EPIGENETICS OF ADAPTIVE PLASTICITY:
AN INVESTIGATION OF PLANT RESPONSES TO ENVIRONMENTAL STRESS WITHIN AND ACROSS GENERATIONS

by

Jacob J. Herman

Faculty Advisor: Dr. Sonia E. Sultan

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I dedicate this dissertation to my grandparents, Glenna and Carl, to my parents, Tiffany and Adrian, and to my wife, Jamie, with love and appreciation.
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Abstract

Environments experienced by parent individuals can profoundly affect offspring phenotypes. These inherited environmental effects can include specific developmental adjustments that improve offspring growth under the conditions that induced them. Such adaptive transgenerational plasticity has garnered much research interest, yet it remains unclear over how many generations these effects persist, if they influence fitness, and how they are inherited. Similarly, the molecular basis of adaptive within-generation plasticity is unclear in most cases. Recent epigenetics research suggests that DNA methylation can mediate adaptive plasticity, both within and across generations. This dissertation combines phenotypic plasticity experiments and DNA methylation analysis in order to address these outstanding questions.

Chapter One reviews examples of adaptive transgenerational plasticity in plants, the potential mechanistic bases of these inherited effects, and their ecological and evolutionary implications. Chapter Two demonstrates that adaptive transgenerational effects of drought stress persist over two generations in the annual plant Polygonum persicaria. These inherited effects enhanced the growth and survival of grandoffspring grown under severe drought stress. Chapter Three shows, through experimental demethylation, that DNA methylation mediates the inherited effects of drought stress in P. persicaria. Furthermore, these methylation-mediated effects of parental drought were genotype-specific. A central conclusion of this study is that genotype, epigenotype, and parental soil-moisture environment interact to
adaptively influence functional traits in \textit{P. persicaria}. Chapter Four examines the relationship between DNA methylation and adaptive within-generation plasticity. Drought stress, low-nutrient stress, and shade each induced DNA methylation changes, as measured by methylation-sensitive AFLP. However, stress-induced methylation changes were not detected in response to each stress in each genetic line. Because genetic lines expressed similar degrees of adaptive plasticity, there was not a consistent association between stress-induced changes in phenotypes and methylation patterns. While this subject requires further study, these results suggest that genotype-specific DNA methylation changes may contribute to the expression of adaptive plasticity. Such genotypic differences underscore the importance of incorporating genetic variation into ecological epigenetics studies.

Together, these studies indicate that interactions between genotype, epigenotype, and environmental signals – including those in previous generations – are a meaningful source of phenotypic variation. Further investigating these interactions represents a promising new direction in evolutionary biology.
Chapter 1: Adaptive transgenerational plasticity in plants: cases studies, mechanisms, and implications for natural populations

Jacob J. Herman and Sonia E. Sultan

Abstract

Plants respond to environmental conditions not only by plastic changes to their own development and physiology, but also by altering the phenotypes expressed by their offspring. This transgenerational plasticity was initially considered to entail only negative effects of stressful parental environments, such as production of smaller seeds by resource- or temperature-stressed parent plants, and was therefore viewed as environmental noise. Recent evolutionary ecology studies have shown that in some cases, these inherited environmental effects can include specific growth adjustments that are functionally adaptive to the parental conditions that induced them, which can range from contrasting states of controlled laboratory environments to the complex habitat variation encountered by natural plant populations. Preliminary findings suggest that adaptive transgenerational effects can be transmitted by means of diverse mechanisms including changes to seed provisioning and biochemistry, and epigenetic

modifications such as DNA methylation that can persist across multiple generations. These non-genetically inherited adaptations can influence the ecological breadth and evolutionary dynamics of plant taxa and promote the spread of invasive plants. Interdisciplinary studies that join mechanistic and evolutionary ecology approaches will be an important source of future insights.

**Introduction**

Effects of parental (usually maternal) environmental stress on the size and development of plant offspring have been well known for several decades. Quantitative geneticists concerned with plant breeding as well as evolutionary biologists studying change by natural selection considered these inherited, induced effects to be “a frequent, and often troublesome source of environmental resemblance” (Falconer, 1981) that masked the genetically-inherited variants that were their central focus. This view of transgenerational environmental effects as a nuisance led researchers to design experiments that minimized the likelihood of detecting these effects, which were treated as experimental error. This approach began to shift in the late 1980’s and 1990’s, when a series of seminal reviews and empirical investigations re-conceptualized these effects as a transgenerational form of individual phenotypic plasticity, and hence a potential source of ecologically and

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2 We use the term *inherited* in its inclusive sense, to indicate all effects transmitted from parent plant to offspring, i.e., effects of parental environment, parental alleles, or epialleles (see Danchin *et al.* 2011).
evolutionarily meaningful variation (Roach and Wulff, 1987; Schmitt et al., 1992; Sultan, 1996; Donohue and Schmitt, 1998; for overviews of both plant and animal studies, see Mousseau and Fox, 1998). Central to this new understanding was the recognition that parent individuals alter specific developmental traits in their progeny in response to particular environmental stresses, and that these alterations may enhance offspring growth and success under those same stresses. Such adaptive transgenerational plasticity is predicted to evolve in cases when the parental environment reliably predicts the offspring environment—e.g., when the offspring are likely to encounter the same specific stresses as the parent(s) (Agrawal et al., 1999; Galloway, 2005).

The past two decades have also seen extraordinary new insights to heritable epigenetic effects on gene expression, particularly the effects of DNA methylation patterns (Grant-Downton and Dickinson, 2005; Henderson and Jacobsen, 2007; Hauser et al., 2011). Although epigenetic mechanisms are a likely means of transmitting environmental effects across generations (Jablonka and Lamb, 1995) and may well be the basis for many cases of adaptive transgenerational plasticity (Rossiter, 1996; Bossdorf et al., 2008), mechanistic studies of epigenetic inheritance and ecological research on transgenerational plasticity have largely proceeded in isolation. As research interest in both epigenetic inheritance mechanisms and adaptive transgenerational plasticity has continued to surge (Jablonka and Raz, 2009; Mousseau et al., 2009), integrating these exciting areas promises important advances.
Here we review recent examples of adaptive transgenerational plasticity in plants in response to various environmental stresses, leading to a multi-species case study in the genus *Polygonum* that illustrates several fundamental points about this aspect of individual environmental response. We then provide a brief overview of the diverse mechanisms that mediate these fascinating responses, and conclude by highlighting some key ecological and evolutionary implications.

**Adaptive transgenerational plasticity: a brief review**

Despite intense interest in functionally adaptive transgenerational effects in plants, to date only a limited number of studies fully document such effects (Donohue and Schmitt 1998; Dyer et al. 2010; see Table One). In part this reflects the particular design challenges of such studies, which must (a) raise genetic replicate parent individuals in contrasting environments (to test for effects of parent environment by holding genotype constant), and (b) monitor expression of ecologically important traits in offspring grown over one or more generations, to test for an adaptive match between parental conditions and offspring performance in like conditions. Below we discuss findings from a number of studies that meet these criteria.

Temperature stress is an environmental challenge that plants often confront in natural populations, and one likely to become even more prevalent as global climate change advances. Whittle and colleagues (2009) reported evidence for adaptive transgenerational responses to heat stress in *Arabidopsis thaliana* that persisted over
at least 2 generations. Using a set of homozygous inbred lines, Whittle et al. found that F₃ Arabidopsis progeny increased reproductive output five-fold under heat stress (30°C) if the F₀ and F₁ generations had also experienced heat stress. This effect persisted into the F₃ generation, even when F₂ plants were grown at a moderate temperature (23°C) (Whittle et al., 2009). However, the precise functional and/or developmental changes to offspring that led to this fitness increase were not documented.

The effects of temperature stress also appear to be heritable and potentially adaptive in the cosmopolitan weed Plantago lanceolata. Two research findings in this system are particularly intriguing. First, the effects of cold temperature treatment persisted across two generations, significantly enhancing seed weight as well as fitness-related leaf and life-history traits in adult grandchild plants (Case et al., 1996). This result makes clear that the expression of transgenerational effects may not be confined to the seedling stage, as is often assumed. (Indeed, adaptive transgenerational effects are differently expressed in the second year of offspring life in this perennial weed; Latzel and Klimešová, 2010). Second, paternal temperature treatment also significantly affected offspring traits via interactions with the maternal temperature environment (Lacey, 1996). Although most cases of transgenerational plasticity are mediated by the maternal plant, and it is likely that direct maternal adjustments are of greatest magnitude, effects of paternal environment may also be relevant in outcrossing species (Roach and Wulff, 1987; Mazer and Gorchov, 1996; Diggle et al., 2010).
Edaphic stresses have been shown to elicit adaptive transgenerational responses in several model systems. Single-factor studies in rice (*Oryza* spp.) and *Arabidopsis* indicate that progeny of both nitrogen-limited and salt-stressed plants have greater tolerance to those stresses, as demonstrated by their enhanced total biomass compared to progeny of non-stressed control plants (Boyko et al., 2010; Kou et al., 2011). Increased tolerance to nitrogen limitation in rice persisted over two progeny generations (Kou et al., 2011).

In addition to abiotic stresses, biotic stresses such as herbivory can induce heritable, adaptive phenotypic responses. In *Raphanus raphanistrum*, the seedling offspring of herbivore-damaged plants (or plants treated with jasmonic acid, an elicitor of plant defenses) had measurably increased resistance to herbivory (evidently due to both trichome density and biochemical defenses; see *Mechanisms of Transgenerational Response*; Agrawal, 2001; 2002). As a result, *Pieris rapae* caterpillars that fed on these seedlings grew 20% less than caterpillars that fed on seedlings of undamaged mother plants (Agrawal et al., 1999), a robust example of transgenerational plant responses likely to influence higher-order community dynamics. Caterpillar herbivory also significantly increased individual seed mass and enhanced early seedling growth in some maternal families, but reduced these traits in other *Raphanus* families (Agrawal, 2001; 2002). Interestingly, the effects of maternal herbivory on seed mass were as large as the effects of genetic line, and consequently as likely to influence natural selection in this system as inherited genetic variation. Similarly, *Mimulus guttatus* plants with experimentally damaged leaves (i.e.,
simulated herbivory) produced offspring with higher densities of defensive leaf trichomes compared to genetically identical offspring of undamaged control plants (Holeski, 2007). Transgenerational responses to herbivory also appear to be adaptive in the colonizing annual Impatiens capensis. Offspring of plants that experienced natural herbivory in the field emerged earlier, grew taller, and had significantly greater biomass than offspring of plants protected from most herbivores (Steets and Ashman, 2010).

Research designed to test plant responses to complex naturalistic stresses can provide insights of direct ecological relevance. For instance, serpentine soils impose at least three distinct environmental challenges to plants: nutrient stress, drought stress, and exposure to high metal concentrations (Dyer et al., 2010). Studies of the invasive grass Aegilops triuncialis identified putatively adaptive transgenerational effects of this edaphic environment on offspring phenology and success (Dyer et al., 2010): offspring of parent A. triuncialis plants grown in serpentine soil flowered earlier (which permits reproduction to occur prior to the onset of summer drought) and grew to a larger size (associated with greater reproductive output) when they were grown in low-nutrient soil, compared to offspring of the same genotypes whose parents had been grown in moist loam (Dyer et al., 2010).

Furthermore, distinct aspects of environmental stress may interact to elicit complex transgenerational responses. In P. lanceolata, the ability of seedlings to express an adaptive, compensatory growth response to disturbance (i.e., simulated
grazing by severe shoot removal) was influenced by both parental disturbance and parental nutrient stress: offspring growth more than compensated for biomass loss due to disturbance if the mother had been either disturbed and grown in nutrient-rich soil, or not disturbed and grown in nutrient-poor soil (Latzel et al., 2010). Such complex, interacting transgenerational effects are likely to be important in natural plant populations, which encounter concurrent variation in multiple abiotic and biotic environmental factors.

An elegant series of field experiments with the forest-edge herb *Campanulastrum americanum* has examined the interplay between adaptive transgenerational plasticity and life history expression in complex natural habitats. When maternal and offspring plants were both grown in either forest light gaps or understory shade, germination fraction and seedling survival were significantly higher, and adult offspring had significantly greater relative leaf areas and produced significantly larger seeds, than ‘mismatched’ combinations of parent and offspring light habitat (Galloway and Etters on, 2007; 2009). Demographic projections indicate that population growth would be three times greater when offspring were grown in the same light environment maternal plants had experienced (a likely scenario given the species’ limited seed dispersal range; Galloway and Etters on, 2007). In addition to this evidence for adaptive transgenerational plasticity in offspring growth and reproduction, this system also exemplifies how maternal environment can adaptively alter life-history expression in the offspring generation, an even more dramatic transgenerational response (Galloway, 2005; Galloway and Etters on, 2007; 2009). C.
Americanum offspring are more likely to express an annual life-history if their maternal parent grew in a forest light gap, where sufficient light is likely available to reproduce within a single season, and a biennial life history if their maternal parent grew in understory shade where a longer growth period is required to successfully reproduce (Galloway and Etterson, 2007). Expression of annual versus biennial life history schedule was also influenced by maternal flowering time and maternal exposure to herbivory (Galloway and Burgess, 2009; Lin and Galloway, 2010), demonstrating the rich complexity of factors involved in transgenerational responses to natural environments.

Transgenerational responses to complex seasonal conditions also play an important role in life history expression in Arabidopsis, which is jointly shaped by maternal and offspring environments. In studies by Donohue and colleagues, seasonal factors that influenced reproductive timing, along with conditions during seed maturation and after dispersal, determined germination timing and offspring life history schedule (Donohue et al., 2005). Companion laboratory studies showed that germination timing was highly responsive to individual environmental variables such as temperature, photoperiod, and canopy shade (reviewed in Donohue, 2009). In order to assess the adaptive value of these maternal seasonal effects, Donohue (2009) used a demographic analysis to test how population growth rates would be affected if maternal environment did not influence offspring germination. She found that population growth would be reduced by as much as 30% if germination (and consequently life-history schedule) were not responsive to maternal environmental
conditions (Donohue, 2009). Related studies showed furthermore that transgenerational plasticity for life-history expression can create selective feedbacks that either promote or constrain evolutionary change (Donohue, 2005).

**Adaptive transgenerational plasticity: A case study**

Experiments with annual species in the genus *Polygonum (sensu latu)* provide a case study of transgenerational plasticity expressed by naturally evolved genotypes exposed to controlled environmental stresses. Field-collected individuals of these primarily self-fertilizing species can be cloned or highly inbred to produce replicate, genetically uniform, highly homozygous parent individuals. These replicate parents can be raised in contrasting glasshouse resource treatments (based on the range of environmental conditions in nature) and allowed to produce selfed progeny (single-seeded achenes). These can be germinated in specified seedling environments to precisely assess the effects of parental environment on offspring development in the absence of confounding genetic variation (Sultan, 2000) and of possible (if unlikely) selective effects at the level of gametophytes, gametes, or embryos (Mazer and Gorchov, 1996). Because these *Polygonum* species are gravity-dispersed, progeny are likely to germinate close to the parent plant, so the likelihood of encountering a similar microsite is high. This experimental system has produced several examples of transgenerational responses to naturalistic resource stresses that enhance seedling success under like stressful conditions, and has revealed variation in these responses
among genotypes, closely related species, and even seed architectural positions. These findings are particularly intriguing because resource deprivation was previously assumed to result only in maladaptive reductions in offspring size (Roach and Wulff 1987). Instead, the Polygonum case study makes clear that the ability to express adaptive transgenerational plasticity is a significant aspect of ecologically important genetic and species diversity (Sultan, 2001; Sultan et al., 2009).

