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SYMPOSIUM

Adaptive Transgenerational Plasticity in an Annual Plant: Grandparental and Parental Drought Stress Enhance Performance of Seedlings in Dry Soil

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Synopsis Stressful parental (usually maternal) environments can dramatically influence expression of traits in offspring, in some cases resulting in phenotypes that are adaptive to the inducing stress. The ecological and evolutionary impact of such transgenerational plasticity depends on both its persistence across generations and its adaptive value. Few studies have examined both aspects of transgenerational plasticity within a given system. Here we report the results of a growth-chamber study of adaptive transgenerational plasticity across two generations, using the widespread annual plant *Polygonum persicaria* as a naturally evolved model system. We grew five inbred *Polygonum* genetic lines in controlled dry vs. moist soil environments for two generations in a fully factorial design, producing replicate individuals of each genetic line with all permutations of grandparental and parental environment. We then measured the effects of these two-generational stress histories on traits critical for functioning in dry soil, in a third (grandchild) generation of seedling offspring raised in the dry treatment. Both grandparental and parental moisture environment significantly influenced seedling development: seedlings of drought-stressed grandparents or parents produced longer root systems that extended deeper and faster into dry soil compared with seedlings of the same genetic lines whose grandparents and/or parents had been amply watered. Offspring of stressed individuals also grew to a greater biomass than offspring of nonstressed parents and grandparents. Importantly, the effects of drought were cumulative over the course of two generations: when both grandparents and parents were drought-stressed, offspring had the greatest provisioning, germinated earliest, and developed into the largest seedlings with the most extensive root systems. Along with these functionally appropriate developmental effects, seedlings produced after two previous drought-stressed generations had significantly greater survivorship in very dry soil than did seedlings with no history of drought. These findings show that plastic responses to naturalistic resource stresses experienced by grandparents and parents can "preadapt" offspring for functioning under the same stresses in ways that measurably influence realized fitness. Possible implications of these environmentally-induced, inherited adaptations are discussed with respect to ecological distribution, persistence under novel stresses, and evolution in natural populations.

Introduction

Developmental plasticity is now understood to play a role in many ecological and evolutionary processes (West-Eberhard 1989, 2003; Sultan 2007; Pfennig et al. 2010; Moczek et al. 2011). Its impact largely depends on how such plasticity influences adaptive diversity and consequent differences in fitness among individual organisms. One particularly intriguing, yet relatively unexplored, form of developmental plasticity occurs when responses to the environment extend across generations to influence the phenotypes of offspring. These effects of parental (usually maternal) environment were initially expected to directly reflect resource levels, with stressed individuals producing low-quality offspring (Falconer 1981; Roach and

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Wulff 1987; Donohue and Schmitt 1998). However, recent studies show that individuals in a number of plant and animal taxa have the ability to adaptively alter their offspring's development in response to environmental stresses, such that the offspring show increased tolerance to the stress in question (Mousseau and Fox 1998; Mousseau et al. 2009; Herman and Sultan 2011; for specific examples, see Sultan 1996; Fox et al. 1997; Donohue and Schmitt 1998; Agrawal et al. 1999; Gustafsson et al. 2005; Lundgren and Sultan 2005; Mondor et al. 2005; Galloway and Etterson 2007; Holeski 2007; Allen et al. 2008; Sultan et al. 2009; Whittle et al. 2009; Dyer et al. 2010; Storm and Lima 2010).

These environmental effects on offspring constitute a developmentally based type of inherited adaptation that can influence the dynamics of selection (Donohue 2009; Bonduriansky and Day 2009) and promote ecological breadth by allowing populations to persist in stressful environments (Sultan 2004; Sultan et al. 2009; Dyer et al. 2010). Adaptive transgenerational plasticity is expected to evolve in cases when (1) dispersal of propagules is spatially limited, and (2) the environment fluctuates over the course of a small number of generations (Galloway 2005; Uller 2008). In such cases, parents and offspring are likely to experience the same environmental challenges, but genetic specialization to those challenges would be unfavorable.

Intriguingly, studies of several plant taxa have found that environmental effects can persist beyond a single generation (Alexander and Wulff 1985; Miao et al. 1991; Case et al. 1996; Wulff et al. 1999; Whittle et al. 2009; Kou et al. 2011). These studies show that traits of seeds, seedlings, and adult plants can be influenced by environments experienced by the grandparental generation, such as thermal stress and variation in nutrient levels; in some cases these effects measurably enhance fitness (e.g., Whittle et al. 2009). Phenotypic variation that stems from the environment experienced during the grandparental, or even more remote, generations may therefore be an underappreciated aspect of adaptive diversity. However, few studies to date have examined the potential for multigenerational inheritance of adaptive stress-induced effects on offspring development, and studies are especially rare in naturally evolved systems subjected to ecologically relevant treatments.

