1   **Transgenerational effects of temperature fluctuations in *Arabidopsis thaliana***

2   Ying Deng[1,2*], Oliver Bossdorf[1], J.F. Scheepens[1,3]

3   [1] Plant Evolutionary Ecology, Institute of Evolution and Ecology, University of Tübingen,

4   Tübingen, Germany.

5   [2] Natural History Research Center, Shanghai Natural History Museum, Shanghai Science &

6   Technology Museum, Shanghai, China.

7   [3] Plant Evolutionary Ecology, Institute of Ecology, Evolution and Diversity, Goethe

8   University Frankfurt, Frankfurt am Main, Germany

9   *** Correspondence:**

10   Ying Deng

11   dengy@sstm.org.cn

14   **ABSTRACT**

15   Plant stress responses can extend into the following generations, a phenomenon called

16   transgenerational effects. Heat stress, in particular, is known to affect plant offspring, but we

17   do not know to what extent these effects depend on the temporal patterns of the stress, and

18   whether transgenerational responses are adaptive and genetically variable within species. To

19   address these questions, we carried out a two-generation experiment with nine *Arabidopsis*

20   *thaliana* genotypes. We subjected the plants to heat stress regimes that varied in timing and

21   frequency, but not in mean temperature, and we then grew the offspring of these plants under

22 controlled conditions as well as under renewed heat stress. The stress treatments significantly

23 carried over to the offspring generation, with timing having stronger effects on plant

24 phenotypes than stress frequency. However there was no evidence that transgenerational

25 effects were adaptive. The magnitudes of transgenerational effects differed substantially

26 among genotypes, and for some traits the strength of plant responses was significantly

27 associated with the climatic variability at the sites of origin. In summary, timing of heat stress

28 not only directly affects plants, but it can also cause transgenerational effects on offspring

29 phenotypes. Genetic variation in transgenerational effects, as well as correlations between

30 transgenerational effects and climatic variability, indicate that transgenerational effects can

31 evolve, and have probably already done so in the past.

32

33 **INTRODUCTION**

34 Plants encounter various environmental challenges in nature, such as episodes of stressful

35 temperatures or low water availability. Many previous studies have investigated how plants

36 respond to contrasting environmental conditions in terms of their fitness and functional traits

37 (e.g. Sultan et al., 1998; Callahan and Pigliucci, 2002; Ibañez et al., 2017; Marais et al.,

38 2017). Although plants generally show reduced fitness under stressful environments, different

39 genotypes often vary in their fitness responses and thus their ability to maintain fitness under

40 adverse environmental conditions (Sultan, 1987, 2000; Ghalambor et al., 2007). This

41 variation in fitness responses is often related to underlying variation in the plasticity of

42 functional traits. For instance, decreased fitness under warmer temperatures may be caused

43 by advanced flowering in the annual *Arabidopsis thaliana* (Ibañez et al., 2017). More

44 generally, there is usually intraspecific variation in plant responses to environmental

45 treatments (i.e. genotype-by-environment interactions, G × E; Sultan, 2000; Pigliucci, 2001),

46 and if such variation exists within populations, then natural selection can act on it, and the

47 trait plasticity can evolve and adapt to local environmental conditions (Sultan, 2000; Groot et

48 al., 2017). If past environments have influenced the evolution of plasticity, we should be able

49 to detect plasticity-environment correlations to identify agents of selection shaping plasticity

50 (Groot et al., 2017; Marais et al., 2017).

51   Organisms may not only respond directly to their current environments, but their

52 phenotypes may also be influenced by the environmental conditions that their ancestors were

53 exposed to (Uller, 2008; Latzel et al., 2014; Groot et al., 2017) – a phenomenon called

54 'transgenerational plasticity' or 'transgenerational effects'. In plants, such transgenerational

55 effects can be physiological and controlled by the mother plant (Herman and Sultan, 2011),

56 for instance through endosperm or seed coat modifications, or they can be epigenetic (Whittle

57 et al., 2009; Rasmann et al., 2012; Suter and Widmer, 2013) and therefore potentially

58 transferable across even more than one generation (Suter and Widmer, 2013; Groot et al.,

59 2017). Through transgenerational effects, plants could prepare (or 'prime') their phenotypes

60 for particular environmental conditions, particularly when offspring are likely to experience

61 similar conditions as their parents, thereby increasing local adaptation (i.e. adaptive

62 transgenerational plasticity; Roach and Wulff, 1987; Mousseau and Fox, 1998ab; Agrawal,

63 2001; Galloway, 2005; Galloway and Etterson, 2007; Uller, 2008; Mousseau et al., 2009;

64 Latzel et al., 2014). However, as with regular (within-generation) phenotypic plasticity,

65 transgenerational effects can only evolve as an adaptation when there is genotypic variation

66 in transgenerational effects and when offspring environmental conditions correlate with

67 parental environmental conditions (Uller, 2008).

68   An increasing number of empirical studies with plants investigated how

69 transgenerational effects may confer adaptation particularly under temperature stress (Sultan

et al., 2009; Herman and Sultan, 2011; Latzel et al., 2014; Groot et al., 2017). For instance, in
a single genotype of the model plant *Arabidopsis thaliana*, transgenerational effects of heat
stress were observed even in the F3 generations where F3 offspring with the same heat stress
in the P1 and F1 generations had a fitness advantage (Whittle et al., 2009). Recently, Groot
and co-workers (2017) showed strong genotypic variation in parental and grandparental
effects of heat stress in 14 *A. thaliana* genotypes.

So far most studies investigating plant responses to altered and/or stressful
environmental conditions – including those studies investigating transgenerational effects –
were performed under controlled conditions but usually with stable treatments that did not
consider the temporal variability of environmental stress, which however plays an important
role in natural ecosystems (Knapp et al., 2002; Schwinning et al., 2004; Shea et al., 2004).
For instance, while global warming is expected to continue (Giorgi et al., 2004; Barros and
Field, 2014), climate anomalies will increase too (e.g. European heat waves in 2003 and
2010), resulting in increasing temporal variability of temperature and, presumably, heat stress
(Schär et al., 2004; Fischer and Schär, 2008; Barriopedro et al., 2011). During climatic
extreme events, the variability aspect itself is often thought to be more important than the
involved changes in means (Katz and Brown, 1992), and some ecosystems have even been
found to be more sensitive to changes in environmental variability than to changes in
environmental means (Knapp et al., 2002).

