1 **No evidence for thermal transgenerational plasticity in metabolism when**

2 **minimizing the potential for confounding effects**

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13

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15 acclimation

16

17 **Abstract**

18 Environmental change may cause phenotypic changes that are inherited across generations
19 through transgenerational plasticity (TGP). If TGP is adaptive, offspring fitness increases with an
20 increasing match between parent and offspring environment. Here we test for adaptive TGP in
21 somatic growth and metabolic rate in response to temperature in the clonal zooplankton *Daphnia*
22 *pulex*. Animals of the first focal generation experienced thermal transgenerational "mismatch"
23 (parental and offspring temperatures differed), whereas conditions of the next two generations
24 matched the (grand)maternal thermal conditions. Adjustments of metabolic rate occurred during
25 the lifetime of the first generation (i.e. within-generation plasticity). However, no further change
26 was observed during the subsequent two generations, as would be expected under TGP.
27 Furthermore, we observed no tendency for increased juvenile somatic growth (a trait highly
28 correlated with fitness in *Daphnia*) over the three generations when reared at new temperatures.
29 These results are inconsistent with existing studies of thermal TGP, and we describe how
30 previous experimental designs may have confounded TGP with within-generation plasticity and
31 selective mortality. We suggest that the current evidence for thermal TGP is weak. To increase
32 our understanding of the ecological and evolutionary role of TGP, future studies should more
33 carefully identify possible confounding factors.

34 **Introduction**

35 Environmental change may cause phenotypic changes that are inherited across generations in the
36 absence of concurrent changes in DNA sequences. More specifically, under transgenerational
37 plasticity (TGP) the offspring environmental reaction norm changes in interaction with the
38 parental [1], or even grandparental environment [2, 3]. If environmental conditions are correlated
39 across generations, parental environments may serve as a reliable cue for the optimal offspring
40 gene expression, and TGP may be adaptive [4, 5]. In this case, TGP represents active phenotypic
41 plasticity, where phenotypes are optimized according to the environment experienced by the
42 parents, in order to maximize the increase (or minimize the decline) in fitness when
43 environments change [6, 7]. However, plasticity may also occur in passive forms [8], in which
44 case it is not a mechanism that has evolved to increase fitness. In this case, it is rather a
45 phenotypic response to environmental conditions that organisms cannot prevent through
46 evolution. Such responses might occur due to resource limitation or environmental stress [sensu
47 8, 9]. Thus, to understand the ecological role of TGP it is not sufficient to demonstrate its
48 presence; one also needs to evaluate whether it confers fitness benefits and preferably link this
49 mechanism to specific patterns in environmental variation. With predictable environmental
50 variation across generations, models that predict TGP could potentially be a special scenario in
51 models on phenotypic tracking [10-13].

52 For ectothermic animals, metabolic activity (metabolic rates, commonly measured as
53 oxygen consumption: VO_2) is under strong control by the ambient temperature through passive
54 plasticity [14, 15]. However, studies of acclimation (within-generation) clearly demonstrate the
55 ability to up- and down-regulate metabolic rate in a more active way, presumably to counteract
56 such passive responses [16, 17]. The failure of adapting to new temperature regimes

57 metabolically could inflict an excessive expenditure of energy or inadequate levels necessary to
58 maintain important physiological functions, which in turn could affect fitness [18, 19]. It is
59 therefore a relevant quantitative trait to study for temperature-mediated TGP, especially as
60 ecological impacts due to climate change becomes increasingly important.

61 Similar to the adaptive properties of within-generation phenotypic plasticity, a TGP
62 response can potentially contribute to momentarily shield a population/species from extinction
63 under changing thermal conditions and allow more time to adapt genetically [20, 21]. Some
64 studies suggest thermal TGP responses in somatic growth rates [22, 23], but the role of metabolic
65 adjustments remains unclear. Two recent studies in coral reef fish [24] and sticklebacks [25]
66 investigated the role of TGP in metabolic capacity for ectotherms. However, the study design
67 applied in those studies cannot rule out the possibility of genetic changes across generations due
68 to effects of selective mortality and/or selective breeding (see *Discussion* for more details).

