Improving our understanding of adaptation by addressing multi-generational non-genetic responses

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41 **Abstract**

42 Populations that face abrupt environmental change reducing their fitness can recover by
43 adaptive genetic evolution over tens to hundreds of generations, but their immediate
44 responses often involve non-genetic mechanisms. When such non-genetic responses span
45 multiple generations, their dynamics may be difficult to distinguish from those of genetic
46 evolution. We here argue that focusing research on such multi-generational non-genetic
47 responses (MGNGR) should be crucial to better understand and predict eco-evolutionary
48 responses to environmental stress. We survey the most salient forms of MGNGR (delayed
49 impact of stress, transgenerational plasticity, and priming), with a focus on how they may
50 impact the dynamics of observed phenotypic change across multiple generations. Analysing
51 the rate, stability, and reversibility of MGNGR, as well as their relative contributions to overall
52 phenotypic responses, and their interactions with genetic changes, should be particularly
53 fruitful towards a more comprehensive deciphering of evolutionary responses to novel or
54 changing environments.

55 **1) Population responses to stressful environments**

56 Understanding how populations respond to environmental change with detrimental impacts 57 on biological function and fitness is a critical goal of basic research in ecology and evolution 58 [1–3], with important applied consequences for conservation, global change research, human 59 health and agriculture[4]. The two main processes allowing organisms to cope with 60 environmental challenges *in situ* (i.e., without dispersing) are phenotypic plasticity, the 61 expression by one genotype of different phenotypes in different environments[5], and genetic 62 evolution, the increase in frequency of beneficial mutations in a population through natural 63 selection. These processes are usually thought to occur over clearly distinguishable 64 timescales, with plasticity taking place within generations, while genetic evolution unfolds 65 across generations. This assumed timescale separation has consequences for predicted 66 responses to environmental stress at the phenotypic and demographic levels. On the one 67 hand, an immediate plastic response, if adaptive, can limit the initial reduction of fitness - and 68 potentially population size - in a novel, stressful environment. On the other hand, adaptive 69 genetic evolution acting on new mutations, standing genetic variation, or a mixture of both[6], 70 leads to gradual changes in traits and fitness that accrue over generations, and might even 71 prevent extinction of a declining population if it happens sufficiently fast[7], a phenomenon 72 known as evolutionary rescue[8]. Such a clear-cut timescale difference between plasticity and 73 evolution, when true, does not preclude interactions among them. For instance, adaptive 74 plasticity may shield phenotypes from selection, reducing the efficiency of the following 75 adaptive genetic evolution[9]. Conversely, non-adaptive or maladaptive plasticity can be the 76 main driver of compensatory evolutionary changes[10,11]. Other effects involve interactions 77 with demography. For instance, adaptive plasticity can allow populations to persist over the 78 first few generations of exposure to environmental stress, until natural selection "takes over" 79 to rescue the population, a phenomenon often described as the Baldwin effect[12].

80 Nevertheless, this timescale separation between plasticity and genetic evolution, although 81 conceptually useful, remains an oversimplified representation of an organism's set of 82 responses to environmental changes. Non-genetic inheritance (NGI)[13,14], defined as any form 83 of inheritance that does not rely on DNA, blurs this separation line, by allowing phenotypic 84 variation (including that induced by the environment) to spill-over from one generation to the 85 next. Transgenerational plasticity (TGP), whereby trait expression depends on the 86 environments experienced by the previous generations, is also increasingly recognized as an 87 important biological phenomenon. Bell & Hellmann[15] recently proposed a useful framework 88 to study such responses, reporting evidence for six different patterns, including bounce-back 89 (visible only in F1 generations) or persistent effects (still visible several generations after 90 stress exposure). Using a more systematic approach, Yin et al.[16] conducted a meta-analysis 91 on TGP, showing their potentially fundamental role in responses to changing environmental 92 conditions across a diversity of taxa (for a more adaptive perspective see[17]).

93 While NGI and TGP are now well-accepted phenomena, their prevalence and contribution to
94 the dynamics of environmental stress responses remain underappreciated. There is
95 accumulating evidence that a diversity of mechanisms can transmit effects of environmental
96 stress across generations in plants, unicellular eukaryotes, and animals[18–20]. Histone
97 modification, patterns of DNA methylation, and the transmission of non-coding RNAs, are
98 some of the well-elucidated mechanisms[21–25]. But NGI may be even more prevalent in
99 unicellular organism, where the lack of a soma/germline divide (at the notable exception of
100 ciliates, see below) means that many aspects of their phenotype, including proteins, gene
101 regulatory factors and epigenetic modifications, are indeed directly transmitted to their
102 descendants over multiple generations[20]. In *Escherichia coli* for instance, the average
103 protein's half-life (~20 hours) is much longer than its generation time of ~20 minutes under
104 ideal conditions[26], and even that estimated under natural conditions[27]. Similarly, the half-life
105 of mRNAs in bacteria is often on a similar order of magnitude as the generation time in ideal
106 conditions[28]. Gene overexpression in the yeast *Saccharomyces cerevisiae* occurs at least 1h
107 after a heat shock event, which overlaps with its doubling time of approximately 90 minutes[29].
108 And in the green microalga *Chlamydomonas reinhardtii*, synthesis of new proteins and lipids
109 in response to shifting temperature can take 24 hours[30], for a generation time of 14h-36h
110 depending on light and temperature conditions[31].