In the initial study, clonal replicates of P. persicaria genotypes raised in very low light produced equally well provisioned seeds as plants given full sun, but reduced the mass of carbon-rich pericarp tissue enclosing the seeds by over 40% (Sultan, 1996). Light-limited parents thus maintained essential offspring provisioning at the cost of reduced longevity in the soil (Sultan, 1996; see also Lacey et al.; 1997). Their offspring also produced more shoot tissue relative to root biomass than offspring of plants grown in full sun (Sultan 1996), a likely pre-adaptation to shade conditions (Salisbury, 1974; Haig and Westoby, 1988). In contrast, clonal replicates of the same parental genotypes raised in nutrient-poor conditions slightly reduced seed provisioning. Despite this reduction, however, the offspring of nutrient-deprived parents increased root allocation to produce (non-significantly) longer root systems than the offspring of genetically identical parents grown in nutrient-rich soil (Sultan 1996), a transgenerational response that would benefit seedlings in nutrient-poor soil by maximizing root uptake surface for mineral ions (Wulff, 1986a).
A later experiment compared the effects of parental drought stress on seedling
development in *P. persicaria*, which occurs in variably dry to moist sites, and *P.
hydropiper*, a closely related species limited to consistently moist habitats (Sultan et al., 1998; Sultan et al., 2009). For several highly inbred genotypes of each species,
aciouses produced by replicate parents grown in either dry or moist conditions were
raised for 21 days in either dry or moist seedling treatments, in a full-factorial split-
brood design (Sultan et al., 2009). Because the rapid production of deep, extensive
root systems is particularly critical for seedlings in dry soil (Hoffman and Isselstein,
2004; Moles and Westoby, 2006), root growth was monitored carefully. Drought-
stressed parent plants of *P. persicaria* produced more well-provisioned seedling
offspring that made longer, more rapidly extending root systems in dry soil than the
offspring of replicate parents of the same inbred lines that had been given ample
water, resulting in significantly greater seedling growth (Sultan et al. 2009; see also
Sultan 1996). The offspring of moist-grown *P. persicaria* plants also produced
slightly (non-significantly) larger seedlings in the moist seedling environment,
evidence of an adaptive match between both moist and dry parental and offspring
environments (Sultan et al., 2009; see Galloway and Etterson 2007). In contrast, *P.
ydropiper* plants transmitted a direct, maladaptive effect of parental drought stress to
their offspring: in this species, drought-stressed parents simply produced smaller
seedlings, with correspondingly slower-extending root systems, that grew less than
the offspring of well-watered parents in both dry and moist seedling conditions
(Sultan et al., 2009). Seedlings of both species increased root:shoot biomass ratio and
specific root length when grown in dry soil; these developmental traits were not influenced by parental environment. Seedling phenotypes in these taxa thus reflected both inherited (transgenerational) and immediate responses to moisture environment (Sultan et al., 2009). Note that the combination of these two modes of plasticity resulted in an earlier and more pronounced adaptive response in *P. persicaria* seedlings than could be achieved via immediate plasticity alone, a crucial benefit of transgenerational effects (see also Agrawal et al., 1999).

Further studies (J.J. Herman, T. Horgan-Kobelski, C. Riggs, and S.E. Sultan, unpublished data) show that the adaptive effects of drought stress in *P. persicaria* persist across two generations: the grandchildren of drought-stressed plants were more well-provisioned, grew larger, and produced deeper and more extensive root systems in dry soil than the grandchildren of well-watered plants of the same inbred genetic lines. Interestingly, this effect remained significant even when seedling biomass was removed as a covariate, showing that production of these enhanced root systems is not simply due to greater provisioning, but reflects a specific developmental adjustment likely mediated by hormonal and/or epigenetic effects shaped by natural selection (Sultan et al., 2009). Higher survivorship in dry soil of seedlings produced after one or two generations of drought stress (J.J. Herman and S.E. Sultan, unpublished data) indicates that these transgenerational effects on development are indeed likely to influence realized fitness in nature, where soil moisture varies spatially and temporally within and among *Polygonum* microsites (Sultan et al., 1998).
A central insight from this body of work is that transgenerational consequences of resource deprivation in plants are not generalized, passive stress effects. Rather, these are specific developmental responses that vary depending on the resource or combination of resources in question and their precise state(s) (Sultan, 2000; e.g. Sultan, 1996; 2001). These transgenerational responses also vary among naturally evolved genomes: even relatively small samples of Polygonum genotypes reveal significant variation for provisioning, germination, and root-length responses to parental drought; germination and achene-mass responses to parental nutrient stress; and developmental effects of parental shade (Schmitt et al., 1992). These differences at the genotype level (documented in analyses of variance as significant genotype-by-parental environment interaction effects) provide the raw material for continued adaptive evolution of transgenerational response patterns, just as genotype-by-environment effects fuel selective evolution of immediate plasticity patterns (Via and Lande, 1985; Sultan, 2007; Sultan, 2011).

Surprisingly, transgenerational effects of parental resource stress can also vary among offspring that develop in different architectural positions on the maternal plant. P. hydropiper is a species that produces achenes in both axial and terminal inflorescences on each plant. Terminally produced offspring of parents grown in simulated shade expressed dramatically different developmental trajectories than terminal offspring of highly inbred replicate plants given full sun: they produced leaves significantly earlier and in greater numbers, grew taller faster, and produced more than double the biomass by day 21 (Lundgren and Sultan, 2005). However,
none of these adaptive transgenerational effects of parental shade on seed
provisioning and shoot development were expressed in the axially produced achenes
(Lundgren and Sultan, 2005). Although position effects on offspring size are
generally interpreted as the result of seed location relative to plant vasculature and
source-sink relations (Diggle, 1995; Imbert, 2002), these more complex positional
differences in transgenerational response may instead reflect an evolved response to
‘prioritize’ terminal achenes, which have greater dispersal ability (see Imbert and
Ronce, 2001; Diggle and Miller, 2004).

As described above, patterns of transgenerational plasticity can also differ
dramatically even among very closely related taxa, such as annual Polygonum species
within a monophyletic subgroup of the genus. Comparisons among four such species
showed significant differences in both the magnitude and direction of effects of
drought and nutrient stress on individual achene mass (Sultan, 2001), seedling
biomass, and rooting depth rate (S. Elmendorf and S.E. Sultan, unpublished data). For
instance, three of the species adaptively increased seedling specific root length (SRL)
and extension rate in response to parental nutrient deprivation compared with
offspring of well-nourished parents, but seedlings in a fourth species reduced SRL
and reached a soil depth threshold 1.5 days slower than offspring of well-nourished
plants in response to the same parental nutrient stress (S. Elmendorf and S.E. Sultan,
unpublished data). Species-level differences in the capacity for adaptive
transgenerational plasticity demonstrate that these response patterns, like other
products of evolution, are shaped by genetic constraints and random forces as well as by natural selection.

**Mechanisms of transgenerational response**

Plastic responses to environmental stress can be transmitted across plant generations via multiple mechanisms, independently or indeed in the absence of DNA sequence variation. It is well known that environmental challenges to a maternal plant can affect the quantity and composition of starch reserves, mRNAs, proteins, hormones, and other primary and secondary metabolites packaged into seeds (Roach and Wulff, 1987; Leishman et al., 2000; Fenner and Thompson, 2005; Moles and Leishman, 2008), and that these seed resources are critical for both germination and initial seedling growth. Recently, it has become clear that stressful parental environments can also induce epigenetic variation, specifically in DNA methylation patterns, that is associated with changes in ecologically important traits, and that may persist for several – and possibly many – generations (Jablonka and Raz, 2009; Hauser et al., 2011). Differences in the duration of various transgenerational effects may reflect differences in their underlying mechanisms: for instance, provisioning effects are likely to be shorter lived than most DNA methylation marks (Latzel and Klimešová, 2010), both within an individual’s life-cycle and across generations.

Below we present a brief overview of these various mechanisms, noting that they are
neither rigidly separate nor mutually exclusive: more than one mechanism or type of mechanism can jointly influence heritable phenotypes.

Transgenerational plasticity via seed provisioning

Seed provisioning refers to the carbohydrate, lipid, protein, and mineral nutrient reserves allocated by the maternal plant to the developing seed (Koller, 1972; Srivastava, 2002). These reserves are mobilized in germinating seedlings to produce the initial shoot and root systems that allow for establishment as a functional individual. Seed provisioning is often reduced when maternal plants are deprived of resources such as light or minerals, resulting in diminished early growth rates, seedling size and competitive ability (Haig and Westoby, 1988; Fenner and Thompson, 2005). In contrast to these maladaptive transgenerational effects, environmentally stressed maternal plants of certain species are able to maintain or even increase seed provisioning (Roach and Wulff, 1987; Schmitt et al., 1992; Sultan, 1996; Donohue and Schmitt, 1998; Sultan, 2001), an adaptive response that can maximize seedling survival. This head start in growth can provide a crucial advantage at this vulnerable life history stage, particularly in stressful conditions (Wulff, 1986b; Agrawal et al., 1999; Moles and Westoby, 2006). For instance, well-provisioned offspring can produce more extensive root systems in dry soil, or larger shoot systems under canopy shade (Silvertown, 1984; Wulff, 1986a; Leishman et al., 2000). In natural populations, the adaptive benefit of such enhanced provisioning may be
limited in two ways. First, resource-deprived maternal plants inevitably produce fewer seeds, even if each one is more likely to successfully establish; and second, trade-offs may exist between increased seed provisioning and decreased persistence in the soil seed bank (Sultan, 1996; Donohue and Schmitt, 1998; Fenner and Thompson, 2005). Hence, transgenerational effects that are mediated via seed provisioning can promote offspring success in stressful conditions, but these benefits will depend on the specific ecological setting.

Transgenerational plasticity via mRNAs, proteins, and hormones

Numerous studies have identified effects of environmental stress on offspring development that are not related to seed provisioning, indicating that other mechanisms commonly mediate this transgenerational aspect of plasticity (e.g. Case et al., 1996; Agrawal, 2001; 2002; Bischoff and Muller-Scharer, 2010; Dyer et al., 2010). As discussed below, seed mass-independent responses to parental stress may be transmitted to offspring via maternally derived proteins and mRNAs or other small RNAs; defensive chemicals or other secondary metabolites; changes in the relative concentrations of hormones; and/or environmentally induced epigenetic marks, such as DNA methylation or histone modifications. Note that maternally derived proteins can affect offspring phenotypes via both as regulatory molecules, as described below, and as nutritive elements (see Transgenerational plasticity via seed provisioning section).
Maternally derived mRNAs and proteins play an important role in the regulation of seed dormancy and germination (Donohue, 2009). Rajjou and colleagues found that non-dormant *Arabidopsis thaliana* seeds can germinate in the absence of post-dispersal transcription, and that there is very little transcriptional activity for the first 16h following germination (Rajjou et al., 2004). This remarkable result shows that stored proteins and mRNAs are adequate for germination of non-dormant seeds in *Arabidopsis*. (Transcription-inhibited seeds did germinate more slowly than control seeds, indicating that *de novo* gene expression is important in regulating germination rate.) Noting that environmental stress can alter many aspects of maternal gene expression, the authors suggest that translation of maternally derived mRNAs may facilitate adaptive growth responses for seeds germinating under stressful conditions (Rajjou et al., 2004).

Maternal environmental stress can also alter seed hormone content and embryonic sensitivity to hormones. For example, experimentally shaded *Amaranthus palmeri* plants increased the abscisic acid (ABA) content of their seeds by 44% (Jha et al., 2010), and maternal drought stress induced changes in both seed ABA content and embryo sensitivity to ABA in *Sorghum bicolor* (Arnold et al., 1991). Because ABA is a central regulator of plant growth throughout the lifecycle, especially under stressful conditions (Holdsworth et al., 2008a; Holdsworth et al., 2008b; Cutler et al., 2010; Peleg and Blumwald, 2011), these results suggest that such hormone adjustments are a likely mechanism for transgenerational environmental effects on offspring growth and development (Sultan, 1996; Baskin and Baskin, 1998).
However, maternal environmental effects on seed hormone content, and the realized impact of such effects on seedling development compared with other factors, are not yet well known. For instance, seed germination is influenced by embryonic gene expression and levels of tissue ABA (Kucera et al., 2005; Donohue, 2009) as well as maternal mRNAs and proteins (Rajjou et al., 2004), but their relative importance as regulators of ecologically appropriate germination behavior is unclear (Donohue, 2009). Although it is challenging to study hormone levels in seed tissues, this potentially important mode of adaptive transgenerational stress response merits further study.

**DNA methylation and histone modifications**

DNA methylation marks tend to silence gene expression by forming densely compact chromatin; these marks are both environmentally sensitive and heritable over multiple (i.e., ≥ 8) generations (Johannes et al. 2009; Reinders et al. 2009; comprehensively reviewed by Jablonka and Raz, 2009 and Hauser et al. 2011). Accordingly, DNA methylation (and possibly other epigenetic mechanisms) is likely to play an important role in regulating transgenerational effects of environmental stress (Kalisz and Purugganan, 2004; Grant-Downton and Dickinson, 2006; Boyko and Kovalchuk, 2011). Recent studies of epigenetic recombinant inbred lines (epiRILs) in *Arabidopsis* provide important insights to these inherited effects on gene expression. These epiRILs were derived from two isogenic parental lines: one
homozygous for a mutation that causes a deficiency in DNA methylation, and the other wild type. As a result, epiRILs have segregating DNA methylation variation, but zero DNA sequence variation (Johannes et al., 2009; Reinders et al., 2009; Teixeira et al., 2009), allowing for the study of quantitative epigenetic variation in plant traits without confounding effects of genetic variation (Richards, 2009). Several important conclusions emerge from the work on epiRILs thus far: (1) stable inheritance of DNA methylation variation can occur at a large number of sites within the genome (for instance, Reinders and colleagues (2009) found 6532 of these sites in one epiRIL); (2) DNA methylation epialleles have a range of transgenerational stabilities; and (3) such variation often has substantial effects on ecologically important, fitness-related traits such as reproductive phenology, germination timing, plant height, and pathogen resistance (Johannes et al., 2009; Reinders et al., 2009; Teixeira et al. 2009).

A wide variety of naturally occurring plant environmental stresses can induce DNA methylation changes, including drought (Labra et al., 2002; Boyko et al., 2010; Wang et al., 2011), flooding (Boyko et al., 2010), nutrient limitation (Boyko et al., 2010; Kou et al., 2011), temperature shock (Boyko et al., 2010), pathogen infection (Boyko et al., 2007; Kathiria et al., 2010), high salinity (Boyko et al., 2010; Verhoeven et al., 2010), heavy metal exposure (Aina et al., 2004), UV radiation (Boyko et al., 2010), and possibly herbivory (Herrera and Bazaga, 2011; Scoville et al., 2011). However, despite intense interest in the subject, relatively few studies have focused on potentially adaptive effects of DNA methylation changes induced by such
ecologically relevant environmental stresses—i.e., on DNA methylation as a mechanism of adaptive transgenerational stress response. In one case, Boyko and colleagues (2010) found that progeny of salt-stressed *Arabidopsis* parents had increased tolerance to salt stress. This adaptive response correlated with inheritance of stress-induced DNA methylation marks, as well as increased frequency of somatic homologous recombination. (Note that heritable changes in homologous recombination rate may not be a general stress response in *Arabidopsis*, as shown by the weak and inconsistent effects of various chemical toxins on progeny recombination rates in two transgenic lines; Pecinka et al., 2009). Further study showed that this response depended on the action of *Dicer*-like proteins that operate in the small RNA pathway (Boyko et al., 2010). Infection with tobacco mosaic virus (TMV) also caused heritable changes in DNA methylation (again associated with increased recombination frequency) and greater pathogen resistance in progeny, possibly due to the higher constitutive and induced levels of *PATHOGENESIS-RELATED GENE1* in progeny of TMV-infected plants (Kathiria et al., 2010). These mechanistic studies are among the first to show that environmentally induced DNA methylation changes are associated with adaptive effects on offspring. Much further study is needed, however, because it is still unclear exactly what signal is transmitted across generations: DNA methylation marks might escape resetting in the germline, or they might be re-instated by the action of transmitted small RNAs or histone modifications (Hauser et al., 2011; Paszkowski and Grossniklaus, 2011).
Verhoeven and colleagues (2010) focused on the inheritance of stress-induced DNA methylation variation in apomictic dandelions (*Taraxacum officinale*), a naturally evolved, non-model system. They exposed genetically identical parent dandelion plants to a variety of environmental stress treatments, and then analyzed changes in DNA methylation that were transmitted to untreated progeny (Verhoeven et al., 2010). Salicylic acid, jasmonic acid, salt stress, and nutrient limitation each induced a considerable number of DNA methylation changes relative to controls, with 74-92% of these changes transmitted to progeny (salicylic acid induced a greater number of heritable changes than the other stresses). Importantly, many of the methylation changes induced by salicylic acid (simulated pathogen attack) were targeted to specific areas of the genome. This divergence in DNA methylation pattern suggests that methylation changes were directed to specific stress-response genes (Verhoeven et al., 2010). A key contribution of this study is that it demonstrates, in a naturally evolved taxon, both the environmental sensitivity of DNA methylation and the potential of DNA methylation to operate as an independent inheritance system. However, because only one genotype was studied for a single offspring generation, and because effects on offspring phenotype were not reported, the ecological and evolutionary relevance of these findings remains to be determined (Verhoeven et al., 2010).