69 Here we test for thermal TGP responses in both metabolism and somatic growth using the
70 crustacean *Daphnia pulex*. *Daphnia sp.* reproduce asexually during growth periods. By using this
71 clonal animal as a model organism, genetic changes occurring from one generation to the next
72 are minimized (being limited to arising new mutations), enabling TGP effects to be studied in
73 isolation. The transparency of the animal and the ovoviviparous system allow maturation status
74 to be assessed continuously, which permits a clear-cut distinction between maternal and
75 offspring environment. We measured metabolic rate and somatic growth responses, the latter
76 being a proxy for fitness [26], through three generations after being transferred to stable new
77 temperatures. Thus, in this case there is a declining mismatch between offspring environment
78 and environment experienced by previous generations when going from the first to the third
79 generation. The interaction between environment in the current and previous generations are thus

80 embedded in the study design and can be tested for by comparing performance in the three
81 generations in the new environment. In the presence of adaptive TGP we predict changes in
82 metabolic rate across generations following a transfer to new temperatures, and corresponding
83 increases in growth rate.

84 Methods

85 *Animal husbandry*

86 Resting eggs (ephippia) of *Daphnia pulex* were sorted from sediment samples collected at Lake
87 Asklundvatnet, central Norway (N 63.588, E 10.729), on November 1st 2013. Temperatures from
88 this lake was logged at 0.5m depth between May 19th – September 9th 2016. Mean temperature
89 during this period was 17.6 °C (range daily mean: 12 – 22.7 °C, see electronic supplementary
90 material S1 for details). The ephippia were dried for one week on a filter paper before cleansing
91 and activation in 5% hypochlorite solution for five minutes. After being rinsed thoroughly with
92 distilled water, the ephippia were left to hatch in filtered lake water under continuous light at
93 room temperature (22 °C).

94 Resulting hatchlings were kept individually in separate 250mL jars where they
95 propagated by asexual reproduction. The animals were kept at a density of 10 animals in 250 mL
96 glass jars and fed three times a week with Shellfish Diet 1800® (Reed mariculture Inc.) at a final
97 concentration of 2 x 10^5 cells ml^{-1}, corresponding to ca. 3.4 mg C L^{-1}. If only juveniles were
98 present, half of this concentration was used. The shellfish diet consisted of four different marine
99 microalgae: *Isochrysis* sp., *Pavlova* sp., *Tetraselmis* sp., and *Thalossiosira pseudonana*. Culling

100 of the populations down to 10 individuals took place once a week when the medium was
101 replaced.

102 These populations (hereafter clones) were kept for 8-15 months (22 to 30 generations) in
103 climate cabinets at 17 ºC on a 16:8 L/D light regime prior to the experiments. This extensive
104 period ensured common garden settings, i.e. no environmental effects on phenotypes from the
105 wild should remain. For sexually reproducing organisms such prolonged artificial rearing could
106 result in evolutionary changes. For asexual organisms, however, the genetic background should
107 stay more constant, being limited to new mutations and possibly within-clone selection at an
108 epigenome level [27]. Although one cannot completely rule out these two selective forces,
109 fixation of new advantageous mutations was made less likely by the random selection of
110 individuals during the weekly culling. Furthermore, in the case of within-clone selection of
111 epigenetic changes, the multiple-generation rearing at 17 ºC of our clones would be expected to
112 produce patterns of gene expression that maximises fitness at that temperature, and hence
113 potentially increasing the ability to detect TGP (i.e. first generation offspring reared at a different
114 temperature should perform worse than subsequent generations, see *Study design, TGP*). The
115 artificial medium used was a modified selenium dioxide version of ADaM [28].