To date, only few studies have examined plant responses to changes in environmental
variability, or genetic variation therein (Parepa et al., 2013, Scheepens et al., 2018),
specifically with respect to the timing (Stone and Nicolas, 1995, 1996; Prasad et al., 1999;
Wang et al., 2016) or frequency (Walter et al., 2009) of stress. To our knowledge, no previous
study tested for transgenerational effects of stress timing and frequency.

94   To address these questions and to better understand the complexity of plant responses to

95   climatic variability (Knapp et al., 2002; Reyer et al., 2013) we carried out a two-generation

96   experimental study with *Arabidopsis thaliana* (L.) Heynh. that tested plant responses to

97   altered timing and frequency of heat stress. To explore intraspecific variation and

98   evolutionary potential, our study included multiple genotypes from different geographic and

99   climatic origins. In the first generation (published in Scheepens et al., 2018) we found that the

100   timing of heat stress had a much stronger effect on the plants than its frequency, that *A.*

101   *thaliana* genotypes significantly differed in their responses to stress timing, and that this

102   intraspecific variation correlated with the precipitation variability at the geographic origins,

103   indicating a possible adaptive evolution of this type of phenotypic plasticity in more variable

104   environments.

105   Here, we report on the results from the offspring generation where we grew plants

106   from 9 of the 11 genotypes included in the parental-generation experiment and tested on the

107   one hand for transgenerational effects of parental stress treatments in a simple common-

108   garden experiment, and on the other hand we subjected a subset of the offspring plants to

109   renewed stress to test the adaptive value of transgenerational effects (reciprocal experiment).

110   As in the parental-generation experiment, we also tested for intraspecific variation in plant

111   responses, correlated this variation with climates of origin, and tested whether increased trait

112   plasticity correlates with fitness robustness, i.e. more stable fitness across treatments.

113   Specifically, we asked the following questions: (1) Are there transgenerational effects of heat

114   stress timing or frequency on the phenotypes of the offspring? (2) If yes, do transgenerational

115   effects affect responses to current stress in an adaptive way? (3) Are there differences among

116   *A. thaliana* genotypes in the magnitudes and/or direction of transgenerational effects? (4) If

117   yes, does this intraspecific variation correlate with environmental conditions at the

118   geographic origins and/or with fitness robustness?

119

MATERIALS AND METHODS

Parental generation experiment

The plant material used here came from a previous study (Scheepens et al., 2018) in which we tested for the direct effects of different temperature stress scenarios, varying in timing and frequency (**Figure 1**), on 11 *Arabidopsis thaliana* genotypes. The 11 genotypes were selected to maximize genetic diversity and came from the "core collection" of the Versailles *Arabidopsis* Stock Center (McKhann et al., 2004). After one week of cold-moist (4 °C) stratification, all seeds were planted into $5 \times 5 \times 4.5$ cm pots with a 9:9:2 mixture of low-nutrient soil, regular potting soil and sterilized sand and placed in a growth chamber with 20/15 °C and a 16/8 h light/dark cycle until one week after germination. For the experimental treatments, we used two identical climate chambers, one set to 20/15 °C ('control chamber'), the other set to 30/25 °C ('stress chamber'), both with a 16/8 h light/dark cycle. A day temperature of 30 °C is known to be stressful for *A. thaliana* and to reduce its fitness (Groot et al., 2017; Scheepens et al., 2018). Light conditions (230 μmol·m^{-2}·s^{-1}) and air humidity (40-60%) were identical in both chambers. The experimental treatments were created by moving different subsets of plants to the stress chamber at different times and intervals. Specifically, we varied the timing and frequency of heat stress periods experienced by the plants (**Figure 1**). To vary timing, we stressed plants either early in their life cycle (plants moved to stress chamber on day 8, right after the first week of seedling establishment), in the middle of most genotypes' life cycle (starting on day 26) or late in the life cycle (starting on day 44). The timing treatment was crossed with a frequency/duration treatment, where heat stress was either applied at low frequency (2 times 6 days of stress, with 6 days in between) or high frequency (4 times 3 days of stress, each time with 2 days in between). Important to

143   note is that in all stress scenarios the plants experienced the same total time in the stress

144   chamber and therefore also the same mean temperature during the experiment (**Figure 1**). In

145   each chamber, the spatial positions of all pots were completely randomized, and were re-

146   randomized every week. We had eight replicate plants of each genotype in each treatment.

147   Altogether, our parental-generation experiment included 11 genotypes × 6 treatments × 8

148   replicates = 528 plant individuals. The experiment ran for approximately 10 weeks. When

149   plants began flowering, we placed their inflorescences into ARACON tubes (Betatech bvba,

150   Gent, Belgium) to prevent cross-fertilization and collect the seeds for the next experimental

151   generation.

152

153   *Offspring generation experiments*

154   We tested for transgenerational effects in two separate experiments, (1) a simple common-

155   environment comparison of offspring from the six parental treatments under control condition

156   (16/8 h light/dark at 20/15 °C), and (2) a reciprocal transplant where we used offspring from

157   only two of the parental treatments, the early and mid-term stress at low stress frequency

158   (**Figure 1**), re-created these two treatments and grew both types of offspring in both

159   environments. We restricted the second experiment to these two treatments because they had

160   the strongest effects in the parental generation (Scheepens et al., 2018). Since in the

161   reciprocal experiment there were 'local' (same conditions as parents) versus 'foreign'

162   (different conditions from parents) plants in each environment, this experiment allowed to

163   test for adaptive transgenerational effects. In both offspring experiments we used nine of the

164   11 genotypes from the previous generation, because of limited numbers of seeds in the

165   remaining two genotypes (**Table 1**; Scheepens et al., 2018), and we stratified and germinated

166   seeds as in the parental experiment. In the first experiment, we had seven replicates per

167   genotype and maternal treatment, for a total of 9 genotypes × 6 parental environments × 7

168   replicates = 378 plants. In the second experiment, there were eight replicates per genotype by

169   treatment combination, with a total of 9 genotypes × 2 maternal environments × 2 offspring

170   environments × 8 replicates = 288 plants. In both experiments, we watered all plants

171   regularly, and re-randomized their spatial positions every week. On day 44, right after the

172   intermediate stress treatment in the reciprocal experiment, we took leaf samples for molecular

173   analyses (not reported here) from 3-4 randomly selected plants from each genotype by

174   treatment combination in each of the two experiments (i.e. from roughly half of the plants).