111 Non-genetic responses to environmental stress can thus span multiple generations, during
112 which they can accumulate gradually, or decay/revert, depending on their mechanism. In
113 other words, they can exhibit dynamics similar to those of rapid genetic evolution (Fig. 1),
114 making these alternatives difficult to distinguish based solely on observations of changes in
115 phenotypes and fitness. To emphasize this similarity of timescale with evolutionary change,
116 we describe any form of NGI and TGP with dynamics that span multiple generations as multi-
117 generational non-genetic responses (MGNGR). We argue that dismissing the temporal
118 dynamics of such MGNGR (whether adaptive or not) and their contribution to fitness, and not
119 clearly distinguishing them from the effects of genetic changes, is likely to limit our ability to
120 infer and predict population responses to changing environments, especially in long-term
121 experimental evolution and common garden experiments. We therefore suggest making
122 MGNGR a study object of their own, by considering them explicitly while designing
123 experiments, deciphering how they interact with adaptive genetic evolution, and how they
124 themselves evolve, in order to improve our understanding of eco-evolutionary dynamics in
125 changing environments.

126

127 2) Major types of multi-generational non-genetic responses (MGNGR)

128 We are interested in population responses to an environmental change that initially causes
129 substantial maladaptation, manifested by a decrease in mean fitness. Unless explicitly stated,
130 we are considering constant conditions following this initial environmental change. The
131 standard scenario for adaptive genetic evolution to such abrupt environmental changes posits

that fitness first declines sharply because of environmental stress, then slowly increases again (Fig. 1 middle), through the establishment and rise in frequency of beneficial mutations (Fig. 1 top). However, different forms of MGNGR might alter the dynamics of adaptive evolution, while closely resembling the genetic mechanisms. We focus below on three main categories that are particularly relevant in this context.

2.1 Delayed impact of stress: time-to-response

The detrimental impact of environmental change on fitness may be delayed, and only manifest some generations after exposure to the stressor(s). Delayed impact of stress may be largely passive, due to purely mechanical or physical reasons that do not involve any specifically evolved response. For example, toxic or harmful molecules (*e.g.*, pesticides) can accumulate passively by slowly permeating into cells, and only start to have measurable detrimental impacts once a tolerance threshold is crossed for the affected cellular functions (*e.g.*, DNA replication, protein synthesis/folding, cell metabolism or structure). This has been shown in *Drosophila melanogaster,* where exposure to silver nanoparticles only started to induce reproductive costs from generation F2 onwards, and these costs increased in the following generations[32]. This was due to the accumulation of oxidative stress and the upregulation of heat shock protein 70, which reduced flies' investment in reproduction, and thereby fecundity. Similarly, nanoparticle exposure of *Caenorhabditis elegans* impaired germ cells, leading to reproductive abnormalities and fecundity reduction only in the F2 generation[33]. Another possible cause of passive delay is when a stressor only acts at a specific stage in the life cycle. For instance, some antibiotics only target newly formed cell membranes[34], such that they cannot express their detrimental effect until cell division occurs.

Alternatively, delayed detrimental impacts of stress could occur because specific mechanisms for coping against stressors cannot be sustained for long, such that their eventual collapse affects survival or reproduction. For instance, specific enzymes, from heat shock proteins to molecular pumps, have intrinsic biophysical and biochemical limits, beyond which they cannot operate[35]. The collapse of tolerance mechanisms could also be mediated by the resources needed to maintain the coping machinery[36,37]. In other words, delays in the onset of stress impacts may be tightly related to the permanency and stability of immediate stress response mechanisms (which we address below). The dynamics of this form of MGNGR will resemble baseline expectations for a fitness decline following environmental changes, but the manifestation of detrimental effects will only start sometime after the stressor exposure (Fig. 2A, yellow line).

2.2 Speed and reversibility of TGP

There is increasing evidence for the importance of transgenerational plasticity (TGP) in the context of environmental change[38], both abiotic[39,40] and biotic[41,42], such as transgenerational acclimation responses to changing temperatures observed in fish[43] and microalgae[44]. Despite attempts to conceptualise TGP and its underlying mechanisms[15], a key aspect that remains understudied is its dynamics over multiple generations. Extending recent arguments about

171 within-generation plasticity[45,46], we suggest that it would be useful to quantify the whole
172 temporal dynamics of TGP, both within the first F1 generation and in the following ones.