116

117 *Study design, TGP*

118 Five of the hatched clones from Lake Asklundvatnet, (abbreviated laboratory names: BP, HF,
119 LP, PM, WH) were used for both metabolic and growth experiments. The parental generation
120 consisted of adult females that had ovulated (released the eggs into their brood chamber within
121 24 hours) for the second or third time. These parents were selected from each clone at their
122 original temperature (17 ºC) and transferred to a stable 12, 17 and or 22 ºC temperature regimes

123 (Fig. 1). Feeding regimes were developed to ensure *ad lib* conditions while at the same time
124 preventing overfeeding at the colder temperature (overfeeding in *Daphnia* cultures causes water
125 quality problems). Based on a pilot study it was found that this was achieved by adjusting food
126 rations down or up by 20% relative to that given at 17 ºC (described above) for the 12 and 22 ºC
127 treatments, respectively.

128 The subsequent first clutch being born by the transferred animals thus became the F1
129 generation. Individuals of this generation thus lived their entire life post-hatching as well as the
130 majority of their post-ovulation egg stage at the new temperature, but were conceived and spent
131 their pre-ovulation (i.e. gonadal) life at the original temperature (17 °C). Upon onset of
132 reproduction by the F1 generation (producing F2), the clone's first week of offspring were
133 discarded to avoid selecting for any potential mutations on age at maturation. Thus, the
134 individuals that comprised the F2 generation were randomly selected from offspring born after
135 one week of clonal reproduction. Epigenetic effects from the grandparental generation (all reared
136 at 17 ºC) may also affect the F2 generation, which required an additional F3 generation to be
137 tested. The F3 generation was created in the same manner as the F2 generation.
138

139 *Study design, maternal effects*

140 One potential caveat with our design relates to maternal effects. Specifically, under TGP we
141 predict F2 to perform better (i.e. grow faster) than F1 in a new environment. However, if growth
142 rate is influenced by an additive maternal effect, and offspring from mothers reared at 17 degrees
143 grow faster than those from mothers reared at *both* lower and higher temperatures, predictions
144 may change. Under this scenario, this would give a positive effect on F1 in the new
145 environments compared to F2, and hence counteract the predicted effect of TGP. Thus, a

146 separate experiment was conducted to test for such maternal effects. In this, mothers that had
147 been born and developed at the high and low temperatures (12 and 22 ºC) were transferred to the
148 intermediate temperature (17 ºC). The growth rates until maturation of their resulting offspring
149 was compared to that of offspring from mothers born and developed at the intermediate
150 temperature. As in the main experiment, the transferred animals were mothers that had ovulated
151 within the previous 24 hours.

152

153 *Metabolic measurements*

154 Oxygen consumption of one animal from each of the five clones from all three temperatures of a
155 given generation (15 in total) were tested in each replicate run at a common temperature of 17 ºC
156 (Fig. 1). This allowed us to quantify metabolic responses of being reared at new temperatures for
157 one, two or three generations. Ten replicate runs were conducted per generation. The body
158 lengths (BL, mm, measured from the base of the caudal spine to the apex of the eye) of the
159 animals were measured to the nearest 0.01 mm using the software ImageJ [29]. Total dry weight
160 (DW, mg) of each individual was calculated using the formula $DW = 0.0084BL^{2.58}$ [30]. The
161 number of eggs in the brood chamber and their development stage were also recorded for each
162 individual. Eggs were defined as late in the development process if it was possible to observe the
163 eyes inside the brood chamber (i.e. development stage 4-5 in [31]), and early if not. Individuals
164 were excluded from the dataset ($n = 56$) if we could not determine the contents of the brood
165 chamber or where the individual had an ephippium present.

