The physiological consequences of varied heat exposure events in adult *Myzus persicae*: a single prolonged exposure compared to repeated shorter exposures.

Behnaz Ghaedi [Corresp., 1] , **Nigel R Andrew** [1]

[1] Centre for Behavioural and Physiological Ecology, Zoology, University of New England, Armidale, NSW, Australia

Corresponding Author: Behnaz Ghaedi
Email address: ghaedi.b@gmail.com

The study of environmental stress tolerance in aphids has primarily been at low temperatures. In these cases, and in the rare cases of high temperature tolerance assessments, all exposures had been during a single stress event. In the present study, we examined the physiological consequences of repeated high temperature exposure with recovery periods between these stress events in *Myzus persicae*. We subjected individuals to either a single prolonged three hour heating event, or three one hour heating events with a recovery time of 24 hours between bouts. Aphids exposed to repeated bouts of high temperatures had more glucose and higher expression of proteins and osmolyte compounds, such as glycerol, compared to the prolonged exposure group. However, aphids exposed to the repeated high temperature treatment had reduced sources of energy such as trehalose and triglyceride compounds than the prolonged exposure group. Recovery time had more physiological costs (based on production of more protein and consumption of more trehalose and triglyceride) and benefits (based on production of more osmolytes) in repeated high temperature treatments. As aphids are known to respond differently to constant versus 'natural' fluctuating temperature regimes, conclusions drawn from constant temperature data sets may be problematic. We suggest future experiments assessing insect responses to thermal stress incorporate a repeated stress and recovery pattern into their methodologies. �

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4 Behnaz Ghaedi[1]*, Nigel R Andrew[1]

5 [1] Centre for Behavioural and Physiological Ecology, Zoology, University of New England, Armidale,
6 NSW, 2351, Australia.

7 Corresponding Author:

8 Behnaz Ghaedi

9 Email address: ghaedi.b@gmail.com

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31 Running title: Heat exposure and associated metabolic changes

32 Authors: Behnaz Ghaedi[1]*, Nigel R Andrew[1]

33 [1]Centre for Behavioural and Physiological Ecology, Zoology, University of New England,
34 Armidale, NSW, 2351, Australia.

35 *Author for correspondence (Ghaedi.b@gmail.com)

36 **Abstract**
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49 osmolytes) in repeated high temperature treatments. As aphids are known to respond differently
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51 temperature data sets may be problematic. We suggest future experiments assessing insect
52 responses to thermal stress incorporate a repeated stress and recovery pattern into their
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55 Keywords: Metabolite, heat exposure, aphid, thermal tolerance, repeated heating

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Introduction

Climate change is one of the most critical threats to biodiversity (Dawson et al., 2011; IPCC., 2014). Human-induced climate change is predicted to increase the frequency of climatic extremes (e.g. heat waves and severe droughts or floods), climatic variability (Lean and Rind, 2008; Rahmstorf and Coumou, 2011) and mean of the thermal environment in regions around the world (Niehaus et al., 2012). Global mean temperature has risen by 0.85°C from 1880 to 2012. All of the warmest 20 years on record have occurred since 1990 (CSIRO-ABM, 2014).

Exposure to different thermal means and variability can have a substantive influence on animal physiological performance (Huey et al., 2012). This may include a modification of performance curve parameters including two critical fitness-influencing components: the upper critical temperature and the thermal optimum (Angilletta, 2009). All insects keep their physiological performance within a specific range of temperatures, and many of their physiological functions may be reduced when exposed to extreme temperatures (Mironidis and Savopoulou-Soultani, 2010). An important aspect of habitat quality is minimal exposure to extreme thermal stress (Huey, 1991). In terrestrial habitats, climate change is strengthening fluctuations and amplitude of temperature variation, which will lead to prolonged adverse temperature exposure in terresterial habitats (Sinclair et al., 2006; Andrew, 2013; Vasseur et al., 2014).

Critical temperature responses can shift somewhat based on an animals environmental thermal experience (Somero, 2010). As insects are ectotherms, their survival, population dynamics, and distribution are influenced by temperature (Chown et al., 2010; Bauerfeind and Fischer, 2014). Therefore investigating impacts of temperature variation on an individual's performance and understanding these plastic responses of populations to temperature is critical (Pörtner et al., 2006); and may have implications for their potential responses to climate change.

Some aphid species, including *M. persicae*, are distributed widely across the globe. The cabbage aphid, *Brevicoryne brassicae* (L.), the turnip aphid, *Lipaphis erysimi* Kalt, and the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphidae) are the three major aphid pests infesting

87   canola in Australia (Gu et al., 2007; Gia and Andrew, 2015). *Myzus persicae* (green peach aphid)

88   is known to transmit over 100 phytopathogenic viruses among 50 different plant families. Many

89   of its hosts include major crops (e.g. sugar beet, beans, brassicas, potatoes, citrus) and on a world

90   wide scale this species is regarded as the most important aphid pest (Blackman and Eastop,

91   1984). Green peach aphid host alternating between the primary peach *Prunus persica* host in

92   winter and various herbaceous hosts, belonging to 50 different families, which include brassicas,

93   potatoes and sugar beet, in summer (Blackman and Eastop, 2000)

94

95   Extreme climatic events such as heat waves and daily fluctuations can impact aphids in various

96   ways: reducing fecundity and population growth; slowing development; and can also affect

97   community structure and interrupt trophic cascades through effects on performance of individual

98   species and through changes in the strength of interactions between them (Davis et al., 2006;

99   Gillespie et al., 2012; Zhao et al. 2014; Colinet et al. 2015). Aphids are more sensitive to acute

100   changes in temperature rather than the duration of extreme temperature exposure, and nymphs do

101   not experience diapause (Davis et al., 2006) , making them a suitable test organism for our study.

102   Studying the effects of climate change on aphids is complex. Many organisms that live in

103   fluctuating thermal environments, such as aphids, display a high degree of thermal plasticity in

104   their response to changing conditions, and have a greater resilience in their ability to recover

105   from ecological perturbations (Davis et al., 2006). In order to understand how aphids perform in

106   a warming climate it is necessary to consider both exposure temperature and exposure duration

107   with or without recovery time.