Here we report the results of three experiments that test for adaptive transgenerational plasticity to naturalistic drought stress over two generations in the generalist plant *Polygonum persicaria* (= *Persicaria maculosa*, Kim et al. 2008). This introduced, colonizing annual is found in a wide range of habitats across much of North America, including dry, variably dry, and consistently moist sites (Sultan et al. 1998). *Polygonum persicaria* meets the two conditions described above for evolution of adaptive transgenerational plasticity, including environmental variation from year to year (i.e., relatively dry vs. wet summers) and the likelihood that offspring will encounter an environment similar to that of their parent—the propagules (one-seeded fruits called *achenes*) simply fall from the parental plant upon ripening and therefore typically germinate in the same spatial microsite (we refer throughout to *parental* environments because offspring are produced by self-fertilization; therefore, the maternal and paternal parents are the same individual). Genotypes of this species can be cloned or highly inbred, allowing for robust examination of transgenerational environmental effects while holding genotype entirely, or almost entirely, constant (Mazer and Gorchov 1996).

Previous studies of *P. persicaria* found that the effects of drought extended across at least one generation to adaptively enhance offspring traits important for functioning in dry soil (Sultan 1996; Sultan et al. 2009). Here we expand this investigation across a second generation by testing all combinations of dry vs. moist parental *and grandparental* soil environment, in the same sample of naturally evolved *Polygonum* genotypes. We measure the effects of these drought-stress histories on ecologically important traits in the offspring such as propagule provisioning and structure, timing of germination, seedling development, and survival in dry soil. Because the vast majority of plant mortalities occur during the seed and seedling stages (Moles and Westoby 2006; Leck et al. 2008), these early phases of the life cycle constitute a stringent selective episode (Moles and Leishman 2008) during which transgenerational effects on offspring phenotypes may have a particularly strong evolutionary impact. The seedling stage is also ecologically critical for *P. persicaria* and other obligately annual plants, since in such taxa population establishment and persistence depends entirely on the success of seedling offspring.

We address the following specific questions: (1) Are there functionally appropriate effects of *grandparental* drought stress on offspring traits, i.e. does transgenerational plasticity persist across two generations? (2) If so, how do alternative sequences of grandparental and parental moisture environment

influence offspring development; for instance, is there a cumulative effect of two generations of drought stress? (3) Are these transgenerational effects adaptive—that is, do they increase the survival of offspring in dry conditions?

Methods

Grandparental and parental generations

Mature achenes were collected in the field in September 1994 from five *P. persicaria* plants in three ecologically distinct natural populations (NAT, Natick, MA; MHF, Northfield, MA; and TP, Dover, MA; for details see Sultan et al. 1998). These achenes were germinated, raised to maturity, and allowed to self-fertilize under uniform glasshouse conditions to produce five inbred (selfed full-sib) genetic lines. In the first experimental generation (= *grandparental generation*), for each inbred line, one seedling was assigned to dry soil and another to moist soil. These grandparental individuals were grown in a fertilized 1:1:1 mixture of sterilized topsoil, horticultural sand, and fritted clay (Turface ^{TM}, Profile Products, Buffalo Grove, IL, USA) in a glasshouse under full summer sun (mean midday PAR $\pm$ SD $= 1239 \pm 108\ \mu mol\ m^{-2} s^{-1}$). Soil treatments were maintained respectively at 13.2% $\pm$ 5.8% (Dry) and 26.6% $\pm$ 4.1% (Moist) soil moisture by mass, corresponding to ~50% and 100% of field capacity for this soil mix. Grandparental plants were grown for 71 days in these treatments before their self-fertilized achenes were collected (for details see Sultan 2001).

Achenes produced in the Dry and Moist grandparental treatments were then grown to maturity in both Dry and Moist soil in a second experimental generation (= *parental generation*), in a full factorial design. Growth treatments were maintained as described above. Parental plants were allowed to self-fertilize such that the resulting offspring represented all five genetic lines in all possible permutations of parental and grandparental moisture treatments. We use the following abbreviations to denote the four possible permutations of these treatments (hereafter, *drought histories*): DD (grandparent Dry / parent Dry), DM (grandparent Dry / parent Moist), MD (grandparent Moist / parent Dry), and MM (grandparent Moist / parent Moist).

Structure, provisioning, and germination timing of offspring (achenes)

Twenty to 25 air-dried achenes from each combination of genetic line × grandparental treatment × parental treatment ($N = 20$ experimental units) were individually weighed on a Cahn C-33 microbalance (Cahn Instruments, Cerritos, CA, USA) and stratified in distilled water at 4°C for 40 days in 96-well tissue-culture trays (BD Falcon, Franklin Lakes, NJ, USA). Stratified achenes were then sown individually on moist filter paper in 24-well tissue-culture trays (BD Falcon, Franklin Lakes, NJ, USA) and germinated in a Conviron growth chamber (Controlled Environments, Winnipeg, Manitoba, Canada) set for a 25°:18°C 14:10 h day:night cycle. Fluorescent lights provided ~500 $\mu mol\ m^{-2} s^{-1}$ PAR during the first two 14 h cycles to cue germination, but were then turned off so that all emergence and growth of seedlings took place in darkness. We censused germination and rerandomized tissue-culture trays at 10 a.m. daily, recording the day of germination (germination timing) of each achene.