166 Oxygen consumption rates were measured in a sealed glass micro plate equipped with
167 planar oxygen sensor spots with optical isolation glued onto the bottom of 128-148 µL (mean:
168 138 µL) wells (Loligo® Systems, Denmark) integrated with a 24-channel fluorescence-based

169 respirometry system, the SDR SensorDish® Reader (PreSens, Germany). Such optode
170 respirometry is known for its simplicity, high throughput, and high temporal resolution and
171 sensitivity (Szela and Marsh 2005). The reader was placed inside a Memmert Peltier-cooled
172 incubator IPP (Memmert GmbH, Germany) that kept a stable 17 °C temperature. Adult daphnids
173 (body lengths 1.7 – 2.9 mm) were measured individually in their respective wells. Pressure
174 influenced the readings greatly, which meant that the measurements had to be done without any
175 lid on the chambers. Oxygen content was therefore measured immediately following the
176 placement of animals into the wells, after which the wells were sealed using a silicone lid, which
177 was suppressed by a lead weight to keep the wells air-tight. At the end of the experimental period
178 this lid was removed before taking the final oxygen measurements. The experimental period (30-
179 60 minutes) depended on the experimental temperature, balancing sufficient consumption (~10-
180 25% reduction in O_2) while being well clear of hypoxia (>4 mg O_2 L^{-1}). Nine of the 24 wells
181 functioned as controls, using a representative amount of medium (ADaM) from which the
182 animals originated in addition to fresh ADaM. The mean change of controls was accounted for
183 when calculating oxygen consumption of the daphnids. Oxygen consumption rate was expressed
184 as µg O_2 h^{-1}.
185
186 Individual oxygen consumption rates (VO_2) were first standardized to a common dry body mass
187 (DW) equal to the mean of all individuals (n=440, $\overline{DW}$ = 0.071 mg) and using our observed
188 relationship between individual DW and VO_2 ($VO_2 = 2.2 \times DW^{0.97}$; $r^2 = 0.23$). The metabolic
189 scaling exponent (0.97, SE 0.08) matches exponents reported in other studies of *Daphnia* [32],
190 and did not differ between the different temperature treatments (tab. S2-1, Supplementary
191 material S2, ANOVA, p = 0.1). Thus, size standardized metabolic rate (sVO_2) for a given
192 individual i was calculated as:

193 $$sVO_{2_i} = e^{\ln(VO_{2_i}) - 0.97(\ln(DW_i) - \ln(\overline{DW}))} \quad (1)$$

194 Based on these size standardized oxygen consumption rates (eq. 1) we calculated relative values
195 (eq. 2) of metabolic rates for individuals reared at the new temperatures as:

196 $$rVO_{2_{ijk}} = sVO_{2_{ijk}} - \overline{sVO_{2_{jk\,17\,°C}}} \quad (2)$$

197 , where indices *j* and *k* represent generation and clone, respectively. The relative metabolic rate
198 thus quantifies the up- or down regulation of metabolism for animals reared at new temperatures
199 compared to their clonal counterparts being maintained at 17 ℃. The distributions of the
200 individual observations, sorted by clone, temperature and generation (sVO_2, eq. 2), can be
201 viewed in supplementary material S3.
202
203 *Growth measurements*

204 Somatic growth (G) was calculated as:

205 $$G = \frac{ln(B_{w2}) - ln(B_{w1})}{t} \quad (3)$$

206 , where B_{w1} and B_{w2} is dry mass (mg) at the start (mean 3.1 x 10^{-3} mg, SE 4.3 x 10^{-4} at 12 ℃,
207 mean 2.9 x 10^{-3} mg, SE 6.9 x 10^{-4} at 22 ℃) and end (mean 4.6 x 10^{-2} mg, SE 6.9 x 10^{-3} at 12 ℃,
208 mean 3.8 x 10^{-2} mg, SE 4.8 x 10^{-3} at 22 ℃). of the growth period, respectively, and *t* is the
209 duration of the growth period in days (mean 13.2, SE 1.2 days at 12 ℃, mean 9.45, SE 2.31 days
210 at 17 ℃ and mean 5.3, SE 0.4 days at 22 ℃). In the TGP experiment all generations were tested
211 simultaneously within each of the two new temperatures, 12 and 22 ℃ (Fig. 1). Similarly, all the
212 animals in the maternal effect experiment were tested simultaneously at 17 ℃. All animals were
213 size measured within 24 hours after birth, visually inspected for maturation status 1-3 times daily
214 and once again measured as soon as they reached maturity. Maturation is defined as the first
215 appearance of eggs in the brood chamber (ovulation). This measure of somatic growth is tightly