108

109   In responding to extreme environmental conditions, insects rely on a combination of different

110   factors: such as molecular processes (gene expression, heat shock proteins, and enzymes);

111   changes in membrane structure; and osmolyte compounds; to survive and recover from

112   unfavourable conditions (Back et al., 1979; Henle et al., 1983; Lin et al., 1984; Kim and Lee,

113   1993; Meng et al., 2004; Wang et al., 2006; Clark and Worland, 2008; Tollarová-Borovanská et

114   al., 2009). Aphids and whiteflies were one of the first insect taxa reported to accumulate polyols

115   in response to high temperatures and also reveal that these compounds could stabilize proteins

116   structure against thermal denaturation (Back et al., 1979). Whole-animal respiration will change

117   when exposed to repeated stress (Lalouette et al., 2011; Yocum et al., 2011) and understanding

118   the implications of this is critical as this will impact on animal survival and body maintenance as

119   changes occur in energy and water usage (Schimpf et al., 2009; Chown et al., 2011). In addition,

120   the upper critical temperature threshold at which an animal loses muscular control (CT_{max}) is a

121   metric of interest as it is useful in predicting phenotypic effects of warming (Huey et al., 2012).

122   Using laboratory estimates of thermal tolerance enables the seasonal abundance and geographic

123   distribution of organisms to be determined (Ju et al., 2013).

124

125   It has become clear that constant temperatures are not useful for study performance thermal

126   response of organisms as they do not mimic the fluctuating temperature conditions which occur

127   within the natural environment (Lamb, 1961; Davis et al., 2006). Constant temperature

128   conditions also underestimate thermal thresholds of individuals and become less accurate

129   compared to fluctuating temperature regimes (Davis et al., 2006; Niehaus et al., 2012). This

130   implies that fluctuating temperature experiments ought to be "normal" while constant

131   temperature insect development studies were "abnormal" experimental conditions (Cloudsley-

132   Thompson, 1953). Fluctuating temperatures enhanced resistance of leaf beetles and fruit-flies to

133   low temperature (Casagrande and Haynes, 1976; Meats, 1976) and that fitness could be more

134   significant in repeated temperatures (Beardmore and Levine, 1963).

135

136   In previous studies much attention has focused on the cold tolerance of *Myzus persicae*

137   (O'doherty and Bale, 1985; Bale et al., 1988; Clough et al., 1990; Howling et al., 1994; Bezemer

138   et al., 1998; Vorburger, 2004) as well as assessments of the effects of temperature on

139   physiological parameters including their thermal tolerance at different latitudes and altitudes

140   (Bezemer et al., 1998; Alford et al., 2012a). There is a paucity of knowledge regarding how

141   aphid physiology is affected by repeated exposure to high temperatures. Here, we assessed aphid

142   metabolic rates after different heating regimes (a single prolonged exposure and repeated short-

143   term exposure with recovery time, and no heat exposure) using flow-through respirometry; we

144   also assessed the upper critical temperature threshold, energy reserves and their osmolyte

145   compound profile, as indicators of aphid stress response after exposure to the different heating

146   regimes.

147

148   We predict that repeated short-term exposure to high temperatures will increase adult aphid

149   thermal tolerance, as recovery time between heat stress bouts will enable metabolic and cellular

150   repairs to occur (Storey and Storey, 2004). We also predict that aphids will accumulate polyols

151   and sugars in response to high temperatures (Hendrix and Salvucci, 1998) which will result in an

152   increased CT_{max}.

153   **Methods and Materials**

154   **Aphids and Diet Chamber Construction**

155   Stock colonies of *Myzus persicae* (green peach aphid), were collected from canola plants at the

156   Laureldale Farm property (University of New England, Armidale, NSW Australia) in 2013.

157   The lab colony were established from an isofemale line (BG_13-001) and maintained on canola

158   variety 'Thunder Bay' plants in a glasshouse with 25±0.5°C temperatures, 65% relative

159   humidity, under a 16:8 h (L: D) photoperiod provided by fluorescent lamp for two years to

160   ensured continuous apomictic parthenogenesis.

161   Since aphids have parthenogenetic embryogenesis (Dixon, 2005) we bred three generations of

162   aphids in the lab to reduce maternal and grand maternal affects: we removed the wingless female

163   adult aphids after 3 days, then the resulting nymphs were left for seven days and we continued

164   this process for three generations. The third generation resulting nymphs were housed at 25°C,

165   65% relative humidity, under a 16:8 h (L: D) in a Thermoline incubator (TRH-300) prior to

166   experimental manipulation.

167

168   **Diet cage preparation**

169   To rear our aphids on artificial diets we used diet cages. Diet cages were constructed from rigid

170   clear PVC plastic tubing with a 12cm diameter (Fig 1). A 7cm x 7cm square of parafilm was

171   extended by force across the top of a cage, and 2 ml of artificial diet was pipetted on top of the

172   parafilm layer. A second 7cm x 7cm sheet of parafilm was then extended over the first sheet,

173   forcing diet across the top surface of the cage, but avoiding leakage over the edge. Then by using

174   a paintbrush, aphids were placed on the underside of the parafilm. The diet cages where then put

175   into a closed transparent box (30×30cm) and put into the incubator prior to the application of

176   treatments.

Artificial Diet

178   The artificial diet used to rear the aphids was based on work by Douglas (1988) and consisted of

179   specific concentrations of phosphate, vitamins, and minerals (Table 1). In the experiments, four

180   replicate groups of 200 larval aphids were maintained on the test diet for 5 days (i.e. from one to

181   6 days of age). The specified amounts of each component (see Table 1) were prepared with

182   distilled deionized water to the total volume of 10ml in a glass container. The pH of the solution

183   was 7.0-7.5. The diet solution was divided into 2ml aliquots, and stored at 4°C s for less than 1

184   week or at – 20°C for less than 3 months. In all experiments, distilled deionized water was used

185   in all of the solutions and the diets of the aphids were changed twice a week for the duration of

186   our study.

Experimental Conditions

188   To investigate the effects of fluctuating thermal regimes (FTR), the experimental design was a

189   simplified version of the experimental protocol of Marshall and Sinclair (2011) and Zhang et al.