Seedlings were harvested 96 h after germination (Sultan 1996) and dried for 1 h at 100°C and ≥72 h at 65°C before weighing on a Cahn C-33 microbalance; because these seedlings were given no light or mineral resources, this early biomass provides a robust estimate of seed provisioning (Sultan 1996). To assess offspring structure, pericarps (fruit walls) were air-dried and weighed, and the proportion of offspring mass in pericarp tissue was calculated (pericarp proportion; pericarp mass/achene mass × 100). Due to measurement error or abnormal development, all data from 11 seedlings were excluded from the analysis and data on seed provisioning and pericarp proportion were excluded for an additional eight and six seedlings, respectively. Because germination was <100%, after these exclusions the final sample sizes were $N = 340$ (seed provisioning), $N = 343$ (pericarp proportion), and $N = 354$ (germination timing).

Seedling growth and root extension in dry soil

Achenes from each combination of genetic line × grandparental treatment × parental treatment were stratified in distilled water at 4°C for 10 weeks and then sown on moist filter paper in petri plates (90 × 15 mm) on a glasshouse bench. Each day, petri-plate positions were rerandomized and germination was censused. One hundred twenty hours after germination, six replicate seedlings from each combination of genetic line × grandparental treatment × parental treatment were transplanted individually into flat plexiglass rhizotrons filled with a 2:2:1 mixture of sterilized topsoil, horticultural sand, and fritted clay (Turface^{TM}), premoistened with 40 ml of distilled water per liter of soil mix. Rhizotrons were

made from 245-mm-square bio-assay dishes (Corning, Lowell, MA, USA) by attaching the lid of each dish with silicone caulk, removing its top with a saw, and drilling four 0.5-cm drainage holes along the bottom edge; these containers were split into two 400 ml growth compartments by rigid plastic vertical dividers. Rhizotrons were mounted at a 50° angle to maximize gravitropic root growth against the transparent front surfaces (Gross et al. 1992; Sultan et al. 2009). Moist chamois were used to cover rhizotron surfaces to maintain cool, dark soil conditions. Seedlings were grown in a randomized complete block design for 23 days, in a dual Conviron growth chamber programmed for a 25°:18°C 14:10 h day:night cycle, with fluorescent lights providing ~500 $\mu mol\, m^{-2} s^{-1}$ daytime PAR. Seedlings were watered individually with distilled water as needed to maintain ~13% soil moisture by mass, corresponding to ~50% of soil field capacity.

Maximum root depth of each seedling at a common, early age (deepest root) was determined by measuring the distance from the deepest visible root to the soil surface on Day 13, when all seedlings had produced visible roots but none had reached the bottom of its rhizotron (Sultan et al. 2009). Seedlings were harvested on Day 23 in treatment and separated into shoot and root tissues. Shoot tissues were oven-dried at 100°C for 1 h and at 65°C for ≥48 h before weighing on a top-loading balance (Mettler-Toledo, Columbus, Ohio; shoot mass). Root systems were hand-washed, stored in 70% isopropanol, and measured on a Comair optical root scanner (Hasker de Havilland, Melbourne, Australia) to determine total root length. After scanning, roots were oven-dried at 65°C for ≥48 h and weighed on a Cahn C-33 microbalance (root mass). Seedling biomass was calculated as the sum of root mass plus shoot mass. Due to measurement error, all data from five plants were excluded, deepest root data were excluded for an additional three plants, and total root length was excluded for one additional plant. Final sample sizes were $N = 115$ (seedling biomass), $N = 104$ (total root length), and $N = 112$ (deepest root).

Survival of seedlings in a naturalistic dry-soil treatment

Achenes from each combination of genetic line × grandparental treatment × parental treatment were stratified in distilled water at 4°C for 40 days and then sown onto moist filter paper in 24-well tissue culture trays and placed in random positions on a glasshouse bench. Each day, trays were rerandomized and germination was censused. Ninety-six hours after germination, 12 replicate seedlings of each combination of genetic line × grandparental treatment × parental treatment were individually transplanted into 6.35-cm clay pots (which allow for naturalistic loss of water vapor) filled with a 1:2 mix of sterilized topsoil and horticultural sand, premoistened with 125 ml of water per liter of soil mix. Nineteen of these seedlings (drawn from all four combinations of grandparental and parental treatment) were given an additional 24–48 h before transplanting, to allow them to reach the same developmental stage as the rest of the seedlings (total $N = 239$ after one transplant loss).