Post-translational modifications of histone proteins can also affect gene expression by altering chromatin structure, and studies in yeast (Grewal et al., 1998), mice (Blewitt et al., 2006), and *Arabidopsis* (Lang-Mladek et al., 2010) suggest that
these modifications can be transferred across generations. Although more than 100 different histone modifications have been identified, the consequences of the vast majority of these modifications are unknown (Bernstein et al., 2007), and it is not yet clear to what extent these changes act independently of DNA methylation effects. One study in *Arabidopsis* found that heat stress and UV-B exposure each induced heritable changes in gene expression that correlated with histone H3 deacetylation in the absence of DNA methylation changes (Lang-Mladek et al., 2010). This effect on gene expression persisted for two progeny generations, but occurred only in small groups of cells within the plant.

*Joint effects of transgenerational plasticity mechanisms*

As noted previously, two or more modes of transgenerational stress response may act in combination to influence offspring phenotypes. For example, Agrawal and coworkers observed both seed mass-dependent and seed mass-independent responses to maternal herbivory in wild radish, *Raphanus raphanistrum* (Agrawal et al., 1999; Agrawal, 2001; 2002). (Seed mass is generally a reliable proxy for seed provisioning; Moles and Leishman, 2008, but see Lacey et al., 1997). Caterpillar herbivory significantly increased the mass of individual seeds produced (see preceding section), but also significantly enhanced seedling growth and leaf trichome density independently of seed mass effects (Agrawal, 2001; 2002). Although the mechanisms of these non-provisioning effects have not been determined with certainty, there is
some evidence that allocation of defensive chemicals and/or defense-inducing hormones to seeds may be involved (Agrawal, 2002; epigenetic mechanisms are a further possibility, but have not been investigated in this system). Scoville et al. (2011) linked increased trichome density in progeny of maternal *Mimulus guttatus* plants subjected to simulated herbivory to epigenetically inherited changes in the expression of a MYB transcription factor. Preliminary evidence suggests that DNA methylation is likely involved in transmitting the high trichome density phenotype across generations (Scoville et al., 2011). These results illustrate how a single environmental stress can induce multiple physiological and epigenetic changes that together enhance offspring performance.

A second way that transgenerational mechanisms can interact is via hormonal effects on epigenetic marks. Hormones can influence gene expression in response to stress via effects on both histone modifications and DNA methylation (Chinnusamy and Zhu, 2009). Studies in *Arabidopsis* and rice have shown that a variety of environmental stresses and resulting hormone signals influence the expression of histone deacetylases that downregulate gene expression (Zhou et al., 2005; Fu et al., 2007; Wu et al., 2008). For example, ABA reduced the expression of the histone deacetylase *AtHD2C* in *Arabidopsis*, causing a decrease in stomatal conductance, which is a critical response to drought stress (Sridha and Wu, 2006). Hormone-mediated changes in histone modifications may also affect DNA methylation via the RNA-directed DNA methylation (RdDM) pathway. The histone deacetylase *HDA6* is both required for the jasmonate response and involved in RdDM, suggesting a
potential role for jasmonate in regulation of DNA methylation via effects on histone acetylation (Aufsatz et al., 2002; Probst et al., 2004; Wu et al., 2008). Any constituent of these environmentally sensitive and interacting pathways – hormone, RNA, histone modification, or DNA methylation – could be transmitted across generations, forming the heritable basis for transgenerational plasticity (Jablonka and Raz, 2009; Hauser et al., 2011). Investigating these interacting pathways offers an exciting research direction that promises to link signal transduction of environmental cues to potentially heritable changes in phenotypic expression.

**Conclusions: Ecological and Evolutionary Implications**

Although a great deal remains to be learned about the ability to express adaptive transgenerational plasticity in diverse plant taxa, populations, and genotypes in response to various abiotic and biotic environmental factors, and about the precise mechanisms, nature, and duration of those responses, existing knowledge leads to a fundamental insight: Because this mode of individual plasticity gives rise to phenotypes that are both adaptive to the conditions that induce them and inherited, it can influence the ecological distribution of plant populations as well as their evolutionary trajectories.

With respect to ecological distribution, taxa capable of such plasticity may be more likely to establish and maintain populations in variably or consistently stressful habitats, because offspring will be specifically pre-adapted to withstand the low
resource levels or other stresses experienced by their parent(s). As noted above, transgenerational plasticity can allow offspring individuals to express a more extreme adaptive phenotype early in life, without undergoing the developmental lag time required for response to the immediate environment (Agrawal et al. 1999; Sultan 1996; Sultan et al. 2009). Because the great majority of plant mortality occurs at the seedling stage (Fenner and Thompson, 2005; Moles and Leishman, 2008), this enhanced ecological tolerance is likely to obtain even in the case of provisioning effects that are expressed only during initial life-cycle stages, or that persist for only a single generation. Indeed, transgenerational effects that persist for one or few generations can substantially influence both phenotypic distributions and population growth rates (Galloway and Etterson, 2007; Donohue, 2009; Inchausti and Ginzburg, 2009).

Existing repertoires of transgenerational plasticity may also allow certain taxa to better tolerate novel stressful environments, such as the global temperature and moisture changes predicted to arise very rapidly due to human activities. This may be of particular benefit in long-lived woody and perennial taxa, in which adaptation by selective evolution is unlikely to keep pace with such rapid environmental changes. Note that these same ecological effects are also likely to promote the spread of invasive plants, which may more successfully withstand environmental stresses encountered in a new, introduced range, and more quickly colonize its diverse habitats, by virtue of transgenerational pre-adaptations. Moreover, transgenerational plasticity may not only hasten the spread of invasive taxa compared with the slower
process of evolutionary adaptation, it may allow for ecological spread in cases where invasive populations lack the genetic potential for such evolutionary change (Hollingsworth and Bailey, 2000; Dlugosch and Parker, 2008; Dyer et al., 2010).

Evolutionary consequences of transgenerational responses are an equally promising area for further investigation. Such responses depart from standard evolutionary scenarios in two key ways (Jablonka and Raz, 2009; Verhoeven et al., 2010). First, transgenerational plasticity gives rise to adaptive heritable variation precisely when it is required in a population, in contrast to randomly occurring genetic variation (Verhoeven et al., 2010). Second, a given environmental stress can induce the same adaptive phenotype in numerous offspring individuals in a population at the same time, again in contrast to a new phenotype arising due to mutation in one or few individuals. In consequence, populations can undergo rapid and wholesale phenotypic adaptation in the absence of allelic (DNA sequence) frequency change (Jablonka and Raz, 2009). Indeed, adaptive adjustments due to transgenerational plasticity may buffer a population against such evolutionary change by permitting existing genotypes to maintain fitness in the face of environmental challenges (Sultan, 2000).

To some extent, the inheritance mechanisms and relative persistence of these environmental effects will shape their evolutionary impact. For instance, because epialleles (such as alternative patterns of DNA methylation) induced by environmental stress influence individual fitness, in cases where they persist across
multiple generations, adaptive evolution could proceed by selection of such epigenetic variants alone. As noted above, such change could take place much faster than standard evolution by allelic substitution (Jablonska and Lamb, 1995; Richards, 2006; Bossdorf et al., 2008). Selection of epialleles may also influence evolution by shifting the relative frequencies of correlated genetic variants (Jablonska and Raz, 2009). In contrast, short-lived effects on provisioning (or transient epigenetic effects) may primarily serve to buffer plant populations from selective change by allowing individuals to express similar, adaptive phenotypes.

A central goal for future transgenerational plasticity research is to integrate studies of inheritance mechanisms with research designed to incorporate ecological and evolutionary realism; e.g., studies in naturally evolved systems that test response to field-based environments. For instance, transgenerational plasticity experiments could be combined with a methylation-sensitive AFLP (MS-AFLP) assay, to test for the involvement of DNA methylation in the production and maintenance of environmentally induced adaptive, heritable phenotypes. As in recent studies of epigenetic variation in natural plant populations (Herrera and Bazaga, 2010; 2011; discussed in Richards et al., 2010; Bossdorf and Zhang, 2011), population genetic analyses could be employed to assess epigenetic differentiation among treatment groups and taxa, and to test for statistical associations between specific epialleles and adaptive phenotypes. Interdisciplinary studies of transgenerational plasticity that focus jointly on the mechanisms of transmission and on the adaptive (developmental
and fitness) consequences of these induced responses will be of particular value in understanding this intriguing aspect of plant plasticity.

Acknowledgments

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Table 1. Examples of adaptive transgenerational plasticity.

<table>
<thead>
<tr>
<th>Parental environment</th>
<th>Species</th>
<th>Offspring trait affected</th>
<th>Number of generations inherited</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>High temperature</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Fruit and seed production</td>
<td>2</td>
<td>Whittle et al., 2009</td>
</tr>
<tr>
<td>Low temperature</td>
<td><em>Plantago lanceolata</em></td>
<td>Seed mass; probability of flowering; leaf area</td>
<td>2</td>
<td>Case et al., 1996; Lacey, 1996</td>
</tr>
<tr>
<td>Nutrient deficiency</td>
<td><em>Oryza sativa</em></td>
<td>Biomass; plant height</td>
<td>2</td>
<td>Kou et al., 2011</td>
</tr>
<tr>
<td>Nutrient deficiency</td>
<td><em>Plantago lanceolata</em></td>
<td>Leaf biomass</td>
<td>1</td>
<td>Latzel et al., 2010</td>
</tr>
<tr>
<td>Nutrient deficiency</td>
<td><em>Polygonum persicaria</em></td>
<td>Root allocation</td>
<td>1</td>
<td>Sultan, 1996</td>
</tr>
<tr>
<td>High salinity</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Germination; seedling growth</td>
<td>1</td>
<td>Boyko et al., 2010</td>
</tr>
<tr>
<td>Drought</td>
<td><em>Polygonum persicaria</em></td>
<td>Biomass; root length, depth, and extension rate; seed provision; germination</td>
<td>1</td>
<td>Sultan, 1996; 2001; Sultan et al., 2009</td>
</tr>
<tr>
<td>Serpentine soil</td>
<td><em>Aegilops triuncialis</em></td>
<td>Shoot biomass; phenology</td>
<td>1</td>
<td>Dyer et al., 2010</td>
</tr>
<tr>
<td>Disturbance (mediated by nutrient environment)</td>
<td><em>Plantago lanceolata</em></td>
<td>Shoot biomass</td>
<td>1</td>
<td>Latzel et al., 2010</td>
</tr>
<tr>
<td>Shade</td>
<td><em>Plantago lanceolata</em></td>
<td>Cumulative fitness of maternal and offspring generations</td>
<td>1</td>
<td>Donohue &amp; Schmitt, 1998</td>
</tr>
<tr>
<td>Shade</td>
<td>Polygonum hydropiper</td>
<td>Seed provisioning; timing/amount of leaves produced; biomass</td>
<td>1</td>
<td>Lundgren &amp; Sultan, 2005</td>
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</tr>
<tr>
<td>Light habitat</td>
<td>Campanulastrum americanum</td>
<td>Germination; seed mass; seedling survival; leaf area; life history</td>
<td>1</td>
<td>Galloway &amp; Etterson, 2007 and 2009</td>
</tr>
<tr>
<td>Herbivory</td>
<td>Raphanus raphanistrum</td>
<td>Seed mass; seedling growth; leaf trichome density</td>
<td>1</td>
<td>Agrawal et al., 1999; Agrawal 2001, 2002</td>
</tr>
<tr>
<td>Simulated herbivory</td>
<td>Mimulus guttatus</td>
<td>Leaf trichome density</td>
<td>1</td>
<td>Holeski, 2007; Scoville et al. 2011</td>
</tr>
<tr>
<td>Herbivory</td>
<td>Impatiens capensis</td>
<td>Emergence; flowering; plant height; biomass</td>
<td>1</td>
<td>Steets &amp; Ashman, 2010</td>
</tr>
<tr>
<td>Viral infection</td>
<td>Nicotiana tabacum</td>
<td>Pathogen resistance; homologous recombination frequency</td>
<td>1</td>
<td>Boyko et al., 2007; Kathiria et al. 2010</td>
</tr>
<tr>
<td>Seasonal environments</td>
<td>Arabidopsis thaliana</td>
<td>Germination timing; life history schedule</td>
<td>1</td>
<td>Donohue et al. 2005; Donohue 2009</td>
</tr>
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Chapter 2: Adaptive transgenerational plasticity in an annual plant: grandparental and parental drought stress enhance performance of seedlings in dry soil

Jacob J. Herman, Sonia E. Sultan, Tim Horgan-Kobelski, and Charlotte Riggs

Abstract

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 versus moist soil environments for two generations in a fully factorial design, producing replicate

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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 non-stressed 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 “pre-adapt” 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 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 (a) dispersal of propagules is spatially limited, and (b) 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 maculata, 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). *P. persicaria* meets the two conditions described above for evolution of adaptive transgenerational plasticity, including environmental variation from year to year (i.e., relatively dry versus 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 versus 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?

**Materials and 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™, Profile Products, Buffalo Grove, Illinois, USA) in a glasshouse under full summer sun (mean midday PAR ± SD = 1239 ± 108 µmol m⁻² s⁻¹). Soil treatments were maintained respectively at 13.2% ± 5.8% (Dry) and 26.6% ± 4.1% (Moist) soil moisture by mass, corresponding to ca. 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 twenty-five air-dried achenes from each combination of genetic line x grandparental treatment x parental treatment (n=20 experimental units) were individually weighed on a Cahn C-33 microbalance (Cahn Instruments, Cerritos, California, USA) and stratified in distilled water at 4°C for 40 days in 96-well tissue-culture trays (BD Falcon, Franklin Lakes, New Jersey, USA). Stratified achenes were then sown individually on moist filter paper in 24-well tissue-culture trays (BD Falcon, Franklin Lakes, New Jersey, USA) and germinated in a Conviron growth chamber (Controlled Environments, Winnipeg, Manitoba, Canada) set for a 25°C:18°C 14:10h day:night cycle. Fluorescent lights provided ca. 500 µmol m⁻² s⁻¹ PAR during the first two 14h 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 re-randomized tissue-culture trays at 10 am daily, recording the day of germination (germination timing) of each achene.

Seedlings were harvested 96h after germination (Sultan 1996) and dried for 1h at 100°C and ≥ 72h 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 x 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 8 and 6 seedlings, respectively. Because
germination was less than 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 x grandparental treatment x parental treatment were stratified in distilled water at 4°C for ten weeks and then sown on moist filter paper in petri plates (90 x 15 mm) on a glasshouse bench. Each day, petri-plate positions were re-randomized and germination was censused. One hundred twenty hours after germination, six replicate seedlings from each combination of genetic line x grandparental treatment x 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™), pre-moistened with 40 ml of distilled water per liter of soil mix. Rhizotrons were made from 245-mm-square bio-assay dishes (Corning, Lowell, Massachusetts, 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-degree 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 d, in a dual Conviron growth chamber programmed for a 25°:18°C 14:10h day:night cycle, with fluorescent lights providing ca. 500 µmol m⁻² s⁻¹ daytime PAR. Seedlings were watered individually with distilled water as needed to maintain ~13% soil moisture by mass, corresponding to ca. 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 1h and at 65°C for ≥ 48h 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 ≥ 48h 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 x grandparental treatment x parental treatment were stratified in distilled water at 4°C for 40 d 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 re-randomized and germination was censused. Ninety-six h after germination, 12 replicate seedlings of each combination of genetic line x grandparental treatment x 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, pre-moistened 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 hours 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 x 15mm) and set in a randomized complete block design in a dual Conviron growth chamber programmed as described above, with fluorescent lights providing *ca.* 500 μmol 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 nine-day experiment. Every day, each seedling received two ml of distilled water at the soil surface and six 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 nine 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 non-significant 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 versus 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 on seedling biomass.