190   (2011). For the repeated heat stress exposure treatment, adult aphids were exposed to one to

191   three diurnal cycles (We left them in the food diet and put them into the incubator) of 1h at 38°C

192   (we didn't control RH) followed by 24h at 25°C, 65% relative humidity (RH). Adult aphids

193   (seven day old individuals) from the third generation reared on the artificial diet were used. For

194   the single prolonged heat exposure treatment, separate groups of adults were exposed to 38°C for

195   3h, to make total heat exposure time equivalent among treatments. Post treatment, this group

196   were also given a 24h-period of recovery at 25°C, 65% RH. Meanwhile, control animals were

197   held at 25°C, 65% RH for the duration of the study.

198   Estimates for the upper critical temperature threshold limits for *M. persicae* have previously

199   found to range from 38.5°C (Broadbent and Hollings, 1951) to 42°C (Hazell et al., 2010). *Myzus*

200   *persicae* are known to survive 1h per day above their CT_{max} of 38.5°C (Davis et al., 2006) and so

201   the FTR was chosen to fluctuate around a value close to their CT_{max}. In general, upper

202   temperatures are difficult to experiment with compared to lower temperatures because their

203   performance curve optima is very close to CT_{max}. A test temperature of 38°C was chosen as there

204   was 60-70% survival following the prolonged thermal exposure for 3 hours.

205 **Quantification of Metabolic Reserves**

206 Twenty-four hours after a heat exposure (Figure 2), four replicate groups of adult aphids were

207 weighed to 0.001mg on a Mettler Toledo XP2U (Switzerland) electronic balance, then

208 homogenized in 80 µl extraction buffer (35mM Tris, 25 mM KCl, 10 mM MgCl2, pH 7.5) with

209 0.1% (v/v) Triton-X-100 after centrifuging for 1 minute at 13,000 rpm at 4°C. Supernatant was

210 then removed and placed in a new tube which was then stored at -20°C until all assays were

211 conducted.

212

213 The assay kits :used included the Sigma triglyceride and glycerol assay kit (Sigma Triglyceride

214 (Sigma:T2449), Free glycerol reagent (Sigma:F6428), Glycerol stock – Sigma:G7793-5 ml at 2.5

215 mg/ml)); the BioRad Coomassie Brilliant Blue microassay method (500-0201), with bovine

216 serum albumin as standard (40–480 mg protein) for protein; and the Sigma glucose assay kit for

217 glucose (Product Code GAGO-20, contains 500 units of glucose oxidase, O-Dianisidine Reagent

218 (Product Code D 2679)) and following trehalose measurement (Porcine Kidney Trehalase

219 (Sigma:T8778) and 0.2 M sodium citrate (5.882 g/100ml), 1 mM Sodium EDTA (37.22 mg/100

220 ml), D+Trehalose dihydrate (Sigma:T0167)).

221

222 For these assays, we measured Glucose, Triglyceride, Glycerol, Trehalose and total protein

223 content in triplicate and we used a visible wavelength spectrophotometer (Epoch: Microplate

224 Spectrophotometer) with absorbance at 544 nm, 540 nm and 750 nm and 96 well plates, with

225 volumes scaled down from the manufacturer protocols (Ridley et al., 2012).

226 **Active Metabolic Rate Measurement (AMR)**

227 Flow-through CO_2-based respirometry was used to record $\dot{V}CO_2$, with a similar experimental

228 setup as described by Terblanche *et al*. (2007). A HiBlow HP40 air pump was used to feed

229 atmospheric air into sodalime (VWR with indicator AnalaR NORMAPUR analytical reagent)

230 and Drierite (W.A. Hammond Drierite Company) scrubber columns, to remove CO_2 and water

231 vapour from the air stream, respectively. The flow rate of the airstream was regulated at 80 ml

232 min^{-1} by a flow control valve (Model 840, Sierra Side-Trak, Sierra Instruments Inc., Monterey,

233 USA), connected to a mass flow controller (Sable MFC-2). Thereafter, air flowed through the

234 zero channel (cell A) of a calibrated (to 6 ppm CO_2 in air) infrared CO_2-H_2O analyzer (Li-7000,

235 Li-Cor, Lincoln, NE, USA). The airstream then flowed over the test animal in the 5ml glass

236   cuvette, which was placed in a programmable water bath (Grant, GP200-R4), programmed using

237   LABWISE software with increasing temperature of 0.25°C min⁻¹ (see Basson & Terblanche,

238   2010). The air leaving the cuvette then entered the analyser through another channel which

239   recorded the difference in CO_2 concentration of the air before and after it flowed through the

240   cuvette, at 1s intervals. Changes in animal position (activity) were recorded using an infrared

241   activity detector (AD-1, Sable Systems, Las Vegas, NV, USA). Aluminium foil was placed

242   around this cuvette to restrict light exposure and to ensure high quality activity recordings

243   (MacMillan et al., 2012).

244   Four aphids per replicate were weighed to 0.001mg on an electronic microbalance before and

245   after the experiment and mean mass used as a covariate in statistical analyses. The output from

246   the CO_2 analyzer and activity data were recorded with the LiCor 7000 software and analysed

247   using Expedata V1.25 software (Sable Systems International, Las Vegas, NV, USA). Volumes of

248   CO_2 in ppm were corrected for baseline drift and then converted to µl CO_2 h⁻¹ using Expedata

249   software. Rates of CO_2 production (in µl CO_2 h⁻¹) were calculated from the whole record by

250   transforming ppm concentration of CO_2 to CO_2 fraction and then multiplying by the flow rate

251   (80mlmin⁻¹). The area under the curve (integral of mlCO_2min⁻¹ *vs* min) was calculated. This area

252   was equal to the volume of CO_2 produced by each replicate in the cuvette, and this volume was

253   divided by the total period of measurement (2.30 h), multiplied by 1000 to give µl CO_2h⁻¹ to give

254   the metabolic rate per aphid per hour (Castañeda et al., 2009). Metabolic rate was measured for

255   each of the four times two replicate aphids after the final exposure in all experimental groups.