Pots were individually placed on inverted petri plate covers (60 × 15 mm) and set in a randomized complete block design in a dual Conviron growth chamber programmed as described above, with fluorescent lights providing ~500 $\mu mol\, m^{-2} s^{-1}$ PAR. Seedlings were kept at 100% of field capacity for 72 h after transplant to prevent transplant shock, and thereafter watered manually, as follows, to maintain very low soil moisture throughout the 9-day experiment. Every day, each seedling received 2 ml of distilled water at the soil surface and 6 ml distilled water introduced via the bottom of the pot (poured into the petri plate cover) to maintain ~2% soil moisture by mass (~9% of field capacity for this soil mix). This treatment mimicked moisture availability to seedlings in the field, where the topmost soil layer holds very little moisture (Sultan et al. 1998). We censused survival daily at 10 a.m. for 9 days, at which point all mortality had evidently occurred. Six seedlings were censored during the course of the experiment due to treatment error, and all seedlings alive at the end of the experiment ($N = 176$) were censored (Kleinbaum and Klein 2005). Censoring is a standard procedure in survival analysis that allows for the use of data for an individual up until the point that the individual leaves the experiment (either due to experimental error or to termination of the experiment). Censored data provide the minimum survival times for individuals in an experimental treatment (Kleinbaum and Klein 2005).

Data analysis

ANOVA with type III sums of squares was used to test for the fixed effects of parental moisture treatment, grandparental moisture treatment, and genetic line as well as all two-way and three-way interactions among these factors (and the effect of block) on seed provisioning, achene structure (pericarp proportion), germination timing, Day-13 deepest root, and Day-23 seedling biomass and total root length.

Block was nonsignificant for all but one trait (seedling biomass) and is not reported. Genetic line was treated as a fixed effect because field genotypes were deliberately drawn from ecologically distinct natural populations and thus do not represent a purely random sample of the species' genetic diversity. Seedling biomass was Box-Cox transformed to meet the assumptions of ANOVA; all other traits were untransformed. For any trait with significant ANOVA results, all pairs of grandparental/parental treatment combinations were compared *post hoc* using Tukey's HSD test (e.g. DD vs. DM; DD vs. MD, DM vs. MD, etc). In order to more powerfully test the specific effects of grandparental and parental drought when results of Tukey's tests were ambiguous (Zar 1999), one-way ANOVA was performed on seedlings from only a given parental or grandparental treatment for certain traits (e.g. to test the effect of Dry vs. Moist grandparental treatment on depth of deepest root in seedlings of parents grown in the moist treatment). We tested for effects of drought history independent of changes in seed-provisioning by including seedling biomass at Day 23 (an indicator of seed provisioning; Kitajima and Fenner 2000; Moles and Leishman 2008) as a covariate in the analysis of total root length.

Survival curves for seedlings from the four different drought histories were calculated, using the Kaplan–Meier product-limit method (Kleinbaum and Klein 2005). Planned comparisons between the four survival curves were performed with a nonparametric log-rank test (Kleinbaum and Klein 2005). All statistical analyses were performed with JMP version 7.0.1 (SAS Institute, Cary, NC, USA).

Results

Offspring structure, provisioning, and germination timing

The combination of grandparental and parental drought stress significantly increased seed provisioning: at 96 h after germination, DD seedlings were 17.4%, 23.7%, and 26.1% larger on average than MD, MM, and DM seedlings, respectively (grandparental environment × parental environment interaction, Table 1; Fig. 1a). The significant main effects on provisioning of both grandparental and parental treatments were driven primarily by the high biomass of DD seedlings; grandparental drought alone (DM) did not increase provisioning, and parental drought (MD) had only a slight effect (provisioning in DM, MD, and MM seedlings was statistically equivalent; Tukey's tests, Fig. 1a). Genetic lines also differed on average and (marginally nonsignificantly) in the effect of prior drought history (effects of genetic line and genetic line × grandparental environment × parental environment, Table 1; see Supplementary Figure 1 for norms of reaction).

Two prior generations of drought stress also resulted in significantly decreased pericarp proportion and earlier germination (Fig. 1b and c). DD achenes had ~10% less pericarp tissue and germinated ~0.5–1 day earlier compared to those from the other treatments (significant grandparental environment × parental environment interactions, Table 1; Fig. 1b and c). The effect of drought-stress history on pericarp proportion varied among genetic lines (genetic line × grandparental environment × parental environment interaction, Table 1; see Supplementary Fig. 1 for norms of reaction). There was also significant genetic variation for the effects of grandparental and parental moisture treatment on germination timing (genetic line × grandparental environment and genetic line × parental environment interactions, Table 1; Supplementary Fig. 1).

Seedling growth and root extension in dry soil

Although the main effects of both grandparental and parental drought stress on seedling biomass were significant (Table 2), grandparental drought increased seedling biomass only in combination with parental drought stress. This combined effect was highly significant: DD seedlings had 45.5% greater biomass after 23 days in dry soil than did MM individuals of the same genetic lines (Fig. 2a). Parental drought alone also (nonsignificantly) increased seedling biomass (21.5% increase in MD vs. MM seedlings; Fig. 2a).