173 An important temporal aspect to consider is the speed of trait change over generations that
174 follow exposure to environmental change, that is, the rate of TGP. This rate is likely to affect
175 short- and long-term responses to stress, and is thus critical to understand and predict when
176 the dynamics of MGNGR is likely to mimic those of adaptation through genetic evolution (Fig.
177 2B orange line). In a simple scenario, we can envisage that following environmental change,
178 the trait changes over generations, until reaching a plateau of stationary trait expression
179 (Fig.3A-B grey line). Populations with a faster rate of TGP (before the plateau) should have
180 faster fitness increase in face of the stressors, and recover more quickly from the initial
181 detrimental impacts of environmental stress, without any genetic evolution. The maximum
182 magnitude of transgenerational plasticity (at the phenotypic plateau), or TGP capacity, in turn
183 determines to what extent genetic evolution is needed at all for adaptation.

184 Another critical aspect of TGP is the stability (or stationarity) and reversibility of responses.
185 Let us first focus on the baseline scenario illustrated in Fig. 3A (E1), where stress exposure is
186 sustained for all generations following the initial environmental change. In this context, a
187 critical question is whether the phenotypic response can be sustained at a phenotypic plateau
188 (stable response), or instead is only transient, and eventually reverts back towards its initial
189 value after some generations of exposure. For instance, it was shown that the pea aphid
190 *Acyrthosiphon pisum* can mount stable TGP responses under persistent ladybird (*Harmonia*
191 *axyridis*) predation across generations, by increasing the production of winged morphs in the
192 progeny compared to unexposed populations[47]. Specifically, after a first high increase in
193 frequency (~75%) at the second generation, winged morphs production then stabilised to a
194 ~45% frequency over 25 generations. Nonetheless, there are several reasons for which TGP
195 may not be sustained after many generations of exposure. First, responses might be costly to
196 sustain because they require important metabolic investments that trade off against other
197 functions of the organism, and/or lead to accumulating metabolic defects (such as free
198 radicals) over generations (which may contribute to the delayed impacts of stress described
199 above). Second, some responses may involve emergency mechanisms, such as heat shock
200 response to thermal stress[48], which last for a few generations before they are replaced by
201 more specific and durable physiological adjustments.

202 In scenarios where the environment changes again immediately or after a few generations,
203 for instance going back to its pre-stress value, the crucial question becomes whether - and
204 how fast - the phenotype can go back to its initial state, *i.e.* how reversible it is (Fig.3B). This
205 was empirically investigated in the yellow monkeyflower plant (*Mimulus guttatus*). The plants
206 increased trichome production in response to wounding that simulated insect damage. Such
207 response was stable for 3 generations in the absence of subsequent damage, before starting
208 to reverse, and eventually decrease to the level of control unwounded plants in generation 4
209 and 5[49]. Interestingly, the results also suggested that both parents contributed to the TGP

210 responses additively, but via different mechanism (maternal methylation *vs.* paternal histone
211 modifications). In the aforementioned study[47], the authors performed additional tests to
212 investigated the reversibility of aphid TGP response. At generation 3, 13, and 22 of the main
213 experiment, they established parallel aphid lines and followed for few generations the
214 production of winged morphs once the ladybird predator was removed. For one generation,
215 winged morph frequency remained high, but then dropped below, and finally converged to
216 the same frequency of the control lines. In contrast, a study on *Daphnia ambigua* showed the
217 absence of reversibility for TGP responses in life-history traits across generations after the
218 environmental stressor (predator cue) was removed[50]. Similarly, temperature-[51] and
219 olfactory-induced[52] TGP responses in *C. elegans* were stable for several generations after the
220 environmental stimulus was removed, respectively up to 14 and 40 generation in the two
221 studies. Differences in the speed of induction *vs.* reversibility of TGP for osmotolerance were
222 found in the unicellular alga *Dunaliella salina*[53]. Transferring populations across salinity levels
223 showed that increasing glycerol content (when going from low to high salinity) was much
224 slower than decreasing it (from high to low salinity), because the mechanisms involved are
225 completely different: increasing glycerol level requires synthesizing it through metabolism,
226 whereas decreasing it can be simply achieved by excretion. The asymmetry between molecule
227 synthesis vs excretion could be a common mechanism. Environmental perturbations can also
228 cause non-genetic changes in gene regulatory networks that are poorly reversible, as shown
229 in *E. coli*[54]. Beyond these examples, little is still known about how reversibility unfolds across
230 generations. Maintaining the machinery to sense the environment and revert the phenotype
231 is likely to have significant costs, as emphasized for within-generation plasticity[37], but
232 multigenerational reversibility also depends on how the mechanisms of NGI are affected by
233 patterns of environmental fluctuations among generations.