Measuring Upper Critical Thermal Limits (CT_{max})

257   Critical thermal limits were determined by subjecting aphids to a regime of increasing

258   temperatures and monitoring their ability to control their movement: loss of muscular controls to

259   determine their CT_{max} threshold. Forty adult aphids were each placed within an individual 5ml

260   plastic tube at a pre-set temperature (25°C). A programmable water bath was set to increase the

261   temperature from 25°C to 35°C with a rate of 0.5°C min⁻¹ then the temperature was increased to

262   45°C with a rate of 0.1°C min⁻¹ to minimize the hardening response across a broad range of

263   upper critical temperatures and to determine the temperature at which walking ceased and aphids

264   succumbed (Hazell et al., 2008; Alford et al., 2012b). Upper critical temperature was measured

265   for 40 adult aphids for each of the three experimental treatments after final heat exposure in all

266   experiments group.

267

268   **Statistical Analysis**

269   All data were analysed for normality and tested for homogeneity of variances for treatment

270   means using the Levene's Test, in the HOVTEST option of GLM procedure within SAS

271   software (2008). A completely randomized design was employed. One-way analysis of variance

272   was performed using the GLM procedure. Tukey post hoc tests were used to compare means ($P<$

273   0.05).

274   **Results**

275   **Glucose Content**

276   The glucose content of the control group exhibited no significant differences over the course of

277   the experiment (Day 1: 1.61±0.02 to Day 3: 1.96±0.06 µg glucose mg^{-1}(Fig .3A). However, adult

278   aphids in both the prolonged and repeated exposure groups experienced a significant increase in

279   glucose content. After a single cycle of 38°C for 1h and 25°C for 24h, glucose content increased

280   significantly, nearly one and half fold for aphids in the repeated exposure group ($P<0.0001$). For

281   the duration of the experiment, the glucose content of aphids in the repeated exposure group

282   peaked at 3.26±0.02 µg glucose mg^{-1} (Fig. 3A). For adult aphids continuously exposed to 38°C

283   for 3h (prolonged exposure treatment), glucose content significantly increased compared to the

284   control group (3.01±0.02 µg glucose mg^{-1}).

285   **Trehalose content**

286   The trehalose content of aphids exposed to repeated heating events significantly decreased from

287   0.44±0.01 to 0.27±0.01 µg trehalose mg^{-1} after the second heating exposure (Fig. 3B). After

288   three cycles of heating, trehalose content of aphids was significantly lower than the control group

289   on the third day of the experiment ($P <0.001$). For aphids exposed to 38°C for 3h, the trehalose

290   content did not significantly differ ($P =0.329$) from that of the control group.

291 **Protein content**

292 Similar to glucose and glycerol content, protein content of aphids that were heated during

293 repeated exposures was significantly higher than in the control group and their prolonged

294 exposure counterparts (P <0.001; Fig. 3C). After one cycle of 1h at 38°C and 24h at 25°C, the

295 protein content of aphids was 17.16±0.06 µg protein mg^{-1}, but this value steadily increased every

296 cycle, and by the end of third cycle protein content was 19.79±0.1 µg protein mg^{-1} (Fig. 3C).

297 Protein content in aphids heated at 38°C for 3h did not significantly differ from the control (Fig.

298 3C).

299 **Glycerol and triglyceride content**

300 Triglyceride content in aphids did not significantly differ between control and the prolonged

301 exposure groups. However there was a significant drop of triglyceride content in aphids after

302 three cycles of repeated heating in the repeated exposure group: triglyceride content in the

303 repeated exposure group was less than half that of control (0.659±0.01) and prolonged

304 (0.597±0.01) aphids and reached 0.324±0.02 µg triglyceride mg^{-1} (P<0.002; Fig. 3E).

305

306 Glycerol content was significantly higher (P<0.0001) in aphids of the repeated exposure group

307 increasing from 1.53±0.01 to 1.96±0.02 µg triglyceride mg^{-1} after three cycles of heating. There

308 was also significantly higher glycerol content in aphids of the prolonged exposure group

309 compared to the control group (P =0.0001; Fig. 3D).

310 **Thermal Tolerance**

311 There was no significant difference in aphid CT$_{max}$ between the thermal treatments (Fig. 4).

312

313 **Active metabolic rate measurements**

314 There was no significant difference in metabolic rate 24h after heating among treatments (P

315 =0.6; Fig. 5A). Aphids from the repeated heating and prolonged heating treatments exhibited

316 significant water loss compared to the control group (P =0.04; Fig. 5b).

317 **Discussion**

318 In the present study, we examined the physiological consequences of either being exposed to

319 repeat short-period high temperatures bouts with recovery time periods between them, or a single

320 prolonged temperature treatment, in the aphid *Myzus persicae* (Figure 2). We observed increased

321 physiological costs and benefits during repeated heating exposure: this group of aphids had more

322 glucose and higher expression of proteins and osmolyte compounds such as glycerol compared

323 to the prolonged exposure group. However the repeated high temperature exposure group also

324 had fewer sources of energy such as trehalose and triglyceride compounds compared to the

325 prolonged exposure group. We found that recovery time had more physiological costs (based on

326 production of more protein and consumption of more trehalose and triglyceride) and benefits

327 (based on production of more osmolytes) for repeated high temperature exposure group, but

328 interestingly we saw no changes in thermal tolerance and metabolic rate across treatments.

329

330 ***Impacts of repeated high temperature on thermal tolerance of aphids***

331 Fluctuating temperature studies are better at predicting the thermal tolerance of aphids than

332 constant temperature studies, even when the mean temperatures are the same between constant

333 and fluctuating regimes (Lamb, 1961); constant temperature studies also underestimate critical

334 temperature thresholds as it is known that fluctuating temperatures develop threshold limits

335 (Casagrande and Haynes, 1976; Meats, 1976). The upper critical temperatures in *M. persicae*

336 start from 38.5°C (Broadbent and Hollings, 1951) to 42°C (Hazell et al., 2010). To our

337 knowledge just two studies (Davis et al. 2006, Gillespie et al. 2012) have assessed fluctuating

338 high temperatures in *M. persicae:* but our study is the first to assess physiology of aphids at high

339 temperatures. Davis et al. (2006) examined the effect of high and fluctuating temperatures on the

340 development of *M. persicae*. They demonstrated that under fluctuating temperatures, *M. persicae*

341 had greater fertility and faster development and had the capacity to survive 1h every day above

342 the CT_{max} of 38.5°C (Davis et al., 2006). These results are in contrast to Gillespie et al. (2012)

343 who found that under heat wave conditions, the growing population in aphids was lower when

344 exposed to heat waves than weather with periodic hot days. So differences in their results may be

345 due to other factors such as changes in mean temperatures.

