726

727 **Figure 4. (A) Relationship between plastic changes in heat knockdown due to direct effect**
728 **of heat hardening (x-axis) versus cross tolerance effect due to desiccation acclimation (y-**
729 **axis). (B) Correlation between plastic changes in heat knockdown as well as starvation**
730 **resistance due to heat hardening in *D. ananassae* of wet or dry season. Percent changes in**
731 **energy budget per fly due to plastic changes are indicated in parentheses.**

732

733 **Figure 5. Schematic representation of stressor induced (heat or drought or starvation)**
734 **plastic changes (accumulation and utilization) of three energy metabolites (trehalose,**
735 **proline and body lipids) in *D. ananassae* female flies of wet (A) or dry (B) season. Slanted**
736 **arrows depict cross-protection effects.**
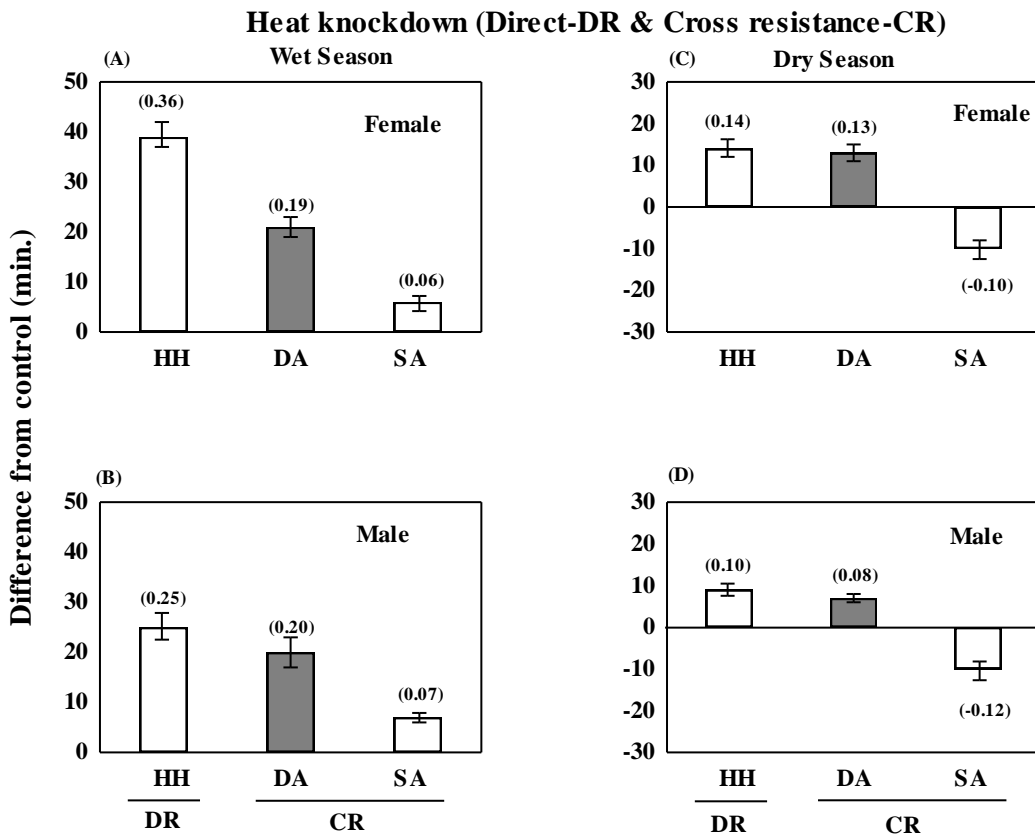