175   Throughout the experiment, we recorded flowering time as the number of days from

176   germination to when the white petals of the first flower became visible. As in the parental

177   experiment, we placed ARACON tubes over the flowering stems to prevent outcrossing and

178   collect seeds. Each plant was harvested one week after its fruits had started to turn yellow.

179   We estimated plant fecundity as the number of fruits >2 mm, and we counted the number of

180   basal shoots and lateral shoots and calculated the ratio of lateral to basal shoot number as

181   index of plant architecture, with lower values indicating more 'shrubby' plants. After that, we

182   separated inflorescences and rosettes, dried them at 60 °C for 72 h and weighed them, and

183   then calculated total aboveground biomass, as well as reproductive allocation as the ratio of

184   reproductive to total aboveground biomass.

185

186   *Statistical analysis*

187   We used linear models to test for the effects of experimental treatments, plant genotypes, and

188   their interactions, on each of the five measured traits: flowering time, plant architecture,

189   aboveground biomass, reproductive allocation and fecundity. For the simple common-

190   environment experiment, the models included plant genotype, timing of parental stress,

191 frequency of parental stress, and all possible interactions, as fixed factors. For the reciprocal

192 experiment, the models included plant genotype, timing of parental stress, timing of offspring

193 stress, and their interactions. Additionally, to account for possible influences of the leaf

194 sampling, all models also included leaf sampling (yes/no) as a fixed factor. To improve the

195 normality of residuals and homogeneity of variance, the flowering time and aboveground

196 biomass data were log-transformed prior to the analyses.

197 In those cases where we discovered a significant genotype by treatment interaction, i.e.

198 genetic variation in plasticity, in either of the two experiments, we additionally tested whether

199 trait plasticities of genotypes were associated with (1) their climates of origin and (2) their

200 fitness robustness. As measure of trait plasticity we used the coefficient of variation (CV) of a

201 trait (Valladares et al., 2006) across all treatments in an experiment (common environment:

202 six parental environments; reciprocal experiment: four combinations of parental and offspring

203 environments). For the climate-plasticity test we extracted climate data for each genotype

204 origin from the WorldClim database (Hijmans et al., 2005), and we used on the one hand

205 several existing bioclimatic variables that describe annual climatic variability [BIO_2 = Annual

206 Mean Diurnal Range, BIO_3 = Isothermality, BIO_4 = Temperature Seasonality (SD), BIO_7 =

207 Annual Temperature Range, BIO_{15} = Precipitation Seasonality (CV)], and on the other hand

208 we calculated several climate variabilities for the specific growing season (see **Table 1**) of

209 each genotype: the SDs of temperature, and the CVs of precipitation, evapotranspiration and

210 climatological water deficit. Additionally, we included latitude from each genotype's origin.

211 To test for relationships between climate variability of origin and the plasticity of *Arabidopsis*

212 genotypes, we calculated Pearson correlations between trait plasticity and the bioclimatic

213 variables, growing-season variabilities and latitude, respectively. For the plasticity-fitness test

214 we calculated the fitness robustness of each genotype as the mean fitness (in terms of number

9

215 of fruits) across treatments divided by the maximum fitness achieved in one of the six

216 treatments, and calculated Pearson correlations between trait plasticity and fitness robustness.

217   All statistical analyses were done in JMP 12 (SAS Institute, Heidelberg).

218

219 **RESULTS**

220 *Common-environment experiment*

221 In the simple common-environment experiment, we found strong genotype differences in all

222 measured traits (**Table 2**), confirming that there was substantial genetic diversity in the

223 studied *A. thaliana* genotypes. The effects of parental stress treatments were much more

224 moderate, and were largely confined to the timing of parental heat stress: Offspring from

225 parents which experienced early stress generally showed an increased ratio of lateral to basal

226 shoots compared to intermediate and late stress (**Figure 2**). For flowering time, the effect of

227 stress timing depended on stress frequency (PT × PF interaction in **Table 2**): at high stress

228 frequency, stress timing had an effect on flowering time, whereas at low stress frequency it

229 did not (**Figure S1**). We found significant genotype by stress timing interactions for

230 flowering time and plant architecture (G × PT interactions in **Table 2**; **Figure 3**), indicating

231 genetic variation in these transgenerational responses. There were no main effects of stress

232 frequency in any of the studied traits, and no genotype by stress frequency interactions. Only

233 for aboveground biomass, there was a significant three-way interaction between plant

234 genotype, parental stress timing and parental stress frequency for aboveground biomass (G ×

235 PT × PF interaction in **Table 2**), indicating complex relationships between these three factors.

236

237   *Reciprocal experiment*

238   When offspring from early and intermediate (low-frequency) stress parents were reciprocally

239   subjected to the same treatments, there were strong effects of offspring environment on all

240   measured traits except for flowering time (OT main effects in **Table 3**), whereas the parental

241   heat stress timing affected only the flowering time of the plants (PT main effect in **Table 3**),

242   with offspring from early-stress parents flowering earlier (**Figure 4**). However, a significant

243   interaction between parental and offspring environment (PT × OT in **Table 3**) indicated that

244   the expression of transgenerational effects on flowering time depended on the offspring

245   environment: the differences between parental treatments were expressed only if the offspring

246   was subjected to early heat stress, but not if heat stress occurred later (**Figure 4**).

247   As in the common-environment experiment, there were significant genotype

248   differences in all of the studied traits (**Table 3**), and there were significant genotype by

249   offspring environment interactions (G × OT in **Table 3**) in four out of the five measured

250   traits, indicating genetic variation in (within-generation) phenotypic plasticity. In addition, we

251   found a genotype by parental environment interaction (G × PT in **Table 3**), indicating

252   genotype-specific transgenerational effects, for flowering time.

253   We did not find a significant parental by offspring environment interaction for plant

254   fecundity (PT × OT in **Table 3**), as would have been predicted for adaptive transgenerational

255   effects. However, there was a significant G × PT × OT interaction, indicating that these

256   interactions are genotype-specific. We therefore tested for a significant PT × OT interaction

257   separately for each genotype. Only in Mt-0 this interaction was significant ($F = 10.38$, $P =$

258   0.003; **Figure S2**), but the results did not confirm our hypothesis. In each offspring

259   environment the plants from the respective *other* parental environment produced more fruits

260   than the ones from the same parental environment, suggesting rather a maladaptive

261   transgenerational effect.