346

347   Extreme temperature exposure in short bursts often increase survival compared to prolonged

348   exposure. At optimum conditions, injury repair can occur by restoring ion homeostasis (Kostal et

349   al., 2007) and replenishing energy levels. In *Drosophila melanogaster,* Krebs and Loeschcke

350   (1994) found similar results for effects of heating in adult female flies by exposing them to 36°C

351   1–3 times with 48h rest between heating exposure. Their results showed that exposure to bouts of

352   stress increased survival temperature to 39°C.

353   Furthermore, recovery time at optimum temperatures may have permitted aphids to recover from

354   the negative impacts of high temperatures (Hazell et al., 2010). In our study, it appears that

355   recovery times between high temperatures can repair injuries, as aphids exhibited higher levels

356   protein and osmolyte in the repeated exposure treatment compared to the prolonged exposure

357   treatment group: but there was no significant difference in their CT_{max} between treatments. One

358   possible explanation is that the proteins and osmolytes were upregulated during the recovery

359   time, but upregulation was not great enough to change their thermal threshold between

360   treatments (Tammariello et al., 1999).One further reason for conservatism of physiological

361   resistance to heat is due to upper thermal tolerance being largely uncorrelated to estimates of

362   natural temperature (Grigg and Buckley, 2013) as a high number of terrestrial organisms are

363   unlikely to evolve physiological resistance to increased heat (Kellermann et al., 2012; Hoffmann

364   et al., 2013). In such cases, development of physiological resistances will be weakened.

365

366   ***Changes in Energy Reserves at Fluctuating Temperatures***

367   Acclimation impacts the structure of lipid layers (Hazel, 1995), sugar or polyol amount (Hendrix

368   and Salvucci, 1998) and metabolic rate (Hoffmann and Parsons, 1997): all of which can

369   influence temperature resistance (Andersen et al., 2010). Whiteflies and aphids appear to be the

370   first reported organisms that collected polyols in response to high temperatures (Hendrix and

371   Salvucci, 1998). Sugars and polyols balance out and stabilize the natural structure of proteins,

372   protecting them from warm denaturation (Back et al., 1979).

373

374   The osmolyte compounds in aphids heated continuously for 3h were less than those aphids

375   exposed to three 1h cycles of repeated high temperatures. Adaptations to surviving high

376   temperatures, such as stress protein production and the synthesis of resources like glucose and

377   trehalose (Hottiger et al., 1994; Jain and Roy, 2009). Accordingly, we anticipated that repeated

378   high temperatures would result in the consumption of vital energy reserves.

379

380   Our results indicate that repeated high temperature exposure is physiologically costly for aphids

381   compared to prolonged high temperature exposure, as there is a significantly lower amount of

382   trehalose and triglyceride production after repeated high temperatures. Trehalose and proteins

383   play important roles in stress responses (Parsell and Lindquist, 1993; Feder and Hofmann, 1999;

384   Salvucci et al., 2000; Jain and Roy, 2009; Smith et al., 2012). Induction of thermal tolerance by

385   trehalose is also inferred from the fact that the level of trehalose is correlated with thermal

386   tolerance (Hottiger et al., 1994): with this in mind we measured the content of these two known

387   compounds in *M. persicae*. Protein content was higher in aphids of the repeated high temperature

388   treatment group, which has a well-defined role as a protective material at high temperatures (Jain

389   and Roy, 2009) and this result is in agreement with previous studies (Huang et al., 2007;

390   Tollarová-Borovanská et al., 2009; Zhang et al., 2011). We hypothesise that in repeated high

391   temperature exposure, protein production is triggered once the aphids are returned to their

392   recovery temperature: a trigger that is not available to aphids exposed to prolonged periods of

393   high temperature.

394

395   An unusual result in our study was that the amount of trehalose decreased in aphids exposed to

396   repeated high temperatures compared to the aphids exposed to prolonged high temperatures. This

397   contradicts the findings of previous studies assessing trehalose after exposure to high

398   temperatures (Hottiger et al., 1994; Jain and Roy, 2009) but does support the findings of Teets et

399   al., (2010). Trehalose may be used to produce an osmolyte, such as mannitol, in aphids which is

400   important at high temperatures (Hendrix and Salvucci, 1998). Trehalose can also function as a

401   reserve carbohydrate in others, which acts as a protectant. For example it has been noted that

402   trehalose accumulates under conditions of environmental stress, such as desiccation and is

403   rapidly metabolized in rehydration. A reserve function could be inferred. Trehalose can also

404   accumulate in response to other stresses, such as heat or osmotic shock (Newman et al., 1993),

405   for example Lalouette et al. (2007) demonstrated that trehalose can be changed to glycogen
406   therefore is related to energy storage functions.

407

408   Triacylglycerols constitute a large part of the aphid lipid assemblage (Itoyama et al. 2000) and
409   serve as a reservoir for fatty acids that can be used for energy production. Very large amounts of
410   triacylglycerol can occur in aphids, comprising 20-30% of fresh body weight (Strong, 1963;
411   Sutherland, 1968). According to expectations, we observed lower amount of triglyceride content
412   after repeated high temperatures compared to the prolonged treatment aphids. One explanation
413   is, these storage lipids are used as a source of metabolite energy for physiological processes:
414   many insects use lipids as an energy source, and fatty acids are stored in the fat body in the form
415   of triacylglycerol (Blacklock and Ryan, 1994; Itoyama et al., 2000). Since glycerol is known to
416   maintain cells from hyperthermic cell death, induced thermal protection and warming protection
417   may be applied by adjustment of either protein or membranes (Henle and Warters, 1982). Our
418   results demonstrated that glycerol in aphids exposed to repeated extreme heat exposure (by the
419   end of the third cycle) is higher than the levels in aphids exposed to prolonged thermal extremes;
420   and this is in accordance with the role of this compound during heat stress (Benoit et al., 2007).