In contrast, either grandparental or parental drought alone increased total root length (DM and MD vs. MM seedlings; Fig. 2b), although these increases were not significant by Tukey's *post hoc* tests. These effects appeared to be additive: roots of DD seedlings growing in dry soil were 49.9% longer than roots of MM seedlings growing in the same soil treatment, and 26.9% and 31.5% longer than DM and MD seedlings, respectively (Fig. 2b). One-way ANOVA confirmed a significant additional effect of grandparental drought on total root length of seedlings with drought-stressed parents ($F_{1,52} = 5.984$, $P = 0.0178$). The overall main effect of grandparental environment on total root length remained significant ($F_{1,78} = 4.446$, $P = 0.0382$) even when biomass of Day 23 seedlings (as an estimate of provisioning) was included as a covariate.

Table 1 Effects of grandparental environment (GPE), parental environment (PE), genetic line (Gen.), and their interactions on seed provisioning (96 h biomass), offspring structure (proportion of achene mass in pericarp), and germination timing

		Seed provisioning			Pericarp proportion			Germination day		
Source of variation	df	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
GPE	1	126872.72	14.49	**0.0002*****	286.464	24.082	<**0.0001*****	1.246	0.739	0.391
PE	1	453153.58	51.77	<**0.0001*****	914.461	76.875	<**0.0001*****	17.979	10.660	**0.0012****
Genetic line	4	183856.19	21.01	<**0.0001*****	96.718	8.131	<**0.0001*****	22.937	13.601	**0.0001*****
GPE × PE	1	191803.89	21.92	<**0.0001*****	441.706	37.133	<**0.0001*****	20.765	12.313	**0.0005*****
Gen. × GPE	4	20001.91	2.29	0.0601†	16.548	1.391	0.237	9.322	5.527	**0.0003*****
Gen. × PE	4	18333.03	2.09	0.0813†	45.967	3.864	**0.0044****	4.497	2.667	**0.0324***
Gen. × GPE × PE	4	20246.11	2.31	0.0575†	62.127	5.223	**0.0004*****	2.996	1.777	0.133
Error		8752.11 (df = 320)			11.8951 (df = 323)			1.6864 (df = 334)		

†$P < 0.10$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

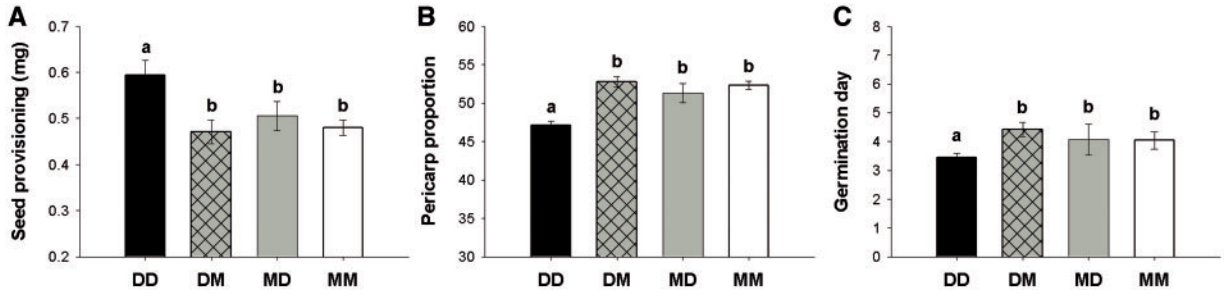

Fig. 1 Grandparental and parental effects on (**A**) seed provisioning (96 h biomass), (**B**) offspring structure (proportion of achene mass in pericarp), and (**C**) germination timing (means ± 1 SE) are shown for offspring of droughted grandparents and parents (DD), droughted grandparents (DM), droughted parents (MD), and moist-grown grandparents and parents (MM). Letters above bars indicate the results of *post hoc* Tukey's tests.

Transgenerational effects of drought on the depth of root systems were similar to those on total root length (Fig. 2c): the deepest roots of DM and MD seedlings were intermediate between those of DD and MM seedlings, with DD seedlings extending their deepest roots 56.5% deeper than MM seedlings. One-way ANOVAs within parental (DM) and grandparental (MD) Moist treatments showed that grandparental drought and parental drought each significantly increased the depth of the deepest root ($F_{1,54} = 8.109$, $P = 0.0062$ and $F_{1,52} = 6.083$, $P = 0.0170$, respectively). We found no evidence of genetic variation for transgenerational effects on these growth traits (genetic line × parental environment, genetic line × grandparental environment, or a three-way interaction; Table 2) apart from a marginally nonsignificant effect of genetic line × grandparental environment on seedling biomass.

Seedling survival in a naturalistic dry treatment

DD seedlings had the highest survivorship, after 9 days in a severe drought treatment (Fig. 3). Only 16% of DD seedlings died compared to 27% mortality for DM and MD seedlings, and 37% mortality for MM seedlings (Fig. 3). Due to the low total number of seedling mortalities (63 out of 239, or ~26%), power was limited to resolve differences between survival curves for different drought histories (Peto et al. 1976; Cuzick 2001). For this reason, we set an overall significance level of $P < 0.10$. The four survival curves differed at this significance level (log-rank test, $P = 0.084$), and planned pairwise comparisons between survival curves revealed a highly significant difference in survivorship between DD and MM seedlings (log-rank test, $P = 0.011$; Fig. 3).