262

263   *Plasticity, climates of origin, and fitness robustness*

264   We found no correlations between climates of origin and trait plasticity in the common-

265   environment experiment (**Table S1**), but in the reciprocal experiment there were several

266   significant climate-plasticity correlations (**Table S2**). The CV of fecundity was negatively

267   correlated with temperature seasonality and annual temperature range, and positively

268   correlated with isothermality (**Table S2**). Thus, genotypes from geographic origins with

269   higher temperature seasonality displayed lower fecundity variation - and therefore greater

270   fitness homeostasis - in response to different stress treatments (**Figure 5A**). The CV of

271   fecundity was also positively correlated with the seasonal CV of evapotranspiration

272   variability (**Table S2**). Moreover, we also found that the CV of aboveground biomass was

273   positively correlated with isothermality and precipitation seasonality (**Figure 5B**), and

274   negatively correlated with latitude and with seasonal CV of climatological water deficit.

275   Finally, the CV of plant architecture correlated negatively with the annual mean diurnal

276   range. Despite significant genotypic variation in the response of flowering time to parental or

277   offspring stress timing, this variation in plasticity did not correlate with any of the climate

278   variables. In both experiments we found that the plasticity of aboveground biomass, but not

279   that of the other traits, was significantly negatively correlated with fitness robustness (**Table**

280   **S3**; **Figure 6**).

281

282   **DISCUSSION**

283   Changes in the temporal variability of environmental stresses are an important aspect of

284   climate change, but we so far know little about the evolutionary consequences for plants:

285   whether plant responses can be transgenerational, if plants harbour intraspecific variation

286   (and thus evolutionary potential) in this respect, and how such transgenerational responses

287   relate to environmental adaptation and fitness. Our study demonstrates that changes in the

288   temporal patterns of heat stress can carry over to the next generation in *Arabidopsis thaliana*,

289   and that there is substantial genotypic variation in the magnitude and direction of these

290   transgenerational effects. Thus, changes in heat stress patterns not only affect plants directly

291   (Scheepens et al., 2018), but also across generations. Several previous studies have reported

292   transgenerational responses to various environmental changes (e.g. Galloway and Etterson,

293   2007; Herman et al., 2012; Groot et al., 2017). For instance, Groot et al. (2017) subjected

294   parental and grandparental plants of 14 *A. thaliana* genotypes to continuous heat stress and

295   found transgenerational effects, as well as genotypic variation therein. The unique aspect of

296   our study is that, while previous studies usually compared stressed and non-stressed plants,

297   we only manipulated the temporal patterns of heat stress, i.e. when the stress occurred and

298   how it was apportioned across time, whereas the total amount of stress (i.e. temperature

299   sums) was identical in all parental environments.

300

Transgenerational effects of stress timing versus frequency

302   Overall, the timing of heat stress had much stronger transgenerational effects than its

303   frequency, consistent with our observations in the parental plants (Scheepens et al., 2018).

304   Variation in parental stress timing consistently affected the architecture, and, depending on

305   the genotype and/or stress frequency, also the flowering time and biomass of offspring plants,

306   whereas the transgenerational effects of stress frequency were only minor.

307   One possibility why stress frequency may play such a little role within and across

308   generations is that plant physiological responses to heat stress may be triggered by the initial

309   stress event, and simply remain 'switched on' afterwards, so that the number or duration of

310   stress events does not matter, at least on the short time-scales of our experiment. A candidate

311   mechanism for this would be heat shock proteins that plants produce to stabilize protein

312   function (Nover et al., 2001; Sung et al., 2003; Swindell et al., 2007), and that may protect

313   plants and their offspring against subsequent heat stress events.

314   In contrast to stress frequency, the timing of parental heat stress influenced several

315   traits of the plant offspring. It is generally well-established that the susceptibility of many

316   plant traits to environmentally-induced developmental changes depends on the life stage. For

317   instance, heat stress during floral bud development determines peg number in peanut (Prasad

318   et al., 1999), in wheat the maximum sensitivity to heat stress for protein accumulation is

319   during the grain filling period (Stone and Nicolas, 1996), and in the herbaceous plants

320   *Andropogon gerardii* and *Solidago canadensis* late-season heat stress causes the greatest

321   reduction in photosynthetic productivity (Wang et al., 2016). The usual explanation for such

322   results is that signaling pathways determining trait changes may only be active during certain

323   developmental periods, but the precise underlying mechanisms are often unknown. Another

324   explanation would be that no active developmental mechanism is involved, but plants are

325   simply more sensitive at some life stages (analogous to 'active' versus 'passive' phenotypic

326   plasticity; van Kleunen and Fischer, 2005). In our experiment, early heat stress occurred at a

327   small seedling stage of *A. thaliana*, whereas in the intermediate treatment the plants were

328   already much larger and well-established. In fact, some were already bolting and/or close to

329   flowering. It is not surprising that heat stress effects differed between these plants. However,

330   all arguments so far, as well as the empirical studies mentioned above, are about within-

331   generation responses to heat stress, whereas in our study we observed transgenerational

14

332   effects. Thus, signaling and developmental regulation alone cannot explain our results, and

333   there must be additional, so far unknown, physiological (Herman and Sultan, 2011) and/or

334   epigenetic (Whittle et al., 2009; Rasmann et al., 2012), mechanisms involved.

335

336   *No evidence for adaptive transgenerational plasticity*

337   In the reciprocal experiment we applied stress treatments to offspring plants to test if

338   transgenerational effects can be adaptive. In our case, if the offspring whose parents

339   experienced the same stress timing as the respective tested one had a higher fitness than the

340   offspring of parents that experienced another stress timing, this would indicate an adaptive

341   transgenerational effect. We found that responses in plant fecundity to current stress timing

342   depended on parental stress timing, but in a highly genotype-specific manner. In fact, the

343   majority of the parent-offspring interactions for separate genotypes were non-significant and

344   only the genotype Mt-0 showed a significant interaction to parental and offspring heat stress

345   timing. However, the pattern was maladaptive, i.e. in contrast to our expectation offspring

346   from parents with the same stress timing had a *lower* fitness. This contrasts with observations

347   of adaptive transgenerational plasticity from previous studies (Galloway and Etterson, 2007;

348   Latzel et al., 2014). The virtual absence of significant interactions across genotypes in our

349   study may have various explanations, such as limited within-population genetic variation in

350   parental effects for the environment to select on, or a lack of selective pressure for adaptive

351   responses under unpredictable temperature stress events.