421

422   Aphids are very sensitive to acute changes in temperature rather than the duration of stress
423   (Davis et al., 2006). We found significant changes in metabolite reserves after repeated high
424   temperature exposure compared to the prolonged temperature exposure treatments with the same
425   duration indicating that the number of extreme high temperature events is more important than
426   the duration of extreme temperatures although the CT_{max} of aphids was constant for both
427   temperature regimes.

428

429   ***Impacts of repeated high temperature on metabolic rate of aphids***
430   Respiration is the first process restricted in animals at low and high temperatures, and is
431   connected to the limitation of blood circulation and ventilation (Portner, 2001). We observed no
432   significant differences in metabolic rate 24h after heating and this is in contrast to previous
433   studies looking at metabolic rates at upper critical temperature (Klok et al., 2004; Boardman et
434   al., 2013). Elevated standard metabolic rate after stressful conditions is a repair cost (Boardman
435   et al., 2013). We measured active metabolic rate with increasing temperatures not resting

436 metabolic rate or standard metabolic rate at 25°C. In addition, we also found that there was a
437 lower rate of water loss after heating: this may be due to aphids closing their spiracles to reduce
438 water loss after heating events, and possibly a strategy for heat resistance.

Perspectives

440 To date, the key assessment of environmental stress tolerance in the aphid, *M. persicae* has been
441 at low temperatures and in most known studies; the aphids have been exposed to a single stress
442 event. In natural conditions, *M. persicae* is exposed to repeated bouts of high temperature
443 punctuated by periods of recovery. Furthermore, because aphids are known to respond
444 differently to consistent versus more 'normal' fluctuating temperatures, conclusions drawn from
445 constant temperature studies may be unreliable. We suggest future experiments incorporate a
446 repeated stress and recovery pattern into their methodologies to fully assess responses to extreme
447 temperature exposure to reflect the natural growing conditions of this species of aphid and also,
448 incorporate biological and fitness studies to physiological studies at the same time to reach
449 comprehensive achievements in this filed.

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457 **Supplemental Information**: https://figshare.com/s/407bb6e98a7c05c3ac3f

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750   Figure Captions:

751   Fig 1. Diet cages were constructed from rigid clear PVC plastic tubes, 12cm in diameter (aphid

752   feeding) and covered with a 7 x 7cm sheet of Parafilm laboratory film.

753   Fig 2. Experimental design, all experiments were performed on adult aphids, that were 6 days old

754   on the first experimental day. Each black rectangle represents a 1h exposure to 38°C. The blue

755   rectangle represents a 3h exposure to 38°C. Adults were kept at 25°C as the control group

756   (yellow line). Red dots indicate sampling points. All samples were collected 24h after final

757   treatments.

758   Fig 3. Mean (± s.e) (A) Glucose, (B) Trehalose, (C) Protein, (D) glycerol and (E) Triglyceride

759   content of *Myzus persicae* during repeated (R1, R2, R3), prolonged (P) and control state (C1, C2,

760   C3). The metabolite content is based on the mean of four replicates, two aphids per replicate for

761   the all metabolites. Post-hoc pairwise differences among treatments indicated by stars.

762

763   Fig 4. Mean (± s.e) thermal tolerance point (CT_{max}) of aphids among control (C), prolonged (P)

764   and repeated (R) exposure treatments.

765

766   Fig 5. Mean (± s.e) (A) CO_2 production (μl CO_2 h-1) and (B) H_2O output rates (μg H_2O h^{-1}) of

767   aphids among control (C), prolonged (P) and repeated (R) exposure treatments. Metabolic rate

768   based on four times two replicate. Post-hoc pairwise differences among treatments indicated by

769   stars.

770

771

772   Table captions

773   Table 1. Concentrations and composition of the artificial diet used to rear *Myzus persicae*.

774   Molecular weight (MW); molar Mass (mM) and mole percent (Mol%).

775

776   Table 2. Energy reserves, osmolytes concentration and protein mass of *Myzus persicae* in three

777   treatment groups: maintained at 25°C (control), heated for a single bout of 3h at 38°C (1ˣ3h) and

778   heated for three bouts of 1h at 38°C (3ˣ1h). Significant values in bold.

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780

781   Figure 1

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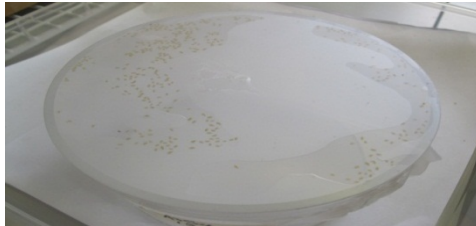
789   Figure 2 – call treatments: prolonged exposure and repeated exposure, heating was set at