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 non-parametric log-rank test (Kleinbaum and Klein 2005). All statistical analyses were performed with JMP version 7.0.1 (SAS Institute, Cary, North Carolina, 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 x 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 non-significantly) in the effect of prior drought history (effects of genetic line and genetic line x grandparental environment x parental environment, Table 1; see Supplemental Fig. 1 for norms of reaction).

Two prior generations of drought stress also resulted in significantly decreased pericarp proportion and earlier germination (Fig. 1b, c). DD achenes had ~10% less pericarp tissue and germinated ~0.5-1d day earlier compared to those from the other treatments (significant grandparental environment x parental environment interactions, Table 1; Fig. 1b, c). The effect of drought-stress history on pericarp proportion varied among genetic lines (genetic line x grandparental environment x parental environment interaction, Table 1; see Supplemental 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 x grandparental environment and genetic line x parental environment interactions, Table 1; Supplemental 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 d in dry soil than did MM individuals of the same genetic lines (Fig. 2a). Parental drought alone also (non-significantly) increased seedling biomass (21.5% increase in MD versus MM seedlings; Fig. 2a).

In contrast, either grandparental or parental drought alone increased total root length (DM and MD versus 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.

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 x parental environment, genetic line x grandparental environment, or a three-way interaction; Table 2) apart from a marginally non-significant effect of genetic line x grandparental environment on seedling biomass.

*Seedling survival in a naturalistic dry treatment*

DD seedlings had the highest survivorship, after nine 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*. 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 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 (see 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 one or two days as shown here, 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 in the Polygonum system to test for possible differences in DNA methylation patterns 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 x 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 “pre-adapts” 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 pre-adaptation can significantly increase the survival of offspring, as was the case in this study (see also
Galloway and Etterson 2007). Second, pre-induced 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; Moczek et al. 2011).

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

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<td></td>
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<td>F</td>
<td>P</td>
</tr>
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*Note:* The table shows the sources of variation, degrees of freedom (df), mean squares (MS), F statistics, and P-values for seed provisioning, pericarp proportion, and germination day.
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). † $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

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Figure 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.
**Figure 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.
**Figure 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, $P = 0.011$).
Supplemental Figure 1. Genotypic norms of reaction for (A) seed provisioning (96 h biomass), (B) offspring structure (proportion of achene mass in pericarp), and (C) germination timing are shown for offspring of droughted grandparents and parents (DD), droughted grandparents (DM), droughted parents (MD), and moist-grown grandparents and parents (MM). Five inbred genetic lines are shown, derived from field genotypes drawn from three natural populations.
Chapter 3: DNA methylation mediates genotype × environment variation for adaptive transgenerational plasticity

Jacob J. Herman and Sonia E. Sultan

Abstract

The environment experienced by parent individuals can profoundly affect offspring phenotypes, in some cases inducing developmental adjustments that enhance offspring growth and survival. Such adaptive transgenerational plasticity is well documented, but the mechanisms responsible for the inheritance of these environmentally induced effects are generally unknown. One possible mechanism is environmentally induced changes to DNA methylation. We tested this hypothesis in the annual plant *Polygonum persicaria*, a species known to express adaptive transgenerational plasticity in response to parental drought stress. Replicate individuals of 12 inbred genetic lines (sampled from natural populations) were grown in dry- versus moist-soil environments, and their offspring were exposed to the demethylating agent zebularine or to control (zebularine-free) conditions during germination. Offspring were then grown under drought stress for three weeks. As previously documented, under control germination conditions the offspring of drought-stressed parents grew longer root systems, larger leaf areas, and attained greater biomass compared to offspring of well-watered parents of the same genetic
lines. Demethylation of offspring DNA removed these adaptive developmental effects of parental drought, but did not significantly alter phenotypic expression in offspring of well-watered parents. Differential seed provisioning, a potential mechanism of transgenerational plasticity in plants, did not contribute to the effect of parental drought on offspring performance. The effect of demethylation on the expression of the parental drought effect varied significantly among genetic lines. These results reveal that DNA methylation mediates the genotype-specific effects of parental environment on adaptively important offspring traits.

Introduction

Parental environment can influence the phenotypes of offspring in diverse taxa (Roach and Wulff 1987; Falconer and Mackay 1996; Bateson et al. 2004; Gluckman and Hanson 2004). Such inherited environmental effects on development or transgenerational plasticity (also known as parental environmental effects) were initially understood to directly reflect resource levels, with resource-deprived parents producing low-quality offspring via maladaptive effects on provisioning to seeds or eggs. While these direct effects of provisioning are indeed widespread, additional aspects of parental environmental effects have increasingly come to light. It is now clear that in some cases stressed parental individuals produce offspring that are developmentally altered in specific ways that mitigate that particular type of stress, resulting in heritable, environmentally-induced adaptation when offspring encounter
similar conditions (Sultan 1996; Mousseau and Fox 1998; Galloway and Eterson 2007; Holeski 2007; Storm and Lima 2010; Salinas and Munch 2012; Walsh et al. 2016). As with other aspects of individual phenotypic plasticity (Scheiner 1993; Des Marais et al. 2013), genotypes differ in their precise patterns and degrees of these environmentally induced effects (Schmitt et al. 1992; Sultan 1996; Stjernman and Little 2011; Alsdurf et al. 2013; Walsh et al. 2015).

Despite the increasing awareness of the potential adaptive value of transgenerational plasticity, in most cases the mechanisms responsible for the transmission of these effects on offspring phenotype remain unclear (Herman and Sultan 2011; Holeski et al. 2012; Salinas et al. 2013). In some systems it is known that adaptive transgenerational effects stem from increases in the provisioning of nutritive resources to seeds or eggs (e.g., Wulff 1986; Fox et al. 1997; Donohue and Schmitt 1998; Agrawal 2001). Increased provisioning can enable greater early growth in offspring, which may increase competitive ability and the likelihood of survival, especially in stressful, low-resource environments (Mousseau and Fox 1998). However, since provisioning-based effects are mediated directly by maternal individuals, environmental effects that persist for multiple generations (e.g., Whittle et al. 2009; Kou et al. 2011; Herman et al. 2012; Shama and Wegner 2014) must stem from mechanisms capable of longer-term stability.

One such mechanism is heritable, environmentally induced changes to DNA methylation patterns in the genome (Bossdorf et al. 2008; Kappeler and Meaney...
In many taxa, the addition or removal of methyl groups on cytosine residues can influence transcriptional activity at specific loci. In plants, numerous environmental stimuli can induce changes to DNA methylation throughout the genome, ranging from biotic stresses such as pathogen infection (Dowen et al. 2012; Pastor et al. 2013; Yu et al. 2013) to abiotic stresses such as drought (Labra et al. 2002; Wang et al. 2011; Zheng et al. 2013; Alsdurf et al. 2015). Such environmentally induced changes in methylation vary by genotype (Dubin et al. 2015), and have been shown to be heritable in a variety of species, in some cases for multiple generations (Jablonka and Raz 2009; Feil and Fraga 2012). Furthermore, heritable methylation variation can have substantial impacts on ecologically important traits. For instance, studies of epigenetic recombinant inbred lines in *Arabidopsis thaliana* showed that DNA methylation variants can be inherited for at least tens of generations, causing substantial heritable variation in complex traits such as primary root length and flowering time in the absence of DNA sequence diversity (Cortijo et al. 2014). This combination of genotype-specific environmental sensitivity, transgenerational stability, and phenotypic impact makes DNA methylation a primary candidate mechanism for adaptive transgenerational plasticity, particularly in plants (Herman and Sultan 2011; Holeski et al. 2012; for discussion of DNA methylation and parental effects in mammals, see Kappeler and Meaney 2010; Daxinger and Whitelaw 2012).

A central yet unresolved question concerns the extent to which genotypes in natural populations vary in their epigenetic responses to the parental environment.
(Schaefer and Nadeau 2015). The prevalence of genotype × parental environment interactions on offspring phenotypes suggests that genotype-specific patterns of epigenetic response may underlie this common form of genetic variation. Such G × E variation provides the substrate for the adaptive evolution of transgenerational plasticity, just as genetic variation for responses to the immediate environment fuels the adaptive evolution of within-generation plasticity (Via and Lande 1985; Sultan 2007; Chevin et al. 2009; Scheiner and Holt 2012). Demonstrating a role for DNA methylation in genotype × parental environment interactions would provide compelling evidence for the evolutionary relevance of environmentally induced epigenetic variation.

Experimental demethylation using pharmacological agents is a well-established method for investigating whether DNA methylation mediates phenotypic expression in diverse animal, plant, and fungal systems (Bossdorf et al. 2010; Boyko et al. 2010; Herrera et al. 2012; Verhoeven and van Gurp 2012; Cook et al. 2015; Akkerman et al. 2016). Several such agents are known to interfere with the DNA methyltransferase enzymes that establish and maintain methylation of cytosine residues, leading to genome-wide reductions in DNA methylation levels. Zebularine is particularly useful as an experimental demethylation agent because its methyltransferase-inhibiting effects are transient and dose-dependent, in contrast to toxic demethylating chemicals such as 5-azacytidine and to mutational approaches which cause long-lasting and drastic changes in methylation (Zhou et al. 2002; Cheng et al. 2003; Marquez et al. 2005; Baubec et al. 2009).
We used zebularine to investigate the functional role of DNA methylation in the adaptive, drought-induced transgenerational plasticity that is differentially expressed by genotypes of the annual plant *Polygonum persicaria* (= *Persicaria maculosa*, Sultan 1996; Kim et al. 2008; Herman et al. 2012). In this system, seedling offspring of drought-stressed parents or grandparents develop more extensive, deeper root systems and have enhanced growth and survival in dry soil (Sultan et al. 2009; Herman et al. 2012). This Eurasian species has successfully spread throughout most of North America, occupying ecologically diverse sites that vary considerably in soil moisture content both spatially and temporally (Sultan et al. 1998). *Polygonum persicaria* has a mixed breeding system with a high natural rate of self-fertilization (Simmonds 1945; Mulligan and Findlay 1970). The lack of inbreeding depression makes it possible to generate highly inbred lines that provide replicate parental individuals of each genotype, to compare genotypic patterns of transgenerational plasticity (Sultan 1996; Herman et al. 2012).

We raised replicate parent plants from 12 inbred genetic lines of *P. persicaria* (initially sampled from natural populations) in both dry and moist soil, and then examined the effects of these parental environments on drought-stressed offspring germinated with or without zebularine, using a concentration known to effect moderate genome-wide demethylation. We also estimated seed provisioning for each offspring individual in the study. This experimental design allowed us to test the following predictions. First, if seed provisioning mediates transgenerational response to drought, then offspring of drought-stressed parents should have greater...
provisioning than offspring of well-watered parents. Second, if DNA methylation regulates the expression of drought-induced transgenerational plasticity in this system (independently or jointly with provisioning), demethylation of offspring DNA should specifically reduce or remove the adaptive effects of parental drought on offspring growth without significantly altering the growth of offspring of well-watered parents (Figure 1). Third, if environmentally induced DNA methylation patterns are genotype-specific, the effect of demethylation on the expression of transgenerational plasticity is expected to vary among genetic lines.

Materials and Methods

Parental generation

Twelve genetic lines drawn from five ecologically distinct natural populations (field environmental data in Sultan et al. 1998) were propagated by self-fertilization and single-seed descent for five generations under uniform, favorable glasshouse conditions (i.e., moist soil, rich mineral nutrient levels, and full sun). In the parental generation, we stratified achenes (1-seeded propagules) from each inbred genetic line for 28 days in distilled water at 4°C to break dormancy and sowed them in vermiculite-filled flats positioned randomly on a glasshouse bench in full sun (March 2014). Individual seedlings were transplanted ~20d after emergence into 1L clay pots filled with a 1:1:1 mix of sterilized topsoil: horticultural sand: fritted clay (Turface™, Profile Products, Buffalo Grove, IL, USA). We assigned one seedling from each
genetic line to a dry soil environment, and another (highly inbred, full-sib) seedling from the same line to a moist-soil environment (12 genetic lines x 1 parent per treatment x 2 moisture treatments = 24 parent plants).

Soil moisture was maintained at 100% of field capacity (~31% moisture by weight) for all plants for one week before the contrasting parental-environment treatments were imposed. Dry and moist-soil environments were maintained at ~42% and ~84% of soil field capacity (respectively) via an automatic watering system, with pot-specific manual watering as needed. Plants were grown in the soil-moisture treatments for 53 d, with individual bench positions re-randomized weekly. Self-fertilized achenes (offspring) were harvested from each parent plant, air-dried, and stored with desiccant at 4°C (Sultan et al. 2012).

**Offspring generation**

Demethylation treatment was imposed at the earliest possible life-cycle stage, during seed germination. A treatment level of 45µM zebularine was chosen to maximize demethylation while minimizing growth disruptions: in a pilot experiment we germinated *P. persicaria* plants on 0.8% agar plates containing all concentrations of zebularine between 5µM and 75µM (in 5µM increments), a range proven effective in studies of *Medicago truncatula* and *A. thaliana* (Baubec et al. 2009), and *Taraxacum officinale* (Verhoeven et al. 2012). Concentrations higher than 50µM stunted seedling growth (J. Herman and S. Sultan, unpublished data). When
transplanted into soil and grown to maturity, plants that had been treated with 45µM zebularine developed normally and reached equivalent final biomass compared to control plants (t-test, \( P=0.44, n=5 \) per treatment). A similar concentration of zebularine (40 µM) reduced global 5-methyldeoxycytidine levels by 15-18% in *M. truncatula* and *A. thaliana*; these reductions were transient, with methylation levels in zebularine-treated plants returning to normal levels after several weeks’ growth in the absence of zebularine (Baubec et al. 2009). We weighed 24 achenes from each parental plant individually on a Cahn C-33 microbalance (Cahn Instruments, Cerritos, CA, USA), placed them into 96-well plates, and submerged them in distilled water at 4°C for five weeks to break dormancy. Achenes were sown in petri plates (8 August 2014) on solidified 0.8% agar containing either 0 or 45µM zebularine (hereafter referred to as Control and Zebularine germination treatments, respectively). Petri plates were placed on a glasshouse bench and their positions were re-randomized daily.

Six days after germination, we transplanted four replicate seedlings from each genetic line × parental environment × germination treatment combination into rhizotrons filled with a 2:2:1 mix of sterilized topsoil: horticultural sand: fritted clay pre-moistened lightly with 40mL of water per liter of soil mix (details in Sultan et al. 2009). The total experimental sample was 192 offspring (12 genetic lines × 2 parental moisture treatments × 2 germination treatments × 4 replicate offspring per line × treatment combination). Seedlings were placed in a randomized complete-block design in a dual Conviron growth chamber (Controlled Environments, Winnipeg,
Manitoba, Canada) at a 25°:18°C 14:10h day: night cycle with c. 500 μmol m⁻² s⁻¹ PAR daytime illumination. A constant low soil moisture level (22% of field capacity) was manually maintained for all seedlings. Seedling positions were re-randomized within blocks each week.