352   Offspring plants that received early stress showed accelerated flowering when their

353   parents had also experienced early stress compared to plants whose parents had experienced

354   intermediate stress. Such advanced flowering may reflect an escape strategy (Franks, 2011),

355   which could enhance the possibility of lineage survival under continuing high temperature

356   conditions (Wahid et al., 2007). Although the induction of earlier flowering by environmental

357   stress treatments is known from previous studies (Balasubramanian et al., 2006; Franks,

358   2011; Ibañez et al., 2017), its transgenerational aspect has so far been rarely studied. Suter

359   and Widmer (2013) detected accelerated flowering in *Arabidopsis thaliana* under control

360   conditions in the fourth generation after heat exposure, but this effect disappeared in the fifth

361   generation after two generations without stress exposure. Groot and co-workers (2017)

362   observed earlier flowering in response to grandparental heat stress, but only in late-flowering

363   genotypes. Our own results confirm that stress exposure can induce earlier flowering also

364   transgenerationally, and thereby contribute to an escape strategy, but that such effects may be

365   restricted to situations with early stress exposure, where plants are still in sensitive

366   developmental stages.

367

368   *Genotypic variation in transgenerational plasticity*

369   So far, few studies have investigated intraspecific variation in transgenerational plasticity

370   under stress conditions (Gaudet et al., 2011; Suter and Widmer, 2013; Nolf et al., 2016; Groot

371   et al., 2017), and our study provides novel evidence for it. Using nine genetically and

372   morphologically diverse genotypes, we found significant genotype × parental treatment

373   interactions both under control conditions and under renewed stress treatments in the

374   offspring generation. Thus, intraspecific variation in environmentally-induced

375   transgenerational responses exists in *A. thaliana*. This genotypic variation among widespread

376   origins suggests evolutionary divergence among populations, which could result from

377   adaptation, genetic drift, or both. We used only a single genotype per population, precluding

378   assessment of within-population variation (or constancy) of responses to experimental

379   treatments. However, the genetic diversity within populations is likely very restricted

380 (Bomblies et al., 2009) whereas genetic diversity is large among the selected populations

381 (McKhann et al., 2004). Therefore, we are confident that the observed patterns reflect

382 evolutionary divergence among populations.

383

384 *Relationships with climates of origin and fitness robustness*

385 We found that plasticity in response to heat stress correlated with a range of climate variables

386 from the genotypes' geographic origins, suggesting that environmental variability at sites of

387 origin might be an important selective factor (Endler, 1986) for the evolution not only of

388 within-generation plasticity (Scheepens et al., 2018) but also of transgenerational plasticity.

389 Interestingly, these relationships were only found under stressful conditions in the reciprocal

390 experiment but not under stress-free conditions in the common-environment experiment.

391   One of the observed plasticity-climate relationships was a negative correlation between

392 plasticity in fecundity and temperature seasonality at sites of origin. The same plasticity was

393 also correlated to two other, closely related, climate variables, isothermality (positive

394 correlation) and annual temperature range (negative correlation). Genotypes from origins

395 with increasing temperature seasonality showed a reduced plasticity in fecundity and thus

396 appear to have evolved a stronger fitness homeostasis in the face of fluctuating temperature

397 conditions, whereas genotypes from origins with more stable temperature regimes evolved to

398 respond more strongly to temperature stress, leading to reduced fitness in our experiments.

399   We also observed a positive relationship between plasticity in biomass and

400 precipitation seasonality, i.e. plants from unpredictable precipitation environments responded

401 more strongly to temperature stress. Since biomass and fecundity are strongly positively

402 correlated in *A. thaliana* (Clauss and Aarssen, 1994), this plasticity-climate relationship

17

403 seems to contrast with the above-mentioned negative correlation between plasticity in

404 fecundity and temperature seasonality. However, precipitation seasonality and temperature

405 seasonality are not correlated in the studied plant origins, so these plasticity-environment

406 correlations may reflect independent evolutionary responses to different aspects of climate

407 variability.

408 The strongest plasticity-environment correlation was between plasticity in

409 aboveground biomass and latitude: plants from higher latitudes responded less to variation in

410 temperature stress. Since increasing latitude is associated with decreasing precipitation

411 seasonality, the latter may be the underlying driver of this relationship. High precipitation

412 seasonality at low latitudes may have selected for strong biomass responses to temperature

413 stress, potentially reflecting escape mechanisms under periods of drought (Franks, 2011).

414 In the parental experiment (Scheepens et al., 2018) we had previously found positive

415 correlations between plasticity and precipitation variability at sites of origin in four out of

416 five traits. We did not find the same relationship in the offspring generation in the current

417 study, even though transgenerational effects were still present in three out of five traits. One

418 possible explanation for this is that the plant responses in the parental generation were

419 passive and/or maladaptive (cf. fitness robustness), and that transgenerational effects caused

420 the offspring generation to respond less in order to retain fitness. We did find correlations

421 between plasticity in plant architecture, aboveground biomass and fecundity and several other

422 climate variables in the reciprocal experiment, indicating a possible adaptive function of

423 these plant responses, and highlighting the general relevance of studying environmental

424 variability for understanding transgenerational plant responses to temperature stress.

425 We found negative correlations between fitness robustness and plasticity in

426 aboveground biomass, but not in other traits, in both experiments. This is similar to our

427   results from the parental plants (Scheepens et al., 2018) and implies that more plastic

428   genotypes show stronger fitness variation in response to (parental and/or offspring)

429   treatments. However, the slopes of these relationships are flatter in offspring compared to

430   parental plants, with fitness robustness values of 0.69-0.95 in the common-environment

431   experiment and 0.72-0.97 in the reciprocal experiment, compared to values of 0.50-0.90 in

432   the parental generation (Scheepens et al., 2018). Therefore, the offspring generation, even

433   when under identical stress, shows an overall improved fitness robustness, which may reflect

434   a transgenerational adaptive response to temperature stress.