790   0.5 degrees C.min⁻¹·

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796 Figure 3

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800   Figure 4

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811   Figure 5

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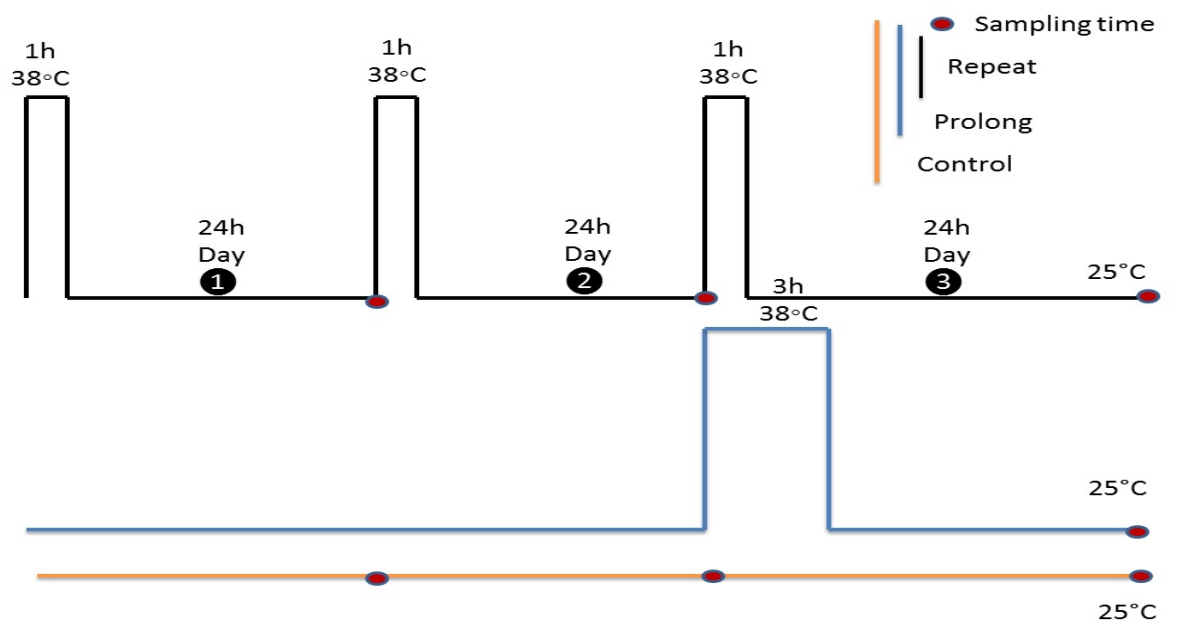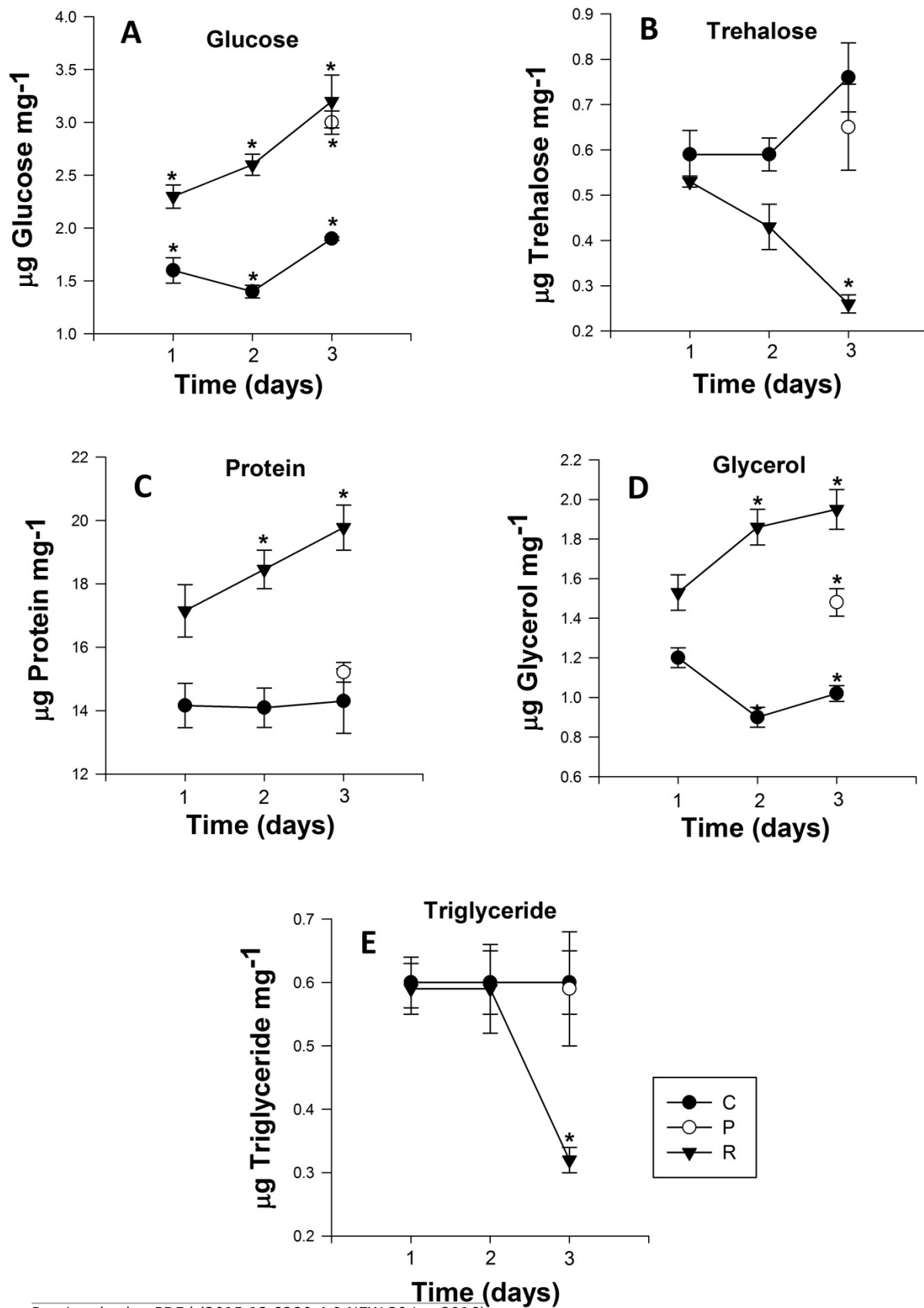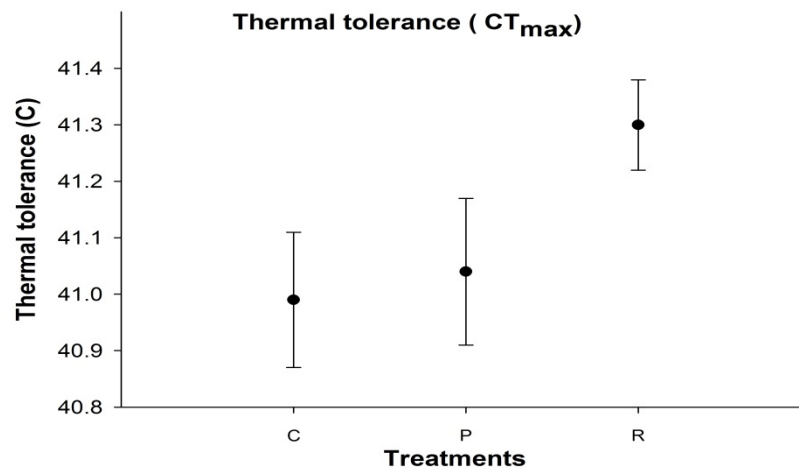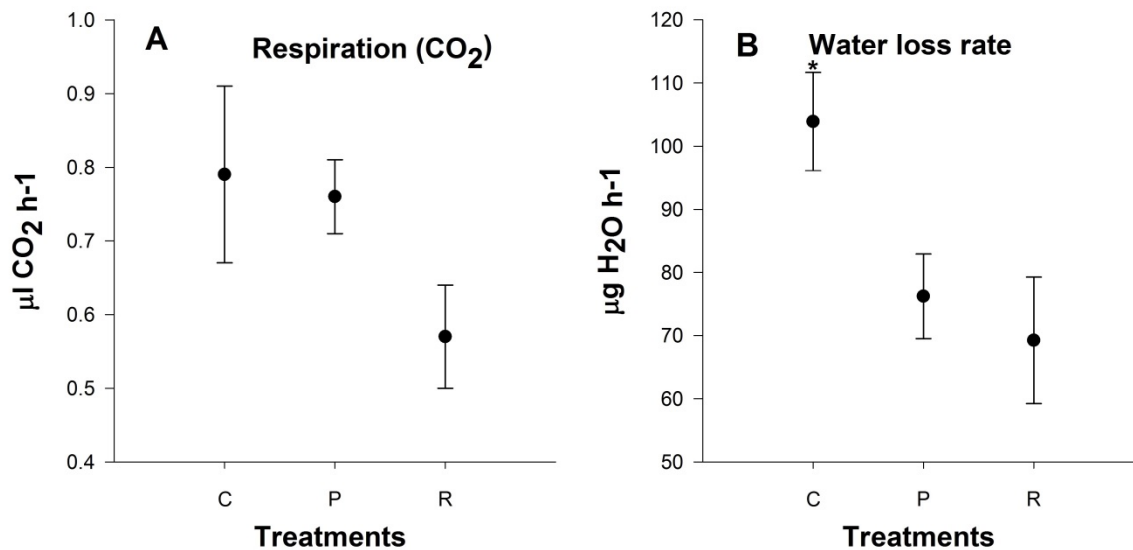
815