Discussion

Inheritance of drought-stress effects across two generations

We studied inbred replicate offspring that differed only in environmental history, in naturally evolved field genotypes of the widespread annual *P. persicaria*.

Table 2 Effects of grandparental environment (GPE), parental environment (PE), genetic line (Gen.), and their interactions on seedling growth after 23 days of drought (deepest root was measured on Day 13)

		Seedling biomass			Total root length			Deepest root		
Source of variation	df	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
GPE	1	2.8×10^{-4}	4.109	**0.0456***	4.077	7.777	**0.0066****	135.532	6.860	**0.0104***
PE	1	8.6×10^{-4}	12.647	**0.0006*****	2.758	5.258	**0.0245***	140.730	7.123	**0.0091****
Genetic line	4	8×10^{-5}	1.177	0.3263	0.347	0.662	0.6202	7.451	0.377	0.8244
GPE × PE	1	6×10^{-5}	0.825	0.3663	0.332	0.632	0.4289	15.239	0.771	0.3822
Gen. × GPE	4	1.5×10^{-4}	2.166	0.0791†	0.930	1.773	0.1425	9.402	0.476	0.7533
Gen. × PE	4	7.2×10^{-5}	1.059	0.3814	0.325	0.619	0.6500	9.563	0.484	0.7474
Gen. × GPE × PE	4	7.4×10^{-5}	1.082	0.3704	0.250	0.476	0.7532	16.315	0.826	0.5123
Error		6.8×10^{-5} (df = 90)			0.525 (df = 79)			19.7559 (df = 87)		

†$P < 0.10$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

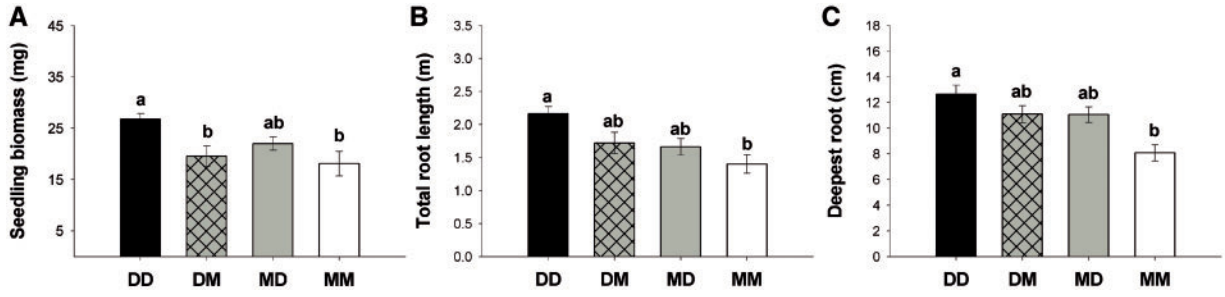

Fig. 2 Grandparental and parental effects on (**A**) seedling biomass (Day 23), (**B**) total root length (Day 23), and (**C**) deepest root (means ± 1 SE) on Day 13 are shown for offspring of droughted grandparents and parents (DD), droughted grandparents (DM), droughted parents (MD), and moist-grown grandparents and parents (MM). Letters above bars indicate the results of *post hoc* Tukey's tests.

Drought-induced changes to ecologically important aspects of offspring structure and development persisted for two generations. Together with studies documenting effects of grandparental temperature and nutrient environment on seedling development (e.g., Alexander and Wulff 1985; Case et al. 1996; Wulff et al. 1999; Whittle et al. 2009; Kou et al. 2011), these results make clear that the grandparental as well as parental environment may influence offspring phenotypes. These transgenerational environmental effects were cumulative: two successive generations of drought stress induced greater provisioning, root growth, and survivorship than did drought in either the grandparental or parental generation alone. In some traits, such as seed provisioning, effects of grandparental stress were evident only when parents were also drought-stressed; in the case of seedling developmental traits, measurable and/or significant grandparental effects occurred even after an intervening, unstressed generation.

Whether transgenerational effects of other environmental stresses persist and interact across two

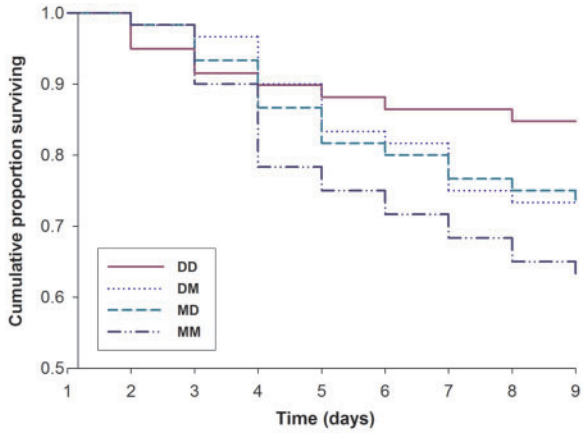

Fig. 3 Kaplan–Meier survival curves are shown for seedlings of droughted grandparents and parents (DD), droughted grandparents (DM), droughted parents (MD), and moist-grown grandparents and parents (MM). Survival curves differed at the $P < 0.10$ level (log-rank test; $P = 0.084$), and a planned comparison revealed a significant difference between survival curves of DD and MM seedlings (log-rank test, $P = 0.011$).