435

436   *Conclusions*

437   Given that changes in temporal environmental variability are an important aspect of climate

438   change, it is important to understand their effects on plants, both in terms of phenotypic

439   plastic responses and of intraspecific evolutionary divergence. To our knowledge, no previous

440   study has tested for transgenerational responses of plants to temporal variability of

441   environmental stresses, rather than their mean changes. We found ample genotypic variation

442   in transgenerational responses to temporal variation in heat stress, suggesting that selection

443   can act on it, and plasticity-environment correlations indicate a possible adaptation to the

444   environmental variability of plant origins.

445

446   **DATA AVAILABILITY STATEMENT**

447   The datasets generated for this study are available on request to the corresponding author.

448

AUTHOR CONTRIBUTIONS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: [WEBLINK]

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625

Conflict of interest:

629   **Table 1.** *Arabidopsis thaliana* genotypes used in this study, and their geographical

630   coordinates and natural growing season (in months; from Scheepens et al. 2018).

631

Name	Country	Latitude	Longitude	Growing season
Bur-0	Ireland	54.1	-6.2	5-8
Can-0	Spain	29.21	-13.48	11-2
Ct-1	Italy	37.51	15.09	12-3
JEA	France	43.68	7.33	3-6
Mt-0	Libya	32.34	22.46	11-2
N13	Russia	61.36	34.15	6-9
Oy-0	Norway	60.39	6.19	5-8
Sha	Tajikistan	38.59	68.79	2-5
St-0	Sweden	59.34	18.06	5-8

632

633 **Table 2.** Results of the common-environment experiment, testing the effects of leaf sampling, parental stress timing, parental stress frequency,

634 genotype, and their interactions, on the flowering time, plant architecture, aboveground biomass, reproductive allocation and fecundity of

635 *Arabidopsis thaliana* offspring. Significant effects ($P < 0.05$) are in bold; df = degrees of freedom.

636

	df	Flowering time		Plant architecture		Aboveground biomass		Reproductive allocation		Fecundity	
		F-ratio	*P*-value	*F*-ratio	*P*-value	*F*-ratio	*P*-value	*F*-ratio	*P*-value	*F*-ratio	*P*-value
Leaf sampling	1	1.03	0.311	1.41	0.236	52.88	**<0.001**	20.54	**<0.001**	32.43	**<0.001**
Parental timing (PT)	2	0.85	0.429	5.96	**0.003**	0.25	0.777	1.35	0.261	1.33	0.267
Parental frequency (PF)	1	0.95	0.331	2.82	0.094	0.33	0.567	0.25	0.615	1.06	0.305
PT × PF	2	5.92	**0.003**	0.12	0.891	0.19	0.831	0.55	0.577	0.16	0.852

30

Genotype (G)	8	260.23	**<0.001**	99.12	**<0.001**	35.65	**<0.001**	174.37	**<0.001**	79.23	**<0.001**
G × PT	16	2.19	**0.006**	2.15	**0.007**	1.30	0.193	1.29	0.202	1.19	0.275
G × PF	8	0.40	0.920	0.54	0.829	1.22	0.287	0.88	0.536	1.30	0.242
G × PT × PF	16	0.97	0.494	1.01	0.441	1.99	**0.013**	1.47	0.109	1.10	0.353

637

638

639　**Table 3.** Results of the reciprocal experiment, testing the effects of leaf sampling, parental stress timing, offspring stress timing, genotype, and

640　their interactions, on the flowering time, plant architecture, aboveground biomass, reproductive allocation and fecundity of *Arabidopsis thaliana*

641　offspring. Significant effects ($P < 0.05$) are in bold; df = degrees of freedom.

642

	df	Flowering time		Plant architecture		Aboveground biomass		Reproductive allocation		Fecundity	
		F-ratio	P-value	F-ratio	P-value	F-ratio	P-value	F-ratio	P-value	F-ratio	P-value
Leaf sampling	1	0.00	0.960	0.14	0.707	18.38	**<0.001**	7.90	**0.005**	11.88	**0.001**
Parental timing (PT)	1	9.92	**0.002**	0.00	0.970	0.21	0.651	0.14	0.708	2.07	0.152
Offspring timing (OT)	1	0.76	0.385	8.08	**0.005**	41.77	**<0.001**	114.43	**<0.001**	17.48	**<0.001**
PT × OT	1	4.74	**0.030**	0.01	0.914	0.23	0.630	0.84	0.360	0.21	0.643

32

Genotype (G)	8	184.29	**<0.001**	14.67	**<0.001**	12.13	**<0.001**	158.91	**<0.001**	57.10	**<0.001**
G × PT	8	3.50	**0.001**	0.50	0.856	0.90	0.517	1.17	0.317	0.86	0.549
G × OT	8	2.07	**0.039**	2.91	**0.004**	5.49	**<0.001**	1.59	0.128	3.97	**<0.001**
G × PT × OT	8	1.82	0.074	0.37	0.937	0.43	0.905	1.28	0.253	2.39	**0.017**