234 **<u>2.3 Trans-generational priming: memory of past responses</u>**

235 Another major class of MGNGR is trans-generational priming, whereby prior exposure of an
236 organism to a stressor (priming) can prepare its descendants to better respond to the same
237 or different stressors upon later exposure (triggering). Trans-generational priming therefore
238 occurs across cycles of stress/non-stress. For instance, the descendants of *E. coli* cells primed
239 with antimicrobial peptides (AMP) exhibited increased persistence when re-exposed to AMP
240 after some generations[55]. Similarly, the four- and five-generation descendants of
241 *Saccharomyces cerevisiae* originally primed with salt exhibited increased resistance to
242 hydrogen peroxide and faster gene expression. The salt priming in the parental generation
243 activated the synthesis of the long-lived cytosolic catalase Ctt1p, which was then propagated
244 through generations by NGI[56]. Exposure of *Arabidopsis thaliana* to caterpillar herbivory
245 primed the descendants for enhanced insect resistance for two generations, due to the
246 production of interfering RNAs[57]. Stress exposure can also prime *E. coli* and/or rewire the
247 regulatory network for several generations[54] without any immediate costs. In an extensive
248 review, which included both within- and trans-generational priming, the authors briefly
249 proposed some temporal scenarios for primed stress responses[58]. For example, the primed

250 organisms can respond faster or earlier, or even produce a stronger response with higher
251 amplitude than the non-primed counterparts upon triggering. Wesener & Tietjen[59] further
252 explored some of these temporal aspects, by modelling population performance under
253 different (trans-generational) priming and stress conditions associated with different costs.
254 Faster and earlier responses were favoured under short and severe/lethal stress, whereas
255 stronger responses were favoured for stress of longer durations. Nonetheless, empirical
256 studies are still limited, especially those with an evolutionary perspective. Understanding
257 whether trans-generational priming reduces the immediate impact of stress just upon next
258 exposure (light green line in Fig 2C), or instead leads to faster TGP in subsequent generations
259 (dark green line in Fig 2C), is of central importance to fully unravel the implications of this
260 process for population dynamics, fitness and evolutionary trajectories. In addition, it would
261 be necessary to measure for how many generations the trans-generational priming could last
262 in the absence of re-exposure to stress.

263 3) Empirical approaches to disentangle MGNGR from genetic responses

264 Because they lead to changes in fitness that unfold over multiple generations, the MGNGR
265 described above can phenomenologically resemble genetically based evolution. To avoid
266 reaching misleading conclusions about eco-evolutionary processes, it is therefore crucial to
267 identify MGNGR and measure their dynamics. We suggest below some approaches and ideas
268 to measure MGNGR while conducting preliminary assays, experimental evolution, and
269 common garden experiments.

270 3.1 Establishment of experimental protocol

271 Preliminary assays and pilot experiments are fundamental steps to optimise protocols,
272 laboratory conditions, and reduce undesirable technical noise or variation. In experiments on
273 stress responses, preliminary tests often serve to identify the levels of stressor (*e.g.*, dose
274 concentration or exposure time) that yield sufficiently strong detrimental effects to be clearly
275 detectable, without leading to rapid extinction of the population. Although it is a necessary
276 part of the scientific approach, this step may in some cases lead to discarding or biasing
277 relevant biological information and variation. For instance, a treatment level leading to rapid
278 population decline might be discarded because the stressor would be deemed too strong for
279 the envisaged protocol. However, longer observations (*e.g.*, over more cycles of batch culture
280 in a microbe) might show fitness recovery through MGNGR (Fig. 2B-C), such that the
281 treatment is actually compatible with long-term evolution. Conversely, a presumably
282 permissive treatment during a short preliminary test could turn out to represent stressful
283 conditions over the longer run, because of delayed impacts of the stressor (Fig. 2A). In both
284 cases, performing preliminary assays for a longer time (*i.e.*, over several cycles of batch
285 culture, or more generations), could allow thoroughly describing a stressor effect on fitness.

286 From a more applied perspective, these considerations are particularly relevant for
287 (eco)toxicological studies[60,61]. Typically, most assays are of short duration (often stopping
288 when any visible effect is observed), and thereby ignore the temporal and transgenerational

components of stress responses[62,63]. For instance, descendants from primed populations might have stronger or faster responses upon exposure to the same or another stressor. Another shortcoming is that these tests often focus on survival without measuring sublethal effects, or other fundamental fitness components such as growth and reproduction over generations[64]. Overall, considering non-genetic aspects by *(i)* implementing longer assays (including across generations), *(ii)* sampling at regular intervals to tackle short-term dynamics, and *(iii)* measuring additional traits, might directly improve risk assessment and policy making in such eco-toxicological studies.

3.2 Experimental evolution

Experimental evolution is a powerful and versatile research approach to test predictions and study eco-evolutionary dynamics and their underlying mechanisms in real time under controlled conditions. Repeated phenotypic assays and measurements over generations allow tracking the dynamics of change in fitness and other traits of interest. However, how MGNGR might act during these long-term experiments and influence their outcomes is still too rarely considered.

A first step towards better inclusion of MGNGR involves simple order of magnitude computations, to assess whether the timescales of observed changes are consistent with genetic evolution in the considered biological system. Beyond differences between model systems, expectations will differ depending on specificities of the experiment, for instance whether genetic evolution has to involve *de novo* mutations (when starting from isogenic populations), or can occur via standing genetic variation[6]. Very rapid fitness dynamics taking place over a few generations are more likely to involve MGNGR than genetic evolution, unless selection is extremely strong and acts on a genetically diverse population. Making these arguments more quantitative requires reliable knowledge about mutation rates, levels of standing genetic variation, and distribution of fitness effects in the studied organism in response to the investigated stressor.