Data collection

We subtracted the mass of the pericarp (retrieved after germination) from the initial mass of the achene to estimate seed provisioning for each individual seedling (offspring). This estimate includes perisperm (nutritive tissue) mass and embryo mass and more accurately captures provisioning than the mass of the entire achene (Sultan 1996; Lacey et al. 1997). After 21d of growth in the drought environment, aboveground tissues from each seedling were separated and dried at 100°C for 1h and then at 65°C for ≥48h before weighing. The first three true leaves of each seedling were scanned on a LI-3100 leaf area meter (LICOR, Inc., Lincoln, NE USA) and weighed after drying to estimate specific leaf area for each seedling (leaf surface area per unit mass; cm² leaf/g leaf). Total leaf area for each seedling was estimated by multiplying this ratio by the total biomass of leaves from that plant. We washed root systems free of all soil mix before measuring total root system length for each seedling with a Comair optical scanner (Hasker de Havilland, Melbourne, Australia). Root systems were dried at 65°C for ≥48h before weighing. We calculated seedling biomass as the sum of shoot and root biomass. Two plants were not included in the
final sample due to insufficient germination and one plant from the control 
germination treatment was removed from the experiment due to abnormal 
development. Pericarps of four plants were missing, six plants were missing data for 
root mass and/or leaf area, and 2 outliers were removed from the analysis. Final 
sample sizes for total root length, leaf area, and seedling biomass were 177, 177, and 
180, respectively.

Data analysis

We used a linear mixed-effect model to analyze effects of parental 
environment (dry vs. moist soil), genetic line, and their interaction on seed 
provisioning, treating genetic line and its interaction with parental environment as 
random effects (variance components estimated by restricted maximum likelihood 
[REML]; Pinheiro and Bates 2000). This approach was also used to analyze the 
effects on offspring (seedling) phenotypes of parental environment (dry vs. moist 
soil), germination treatment (zebularine vs. control), genetic line, and all two- and 
three-way interactions among these factors, again treating genetic line and its 
interactions as random effects. A significant parental environment × germination 
treatment interaction would indicate that the demethylation treatment altered the 
expression of transgenerational plasticity (i.e., that the effect of dry vs. moist parental 
environment differs if offspring are germinated in zebularine rather than control 
conditions). Seed provisioning was included as a covariate in the analyses, and spatial
block was included in the model as a fixed effect.

We did not test the significance of the random effect of genetic line and its interactions because there is no generally accepted method to do so for complex mixed models involving high-order random effects (Drikvandi et al. 2013). The most common approach to significance testing of random effects in linear mixed models uses the likelihood ratio \( \chi^2 \) test, yet this test is well known to be overly conservative because the distribution of the test statistic does not conform to a single \( \chi^2 \) distribution, but instead conforms to a mixture of \( \chi^2 \) distributions with different degrees of freedom depending on the difference in parameters in the models under comparison (Pinheiro and Bates 2000; Dominicus et al. 2006; Visscher 2006; Bolker et al. 2009). Currently there is no well-established method for determining the correct mixture of \( \chi^2 \) distributions for correctly testing the significance of high-order random effects such as three-way interactions (see discussion and references in Visscher 2006; Bolker et al. 2009; Drikvandi et al. 2013). A straightforward alternative is to determine whether the random effects account for substantial trait variation (e.g., Martin and Pfennig 2010; Oosthuizen et al. 2015; Lévesque et al. 2016). To assess the effect size of each random effect, we expressed the variance for each random effect as a percentage of the remaining variance that was not explained by fixed effects (random effect variance / [sum of all random effect variances + residual variance])\(^*\)100, see Martin and Pfennig 2010). If the three-way interaction of genetic line \( \times \) parental environment \( \times \) germination treatment explained a substantial
percentage of remaining variance that would indicate that the effect of demethylation on the expression of transgenerational plasticity varied among genetic lines. We set a threshold of ≥10% of variance explained to indicate a biologically meaningful source of variation, but we report the actual percent of variance explained for each effect so that the reader may set this threshold however s/he sees fit. We verified (qualitatively) the random effects results obtained from linear mixed-effects models by running similar analyses in a mixed ANOVA framework. (Note however that linear mixed models provide more robust estimates of random effects than mixed ANOVA, especially when there are missing data, (Shaw 1987; Pinheiro and Bates 2000). We also ran linear mixed models within each germination treatment to calculate the percentage of variance explained by the genetic line × parental environment interaction.

We used one-way ANOVA to test a priori hypotheses (shown in Figure 1) regarding the effect of parental drought vs. moist-soil environments on offspring within each germination treatment. Visual inspection of the results suggested that genetic lines that most strongly increased seedling biomass in response to parental drought under control germination conditions were also the most inhibited in their growth when demethylated. We used Spearman’s rank correlation coefficient to test the significance of this apparent negative correlation (Zar 1999). Mixed-model ANOVAs were performed in JMP 11 (SAS Institute, Cary, NC, USA). All other analyses were conducted with R version 3.1.2 (R Core Team 2015). The nlme package was used to perform linear mixed-effects models (Pinheiro et al. 2012).
Results

Parental drought did not increase seed provisioning

Offspring of drought-stressed and well-watered parents had equivalent seed provisioning on average (Figure 2, parental environment, $F_{1,172}=0.547$, $P=0.461$). Offspring provisioning did not significantly contribute to variation in any seedling trait (Table 1).

Demethylation removed the adaptive effect of parental drought stress

On average, the zebularine (demethylating) germination treatment did not alter any seedling traits in offspring of well-watered parents (Figure 3a, b, c), but did alter the development of seedlings of drought-stressed parents (cf. significant interaction between parental environment and germination treatment for total seedling root length, leaf area, and biomass, Table 1). Under control germination conditions, as found in previous studies, the offspring of drought-stressed parents had on average ~20% longer root systems (Figure 3a, $F_{1,90}=5.218$, $P=0.025$), ~23% greater leaf area (Figure 3b, $F_{1,89}=5.04$, $P=0.027$), and ~16% greater biomass (Figure 3c, $F_{1,91}=4.749$, $P=0.032$) compared to offspring of the same genetic lines whose parents had been well watered. In contrast, when germinated in the presence of zebularine, offspring of drought-stressed parents had ~17% lower biomass (Figure 3c, $F_{1,91}=8.294$, $P=0.005$) as well as non-significantly shorter roots and lower leaf area.
(11% and 13% reduction, Figure 3a, b, respectively) compared to either control-germinated or zebularine-treated offspring of well-watered parents.

Demethylation reduced the positive growth effects of parental drought stress on seedling traits more strongly than expected, resulting in reduced rather than equivalent root system length, leaf area, and seedling biomass compared to offspring of non-stressed parents as described above (compare Figure 1 to Figure 3a, b, c; significant main effect of germination treatment, Table 1). We examined germination dynamics to test a possible explanation for this unexpected effect: that offspring of drought-stressed parents may have experienced a more extreme zebularine treatment than those of well-watered parents by virtue of germinating later and consequently remaining longer in the petri plate. This was not the case: there was no difference in germination timing (i.e., number of days between sowing and germination) between offspring of drought-stressed vs. well-watered parents in the zebularine germination treatment \(F_1, 177=0.398, P=0.529\). In fact, 114 of the 190 transplanted seedlings germinated on the same day, and this subsample of seedlings displayed the same pattern of response differences observed in the full dataset (Figure S3), indicating that offspring from both parental environments received equivalent demethylation treatments.
*Genetic lines varied in both the effect of parental drought and its alteration by demethylation*

The response patterns of most genetic lines were qualitatively similar to the general pattern explained above: in most cases, parental drought increased offspring total root length, leaf area, and biomass, and the zebularine demethylation treatment inhibited this effect (Figures 4, S1, S2). However, there was substantial variation among genetic lines in the magnitude of the parental drought effect and its alteration by demethylation. Under control germination conditions, parental drought increased seedling biomass between 5.8% and 48.8% in 11 of 12 lines (Figure 4a-j), while slightly decreasing biomass in one line (Figure 4l) by 4.7% (within the control germination treatment, *genetic line × parental environment* interaction accounted for 23.6% of variance in biomass after accounting for fixed effects). Lines in which control-germinated offspring most sharply increased biomass in response to parental drought stress were also the most growth-inhibited by parental drought stress when demethylated (Figure 5; Spearman’s rank correlation ρ =-0.776, P=0.002). In offspring of well-watered parents, demethylation caused either no reduction or a slight growth reduction in most lines but *increased* biomass substantially in one genetic line and very slightly in two lines (Figure 4a, b, c). In two other lines, demethylation had similar effects on offspring from both drought-stressed and well-watered parents (Figure 4k, l). This complex pattern of genetic variation for the effects of parental soil-moisture treatment and DNA methylation status is reflected in the three-way interaction between *genetic line, parental environment,* and
germination treatment, which explained ~30.5% of the remaining variance in seedling biomass after accounting for fixed effects (Table 1). Variation in total seedling leaf area revealed very similar patterns of genotype-specific differences in parental environment × germination treatment effects, explaining ~24% of the remaining variance in this trait after accounting for fixed effects (Figure S2, Table 1). Effects of parental drought and demethylation on total root length varied less among genetic lines (genetic line × parental environment × germination treatment explained 17.1% of remaining root length variance, Figure S1, Table 1). Variance components for the three-way interactions estimated by mixed-model ANOVAs were qualitatively similar (though slightly smaller) to those estimated by REML in linear mixed models: after accounting for fixed effects, the remaining variance explained by the three-way interaction was 28.59% for seedling biomass ($P=0.007$), 15.19% for leaf area ($P=0.064$), and 10.47% for total root length ($P=0.143$).

**Discussion**

*DNA methylation mediates adaptive transgenerational plasticity in P. persicaria*

Results from the control germination treatment showed that drought-stressed *P. persicaria* parents produced drought-adapted offspring, compared with parent plants of the same genetic lines that had been given ample moisture. This finding confirmed previous studies documenting adaptive transgenerational plasticity to drought stress in this system. Earlier work showed that offspring of drought-stressed
parents developed longer root systems, and extended them at a faster rate into dry soil, compared to offspring of the same inbred genetic lines whose parents were well watered (Sultan et al. 2009). Consequently, those offspring had better access to the limited amount of moisture available in their own dry-soil conditions, and produced more biomass than offspring of well-watered parents. Note that seedling biomass is a reliable proxy for survival in this species (Herman et al. 2012), and is a generally accepted indicator of fitness in seedlings since early growth differentials generally become even greater under natural conditions (Reekie and Bazzaz 2005; Moles and Leishman 2008). These effects of drought stress were found to persist for two generations, increasing the survival of grandoffspring grown under severe drought conditions (Herman et al. 2012). Such multigenerational inheritance confirms that these developmental effects are truly transgenerational, rather than direct influences of the parental-plant environment on its developing seeds.

In the present study, control-germinated offspring of drought-stressed parents likewise produced extensive root systems in dry soil, allowing them to support greater leaf areas and produce more biomass than offspring of well-watered parents grown in the same dry conditions. These results add to a growing number of cases in which environmental stress induces inherited phenotypic effects that enhance offspring performance under the same stress (e.g., Agrawal et al. 1999; Galloway and Etterson 2007; Holeski 2007; Storm and Lima 2010; Scoville et al. 2011; Salinas and Munch 2012; Walsh et al. 2015).
Experimental demethylation of offspring DNA removed these adaptive effects of parental drought, indicating that DNA methylation is required for the expression of these inherited environmental effects on offspring development. Adaptive transgenerational plasticity in this system derives much of its benefit from the initial advantage that parental exposure to drought confers on offspring in dry soil at the outset of their growth and development (Sultan et al. 2009). These inherited effects cause seedlings to enhance root extension immediately after emergence without experiencing a lag time between sensing dry conditions and initiating a response. We removed this initial advantage by briefly demethylating offspring with zebularine during seed germination. It is possible that zebularine could have had effects on the Polygonum genome in addition to demethylation, such as mobilization of transposable elements (Baubec et al. 2009), resulting in generalized changes to seedling development. However, demethylation-induced transposition or other systemic effects would have occurred in offspring produced in both parental environments, so the specific effect of zebularine on offspring of drought-stressed parents suggests that such accessory treatment effects were not appreciable. Rather, the results indicate that it is specifically the inherited effect of parental drought stress that is removed when DNA methylation levels are experimentally reduced.

DNA methylation is but one process among a suite of mechanisms that can independently, or jointly, transmit environmental effects across generations. Such mechanisms also include environmentally induced changes in provisioning of seed or egg resources such as starches and proteins, changes to cytoplasmic factors including
hormones, defensive chemicals, and other secondary metabolites, and heritable changes to epigenetic regulatory molecules such as small RNAs and histone modifications that work in concert with DNA methylation to regulate gene expression (Bonduriansky and Day 2009; Boyko and Kovalchuk 2011; Herman and Sultan 2011; Holeski et al. 2012; Duncan et al. 2014). In plants, altered provisioning to nutritive seed tissues is often expected to be the primary mechanism of both beneficial and maladaptive effects of parental environment, and indeed is a major source of variation in many cases (Roach and Wulff 1987; Rossiter 1996). We found no evidence that changes to seed provisioning accounted for the inherited effects of parental drought in this study. Previous studies of *P. persicaria* that used smaller genotypic samples found some evidence for drought-induced changes in provisioning (Sultan 1996; Herman et al. 2012), suggesting that this effect occurs in some genetic lines but is not predominant in the species. In one study, adaptive transgenerational effects of drought remained after effects of provisioning were removed via covariate analysis, indicating that the expression of transgenerational plasticity in this system relies on additional mechanisms (Herman et al. 2012). Studies in other plant systems have also identified effects of parental environment that are independent of seed provisioning (Case et al. 1996; Agrawal 2002; Bischoff and Muller-Scharer 2010; Dyer et al. 2010), suggesting that other transgenerational regulatory mechanisms may be involved in many cases of transgenerational plasticity in plants.

Despite a surge of new findings about the role of DNA methylation in regulating environment-specific gene expression in plants and animals (Feil and
only a few studies have tested whether methylation is involved in mediating adaptive transgenerational plasticity. In a study of the perennial plant *Boechera stricta*, Alsdurf and colleagues (2015) found that parental drought induced changes in DNA methylation that correlated with increased drought-tolerance in offspring. These transgenerational changes also correlated with decreased production of defensive chemicals in offspring, thus implicating epigenetic inheritance in an ecologically meaningful trade-off between offspring tolerances to different stresses. A recent study of simulated herbivory in *Mimulus guttatus* found that demethylation of offspring DNA removed potentially adaptive maternal, but not paternal, effects on leaf trichome density in offspring, suggesting that distinct epigenetic mechanisms regulate inheritance of these effects (Akkerman et al. 2016). DNA methylation has also been implicated in transgenerationally-induced tolerance of salt stress in *A. thaliana* (Boyko et al. 2010; Bilichak et al. 2012), nutrient deficiency in rice (Kou et al. 2011), and resistance to pathogen attack in *Nicotiana tabacum* (Kathiria et al. 2010). Similarly, a recent study of the aquatic invertebrate *Artemia* found that heritable, heat-shock induced resistance to both temperature stress and pathogen infection correlated with heritable changes in DNA methylation and histone modifications (Norouzitallab et al. 2014). These initial studies suggest that adaptive transgenerational plasticity that is regulated by DNA methylation may be phylogenetically widespread.
In the present study, a sample of *P. persicaria* genetic lines altered offspring to varying degrees in response to parental drought stress. These genotype-specific environmental responses were mirrored by genotypic differences in the degree to which demethylation changed the expression of the parental drought effect. These results suggest that parental drought stress induces genotype-specific changes to DNA methylation in offspring, and that it is these methylation changes that underlie the adaptive transgenerational phenotypic effects of the stress.