216 correlated with the intrinsic population growth rate *r* [26], and thus represents a measure of
217 fitness. Protocols for feeding and measurements followed those used in the metabolism
218 experiment (see above), except that animals were reared individually in 50 mL plastic tubes.
219

220 *Statistics*

221 When analysing metabolism; temperature during development, number of generations since the
222 transfer to the new temperature, clone identity (all categorical variables), and number of eggs at
223 early or late embryonic stages (continuous variables) were included as fixed effects in the full
224 model. To test for G×E, TGP×Environment and G×TGP interactions, the interactions between
225 clone and temperature, generation and temperature, and clone and generation were also included.
226 Replicate number (15 animals representing all clones and temperatures, within a generation,
227 were tested simultaneously) was included as a random effect (categorical variable).

228 For the main somatic growth analyses (investigating TGP effects), fixed effects in the full
229 model included the number of generations, clone identity (both categorical), as well as the
230 interaction between these two variables. As we were unable to start growth experiments for all
231 individuals on the exact same day, experiment start date (categorical variable) was tested as a
232 random effect. Within-clutch correlations were also analysed, using clutch ID as a random
233 intercept. For the experiment at 22 ºC, initial analyses suggested a bimodal distribution of growth
234 rates which corresponded to the distributions of age at maturity (5 vs. 6 days, mean 5.25, SE
235 0.46). Thus, considerable noise appeared to be due to an insufficient frequency of maturity
236 checks at this temperature. Since all clones and generations were represented among both age
237 classes (5 vs. 6 days), we included the number of days over which growth was measured as a
238 fixed effect for this temperature treatment. For the subsequent 12 ºC experiment the maturity

239 status was checked more regularly, and in addition the range of ages at maturity was
240 considerably larger (10-17 days, mean 13.2, SE 1.2 days). Thus, in this treatment no such effects
241 were noticed.

242 In the separate growth experiment at 17 ºC (investigating additive maternal effects), the
243 number of generations-variable was replaced with maternal temperature (categorical).
244 Experimental start date was similarly treated as a random effect. However, clutch ID was in this
245 experiment not used as a random effect for the maternal effect experiment, as only 1-2
246 individual(s) represented a given clutch. Maturation status was checked 2-3 times daily and
247 growth showed no signs of statistical noise due to maturation time, suggesting that it was not too
248 infrequent.

249 All statistical analyses were done in the statistical software *R,* v. 3.2.1 [33]. Linear mixed
250 models were fitted using the *lme4* package [34, 35]. Models that included significant random
251 effects had estimates fitted with restricted maximum likelihood (REML). Model selection was
252 conducted using a backwards selection procedure where variables were removed sequentially
253 until no further simplification could be made without causing a significant decrease in log-
254 likelihood (i.e. log-likelihood ratio test, [35], where the degrees of freedom were estimated by
255 Satterthwaite approximation using the *lmerTest* package [36]). Thermal autocorrelation analysis
256 of the study site was analysed using the *acf* package in *R*, using daily mean temperatures from
257 the summer of 2016 (see supplementary material S2 for correlogram). For details regarding this
258 analysis please see [37, 38].

259 **Results**

260 *Metabolism*

261 Overall across all generations, animals living at a lower temperature had up-regulated their
262 metabolism (mean $sVO_2 = 0.201$ µg O_2 h^{-1}, SE 0.007), and animals living at higher temperatures
263 had down regulated their metabolism (mean $sVO_2 = 0.142$ µg O_2 h^{-1}, SE 0.007), compared to the
264 control group being maintained at 17 °C (mean $sVO_2 = 0.162$ µg O_2 h^{-1}, SE 0.007).