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Fig. 1. Plastic changes (acclimated trait value – control) in heat knockdown (minutes) as a consequence of heat hardening (direct effect) and due to cross tolerance effects of desiccation - DA or starvation – SA in *D. ananassae* of wet or dry season. Relative acclimation capacity (RAC) value is shown on top of each bar.
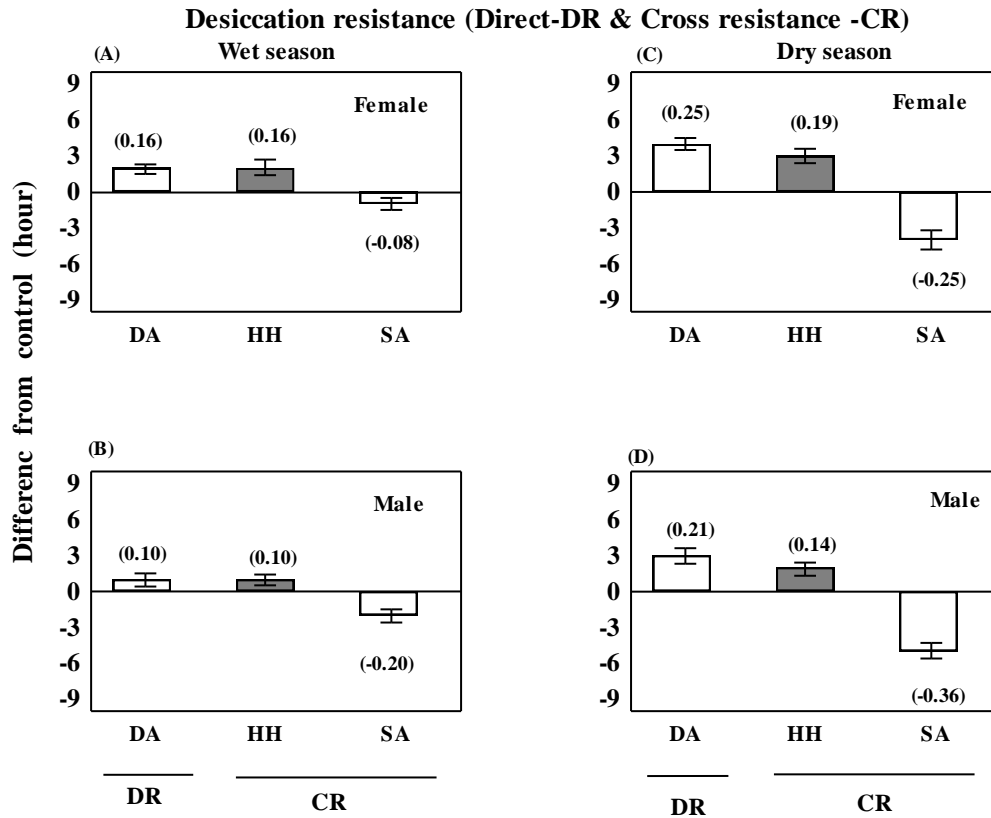
Fig. 2. Wet or dry season as well as sex specific changes in desiccation resistance (h) as a consequence of desiccation acclimation (direct effect) and due to cross tolerance effects of heat hardened - HH or starvation acclimated -SA in *D. ananassae* of wet or dry season.
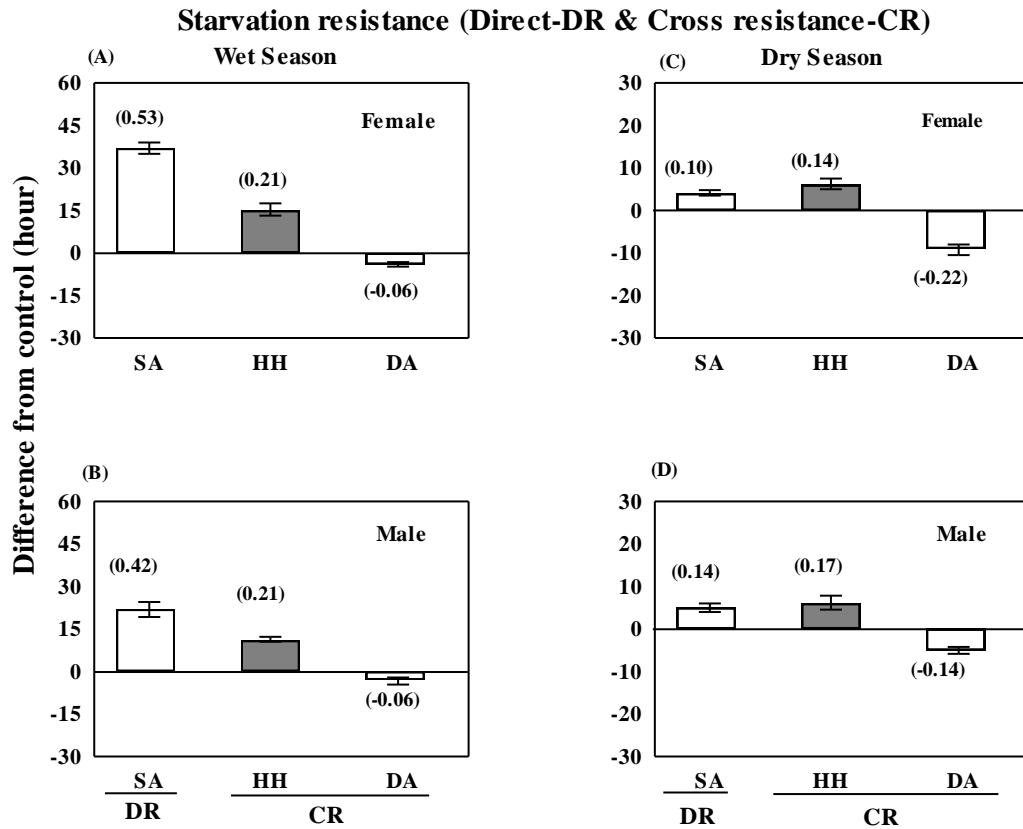
Fig. 3. Comparison of plastic changes in starvation resistance (h) due to starvation acclimation (direct effect) and cross tolerance effects of heat hardened - HH or desiccation acclimated – DA in *D. ananassae* of wet or dry season.