Another quantitative aspect to consider is the repeatability of observed responses. Because of stochasticity in the origin of mutations and random genetic drift, genetic responses to selection are not expected to occur exactly at the same time and with identical effects (*e.g.* speed of fitness recovery) in different replicated lines, such that high repeatability of responses is more consistent with MGNGR. However, this argument should be used with caution. Highly polygenic traits could have highly repeatable evolutionary responses at the phenotypic level, despite being underlaid by very unpredictable genetic changes because of high redundancy in their genetic architecture[65,66]. Furthermore, the (possibly transient) presence of hypermutator strains, mutation hotspots, or any other sources of mutation bias, could increase the repeatability of evolutionary outcomes even at the molecular level[67,68].

The relative contributions of MGNGR *vs.* allele frequency changes to phenotypic changes at different timescales can be investigated by using a combination of omics analyses (*e.g.*,

327 transcriptomics and genomics *vs.* epigenomics) with phenotypic assays over time. In practice,
328 this would require assessing the genetic and epigenetic composition of the population at
329 many timesteps, to track the relative frequencies of genomes and epigenomes, together with
330 phenotypic traits and/or fitness. Nevertheless, even when simultaneous genomic and
331 phenotypic change is observed, showing that the former explains the latter is challenging
332 when only using population-based measurements. More progress can be achieved by
333 isolating genotypes, for instance by creating clonal populations for microbes, before
334 phenotyping them. However even when this is feasible, deciphering the genotype-phenotype
335 map remains extremely difficult[69,70], as it requires more resolution (*e.g.* low linkage
336 disequilibrium) than is typically available in experimental evolution designs. Introducing a
337 mutation of interest in the ancestral background to isolate its effect (reverse genetics) is
338 another possible approach with model bacteria. Yet, this remains labour intensive, and the
339 dynamics are intractable without reducing and precisely controlling the number of mutations
340 to test.

341 In addition to jointly tracking genetic and phenotypic change over time, more insights on the
342 role of MGNGR in experimental evolution can be achieved by performing more complete
343 assays, beyond the conditions from the evolution experiment. In particular, sampling lines at
344 several points during experimental evolution, and re-exposing them to transfers from control
345 to stress conditions, would help identify whether the initial response in the experiment was
346 mediated by genetic or non-genetic mechanisms. Nonetheless, this approach would already
347 require somewhat precise knowledge about the speed of reversibility (see above) of the
348 response of interest in the model system. This would also imply some underlying knowledge
349 of how many generations the study organisms should be maintained in control before
350 switching back to stress conditions.

351 **3.3 Common-garden experiments**

352 Common garden and transplant experiments are standard protocols to evaluate whether
353 populations from different experimental or natural origins show patterns of local genetic
354 adaptation. By placing individuals from different origins in the same environmental
355 condition(s), environmental and non-genetic effects are expected to be removed, thus
356 quantifying the genetic basis of trait variation. Typically, this procedure allows controlling for
357 the potential misleading outcomes due to parental effects (maternal and paternal), which
358 have been recognised for a long time[71,72]. However, controlling for one generation (parental)
359 might not be enough. As emphasised above, transgenerational effects are common, and they
360 might lead to wrong inference even for well-designed studies that only control for parental
361 effects. The extent to which MGNGR have to be accounted for, and consequently the required
362 number of generations of common garden, depend on the question asked and the organism.
363 The duration of the common garden should account for the possibility of long MGNGR when
364 these are suspected, while limiting the opportunity for *de novo* mutations to arise.

365 A productive way forward would be to systematically measure the dynamics of traits during
366 the common garden phase. In particular, transferring an organism to standardised laboratory
367 conditions might represent a complex set of changing environmental conditions *per se*, which
368 could trigger undesired MGNGR. In fact, due to historical contingency, individuals or lines
369 from different localities or treatments might react differently to the common garden, leading
370 to a confusion between genetic adaptation and transgenerational effects. Priming might be
371 the most problematic MGNGR in this context, as it could lead to phenotypic effects in
372 responses to environments that differ from those where populations have been sampled.
373 Priming could be particularly difficult to distinguish from the consequences of recent
374 evolutionary history of stress exposure, *i.e.*, the "ghost of selection past"[73], making the explicit
375 study of the timing of adaptation through multiple generations particularly important in eco-
376 evolutionary studies.

377 Here again, more progress can be made by explicitly addressing MGNGR. For instance,
378 samples can be transferred from different natural environments (or evolutionary treatments)
379 to control condition / common garden, and then back from control to treatment, to measure
380 the rate of phenotypic change in response to these environmental changes. Additionally,
381 changing the environment gradually *vs*. abruptly (or modifying stress intensity and duration)
382 could highlight differences in transient dynamics and potential costs.

383 **4) The evolution of transgenerational effects**

384 Although the main accepted mechanism of adaptive evolution (at least in the long run) is
385 change in allele frequency by natural selection[74], MGNGR can lead to dynamics of phenotypic
386 change over generations that may mimic patterns of genetic evolution. In addition, MGNGR
387 can produce variation on which selection may act, and may themselves vary genetically, and
388 thus evolve. We propose some promising basic research questions on the evolutionary
389 consequences of MGNGR, to hopefully motivate more theoretical and empirical studies and
390 stimulate further discussion.