265 When analysing metabolic rate in individuals from new temperatures relative to that of
266 their counterparts being kept at their original temperature (rVO2), the random effect of replicate
267 number could not be removed from the model without causing a significant decrease in log-
268 likelihood ($p < 0.001$). All interactions tested for could be removed (generation × temperature p
269 $= 0.23$, generation × clone $p = 0.09$, clone × temperature $p = 0.16$, supplementary material S3).
270 The effect of rearing temperature was significant ($p < 0.001$), whereas the number of generations
271 kept at the new temperature had no effect ($p = 0.73$). Thus, adjustments of metabolic rate
272 occurred during the first generation in a new thermal environment but then remained stable in
273 subsequent generations (Fig. 2a). The final model also included a significant positive effect of
274 the number of eggs at a late development stage ($p < 0.001$), increasing the oxygen consumption
275 by 0.010 µg O2 $hour^{-1}$ egg^{-1} (SE 0.001). There was no such effect of number of eggs at an early
276 development stage ($p = 0.14$). Finally, there was a minor significant effect of clone identity ($p <$
277 0.05, Fig. 2b).

278

279 *Growth, TGP*

280 In the growth data for animals experiencing 12 °C, there was no significant effect from using
281 clutch ID as a random effect ($p \sim 1$), whereas experiment start date effects were significant ($p <$
282 0.001). No significant interaction occurred between clones and TGP effects ($p = 0.12$). There
283 was no general response from TGP, with approximately equal growth in all generations (Fig. 3a,
284 $p = 0.55$). The different clones showed significant difference in growth at 12 °C (Fig. 3b, $p <$
285 0.01). At 22 °C, the effect of clutch ID was significant ($p < 0.001$), as was the effect of
286 experimental start date ($p < 0.01$). The interaction between clone and generation number did not
287 show a significant influence on the growth ($p = 0.32$). Genetic differences and TGP were absent,
288 as neither the generation number variable nor clone variable proved significant in the final
289 models (Fig. 3c & 3d, $p = 0.10$ and $p = 0.12$, respectively).

290

291 *Growth, maternal effects*

292 Temporal laboratory fluctuations (experiment replicate number, see *Methods*) were also present
293 in the growth experiment at 17 ºC ($p < 0.001$). As in the analyses of growth at 12 and 22 °C,
294 animals grew equally at 17 °C regardless of maternal temperature (Fig. 4a, $p = 0.46$). There were
295 no differences among the clones in growth (Fig. 4b, $p =0.65$) at 17 ºC, nor any significant
296 interaction between clone and maternal temperature ($p = 0.28$).

297 Discussion

298 In the present study, we tested for thermal TGP responses in *Daphnia*. We found no evidence for
299 such effects, neither for metabolic rate nor for somatic growth rate. For the metabolic rates, up-
300 and down-regulation was observed for the low and high temperature treatments, respectively.
301 These patterns are consistent with the cold-adaptation hypothesis, whereby ectothermic

302 organisms living in cold environments are expected to up-regulate their metabolism relative to
303 those living in warmer environments when tested at a common temperature. This represents a
304 mechanism to counteract the direct passive effect of reduced temperature on their ability to feed,
305 grow and reproduce [16, 17]. However, the patterns were consistent for all three generations,
306 such that the level of up- or down-regulation was just as great for the first generation (having
307 experienced only within-generation plasticity) as for the two subsequent generations (where
308 additional TGP effects would be expected). Thus, the full possible extent of such adaptation was
309 obtained through acclimation occurring during post-ovulation egg development and/or the
310 juvenile stage. Consistent with this, we observed no tendency for increased somatic growth over
311 the three generations when reared at new temperatures. Finally, we found no evidence for effects
312 of temperatures experienced by mothers on the growth rate of their offspring. The negative
313 results with respect to a TGP response was therefore not caused by counteracting additive
314 maternal effects. Since our measure of somatic growth rates is highly correlated with fitness (i.e.
315 population growth rate r [26]), this also suggests an absence of thermal TGP responses in traits
316 other than metabolic rate that would provide fitness benefits.