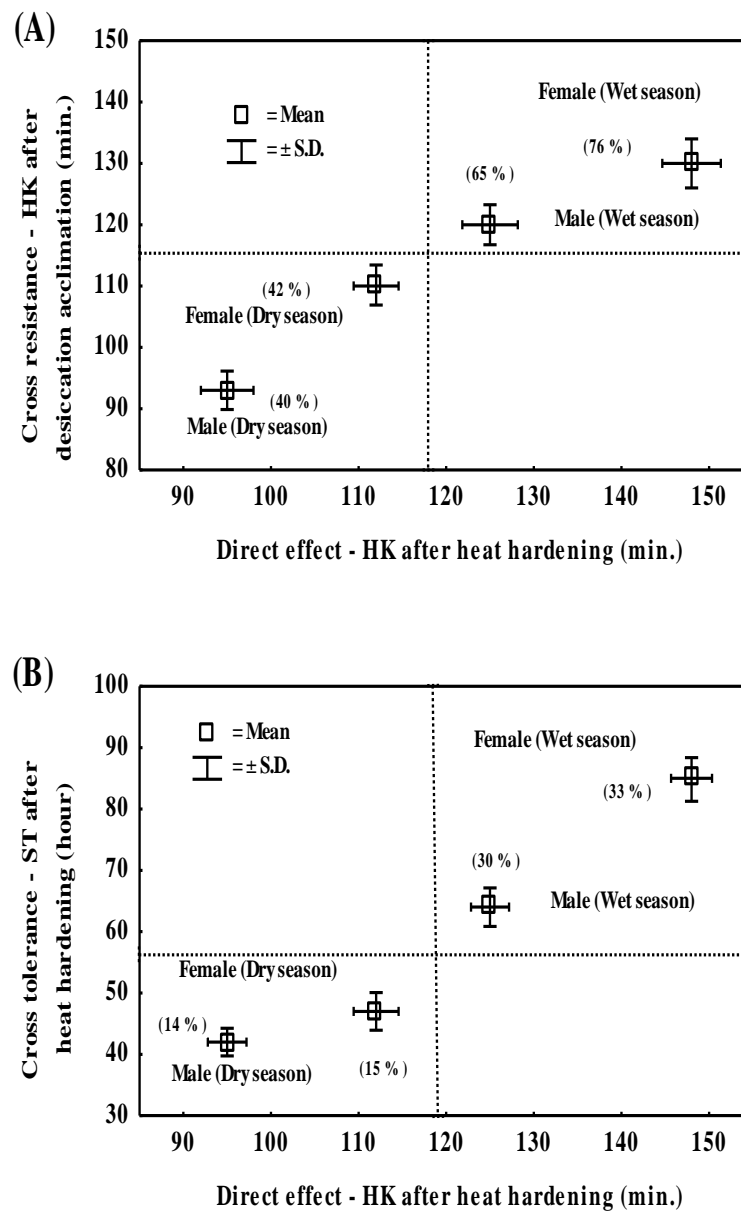**Fig. 4. (A) Relationship between plastic changes in heat knockdown due to direct effect of heat hardening (x-axis) versus cross tolerance effect due to desiccation acclimation (y-axis). (B) Correlation between plastic changes in heat knockdown as well as starvation resistance due to heat hardening in *D. ananassae* of wet or dry season. Percent changes in energy budget per fly due to plastic changes are indicated in parentheses.**

(A) Cross-protection for wet-season female flies		Accumulation (+)	Utilization (-)	Proline* + or -
(a)	Heat hardening (2 hr)	Lipids (79%) ⬆	(-72%) Trehalose ⬇	No
(b)	Desiccation acclimation (5 hr)	Trehalose (115%) ⬆	___	No
(c)	Starvation acclimation (25 hr)	___	Lipids (-42%) ⬇	No

*Lack of changes in the proline content in the wet season female flies

(B) Cross-protection for dry-season female flies		Accumulation (+)	Utilization (-)
(a)	Heat hardening (2 hr)	Lipids (48%) ⬆	Trehalose (-15%) ⬇ Proline (-50%) ⬇
(b)	Desiccation acclimation (5 hr)	Trehalose (30%) ⬆ Proline (60%) ⬆	___
(c)	Starvation acclimation (25 hr)	___	Lipids (-20%) ⬇

Fig. 5. Schematic representation of stressor induced (heat or drought or starvation) plastic changes (accumulation and utilization) of three energy metabolites (trehalose, proline and body lipids) in *D. ananassae* female flies of wet (A) or dry (B) season. Slanted arrows depict cross-protection effects.