391 A first critical question towards understanding evolution of MGNGR is how selection operates
392 on them. We still know little about which pattern of environmental change favours each type
393 of response and why, but theory has started exploring this problem[14,75]. Furrow & Feldman[76]
394 found that slow temporal environmental fluctuations can lead to the evolution of more
395 faithfully transmitted transgenerational effects (and conversely), providing that their
396 underlying mechanisms entail little costs (see also[77]). Similarly, other mathematical models
397 showed that transgenerational effects can rapidly evolve, depending on the accuracy of the
398 environmental stressor as a predictor of future (strong) selective pressures[78,79]. More
399 recently, a population-genetic model of two habitats interconnected by dispersal found that
400 adaptive transgenerational effects were likely to evolve under moderate dispersal, and when
401 the direction of selection differed between habitats[80]. However, to our knowledge little
402 attention has still been given to the evolution of dynamic aspects of MGNGR, such as the rate

of TGP, the stability and reversibility of responses across generations, or the duration of priming.

Another fundamental question is the genetic basis and heritability of these processes. In fact, although field work has shown the importance and contribution of transgenerational mechanisms in generating phenotypic variation in natural populations[81], these studies remain mainly correlative and it remains unclear whether transgenerational effects are genetically encoded. Nonetheless, there is growing evidence that epigenetic variation is genotype dependent. In *Arabidopsis thaliana*, the disruption of methylation-sensing gene regulatory circuit in engineered mutant plants caused genome-wide methylation losses, which ultimately led to abnormal phenotypes that worsened across generations[82]. These results highlight the presence of genetic basis for stable and long-term epigenetic inheritance, and confirm previous findings in the same plant species suggesting the presence of genetic control on epigenetic marks[83]. Analysis from two publicly available data sets in humans (247 sequenced) further determined that the methylation patterns were likely under the control of DNA sequence[84]. Supporting the idea that transgenerational effects can evolve by natural selection, laboratory experiments demonstrated the presence of genetic variation for such processes. For instance, genotypic-specific TGP responses to temperature was found for several phenotypic traits in *A. thaliana*[85]. Similarly, it was shown that in the ciliate *Tetrahymena thermophila* the TGP of traits related to dispersal was determined by their genotypes[86].

Beyond the evolution of transgenerational effects, a key question is how they influence “standard” genetic evolution. First, heritable non-genetic phenotypic changes can mask genotypic variation from selection, thereby modifying evolutionary trajectories[20]. In addition, some mechanisms of non-genetic inheritance can interact with the origination of genetic variation. In particular DNA methylation, by influencing mutation rate and transposon insertion, can affect genome stability, and therefore directly contribute to DNA sequence evolution[87,88]. These combined influences of epigenetics on selection and mutation could lead to potentially strong positive effects on adaptive evolution, opposite to the abovementioned buffering hypothesis. Such interactions between transgenerational effects and genetic evolution have been investigated in a few experimental studies. An evolutionary experiment with an engineered strain of *Saccharomyces cerevisiae* showed that transgenerational effects can modify rates of evolutionary adaptation[89]. Intermediate levels of transgenerational gene silencing of the URA3 gene locus, responsible for the production of uracil (an essential component for cell growth), enabled better survival and faster adaptation to a novel environment[89]. This occurred because transgenerational silencing increased the effective population size, thereby facilitating the appearance of new mutational targets and alleles that could accelerate adaptation. Furthermore, the transgenerational gene silencing was rendered more stable and strongly heritable by the novel alleles introduced by mutation. Using the same strains, the authors additionally showed that transgenerational gene silencing provided an adaptive advantage under fluctuation regimes[90]. In another study, populations of the

unicellular green alga *Chlamydomonas reinhardtii* were evolved in three different environments (high salt, low phosphate, and high CO_2) for two hundred asexual generations[91]. The populations genetically adapted in all environments and increased their fitness. The same treatment was additionally applied to algal populations in which the authors genetically and chemically reduced the amount of non-genetic variation produced and transmitted. Decreasing non-genetic variation reduced or impeded adaptation to the high salt and CO_2 environments, but had little role in adaptation to low phosphate. Overall, these results highlight the role of transgenerational effects in adaptive evolution, and how this might depend on the environmental context. The consequences on non-genetic inheritance may even scale up to the macro-evolutionary scale. For instance, epigenetic variation is a good predictor of behavioural isolation and divergence in the fish genus *Etheostoma*[92], and may thus influence speciation, consistent with conceptual and theoretical findings[93–95].

Interestingly, differences between MGNGR and genetic adaptation might sometimes not be fundamentally clearcut, even conceptually (not only experimentally), and cases exist where MGNGR and genetic adaptation mechanistically cross each other. Ciliates, unicellular eukaryotes characterised by nuclear dimorphism (germline and somatic), are an extraordinary example in which TGP might actually occur through genetic mechanisms. In brief, genetic mutations can occur in both their nuclei, but during sexual reproduction the somatic nucleus is lost, with (almost) no transfer to descendent cells. Thus, genetic evolution in the somatic nucleus can be considered a form of TGP at the scale of their sexual generations[96].