317 Our results are inconsistent with previous studies reporting the presence of thermal TGP
318 responses in animals [e.g. 22-24, 39]. One potential explanation of this discrepancy could be a
319 difference in thermal autocorrelation patterns between study systems. One obvious prerequisite
320 for expecting the evolution of TGP as an adaptive mechanism is that the environment
321 experienced by mothers has some predictive value for the environments their offspring will
322 encounter. In Lake Asklundvatnet the water temperature on a given day is significantly
323 positively correlated within a lag of up to 12 days, which is a period that exceeds the age of first
324 reproduction during considerable periods of the summer (maturation time 5-14 days between 12

325 and 22 °C, electronic supplementary material S1). Thus, the thermal properties of our study
326 system are similar to the temperature autocorrelation patterns observed in other study systems,
327 both where thermal TGP in ectotherms is shown to be present [40] and absent [38]. Both these
328 marine study systems showed significant autocorrelation in temperature for 9 and 15 days,
329 respectively, which were relevant biological timescales for the ectotherms studies.

330 In attempting to explain the discrepancies between our results and previous studies
331 demonstrating thermal TGP we suggest that one should consider the potential for confounding
332 effects that may arise in tests of thermal TGP. The suggested confounding effects are not
333 highlighted as to deny the existence of TGP, but intend to exemplify certain issues with
334 commonly applied study designs in this field. This is necessary to improve the ability of future
335 studies to disentangle TGP effects from other sources of variance. First, when animals are moved
336 back and forth between temperatures to test for interactive effects, focal traits could be measured
337 during different temporal periods due to logistical reasons. When measurements are disjointed on
338 a temporal scale, significant differences might appear between treatments due to laboratory
339 fluctuations [this study, 38]. Second, for highly fecund sexually reproducing organisms (such as
340 those used in [3, 22]) there is often extensive mortality either at birth or through life (e.g. ~50-
341 75% in [3], 60-90% in [40]), and/or individuals might fail to mature and/or reproduce at certain
342 temperatures [24]. Even if some members within each family survive and reproduce, it will
343 remain unknown to what extent genetic composition among treatment groups and generations
344 remain constant. For clonally reproducing organisms, effects of selective mortality/breeding can
345 usually be excluded. To some degree, one may also exclude genetic changes in strains of
346 sexually reproducing organisms that are fully homozygous (e.g. *Arabidopsis thaliana* [41]).
347 Finally, studies that aim to apply a certain environmental treatment to the parental generation

348 may inadvertently expose early stages of the offspring generation to the same treatment. This
349 may be particularly important for organisms with short life cycles. For example, for studies of
350 *Daphnia*, a period of 24h post-birth may represent ~20% of the juvenile life span (and 100% of
351 the embryonic development). Yet, it is not uncommon for experimental designs to allow F1
352 offspring periods of 12-24 h following birth in the same environment as the parental generation
353 before transferring the offspring to the new environment, and then use the comparison of
354 phenotypes of F1 from different parental environments as a test of TGP. This design has recently
355 been used to show thermal TGP in growth/fitness for *Daphnia* [23]. However, an alternative
356 explanation could be that poikilothermic individuals having completed their egg development as
357 well as the initial period after birth at the same temperature as their mothers perform best at that
358 temperature due to within-generation phenotypic plasticity. Late transfer of offspring and within-
359 generation phenotypic plasticity could also potentially confound other studies that investigate the
360 role of TGP in asexual animals with respect to environmental variables such as food
361 concentration [42, 43], pathogen abundance [43-45], exposure to toxic substances [46], predator
362 presence [47] and temperature and salinity [48].