643
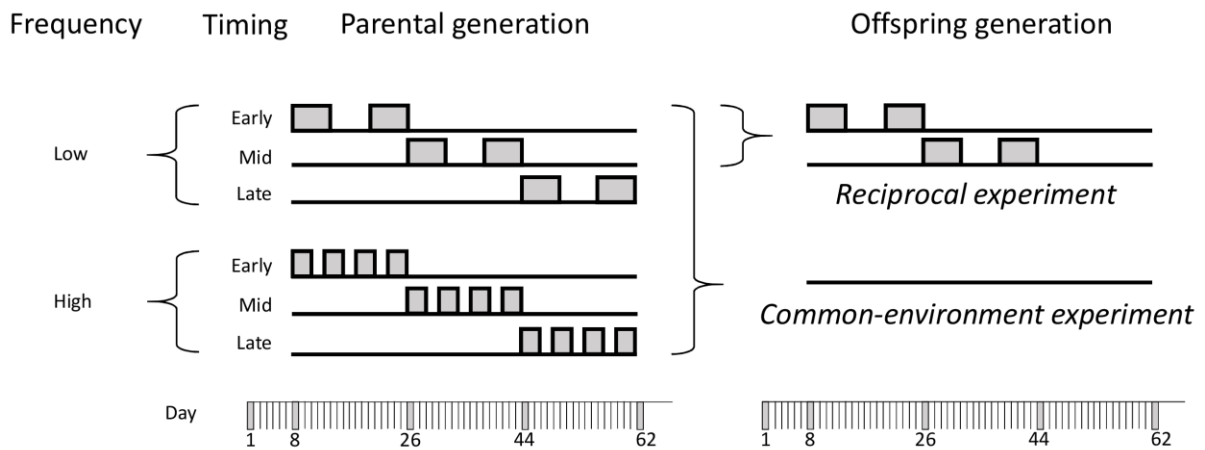
Figure 1. Experimental design of the parental-generation experiment (left) and the two offspring experiments (right) with *Arabidopsis thaliana*, with periods of 30 °C heat stress indicated in grey. In the offspring generation, plants from all parental treatments are grown in a constant control environment (common-environment experiment), and plants from two parental stress treatments are subjected to the same two treatments again (reciprocal experiment).
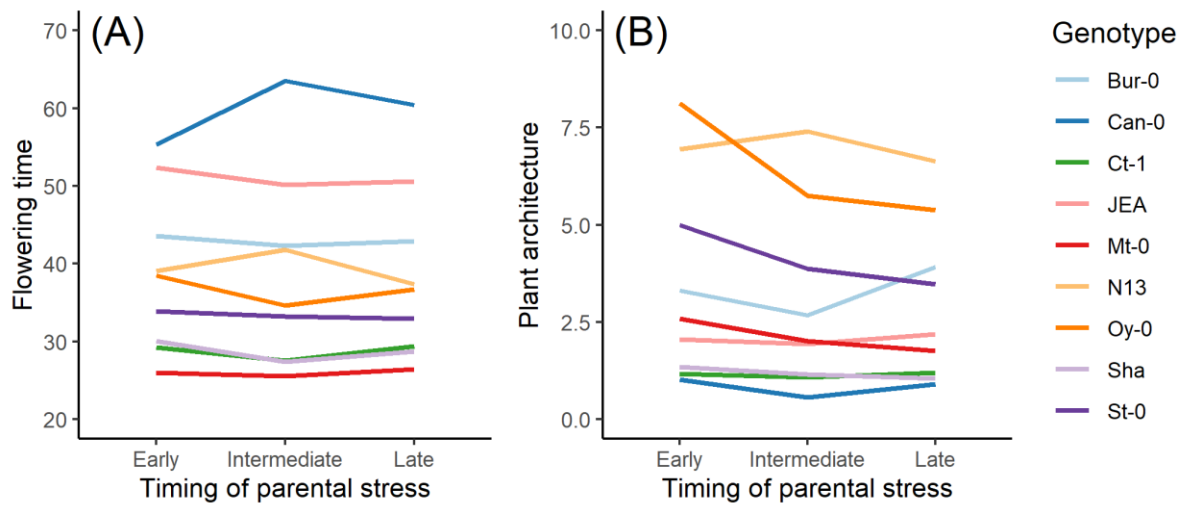
651
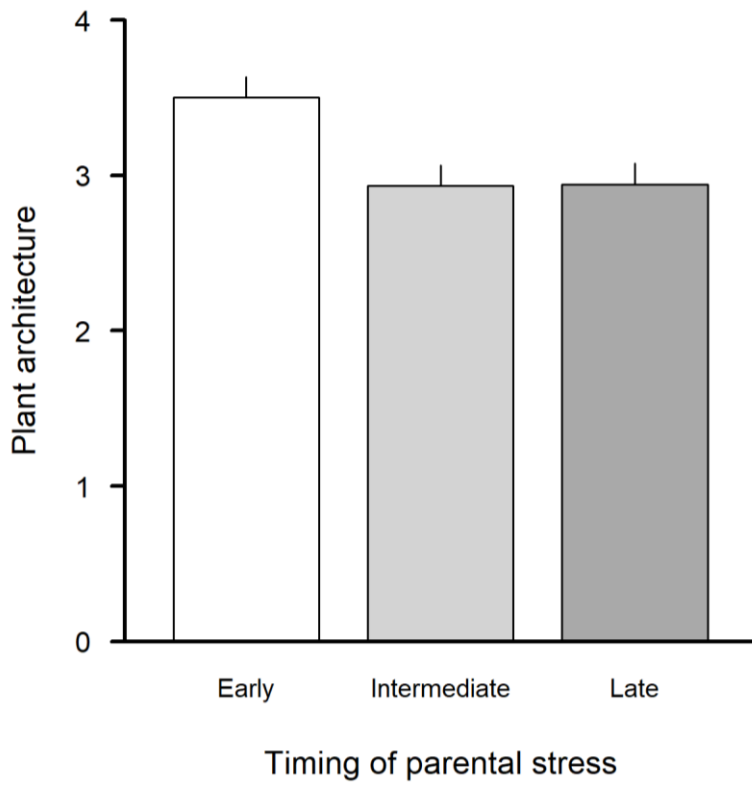
Figure 2. Effects of parental stress timing on plant architecture (number of lateral shoots / number of basal shoots) of *Arabidopsis thaliana* in the common-environment experiment.

654

Figure 3. Genotypic variation in the transgenerational responses of flowering time (A) and plant architecture (number of lateral shoots / number of basal shoots; B) of nine *Arabidopsis thaliana* genotypes to different timing of parental heat stress in the common-environment experiment.

661
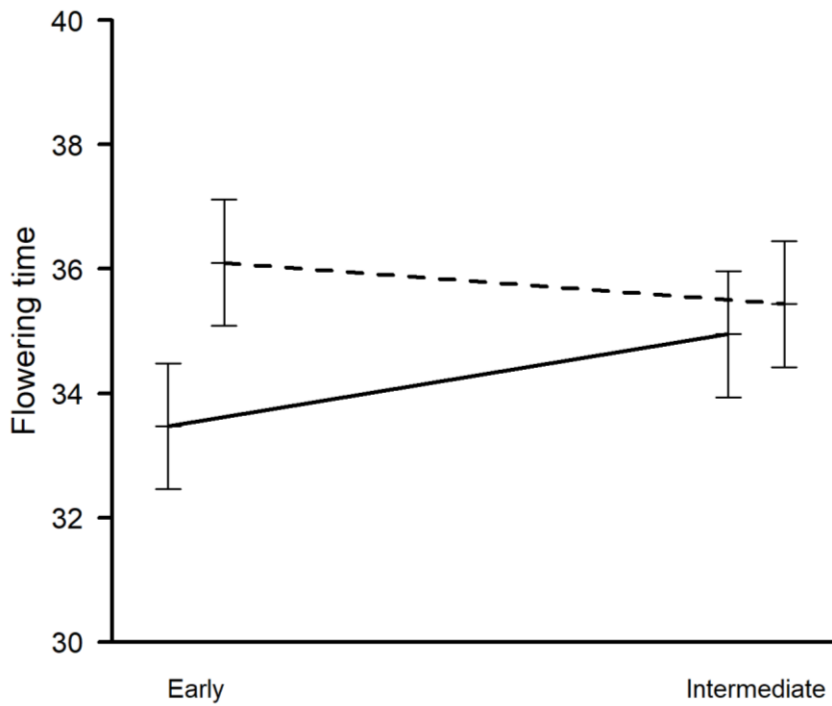
Figure 4. Effects of parental and offspring heat stress timing on flowering time in *Arabidopsis thaliana* in the reciprocal experiment. Solid line – early parental stress; dashed line – intermediate parental stress.