generations in this, or other, systems remains to be determined by further studies that factorially test plastic responses to grandparental and parental environment. Although the multiple genetic lines, environments, and generations required lead to very large experiments, substantial replication is also required to provide adequate statistical power to test these effects. To our knowledge, the only other transgenerational study to factorially vary both grandparental and parental environment (Miao et al. 1991) tested an environmental *supplement* rather than a stress, using the cosmopolitan weed *Plantago lanceolata*. Consistent with the inheritance pattern documented here, these authors found that, in certain traits and competitive conditions, two successive generations of nutrient addition more strongly affected the phenotypes of offspring than did adding nutrients during either generation alone. In contrast to the specific, adaptive effects of drought stress that we report here, the results of repeated nutrient enhancement may simply reflect a passive, resource-based effect, in which resource-rich individuals produce higher-quality offspring (Roach and Wulff 1987).

Transgenerational effects of drought stress on function and fitness

Our results show that inherited developmental effects of drought stress in *P. persicaria* enhanced specific traits that contribute to the success of offspring in dry soil conditions. One such trait is provisioning, which refers to the carbohydrates, lipids, and minerals stored in the seed by the maternal plant (Roach and Wulff 1987; Srivastava 2002). Since these reserves are the sole source of energy for the initial production of roots and shoots, increased provisioning enhances seedlings' early growth and raises the likelihood of successful establishment (Kitajima and Fenner 2000; Moles and Westoby 2006). The substantial provisioning enhancement that resulted from two successive generations of drought stress in *P. persicaria* would likely be particularly advantageous in dry soil, where seedlings must immediately extend deep roots to reach moist soil if they are to survive (Salisbury 1974; Wulff 1986; Moles and Leishman 2008). Seeds with greater provisioning can also emerge from greater soil depths, where moister, more favorable conditions for germination occur (Leishman and Westoby 1994).

Combined grandparental and parental drought stress also resulted in a change in achene (offspring) structure, namely a reduction in the relative mass of stony pericarp (fruit wall) tissue enclosing the seed. Evidently as a result of their thinner pericarps (Sultan 1996), these achenes germinated significantly faster than did those with no history of drought in their immediate ancestry. Such differences in the timing of germination can provide a competitive advantage that is particularly important in resource-limited environments, by allowing early germinants to preempt soil moisture and nutrients and to overtop neighbors (Kitajima and Fenner 2000). Advancing germination, even by only 1 or 2 days, can lead to dramatic differences in seedling biomass (Morse and Schmitt 1985), survival (Howell 1981), and reproductive fitness (Kalisz 1986).

Along with these adaptive adjustments in offspring provisioning, structure and germination, grandparental and parental drought stress resulted in specific, functionally appropriate modifications to seedling development in dry soil. By increasing the total length of seedling root systems and their rate of extension into deep soil, these transgenerational responses allow the plant to quickly access available moisture, thereby maximizing both the growth and survival probability of seedlings in dry soil (Hoffman and Isselstein 2004; Moles and Westoby 2006). The adaptive value in dry conditions of rapid, deep root extension and other transgenerational effects of combined grandparental and parental drought stress was confirmed by the significantly greater biomass, longer survival times, and greater survivorship of seedlings with this drought history compared with seedling offspring of well-watered parents and grandparents. These findings add to a growing body of research in the *Polygonum* system that demonstrates functionally adaptive transgenerational plasticity in response to a range of naturalistic light, nutrient, and soil moisture stresses (reviewed by Herman and Sultan 2011). Such inherited environmental effects interact with immediate plastic responses of seedlings to their growth conditions to result in rapidly expressed adaptive phenotypes (Sultan et al. 2009).

Possible mechanisms of transgenerational plasticity

The larger, deeper root systems produced by offspring of drought-stressed *P. persicaria* grandparents and parents evidently reflect increased seed provisioning, a known plastic response to drought in this species (Sultan 1996, Sultan et al. 2009) that was confirmed by the present study. However, such provisioning is mediated directly by the parent, so grandparental effects must result from other mechanisms that are not directly resource-based. Consistent with this view,

grandparental drought resulted in increased *Polygonum* seedling root length even across an intervening unstressed parental generation, and a covariate analysis confirmed that this specific developmental effect remained significant, independent of changes in the provisioning of seeds. Indeed, environmental stresses experienced during the parental generation can also lead to effects on growth independent of provisioning (e.g., Case et al. 1996; Agrawal 2001; Bischoff and Muller-Scharer 2010; Dyer et al. 2010).