Studies of transgenerational environmental effects commonly reveal variation among genotypes for those effects (i.e., genotype × environment variation for transgenerational plasticity; e.g. Schmitt and Wulff 1992, Sultan 1996, Stjernman and Little 2011). Our results show that, in some cases, such transgenerational genotype × environment variation may result from genotype-specific differences in environmentally induced, heritable DNA methylation changes. Since DNA methylation occurs primarily at cytosine bases in eukaryotes, variation among genetic lines in cytosine content in or near either key regulatory sequences or protein-coding genes constitutes genetic differences in the potential for methylation changes at those loci (Gutierrez-Arcelus et al. 2013; Duncan et al. 2014; Ladd-Acosta and Fallin 2016). The genetic lines in our sample may have varied in their susceptibility to environmentally induced methylation changes due to this type of local sequence-based constraint. The recent finding that, in ants, quantitative differences in DNA
methylation levels are associated with quantitative phenotypic variation suggests that the genotype-specific frequency of methylated sites may influence development (Alvarado et al. 2015). Environmentally induced methylation patterns at specific loci can also be influenced by allelic variation at distant loci throughout the genome. For instance, a recent study of within-generation environmental effects in 150 Swedish A. thaliana accessions found that both cis and trans genetic variants substantially influenced temperature-induced changes in DNA methylation at hundreds of transposable elements (Dubin et al. 2015; see Teh et al. 2014 for similar findings in humans). Since such methylation changes are often associated with changes in gene expression, the sequence-specific nature of those changes points to DNA methylation as a source of genotype x environment interaction: in other words, induced methylation changes may translate specific environmental signals into genotype-specific adjustments in trait expression. When such environmentally induced methylation changes are heritable (which appears to be especially common in plants, Jablonka and Raz 2009; Feil and Fraga 2012), they may also underlie the expression of genotype × parental environment variation.

Our results indicate that DNA methylation plays an integral role in the expression of genotype-specific effects of parental drought, yet it remains unclear what precisely is induced by parental drought and inherited by offspring. It is possible that drought induces targeted changes in DNA methylation in parents that are transmitted through meiosis to promote the expression of adaptive phenotypes in offspring (Jablonka 2013). In this case, genetic variation for transgenerational
plasticity could reflect either (a) differences among genetic lines in their potential for methylation changes (as described above), (b) the transgenerational stability of those changes, or (c) genotypic differences in their phenotypic impact (or some combination of these effects). In support of possibility (b), a recent study of DNA methylation transmission through male gametes in the perennial plant *Helleborus foetidus* documented genetic variation for the ability of methylation marks to persist unchanged through meiosis (Herrera et al. 2014). However, changes in DNA methylation may not themselves be inherited, but instead may be reconstituted by inheritance of other factors such as small RNAs or hormones. These factors may also be subject to genetic variation in their inducibility, transmissibility, and/or influence on phenotypes.

The distinct pattern of genotype × parental environment × demethylation effects in our study may offer additional clues to the nature of these interaction effects: genetic lines that most strongly increased biomass in response to parental drought were also most strongly inhibited in their growth when offspring of droughted parents were demethylated. This growth reduction resulted in significantly lower rather than equivalent biomass compared to offspring of well-watered parents (on average). Demethylation may have revealed a maladaptive developmental effect of parental drought that is normally overcompensated by the effects of drought-induced DNA methylation. Alternatively, the genetic lines that most strongly increased offspring biomass in response to parental drought stress may have had the fastest root extension rates at the outset of growth. By initially taking up more water,
these large-rooted seedlings would have acquired more zebularine immediately after germination compared to seedlings with slower root extension rates, potentially reducing seedling growth due to more extreme demethylation effects than those experienced by smaller-rooted seedlings. However, greater-than-expected reductions in growth did not occur in all cases, indicating that the removal of the parental drought effect by demethylation did not stem simply from enhanced uptake of zebularine.

The genetic differences that we observed in the degree of adaptive response to parental drought may also stem from (epi)genetic constraints that limit the ability of some genotypes to express adaptive transgenerational responses. As a result of genotype-specific constraints, genotypes may differ in the specific combinations of plastic trait adjustments they express in response to a given stress (Heschel and Sultan 2004). Genetic lines that did not express adaptive transgenerational responses to drought (and those that did so weakly) may express particularly effective within-generation plastic responses to drought stress, while genetic lines that strongly expressed the adaptive parental drought effect may be less efficient at mounting an immediate response to moisture limitation. More broadly, genetic differences in the expression of within- versus transgenerational plasticity may derive from differences among source populations in historical exposure to particular regimes of environmental change (Herman et al. 2014). Recent theory predicts that adaptive within-generation responses will evolve when there is high temporal environmental variability, whereas adaptive transgenerational plasticity is likely to evolve when
environments are stable over generations such that parents and offspring experience similar conditions (Leimar and McNamara 2015; Uller et al. 2015).

The present study demonstrates that DNA methylation mediates genotype × environment interactions for adaptive transgenerational plasticity. This research contributes to the emerging picture that genetic variation, epigenetic variation, and environmental cues interactively contribute to adaptive diversity. Further exploring the interactions between these factors in naturally evolved systems represents a promising new direction in evolutionary biology.

Acknowledgments

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Table 1. Effects of parental environment (PE; drought vs. moist-soil), germination treatment (GT; demethylation vs. control), genetic line, and their interactions on total root system length, seedling biomass, and leaf area from linear mixed-effects models. An estimate of seed provisioning was included as a covariate. The variance for each random effect is expressed as the percentage of the variance that was unexplained by fixed effects (% Var. = (random effect variance / (sum of all random effect variances + residual variance))*100). Significance levels for fixed effects indicated as † P < 0.10, * P < 0.05, ** P < 0.01, *** P < 0.001.
<table>
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<tr>
<th>Fixed effects</th>
<th>Total root length (N=177)</th>
<th>Leaf area (N=177)</th>
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**Figure 1.** Predicted effects of parental drought and demethylation on offspring grown in dry soil. If drought-induced transgenerational plasticity is mediated by DNA methylation, germinating offspring in the presence of the demethylating agent zebularine should remove the adaptive effect of parental drought on seedling growth without significantly altering growth in offspring of well-watered parents (see *Introduction* and *Materials and Methods*).
Figure 2. Parental drought vs. moist-soil environment did not significantly influence offspring seed provisioning (means ± SE).
Figure 3. Demethylation of offspring DNA removes the adaptive effect of parental drought on total root length (a), leaf area (b), and total biomass (c) of individual seedling offspring grown in dry soil. Means ± SE are shown for offspring exposed to 0 µMol (control) or 45 µMol zebularine during germination. Asterisks indicate significance of the parental drought effect (one-way ANOVA separately testing the effect of parental environment on control-germinated and zebularine-germinated seedlings, * P < 0.05, ** P < 0.01, NS, non-significant, see Results for details).
**Figure 4.** Genetic variation for the effect of parental drought on seedling biomass and its alteration by demethylation, reflecting the interaction between *genetic line*, *parental environment*, and zebularine vs. control *germination treatment* in the linear mixed-effect model (see Table 1). Each plot displays means ± SE for one genetic line.
**Figure 5.** Genetic lines that most strongly increased seedling biomass in response to parental drought when germinated under control conditions were also the most inhibited in their growth when germinated in the presence of zebularine. The x-axis represents the percent increase in seedling biomass due to parental drought when followed by the control germination treatment. The y-axis represents the percent decrease in seedling biomass due to parental drought when followed by germination in demethylating (zebularine) conditions. Each point represents the average percent change in seedling biomass for one genetic line (based on 3-4 replicates in each germination treatment). Significance test is shown based on Spearman’s rank-correlation coefficient (see *Methods, Data analysis*).
Spearman's $\rho = -0.776$
$P = 0.002$
**Supplemental Figure 1.** Genetic variation for the effect of parental drought on total seedling root length and its alteration by demethylation, reflecting the interaction between genetic line, parental environment, and zebularine vs. control germination treatment in the linear mixed-effect model (see Table 1). Each plot displays means ± SE for one genetic line, presented in the same order as in Figure 4.
**Supplemental Figure 2.** Genetic variation for the effect of parental drought on seedling leaf area and its alteration by demethylation, reflecting the interaction between *genetic line, parental environment,* and zebularine vs. control *germination treatment* in the linear mixed-effect model (see Table 1). Each plot displays means ± SE for one genetic line, presented in the same order as in Figure 3.
**Supplemental Figure 3.** Effects of parental environment and offspring demethylation on the biomass (means ± SE) of 114 seedlings that germinated on the same day (out of 190 total transplants) are consistent with analyses of the full dataset, ruling out the possibility that the lower biomass of offspring of drought-stressed parents (compared to offspring of well-watered parents) resulted from slower germination and consequently longer exposure to zebularine. Asterisks indicate significance of the parental drought effect (one-way ANOVA separately testing the effect of parental environment on control-germinated and zebularine-germinated seedlings, **P < 0.01, NS, non-significant, see Results for details).
Chapter 4: Environmental stresses induce adaptive phenotypic plasticity and genotype-specific changes in DNA methylation in an annual plant

Jacob J. Herman and Sonia E. Sultan

Abstract

Organisms respond to environmental stresses by altering their own physiology and development, often in ways that are functionally appropriate to the environments that induce these changes. Although such adaptive phenotypic plasticity is recognized to play an important role in the ecology and evolution of natural populations, the molecular mechanisms that mediate these adaptive responses are poorly understood in most cases. Recent epigenetics research suggests that DNA methylation may be an important mediator of adaptive plastic responses to environmental stresses, yet few studies have tested this hypothesis under ecologically meaningful conditions in naturally evolved taxa. Furthermore, little is known about genetic differences in the effects of environmental stresses on DNA methylation patterns. Here we take a first step in examining the relationship between DNA methylation and adaptive plasticity in multiple inbred genetic lines of the annual plant Polygonum persicaria. Replicate individuals of five genetic lines were raised in each of four ecologically realistic greenhouse environments: high resource conditions (favorable levels of light, soil

1 This chapter is a draft of a work in progress.
moisture, and soil nutrients), drought stress, low nutrient stress, and simulated
understory shade. We measured the effects of each environmental stress on key
functional phenotypes in order to confirm that genetic lines responded to stress
adaptively. We epigenotyped each individual plant using the methylation-sensitive
AFLP method in order to test for correlations between adaptive plastic responses and
DNA methylation changes. We found that each environmental stress induced both
adaptive plasticity and DNA methylation alterations. Genetic lines expressed broadly
similar adaptive plastic responses to each environmental stress. The effects of drought
stress and shade on overall DNA methylation levels differed significantly among
genetic lines. However, Analysis of Molecular Variance (AMOVA) did not
consistently detect stress-induced differentiation in MS-AFLP profiles in each genetic
line in response each environmental stress. Because genetic lines differed in their
methylation responses to stress, but expressed similar degrees of adaptive plasticity,
there was not a consistent association between adaptive plasticity and stress-induced
methylation changes in our sample. While the relationship of DNA methylation to
adaptive plasticity in this system requires further study, these results suggest that
genotype-specific DNA methylation changes may contribute to the expression of
adaptive plasticity. Such genotypic differences underscore the importance of
explicitly incorporating genetic variation into ecological epigenetics studies.
Introduction

Individual organisms in natural environments must frequently cope with stressful environmental conditions. Indeed, as climate change progresses, environmental stresses are predicted to become more variable, frequent, and severe (IPCC 2014). The ability of natural populations to persist under stressful environmental conditions depends in part on the capacity of individual organisms to adjust their expression of key functional traits in response to stress, in order to reduce the negative fitness impacts of unfavorable environmental conditions (Sultan 2007; Nicotra et al. 2010). Although the ecological and evolutionary impacts of such adaptive plasticity have been studied extensively (West-Eberhard 2003; Matesanz et al. 2010; Pfennig et al. 2010; Moczek et al. 2011; Sultan 2015), relatively little is known about the molecular mechanisms that mediate adaptive phenotypic responses to environmental signals.

Recent epigenetics research suggests that environmentally induced changes in DNA methylation patterns may be an important molecular component of adaptive plastic responses to environmental stress. In eukaryotes, gene expression is correlated with the level of cytosine methylation in regulatory regions and protein-coding genes (Henderson and Jacobsen 2007; Schaefer and Nadeau 2015). DNA methylation states at these genomic sites are often dependent on the environmental conditions in which the organism develops (Angers et al. 2010; Duncan et al. 2014). In plants, the effects of environmental stresses on DNA methylation have been studied primarily in model and crop systems. Stresses known to induce DNA methylation changes in these
systems include drought (Wang et al. 2011; Zheng et al. 2013), nutrient limitation (Kou et al. 2011), pathogen infection (Kathiria et al. 2010; Dowen et al. 2012), high salinity, temperature shock, and UV radiation (Boyko et al. 2010). Such DNA methylation changes have been associated with induced expression of critical stress response genes (Garg et al. 2015; Yong-Villalobos et al. 2015), thus implicating DNA methylation in the expression of adaptive phenotypic plasticity.

In non-model plants, an extensive literature has developed showing that DNA methylation variation is plentiful in natural populations and often correlates with naturally occurring environmental gradients (e.g., Herrera and Bazaga 2011; Richards et al. 2012; Medrano et al. 2014; Schulz et al. 2014; Foust et al. 2016). Yet there have been relatively few controlled-environment studies focused on stress-induced changes in DNA methylation in non-model plants (for examples, see Labra et al. 2002; Aina et al. 2004; Gao et al. 2010; Verhoeven et al. 2010). Furthermore, few studies of model or non-model plants have examined DNA methylation in the context of functionally adaptive phenotypic responses to ecologically realistic environmental stresses. It is critical to address these issues in non-model systems, because model species such as *A. thaliana* have genomic features relevant to epigenetic regulation that may be unrepresentative of plants at large, such as low numbers of transposable elements relative to genome size (Tenaillon et al. 2010).

The extent to which genotype modulates environmentally induced DNA methylation changes is also relatively unexplored in both model and non-model
organisms. Such genetically mediated environmental effects on methylation may comprise a component of the well-known phenomenon of genotype × environment interaction on phenotypes. The presence of this form of genetic variation is a key condition for the adaptive evolution of phenotypic plasticity (Scheiner 1993; Pigliucci 2001). Initial investigations of genotype × environment interactions on DNA methylation variation in *A. thaliana* (Dubin et al. 2015) and humans (Teh et al. 2014) indicate that such interactions are extensive in these systems.

In this study, we took a first step in examining the relationship between DNA methylation and adaptive plasticity in multiple genotypes of the annual plant *Polygonum persicaria* (= *Persicaria maculosa*, Kim et al. 2008). Native to Eurasia, this species has spread throughout most of North America, occupying sites that vary widely in soil moisture, nutrient content, and light availability (Sultan et al. 1998). Genotypes of this species express adaptive physiological, allocational, morphological, and reproductive plasticity in response to drought stress, nutrient limitation, and shaded conditions (e.g., Sultan and Bazzaz 1993a, b, c; Heschel et al. 2004). Such adaptive plasticity allows genotypes of *P. persicaria* to maintain reproduction in diverse environmental conditions, contributing to this species’ broad ecological distribution (Sultan 2001; Griffith and Sultan 2012). This system therefore provides a rich ecological context in which to examine effects of environmental stress on DNA methylation patterns. *Polygonum persicaria* has a mixed breeding system with a high natural rate of self-fertilization (Simmonds 1945; Mulligan and Findlay 1970). The lack of inbreeding depression makes it possible to generate highly inbred lines that
provide replicate individuals of each genotype, to compare genotypic patterns of environmental effects on phenotypes and DNA methylation patterns.