Table 1. Data on seasonal differences in basal levels of stress resistance to heat, or desiccation or starvation stress and energy metabolites (trehalose, proline and body lipids) of _D. ananassae_ flies grown under season specific simulated conditions (rainy or autumn). For each trait, data are mean ± s.e.m for three replicates of thirty flies (n = 90).

	Traits	Sex	Wet season	Dry season	Fold	_t_-test
(a)	Heat Knock-down (min.)	F	109 ± 2.32	98 ± 3.12	1.11	12.78***
		M	100 ± 4.13	86 ± 2.20	1.16	23.66***
(b)	Desiccation resistance (h)	F	12 ± 0.56	16 ± 1.02	1.33	5.09***
		M	10 ± 0.37	14 ± 0.78	1.40	4.48***
(c)	Starvation resistance (h)	F	70 ± 2.13	41 ± 3.42	1.70	15.25***
		M	53 ± 1.09	36 ± 3.02	1.47	32.22***
(d)	Trehalose (μg mg^{-1} fly^{-1})	F	11.20 ± 0.25	24.74 ± 0.44	2.20	18.72***
		M	17.71 ± 0.13	22.31 ± 0.35	1.26	12.20***
(e)	Proline (μg mg^{-1} fly^{-1})	F	13.48 ± 0.11	48.55 ± 1.06	3.60	27.12***
		M	18.19 ± 0.29	52.48 ± 1.48	2.89	45.40***
(f)	Body lipids (μg mg^{-1} fly^{-1})	F	190 ± 3.55	123 ± 2.56	1.54	56.30***
		M	140 ± 3.02	108 ± 2.26	1.30	43.21***

*** $P < 0.001$

Table 2. Seasonal changes in the sum of energy budget per fly (for trehalose, proline and body lipids) due to direct-adult acclimation effects of heat hardening (2 h), desiccation acclimation (5 h) and starvation acclimation (25 h) of wet or dry season flies of *D. ananassae*. For each trait, data are shown as J mg^{-1} fly^{-1} along with percent change in parenthesis as compared with control (non acclimated flies).

	Sum of energy budget (females)		Sum of energy budget (males)	
	Wet season flies	Dry season flies	Wet season flies	Dry season flies
HA	13.66 ± 0.91 (+ 72.85)	7.94 ± 0.33 (+ 29.63)	9.91 ± 0.98 (+ 61.44)	7.09 ± 0.15 (+ 27.17)
DA	8.17 ± 0.32 (+ 3.35)	6.85 ± 0.29 (+ 11.72)	6.37 ± 0.37 (+ 3.73)	6.25 ± 0.24 (+ 12.35)
SA	4.76 ± 0.16 (- 39.73)	5.23 ± 0.11 (- 14.66)	4.18 ± 0.28 (- 31.92)	4.82 ± 0.36 (- 13.41)
Cont	7.91 ± 0.24	6.13 ± 0.22	6.14 ± 0.33	5.57 ± 0.28

Heat hardening = HA; Desiccation acclimation = DA; Starvation acclimation = SA; Cont = Control

Conversion factors include 17.6 J mg^{-1} for trehalose, 39.3 J mg^{-1} for lipids, 17.8 J mg^{-1} for proline (Schmidt-Nielsen. 1990).

Table 3. Rate of metabolite change (regression slope values) as a function of different durations of heat hardening or desiccation acclimation (1h or 2h or 3h or 4h and 5h); and starvation acclimation (10h or 15h or 20h or 25h and 30 h) for Wet and Dry season flies of _D. ananassae_.

Rate of change in each metabolite under heat, desiccation or starvation stress				
	Sex	Wet season	Dry season	_t_-test
(a) Heat hardening (µg min^{-1})				
Trehalose	F	-0.07	-0.03	0.011**
	M	-0.12	-0.02	0.016***
Proline	F	-----	-0.20	-----
	M	-----	-0.23	-----
Body lipids	F	+1.25	+0.49	0.020***
	M	+0.85	+0.43	0.036***
(b) Desiccation acclimation (µg h^{-1})				
Trehalose	F	+2.14	+1.14	1.03***
	M	+3.02	+0.96	0.98***
Proline	F	-----	+4.86	-----
	M	-----	+4.79	-----
(c) Starvation acclimation (µg h^{-1})				
Body lipids	F	-3.20	-0.89	1.01***
	M	-2.00	-0.80	0.94***

Slope values represent rate of change of metabolites as a function of time. **$P < 0.01$, *** $P < 0.001$