Such findings suggest that other biochemical and/or epigenetic mechanisms may independently, or jointly, mediate certain transgenerational responses to environmental stress (see discussion and references by Herman and Sultan 2011). Possible mechanisms include the action of transmitted hormones, RNAs, and regulatory proteins, as well as chromatin marks such as DNA methylation and histone modifications (reviewed by Bonduriansky and Day 2009; Jablonka and Raz 2009). We are currently conducting a preliminary methylation-sensitive AFLP study of the *Polygonum* system to test for possible differences in patterns of DNA methylation induced by different grandparental and parental drought histories. Studies combining investigations of potential regulatory factors with inheritance patterns of transgenerational responses will be critical for understanding this ecologically significant aspect of individual plasticity.

Genetic diversity for transgenerational plastic responses

Our sample of five genetic lines from three field populations showed significantly different responses to grandparental and/or parental soil moisture environments with respect to achene structure and consequently germination timing. This result is consistent with previous studies documenting genetic diversity for transgenerational plasticity in annual *Polygonum* species (Sultan 1996, 2001). Such variation in transgenerational norms of reaction provides the raw material for further evolution of adaptive transgenerational plasticity (Schmitt et al. 1992; Wulff et al. 1994; Case et al. 1996), just as genetic variation in response to immediate environments (i.e., $G \times E$ variation) fuels evolution of within-generation plasticity (Via and Lande 1985; Sultan 2007). The three-way interaction of genetic line, grandparental environment, and parental environment constitutes a particularly complex type of genetic variation, the expression of which is contingent on the environments encountered by two previous generations. Indeed, it is possible that such variation can encompass more than the two generations investigated here, and that genotypic norms of reaction can vary across multiple, successive environments.

Interestingly, there was very little evidence for genetic variation in transgenerational effects on seedling growth characteristics. The predominance of inherited environmental effects over genotypic effects on ecologically critical aspects of seedling growth, such as extension and total length of roots, suggests that natural selection could act primarily on environmentally determined phenotypic variation during this key life-history stage, including variation stemming from the environment experienced by grandparents.

Ecological and evolutionary implications

Together with previous work on *P. persicaria*, these findings demonstrate two key ways that adaptive transgenerational plasticity can contribute to phenotypic flexibility, ecological tolerance, and evolutionary potential in natural systems. First, transgenerational induction of functionally appropriate phenotypes effectively "preadapts" offspring to withstand an environmental challenge that was encountered by parents and grandparents, without the developmental lag time required for an immediate plastic response (Uller 2008; Sultan et al. 2009). In the case of severe stresses such as dry soil that can lead to early mortality, such preadaptation can significantly increase the survival of offspring, as was the case in this study (see also Galloway and Etterson 2007). Second, preinduced offspring may be capable of more extreme developmental outcomes than are produced solely by means of within-generation plasticity (Agrawal et al. 1999), potentially accommodating a broader range of habitats. Accordingly, the capacity for environmentally induced, inherited adaptations, such as those documented here, may increase a species' ecological distribution to include more variable or more stressful habitats. Indeed, multi-species comparisons in the genus *Polygonum* suggest that interspecific differences in patterns of adaptive transgenerational plasticity contribute to the species' contrasting ecological distributions in the field (Sultan et al. 1998; Sultan 2001; Sultan et al. 2009).

Both experimental and theoretical explorations of transgenerational plasticity point to potential evolutionary implications (discussed by Bonduriansky and Day 2009), including effects on rates of population growth (e.g., Galloway and Etterson 2007; Donohue 2009; Inchausti and Ginzburg 2009), and on the rate of evolution and direction of selection (Kirkpatrick and Lande 1989). Unlike the random and rare

occurrence of new genetic variants, transgenerational plasticity provides adaptive, heritable variation when it is needed, and in numerous offspring individuals, so a population can undergo rapid phenotypic adaptation without allele frequency change (Jablonka and Raz 2009; Verhoeven et al. 2010). In consequence, such plasticity may promote the spread of invasive species, which often have reduced genetic variation due to population bottlenecks upon introduction to a new geographic range (Dyer et al. 2010).

More subtle evolutionary impacts within populations are also of great theoretical and empirical interest. Effects of parental environment can determine which genes are exposed to natural selection by regulating the genes involved in the expression of offspring phenotype (Donohue et al. 2008; Donohue 2009). Persistent transgenerational environmental effects, such as those described in this study, may also obscure genetic differences among individuals (Platenkamp and Shaw 1993), promoting the maintenance of genetic variation that could be expressed should populations experience novel environmental conditions. Note that by increasing parent-offspring resemblance, transgenerational plasticity that is not identified as such can lead to inflated estimates of genetic variation, heritability, and selective change.

At the level of distinct populations, consistent environmental differences can lead to plastic changes that promote reproductive isolation and hence evolutionary divergence (Bonduriansky and Day 2009). More broadly, transgenerational plasticity can increase the range of phenotypic variation available both within and among populations for subsequent adaptive evolution (Badyaev 2008; Badyaev and Uller 2009; see West-Eberhard 2003 and Moczek et al. 2011).

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Supplementary Data

Supplementary Data are available at *ICB* online.

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