We suggest that a productive future line of research would be to investigate how the dynamics of MGNGR influence their effects on genetic evolution. For instance, MGNGR that are both rapidly induced and stable through time could be expected to have more long-lasting influences on genetic evolution. This could be investigated by manipulating the dynamics of MGNGR through engineering where feasible[91], or mathematical modelling[97,98]. The development of new theoretical work could help refine predictions and expectations , or even propose novel mechanisms. For instance, a recent model simulating gene silencing/activation *via* DNA-methylation and de-methylation demonstrated that epigenetic mutations could enable the evolution of phenotypic plasticity[99]. In fact, the simulations showed that when such epigenetic mechanisms are genetically encoded, they can favour the evolution of phenotypic plasticity, particularly under periodically changing environments. Extending similar models to allow for epigenetic inheritance would allow investigating how transgenerational effects, possibly accumulating over generations, evolve and interact with evolution of purely genetic effects. The development of such theory would guide future empirical work on these questions, by suggesting experimental design strategies, and informing on specific ecological conditions favouring each of these phenomena.

5) Concluding remarks

Although their relevance for adaptation is still being debated[74], NGI and TGP are an integral part of population responses to environmental change[14,20,38]. Evidence is mounting that such responses are not only repeatable and widespread, but also can span multiple generations (which we describe as MGNGR), and thus take place over similar timescales as rapid genetic evolutionary responses. Modern techniques allowing for precise in-depth investigations of the underlying mechanisms are now available to go a step further in our comprehension of the many forms of MGNGR, provided we make them an object of study rather than a mere nuisance parameter. Here, we highlighted some major types of MGNGR, and proposed empirical assays that could help identify such effects and understand their consequences. Critical insights could be gained by jointly tracking changes in genotypes frequencies and within-genotypes phenotypic changes, in common gardens[100], evolutionary experiments, or natural populations. This would provide precious information on the extent of these effects, and on their relative contributions to short- and long-term responses to environmental change. In the current context of global changes, explicitly considering the contribution of MGNGR to population responses to environmental changes, and potentially of adaptation, may prove particularly important.

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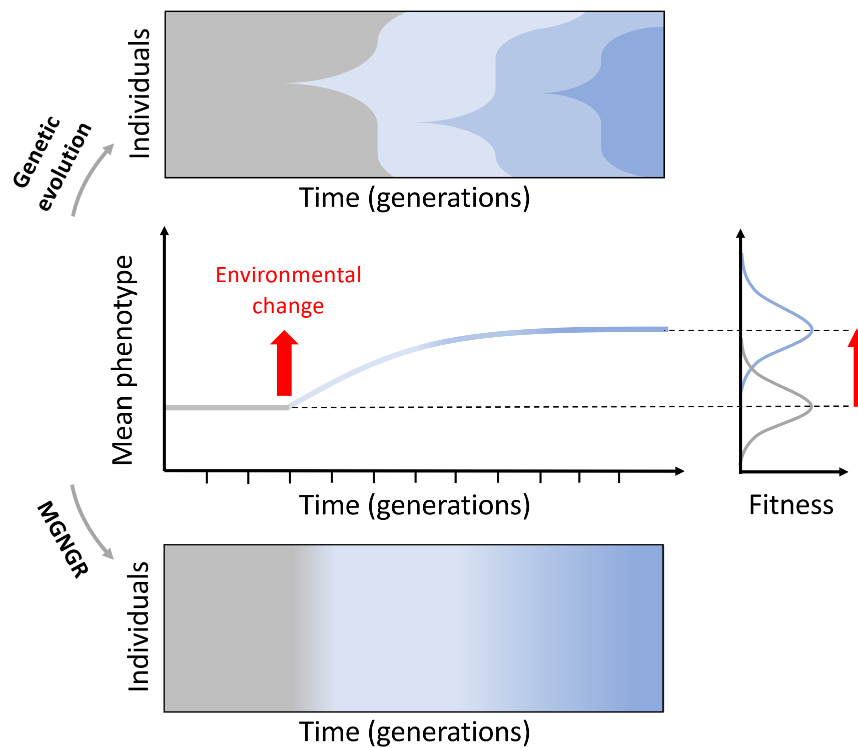
746