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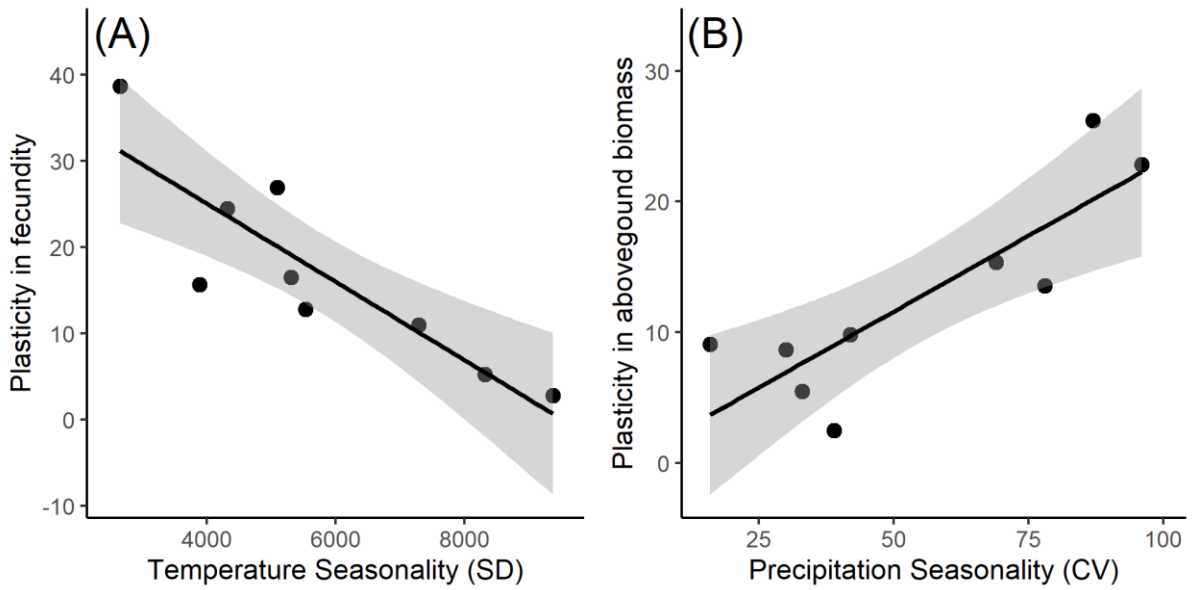
Figure 5. Relationships between trait plasticities and climates of origins for nine *Arabidopsis thaliana* genotypes in the reciprocal experiment. (A) Correlation between temperature seasonality (SD) and plasticity in fecundity. (B) Correlation between precipitation seasonality (CV) and plasticity in aboveground biomass. The plasticity values are coefficients of variation across experimental treatments. The grey areas indicate the 90% confidence intervals of the correlations.

674
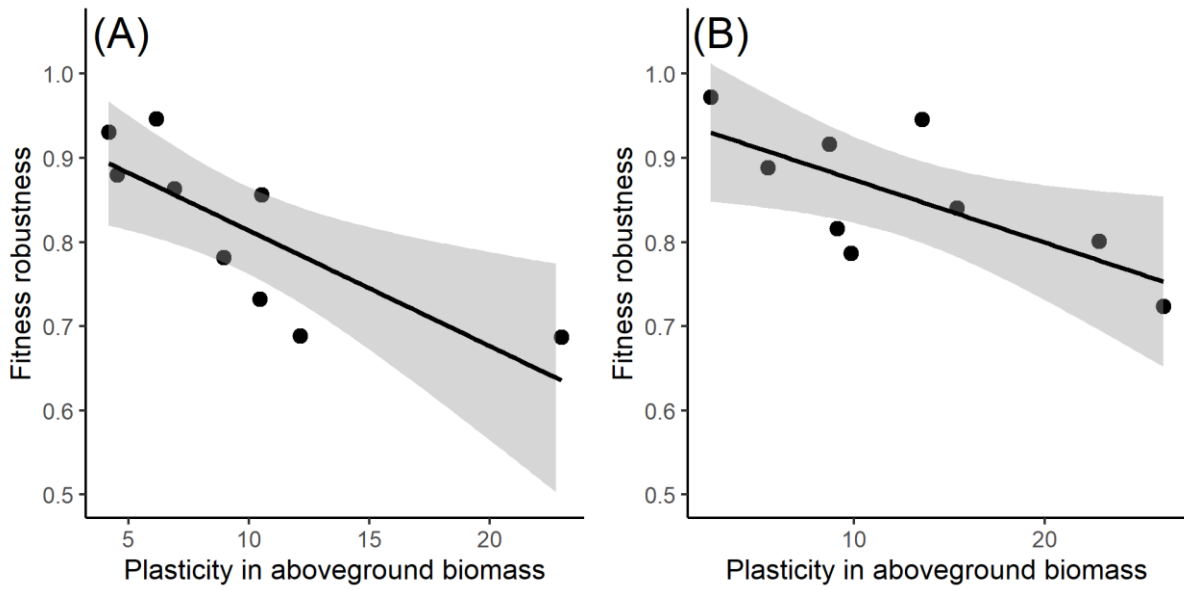
Figure 6. Relationships between fitness robustness across environments and plasticity in aboveground biomass for nine genotypes of *Arabidopsis thaliana* in the common-environment experiment (A) and in the reciprocal experiment (B). The grey areas indicate the 90% confidence intervals of the correlations.

679