816   Table 1

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Amino Acid	MW	Mol%	150mM	Sucrose mix	1000 mM
Alanine	89.09	3.8	50.8mg	ascorbic acid	10mg
Asparagine	150.1	9.5	213.9mg	citric acid	1mg
Aspartate/Aspartic Acid	133.1	9.5	189.7mg	$MgSO_4$	11mg
Cysteine	157.6	1.8	42.5mg	sucrose	3400mg
Glutamic Acid	147.13	5.6	123.6mg	**Mineral stock**	
Glutamine	146.1	11	241.1mg	$FeCl_3$	13.1mg
Glycine	75.07	0.8	9.0mg	$CuCl_2. 2H_2O$	1.7mg
Proline	115.1	3.8	65.6mg	$MnCl_2.4H_20$	4mg
Serine	105.09	3.8	59.9mg	$ZnCl_2$	13.6mg
Tyrosine	181.2	0.4	10.9mg	**Vitamin stock**	
Arginine	210.66	9.5	300.2mg	Biotin	0.1 mg
Histidine	209.6	5.8	182.4mg	Pantothenate	5mg
Isoleucine	131.18	5.8	114.1mg	folic acid	2mg
Leucine	131.18	5.8	114.1mg	nicotinic acid	10mg
Lysine	182.6	5.8	158.9mg	pyridoxine	2.5mg
Methionine	149.2	1.9	42.5mg	thiamine	2.5mg
Phenylalanine	165.2	1.9	47.1mg	choline	50mg
Threonine	119.1	5.8	103.6mg	myo-inositol	50mg
Tryptophan	204.2	1.9	58.2mg	**Phosphate**	
Valine	117.1	5.8	101.9mg	$K_2PO_4.3H_20$	150mg

818

819 Table 2
820

| Treatments | | | | Contrasts | | | | | | | | | | | |
| Measurements | Treatments | | | C₃_R₃ | | | C₃_P | | | P-R₃ | | | C₃_R₃_P | | |
	D.F	F Value	P-value	D.F	F Value	P-Value	D.F	F Value	P-Value	D.F	F Value	P-Value	D.F	F Value	P-Value
Glucose	6	22.31	**<0.0001**	1	39.71	**<0.0001**	1	25.85	**<0.0001**	1	1.48	0.2262	2	43.22	**<0.0001**
Trehalose	6	4.40	**0.0005**	1	21.06	**<0.0001**	1	0.96	0.3297	1	13.03	**0.0005**	2	10.34	**0.0017**
Protein	6	15.85	**<0.0001**	1	84.09	**<0.0001**	1	32.54	0.3719	1	12.01	**<0.0001**	2	73.75	**0.0004**
Glycerol	6	23.24	**<0.0001**	1	67.76	**<0.0001**	1	16.08	**0.0001**	1	17.82	**<0.0001**	2	49.96	**<0.0001**
Triglyceride	6	3.57	**0.0029**	1	16.02	**0.0001**	1	0.55	0.4614	1	10.65	**0.0015**	2	7.49	**0.0073**

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822

| Measurements | Contrasts | | | | | | | | | | | |
| | C_3-P-R_3 | | | C_3-P | | | C_3-R_3 | | | P-R_3 | | |
	D.F	F- Value	P-Value	D.F	F- Value	P-Value	D.F	F- Value	P-Value	D.F	F- Value	P-Value
CO_2	2	0.48	0. 62	1	0.07	0.967	1	4.65	0.481	1	3.55	0.312
H_2O	2	3.61	**0.04**	1	11.24	**0.012**	1	17.69	**0.005**	1	0.73	0.872
CT_{max}	2	1.63	0.127	1	3.61	0.759	1	0.09	0.059	1	2.54	0.113

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