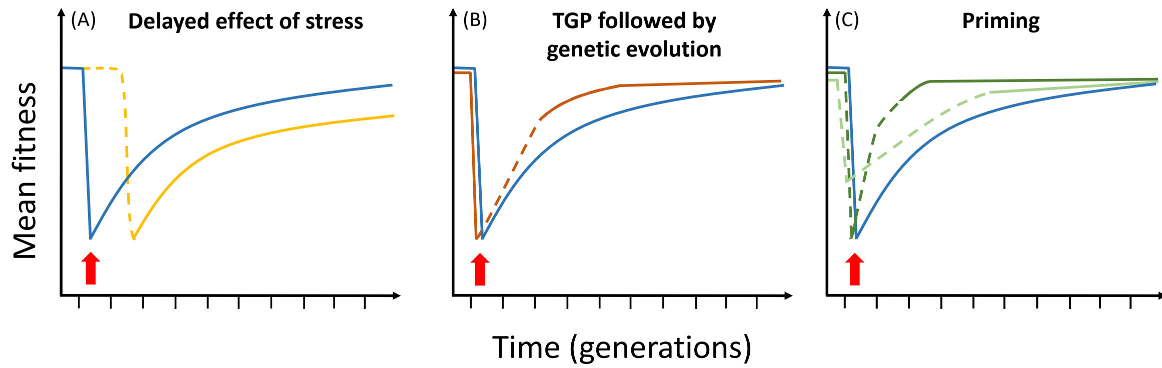
747 **Figures**



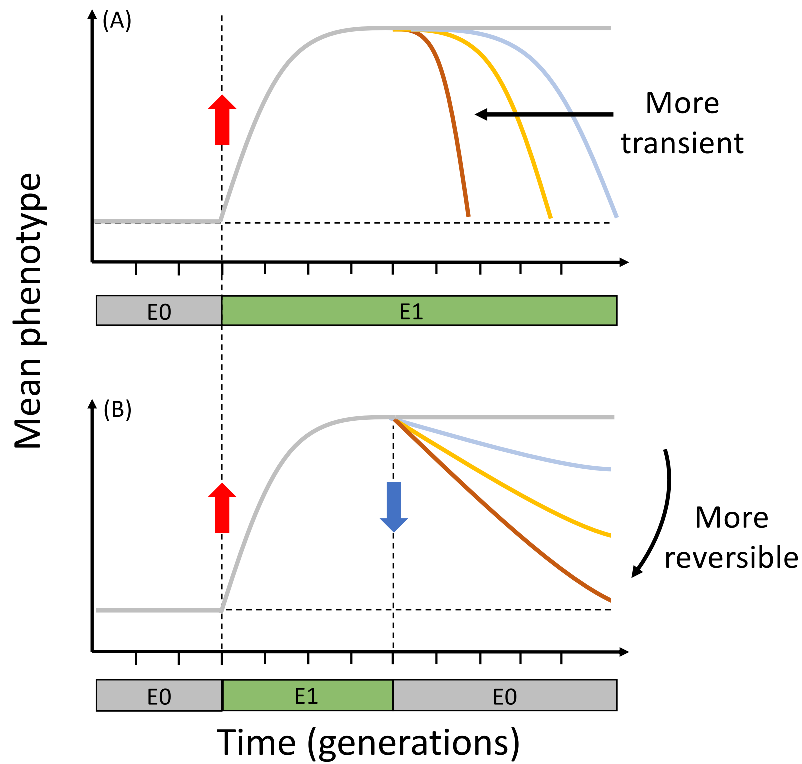
748

749 **Figure 1** Phenotypic response to environmental change can result from either genetic evolution (top panel) or
750 multi-generational non-genetic response (MGNGR, bottom panel). The line in the middle panel represents the
751 dynamics of the mean value of a phenotypic trait, following an environmental change (red arrow). The curves
752 on the right depict the fitness landscape before and after environmental change, with an optimum phenotype
753 shifted upwards (grey to blue fitness landscape). The Muller plot in the top panel represents the canonical case
754 of genetic evolution caused by changes in the genetic composition of the population, with an appearing colour
755 denoting a new mutation/genotype. The bottom panel illustrates MGNGR, in which the environment
756 experienced by one generation gradually impacts the phenotype of the following generations (from light grey
757 to dark blue), homogeneously in the population.

758



759
760 **Figure 2** Examples of dynamics of mean population fitness over time after an environmental change (bottom
761 red arrow), under different mechanisms of MGNGR. The blue line in all panels illustrates the baseline scenario,
762 with an instantaneous effect of stress reducing mean fitness, followed by adaptation via genetic evolution. The
763 coloured lines illustrate different forms of MGNGR mechanisms. Their effects are shown with dashed lines, and
764 are followed by genetic evolution in full lines. (A) Delayed effect of stress (yellow). (B) Dynamic TGP (orange).
765 (C) Priming effect of previous stress exposure on initial fitness drop (light green), or on rate of fitness recovery
766 by dynamic TGP (dark green).
767



768
769 **Figure 3.** Stability and reversibility of phenotypic responses across generations. (A) Transient dynamics occur
770 when the phenotypic trait goes back to its initial state, even though the environment has remained unchanged
771 following the initial environmental shift (from E0 to E1), indicated by the red arrow. The grey line is a non-
772 transient phenotypic response, while coloured lines show increasingly transient responses from light blue to
773 orange. (B) Reversibility is the tendency of a phenotype to go back to the initial state after the environment has
774 changed back (from E1 to E0), as indicated by the blue arrow. The grey line shows an irreversible phenotype,
775 while coloured lines show increasingly reversible responses from light blue to orange.