363 With our approach, where we transferred the parental generation to contrasting
364 environments shortly after ovulation of eggs (rather than transferring their offspring at birth), and
365 tested for changes in phenotypes of clones over the subsequent generations, we minimize the
366 confounding effects identified above. To our knowledge, only in two previous studies have
367 "pregnant" *Daphnia,* instead of neonates, been introduced into a new environment to study TGP.
368 These studies show TGP effects of maternal predation exposure on offspring defensive
369 morphology[49], and maternal food and photoperiod exposure on *ephippia* production by
370 offspring [50]. Thus, there seem to be a clear potential for evolution of adaptive TGP in

371 *Daphnia*. The fact that we fail to find any differences among generations in the present study
372 suggests that thermal TGP responses to maternal or grand maternal temperature are weak or
373 absent with the given experimental settings. Within-generation phenotypic responses to
374 temperature (e.g. metabolic rate [this study], haemoglobin concentrations [51]) could be
375 sufficiently rapid, and the costs of responding rapidly sufficiently low, so that any additional
376 TGP responses would be of marginal adaptive value.

377 In conclusion, our study provides strong evidence for within-generation phenotypic
378 plasticity in *Daphnia* metabolic rates as a response to temperature regimes, but no indication that
379 thermal TGP plays a role in improving fitness under changing temperatures. We suggest that
380 future studies of thermal TGP should use designs that clearly separate between these two sources
381 of variation, as well as avoiding effects of selective mortality, and caution that failing to do so
382 may lead to an overly optimistic view on the ability of organisms to adapt to changing climates
383 through TGP.

384 **Competing interests**

385 The authors have no conflicting interests to declare.

386 **Data accessibility**

387 Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.fp00p

388 **Author contributions**

389 ØK participated in the study design, method development, animal husbandry, experimental
390 procedures, data and statistical analysis and writing the manuscript. CB helped with the study
391 design, method development, statistical analysis and manuscript revisions. SE initiated the
392 project, developed the study design and methods, revised the manuscript and participated in the
393 data and statistical analysis. All authors gave final approval for publication

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401

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530

531 **Figure captions**

532 **Figure 1 Schematic representation of the experimental design. Metabolic rate (at 17 ºC)**
533 **and growth (at 12 and 22 ºC) were measured for 440 and 368 animals, respectively. Five**
534 **unique clones of *D. pulex* were represented in each temperature treatment, and represented**
535 **approximately equally at all factorial levels. This number of levels and animals yields ~10**
536 **inds. clone^{-1} generation^{-1} temperature^{-1} for metabolism measurements and ~13 inds. clone^{-1}**
537 **generation^{-1} temperature^{-1} for growth measurements. Online version in colour.**

538 **Figure 2 Size-standardized metabolic rate (rVO_2, mean ± SE, measured at 17 ºC) of *D.***
539 ***pulex* reared at two new temperatures for one, two or three generations relative to controls**
540 **being maintained at the original temperature (17 ºC). Deviations from the zero baselines**
541 **thus indicate an up- (positive values) or down-regulation in VO_2. (a) Generation-specific**
542 **mean rVO_2 across all clones (b) Clone-specific rVO_2 means across all generations**
543

544 **Figure 3 Mean (± SE) growth until maturity (G, eq. 3) in *D. pulex*. (a) and (b): Growth, in**
545 **the 1st and two subsequent generations after being introduced to new thermal**
546 **environments; 12 ºC (a) and 22 ºC (b), with n = 205 and n = 162 animals, respectively. The**
547 **ancestors had reproduced asexually for 12-15 months at an original temperature (17 ºC)**
548 **prior to 1st generation. (c) and (d): Growth for the different clones at 12 ºC (c) and 22 ºC**
549 **(d).**
550

551 **Figure 4**
552 **Mean (± SE) growth until maturity (G, eq. 3) in *D. pulex* at 17 ºC (total n = 327 inds.; (a):**
553 **77-128 inds. temperature^{-1}; (b): 58-71 inds clone^{-1}). The maternal generation (n = 226) were**

554 **all conceived at 17 ºC, but spent their life (including embryogenesis) at different maternal**

555 **temperatures.**

556