We conducted a plasticity experiment focused on the effects of ecologically realistic levels of drought stress, nutrient limitation, and shade conditions on both functional phenotypic expression and DNA methylation variation in five *P. persicaria* genetic lines. We used the *methylation-sensitive* AFLP (MS-AFLP) method to test for stress-induced differentiation in DNA methylation profiles. MS-AFLP is a useful method for screening genome-wide DNA methylation at anonymous marker loci in organisms that lack a reference genome (Salmon et al. 2008; Richards et al. 2012; Medrano et al. 2014; Preite et al. 2015; Foust et al. 2016). We addressed the following specific questions: do drought stress, nutrient deprivation, and shade induce changes in DNA methylation patterns in *P. persicaria* genomes, compared to methylation patterns under favorable conditions? If so, do such stress-induced changes in DNA methylation patterns vary among genetic lines, i.e., are there genotype × environment interactions on DNA methylation patterns? And is there a consistent association among genetic lines between stress-induced changes in DNA methylation and stress-induced changes in phenotypic expression?
Materials and Methods

Plant material and growth conditions

The five genetic lines used in this experiment were drawn from three ecologically distinct natural populations and were propagated by selfing and single-seed descent for four generations under common, favorable glasshouse conditions prior to this experiment. We stratified one hundred fifty achenes (one-seeded fruits) from each line in de-ionized water at 4°C for five weeks in order to break dormancy. Achenes were sown into flats of vermiculite and placed in random positions on a glasshouse bench, with flat positions re-randomized every other day. Five days post-emergence, 48 seedlings were randomly selected from each line and transplanted individually into 1L clay pots filled with a 1:1:1 mix of sterilized topsoil: horticultural sand: fritted clay. Seedlings were maintained at 100% of soil field capacity (i.e., the maximum amount of water the soil can hold against gravity) and 70% of full sunlight for 48 hr after transplanting to facilitate establishment. After 48 hr, we randomly assigned 12 replicate seedlings from each line to each of four glasshouse environments: high-resource (high light, moist soil, rich nutrients), drought (high light, dry soil, rich nutrients), low-nutrient (high light, moist soil, poor nutrients), and simulated understory shade (low light, moist soil, rich nutrients). Accordingly, the total sample size was 240 plants (5 genetic lines x 4 environmental treatments x 12 replicate plants / genetic line / treatment = 240). After removal of several plants due to abnormal development, there were 10-12 replicate plants per genetic line and
environmental treatment combination. Plants were grown in a randomized complete-block design June-August 2012.

Soil-moisture treatments were maintained at 100% of field capacity (~30% moisture by weight) for moist-soil environments (high-resource, low nutrient, and shade) and 50% of field capacity (~15% moisture by weight) for the drought-stress environment throughout the experiment. This drought treatment ensures that all plants wilt at least once per day, but does not cause mortality. Plants were watered with an automatic system that delivers reverse-osmosis filtered water to pots individually via plastic tubes. Manual watering was provided as needed.

Plants in rich nutrient environments (high-resource, drought, and shade) received 2.5 g of slow-release 15:8:12 NPK fertilizer per pot. Plants in the low-nutrient treatment did not receive added fertilizer. This protocol ensures low levels of macronutrients in the low-nutrient environment, as the sand and clay (2/3 of the growth medium) provide little to no nutrients (Sultan 2001).

Plants in high light environments (high resource, drought, and low nutrient) received ~1060 µmol m$^{-2}$ s$^{-1}$ PAR at midday. Plants assigned to the simulated understory shade environment were placed under metal frames covered with a neutral-density shade cloth (PAK Unlimited Inc., GA USA), limiting light levels to ~130 µmol m$^{-2}$ s$^{-1}$ PAR at midday. In order to simulate sunflecks characteristic of an understory environment, rows of equidistant 3.5 cm holes were cut in the neutral shade cloth such that each plant received an ~15 min. sunfleck at noon each day
(Matesanz and Sultan 2013). We simulated understory light quality (i.e., reduced red:far red ratio) by placing strips of transparent green plastic (#138, Lee Filters, Burbank, CA USA) on top of the neutral shade cloth between the rows of sunfleck holes.

We began weekly collections of ripe achenes after plants were in environmental treatments for six weeks. Plants were harvested after eight weeks in treatment (i.e., at the onset of senescence) by collecting all remaining achenes and reproductive tissue, removing all leaves, and removing shoot tissue at the root:shoot junction. Leaf area was measured on a subsample of three fully expanded leaves taken from the main axis of each plant on a LI-3100 leaf area meter (LICOR, Inc., Lincoln, NE USA). Stems and leaves were dried separately at 100°C for 1 hr and then 65°C for ≥48 hr before weighing. Specific leaf area was calculated by dividing leaf area by leaf mass, and leaf area ratio was calculated as total estimated leaf area (TELA; specific leaf area × mass of all leaves) divided by total plant biomass. Root systems of six replicate plants from each combination of genetic line and environment were washed to remove all soil matter; total root system length was measured for four of these plants using a Comair optical root scanner (Hasker de Havilland, Melbourne, Australia). Root systems were dried at 65°C for ≥48 hr before weighing. Root length ratio was calculated by dividing total root system length by total plant biomass. Root:shoot biomass ratio was calculated by dividing root mass by shoot mass.
**AFLP genotyping and MS-AFLP epigenotyping**

We performed AFLP genotyping in order to confirm genetic similarity of replicate plants within each genetic line. Contingent on high genetic similarity among replicates of the same genetic line, MS-AFLP differences among replicates can be considered environmentally induced or random with respect to DNA sequence (Verhoeven et al. 2010). We collected four fully expanded leaves on the primary axis of each plant after 6 weeks in treatment for DNA analysis and immediately stored them at -80°C. Total genomic DNA was extracted using the Qiagen DNEasy Plant Minikit (Qiagen, Valencia, CA) following the manufacturer’s protocol, replacing TE buffer with water in the DNA elution step. We followed a standard AFLP protocol (Richards et al. 2012) in which we multiplexed fluorescently labeled EcoRI+AGC (FAM) and EcoRI+ACG (HEX) primers with unlabeled MseI+CAA primers in the selective amplifications. Samples were processed randomly with respect to genetic line and environmental treatment at each step of the protocol (including DNA extraction). We sent selective PCR products to the Iowa State University DNA Facility for fragment detection on an ABI 3130XL DNA analyzer (ThermoFisher, Waltham, MA). Each 96-well plate included negative and positive controls in order to confirm absence of contamination and consistency of fragment detection across plates, respectively. All enzymes were obtained from New England Biolabs (Ipswich, MA), and adaptors and primers were synthesized by Integrated DNA Technologies (Coralville, IA).
We scored AFLP data with the R package RawGeno v. 2.0-1 (Arrigo et al. 2009). We set a conservative average peak threshold of 150 rfu for bin inclusion in the dataset, following the recommendation in the package documentation. We manually verified the automatically designated bins using the package’s graphical user interface (adjusting bin width as necessary), scoring only fragments >100 bp in length to reduce the likelihood of fragment-size homoplasy (Pompanon et al. 2005). Fragment presence and absence were scored as “1” and “0”, respectively. We refer to AFLP and MS-AFLP fragments as “loci”, following standard AFLP terminology. We estimated scoring-error rates for each primer combination by performing duplicate analyses for 26 plants (10.8% of the total sample size) starting at the DNA extraction step, calculating the error rate as the ratio of the number of differences between duplicate samples to the product of the number of duplicated plants and the number of loci (Medrano et al. 2014). Bins with >2 discordances among duplicated samples were considered unreliably scorable and were removed from the analysis (Preite et al. 2015). There was only one difference between duplicates in the final AFLP dataset, yielding an overall AFLP scoring-error rate of 0.03%.

The MS-AFLP protocol was identical to the AFLP protocol (using the same DNA extractions), except the methylation-sensitive enzymes HpaII or MspI were used in separate reactions in place of Msel. We performed two sets of selective PCRs with the unlabeled primers HpaII/MspI+TCAT and HpaII/MspI+TCAC, each multiplexed with the fluorescently labeled EcoRI primers described above (the same primers are used for both HpaII and MspI in MS-AFLP assays because these enzymes
share the same restriction site). To score MS-AFLP fragment presence and absence, we used the same semi-automated scoring approach described above for AFLPs. Both \textit{HpaII} and \textit{MspI} cleave at 5’-CCGG, but they are differentially sensitive to cytosine methylation at this sequence. \textit{HpaII} does not cleave when the inner or outer cytosine is methylated on both strands, whereas \textit{MspI} does not cleave when the outer cytosine is fully or hemi-methylated. When both cytosines are unmethylated, both enzymes cleave; when both cytosines are methylated, neither enzyme cleaves. Following standard methods, we condensed the presence/absence scores for each locus into a binary score of 1 for “methylated” and 0 for “non-methylated” (as in Richards et al. 2012; Herrera et al. 2014; Foust et al. 2016). Cases where fragments were absent in both enzyme samples were treated as missing data because both hypermethylation and mutation of the restriction site can produce this cutting pattern (Salmon et al. 2008; Herrera and Bazaga 2010; Schrey et al. 2012). We estimated MS-AFLP scoring-error rates for each primer combination by running duplicate analyses for 28 plants (11.7% of total sample size), starting at the DNA extraction step. We calculated the error rate as the ratio of the number of discordances between \textit{HpaII} and \textit{MspI} scores to twice the product of the number of duplicated plants and the number of loci (Medrano et al. 2014). MS-AFLP scoring-error rates per primer combination ranged from 0.55% - 8.09%, yielding a mean error rate (±SE) of 2.39 ± 0.43%. This error rate is within the typical range of 2-10% for AFLP and MS-AFLP studies (Bonin et al. 2004; Medrano et al. 2014; Alsdurf et al. 2016).
**Phenotypic analyses**

We used ANOVA to compare functional trait expression in high resource vs. stressful (drought, low nutrient, or shade) environmental conditions. Separate ANOVAs were conducted for each pairwise combination of high resource environment and stressful environment (i.e., one ANOVA each for high resource vs. drought, high resource vs. low nutrients, and high resource vs. shade comparisons, as in Zhang et al. 2013). These analyses were performed separately for each genetic line to facilitate comparisons with MS-AFLP results at the level of genetic line. Genetic line was treated as a fixed effect because genotypes were deliberately drawn from ecologically distinct natural populations, and thus do not represent a purely random sample of the species’ genetic diversity. Spatial block was included in these models as a fixed effect. Analyses were conducted with R version 3.1.2 (R Core Team 2015).

**MS-AFLP analysis**

We performed analyses separately for each self-fertilized genetic line in order to control for sequence-based differences in DNA methylation while testing for environmentally induced changes in methylation. We tested for environmentally induced differentiation in DNA methylation profiles with Analysis of Molecular Variance (AMOVA) in the R package msap (Pérez-Figueroa 2013). For each genetic line, we ran separate AMOVAs for each pairwise combination of high resource vs. stressful environment (drought, low nutrients, shade) to test for differentiation in
DNA methylation profiles due to environmental treatment ($\Phi_{PT}$ was calculated as a measure of epi-haplotypic differentiation between environments). Locus-by-locus AMOVAs were performed in GenAlEx 6.5 (Peakall and Smouse 2012) to test for environmentally induced methylation differentiation at individual loci. We corrected for multiple locus-by-locus tests using a false-discovery rate of 0.05 (Benjamini and Hochberg 1995).

We tested environmental stress effects on the overall proportion of variable (i.e., polymorphic) loci that were in the methylated state, averaged across replicates within each genotype and a given environmental treatment. We performed ANOVA testing the fixed effects of genetic line, environment, genetic line $\times$ environment interaction, and block on this proportion. Separate ANOVAs were performed for each pairwise combination of high resource and stressful environment. Note that, because MS-AFLP provides estimates of methylation levels in a specific sequence context, the actual genome-wide proportion of methylated cytosines likely differs from the proportion estimated by this method (Alonso et al. 2016).
RESULTS

Phenotypic responses to environmental stresses

Drought

Drought stress significantly altered phenotypic expression in each of the five genetic lines (Table 1). For instance, in genetic line MHF 1, drought stress increased root: shoot biomass ratio, root length ratio, and the ratio of root mass: Total Estimated Leaf Area (TELA) by 67%, 147%, and 34%, respectively (Figure 1). Drought-induced increases in trait values were broadly similar for the other genetic lines, with the exception of a more moderate increase in root length ratio expressed by genetic line NAT1 (significant genetic line × environment interaction, Table S1).

Low nutrients

Genetic lines NAT1 and NAT2 significantly increased both root: shoot biomass ratio (by 68% and 46%, resp.) and root length ratio (by 33% and 61%, resp.) in response to low-nutrient stress (Figure 2; Table 1). By contrast, lines MHF2 and TP2 significantly increased root: shoot biomass ratio only in response to low nutrients (by 32% and 37%, resp.), and line MHF1 did not significantly alter either trait in response to nutrient deprivation (Figure 2; Table 1). However, when analyzed together, the genetic line x environment interaction was non-significant (Table S1).
Shade

Simulated understory shade induced significant increases in both specific leaf area and leaf area ratio in all five genetic lines (Table 1, Figure 3). Compared to trait values in the high resource environment, shade increased specific leaf area by 121% (MHF1) to 126% (MHF2), and leaf area ratio by 351% (MHF1) to 360% (TP2). Effects of shade on these traits were similar among genetic lines (non-significant genetic line x environment interaction, Table S1).

AFLP genotypes

The four selective AFLP primer combinations produced 111 reliably scorable loci among the five genetic lines. Three of these loci were variable (2.7% of total). Replicate individuals of the same genetic line shared the same AFLP genotypes at these loci (as expected based on 4 prior generations of self-fertilization), with a few exceptions. Replicates of genetic line MHF1 shared identical AFLP genotypes with the exception of a single difference in one replicate at one locus (locus CAA-ACG-410). Replicates of genetic line MHF2 shared identical AFLP genotypes with the exception of differences at one locus (CAA-ACG-452) in two replicates. (Note that the AFLP procedure was duplicated for one of these MHF2 replicates for determination of scoring error rates; locus CAA-ACG-452 was not present in the duplicated sample, raising the possibility that the presence of this locus in only two MHF2 replicates represents technical error rather than genetic differences in these
two plants.) Replicates of genetic line NAT1 shared identical AFLP genotypes with no exceptions. Replicates of genetic line NAT2 shared identical AFLP genotypes with the exception of differences at two loci in three replicates (loci CAC-AGC-220 and CAA-ACG-410). Replicates of genetic line TP2 shared identical AFLP genotypes with no exceptions. Genetic lines MHF2, NAT1, and NAT2 each had unique AFLP genotypes at the variable loci. Genetic lines MHF1 and TP2 shared the same AFLP genotypes at these loci. However, MHF1 and TP2 differed significantly in leaf trait expression (e.g., effect of genetic line on specific leaf area, $F_{1,39}=23.054$, $P<0.0001$), confirming that they are genetically distinct.

*Effects of environmental stresses on MS-AFLPs*

The four selective MS-AFLP primer combinations produced different numbers of MS-AFLP loci in each genetic line, ranging from 159 loci in TP2 to 217 loci in MHF1 (Table 2). The number of loci that varied in methylation status depended on the specific combination of genetic line and environmental stress (Table 2). For instance, in the high resource vs. drought comparison, the number of polymorphic MS-AFLP loci ranged from 33 in MHF2 (18% of total MHF2 loci) to 64 in MHF1 (29% of total MHF1 loci).

AMOVA over all loci revealed modest but significant stress-induced differentiation in DNA methylation in four out of five genetic lines (Table 3). However, each stress did not induce significant DNA methylation differentiation in
each genetic line. Compared to the high resource environment, simulated understory shade induced significant methylation differentiation among replicates within genetic lines MHF1, NAT2, and TP2; in these lines, environmental differences explained 7.9%, 12.5%, and 12.1% of MS-AFLP variation, respectively (Table 3). Drought stress induced significant methylation differentiation compared to the high resource environment in genetic lines MHF2 and NAT2. In these genetic lines, environment (high resource vs. drought conditions) explained 7.9% and 18.9% of MS-AFLP variation, respectively. The Low nutrient environment, by contrast, induced significant methylation differentiation in genetic line TP2 only. In this case, environmental variation explained 7.4% of MS-AFLP variation. Significant AMOVA results for all environmental comparisons stemmed from the accumulation of modest environmentally induced differences in methylation across multiple loci, rather than from strong environmental differentiation at few or many loci. Locus-by-locus AMOVAs revealed one locus in one genetic line that was significantly differentiated between environments after correcting for false discoveries: in genetic line MHF1, methylation states at locus TCAT-ACG-213 differed significantly between the high resource and shade environments ($P=0.048$). At this locus, 8 of 11 replicate MHF1 high-resource plants were methylated while all MHF1 shade-treated plants were unmethylated.

The main effect of environment on the proportion of loci in the methylated state was not significant for any of the pairwise comparisons between high resource and stressful environment. Instead, methylation proportion varied depending on the
specific combination of genetic line and environmental treatment. For instance, in genetic line NAT2, the proportion of methylated loci (averaged across replicate plants within each environment) increased from 23% in high resource conditions to 40% under drought stress ($F_{1,21}=9.979$, $P=0.005$). By contrast, drought (non-significantly) decreased methylation proportion from 47% to 41% in genetic line MHF2. This genetically variable effect of drought stress was reflected in a significant genetic line $\times$ environment interaction on methylation proportion ($F_{4,103}=3.00$, $P=0.022$; Figure 4). The effect of shade on methylation proportion also differed significantly among genetic lines (genetic line $\times$ environment interaction, $F_{4,100}=2.92$, $P=0.025$; Figure 4). The low nutrient environment did not significantly affect methylation proportion (non-significant effects of environment and genetic line $\times$ environment interaction). On average, methylation proportion differed significantly among genetic lines (genetic line effect was significant at $P<<0.0001$ in each of three ANOVAs testing the effects of high resource environment vs. drought, low nutrients, and shade on methylation proportion).

**Discussion**

*Adaptive phenotypic plasticity in response to drought stress, low-nutrient stress, and shade*

*Polygonum persicaria* genetic lines expressed adaptive plasticity in response to each environmental stress analyzed in this study, in accordance with expectations
based on plant ecophysiological requirements (Fitter and Hay 2002). In response to
drought, all *P. persicaria* genetic lines significantly increased biomass allocation to
root tissue and developed proportionally longer root systems relative to individual
plant biomass compared to plants of the same genetic lines grown in moist soil. These
drought-induced developmental adjustments resulted in significantly larger root
systems relative to an individual plant’s total leaf area, allowing drought-stressed
plants to maximize water uptake relative to transpirational water loss. In response to
nutrient deprivation, these genetic lines also increased biomass allocation to root
tissue, and in some cases relative root length, thus mitigating the growth-limiting
effects of the low nutrient environment by increasing nutrient-foraging ability. In
response to simulated understory shade, *Polygonum* genetic lines maximized their
ability to intercept light by making broader leaves per unit mass of leaf tissue,
resulting in increased photosynthetic surface area relative to total plant biomass
compared to the same genetic lines grown in full sun. These findings are consistent
with previous research on adaptive plasticity in *P. persicaria* showing that these
particular environmental stresses elicit adaptive phenotypic adjustments in key
functional traits, allowing stressed plants to mitigate the fitness reductions inherent to
growth and development under stressful conditions (e.g., Sultan and Bazzaz 1993a, b,
c; Sultan 2001; Heschel et al. 2004; Griffith and Sultan 2005; Griffith and Sultan
2012).
Genotype-specific effects of drought stress, low-nutrient stress, and shade on DNA methylation

In addition to these adaptive effects on functional trait expression, we found that drought stress, nutrient deprivation, and shade also induced DNA methylation changes in *Polygonum* genomes. However, AMOVA did not consistently detect differentiation in MS-AFLP profiles in response to each environmental stress in each of the five genetic lines analyzed. Depending on the particular stress and genetic line, development in high resource vs. stressful conditions accounted for 7.4% to 18.9% of MS-AFLP variation. Considering that these levels of differentiation were modest and inconsistently detected, we examined the possibility that the significant AMOVA results could be due to Type I error. We performed 15 AMOVAs on MS-AFLP profiles in total (5 genetic lines x 3 environmental comparisons). Six of these tests were significant at $P < 0.05$, whereas one test might be expected to be significant due to chance (15 tests $x$ 0.05 = 0.75 test). These results therefore indicate that environmental stresses did induce changes in DNA methylation profiles in our sample. Furthermore, because the genetic lines we used were produced by four generations of self-fertilization with single-seed descent, individuals of the same inbred genetic line generally had identical AFLP genotypes. Accordingly, genetic differences among replicate plants of the same inbred genetic line were not a substantial source of error in our analyses (Verhoeven et al. 2010). Note that an experimental demethylation study in *P. persicaria* provides further evidence for a role of DNA methylation in stress response in this system. This study found that DNA
methylation mediated genotype x environment interactions for transgenerational
effects of parental drought on offspring growth in dry soil (Herman and Sultan,
unpub.).

Other studies in both model and non-model plants have demonstrated that
drought (Labra et al. 2002b; Wang et al. 2011; Rico et al. 2014; Alsdurf et al. 2016),
nutrient deprivation (Kou et al. 2011; Secco et al. 2015), and light conditions (Greco
et al. 2013; Kuo et al. 2015) induce changes in DNA methylation. Recent studies that
integrated whole-genome DNA methylation analysis with RNA-seq found that
drought (Garg et al. 2015) and nutrient deprivation (Yong-Villalobos et al. 2015)
induced changes in DNA methylation that correlated with the expression of critical
stress-response genes. Additionally, experimental manipulations of DNA methylation
altered phenotypic responses to nutrient environment (Bossdorf et al. 2010) and light
conditions (Tatra et al. 2000). These studies, together with research in fungi (Herrera
et al. 2012) and ants (Alvarado et al. 2015), suggest that environmentally induced
DNA methylation changes are an important and phylogenetically widespread
component of phenotypic responses to environmental conditions.

There are several possible explanations for the inconsistent AMOVA results
in this study. In cases where AMOVA results were non-significant, it is possible that
DNA methylation is simply not responsive to those particular environmental stresses
in those genetic lines. However, we view this possibility as unlikely, considering the
weight of evidence that DNA methylation mediates stress responses in plants. Our
analysis of the overall proportion of methylated loci suggests that environmental stress may have influenced DNA methylation in certain cases where AMOVA results were non-significant. For instance, methylation proportion changed from ~41% to ~47% in response to drought in genetic line MHF1, but this difference was non-significant. It is possible that methylation differentiation may have occurred in all genetic lines in response to each stress, but those changes may have gone undetected in some lines due to the limited resolution of the MS-AFLP technique.\(^2\) MS-AFLP does not provide full coverage of the genome, but instead detects DNA methylation only in the CCGG context at a limited number of sites determined by the PCR primers. If *Polygonum* genetic lines differed in the presence of CCGG sequences at genomic sites that were differentially methylated in response to environment, MS-AFLP could have detected such differential methylation in only those genetic lines with CCGG sequences at those sites. This scenario may have contributed to the AMOVA results in this study. Indeed, although there was overlap in the polymorphic loci identified among genetic lines, many polymorphic loci were unique to particular genetic lines, confirming the genotypic specificity of DNA methylation in this system.

Consistent with this interpretation, the effects of drought and shade on the proportion of methylated loci differed among genetic lines (i.e., there were significant

\(^2\) A second, non-mutually exclusive possibility is that AMOVA may not have had the power to resolve methylation differentiation given high methylation variation within each environment and relatively small sample sizes (n=20-24 for each AMOVA). Furthermore, previous studies have shown that certain environmental stresses can increase variation in DNA methylation (e.g., Verhoeven et al. 2010; Nicotra et al. 2015). This possibility is currently under investigation.
genotype × environment interactions on methylation proportion in response to these stresses). These findings are consistent with other studies showing genome-wide changes in the overall frequency of DNA methylation in response to environmental cues (Labra et al. 2002; Aina et al. 2004; Mason et al. 2008), including genotype × environment interactions on genome-wide methylation levels (Asselman et al. 2015; Dubin et al. 2015). However, relatively few studies have demonstrated genotype × environment interactions on DNA methylation, either at the genome-wide level or at specific loci (Asselman et al. 2015). A study of 150 Swedish A. thaliana accessions found that both cis and trans genetic variants substantially influenced temperature-induced changes in methylation at hundreds of transposable elements (Dubin et al. 2015). Such changes in transposon methylation may be relevant for adaptive plasticity since the methylation status of transposons can influence the expression of nearby genes (Mirouze and Vitte 2014). Similarly, in a study of humans, Teh et al. (2014) found that interactions between genotype and the uterine environment explained substantially more variation in neonate methylomes than did genotype or environment alone. Studies of rice (Wang et al. 2011), horned beetles (Snell-Rood et al. 2012), and earthworms (Kille et al. 2013) also indicate that genotype × environment interactions are a source of inter-individual differences in DNA methylation.
Relationship of DNA methylation to adaptive plasticity

In the present study, all genetic lines expressed similar phenotypic responses to each stress. Because stress-induced DNA methylation changes were detected in only some genetic lines, there was not a consistent association between adaptive plasticity and methylation changes in this study. It is possible that this result reflects a lack of involvement of DNA methylation in these phenotypic responses. However, this result is also consistent with the scenario outlined above in which genotype-specific methylation changes did occur in all genetic lines, but were inconsistently detected by MS-AFLP. Under this latter scenario, genotype-specific methylation responses may have allowed different genotypes to achieve similar functional phenotypes. Such convergence on functional phenotypic expression by different genotypes may be achieved by the methylation-mediated expression of different alleles or genes in different genetic lines. Transcriptomic data from other systems are consistent with this hypothesis. For instance, a study of *Populus* showed that drought caused divergent changes in gene expression between two genotypes, yet these genotypes converged on adaptive expression of several traits relevant to drought tolerance, such as increased water use efficiency (Cohen et al. 2010). Moreover, these genotypic differences in drought-induced gene expression were apparent in leaf tissue, but not in roots. *Polygonum* genetic lines may have similarly diverged in patterns of methylation-mediated gene expression in the leaf tissue that we sampled. These considerations underscore the importance of not only analyzing multiple genotypes in ecological epigenetics studies, but also analyzing multiple tissues.
Indeed, transcriptional changes that are mediated by DNA methylation are known to be tissue-specific (Widman et al. 2013).

It is critical to note that determining the precise role of specific DNA methylation changes in the adaptive plasticity of complex traits will require integrative approaches that examine environmental effects on multiple levels of biological organization. Such studies are necessary because the relationship of DNA methylation changes to complex phenotypes may be obscured by subsequent regulatory interactions. Systems for cue perception and signal transduction include multiple sensors and signaling molecules that transmit information about perceived cues to the nucleus (Sultan 2015 and references). Environmentally responsive methylation marks may be involved in the expression of these regulatory factors that are situated at the beginning of an organism’s response to environmental signals. Once a signal is received in the nucleus, DNA methylation changes drive changes in gene expression, but these methylation changes may not simply correlate with high-order phenotypes due to subsequent editing of mRNA transcripts or post-translational modifications of proteins. In some cases, local DNA methylation changes may be a consequence, rather than a cause, of stress-induced changes in the expression of nearby genes (Secco et al. 2015). Furthermore, regulation of stress responses at the molecular level must be integrated at the level of cells, tissues, and organs. Considering this complexity, specific, functionally important DNA methylation changes may be most apparent at the level of particular cell populations and tissues.
Dissecting these details will require methylation analyses with greater resolution than MS-AFLP. Recently developed reduced-representation sequencing methods enable the simultaneous detection of SNPs and single-cytosine methylation polymorphisms in non-model species that lack a reference genome (Trucchi et al. 2016; van Gurp et al. 2016). It will be essential to employ such techniques in order to further delineate the interacting roles of genotype, epigenotype, and environmental stress in the expression of adaptive plasticity.

Acknowledgments

We thank Caleb Corliss, Justin Raymond, Zachary Steinman, and Nora Vogel for providing assistance in the greenhouse. Special thanks to Aaron Schrey and Christina Richards for training on MS-AFLP. Frederick Cohan, Christina Richards, and Michael Singer provided helpful feedback on this manuscript. Funding was provided by a Sigma Xi Grant-In-Aid of Research, the New Phytologist Trust, and Wesleyan University.
Table 1. Effects of environment on functional trait expression. For each genetic line, three separate ANOVAs were performed testing the effects of high resource vs. drought, low nutrient, or simulated understory shade environments on phenotypes. *F*-ratios for the effect of environment are shown; significance is denoted as †*P* < 0.10, *P* < 0.05, **P* < 0.01, ***P* < 0.001, ns, not significant. Sample sizes for each ANOVA varied from 8-24 depending on the trait (see Methods). (Residual df = 9 [root: shoot biomass ratio, root mass: TELA], 5 [root length ratio, leaf area ratio], and 18-21 [depending on genetic line, specific leaf area]).
<table>
<thead>
<tr>
<th>ANOVA</th>
<th>MHF1</th>
<th>MHF2</th>
<th>NAT1</th>
<th>NAT2</th>
<th>TP2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High resource vs. Drought</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root:shoot ratio</td>
<td>53.67***</td>
<td>45.05***</td>
<td>8.52*</td>
<td>42.33***</td>
<td>67.81***</td>
</tr>
<tr>
<td>Root length ratio</td>
<td>37.00**</td>
<td>21.82**</td>
<td>6.98*</td>
<td>23.98**</td>
<td>29.57**</td>
</tr>
<tr>
<td>Root mass: TELA</td>
<td>11.27*</td>
<td>19.17**</td>
<td>4.97†</td>
<td>6.7124*</td>
<td>17.05**</td>
</tr>
<tr>
<td><strong>High resource vs. Low nutrients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root:shoot ratio</td>
<td>2.74&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>9.52*</td>
<td>23.14***</td>
<td>8.96*</td>
<td>17.28**</td>
</tr>
<tr>
<td>Root length ratio</td>
<td>2.58&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>0.21&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>8.45*</td>
<td>9.22*</td>
<td>0.93&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>High resource vs. Shade</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf area ratio</td>
<td>434.53***</td>
<td>293.18***</td>
<td>625.38***</td>
<td>194.28***</td>
<td>722.85***</td>
</tr>
<tr>
<td>Specific leaf area</td>
<td>581.87***</td>
<td>686.38***</td>
<td>275.44***</td>
<td>325.69***</td>
<td>471.83***</td>
</tr>
</tbody>
</table>
Table 2. Total number of MS-AFLP loci and number of MS-AFLP loci that varied in methylation status (i.e., were polymorphic) within each environment-specific dataset (including plants in stressful and favorable treatments for soil moisture, soil nutrients, and light conditions) in each of five genetic lines.

<table>
<thead>
<tr>
<th>Genetic Line</th>
<th>MHF1</th>
<th>MHF2</th>
<th>NAT1</th>
<th>NAT2</th>
<th>TP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total No. MS-AFLP loci</td>
<td>217</td>
<td>179</td>
<td>191</td>
<td>214</td>
<td>159</td>
</tr>
<tr>
<td>No. polymorphic loci (~% of total)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>High resource, Drought</strong></td>
<td>64 (29%)</td>
<td>33 (18%)</td>
<td>46 (24%)</td>
<td>60 (28%)</td>
<td>35 (22%)</td>
</tr>
<tr>
<td><strong>High resource, Low nutrients</strong></td>
<td>72 (33%)</td>
<td>35 (20%)</td>
<td>45 (24%)</td>
<td>70 (33%)</td>
<td>35 (22%)</td>
</tr>
<tr>
<td><strong>High resource, Shade</strong></td>
<td>68 (31%)</td>
<td>36 (20%)</td>
<td>47 (25%)</td>
<td>61 (29%)</td>
<td>36 (23%)</td>
</tr>
</tbody>
</table>
Table 3. AMOVA testing the effects of High resource vs. Drought, Low nutrient, or Simulated understory shade environments on MS-AFLP profiles, performed separately in each of five *P. persicaria* genetic lines. Φ_{PT} values are shown based on 9,999 permutations, with significance levels indicated as † *P* < 0.10, * *P* < 0.05, ** *P* < 0.01, ns, non-significant. Sample sizes for each AMOVA vary from 22-24, depending on genetic line and environmental comparison (see *Methods*).

<table>
<thead>
<tr>
<th>AMOVA</th>
<th>Genetic Line</th>
<th>MHF1</th>
<th>MHF2</th>
<th>NAT1</th>
<th>NAT2</th>
<th>TP2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High resource, Drought</strong></td>
<td></td>
<td>0.008^{ns}</td>
<td><strong>0.079</strong></td>
<td>0.021^{ns}</td>
<td><strong>0.189</strong></td>
<td>0.030^{ns}</td>
</tr>
<tr>
<td><strong>High resource, Low nutrients</strong></td>
<td></td>
<td>0.000^{ns}</td>
<td>0.014^{ns}</td>
<td>0.000^{ns}</td>
<td>0.020^{ns}</td>
<td><strong>0.074</strong></td>
</tr>
<tr>
<td><strong>High resource, Shade</strong></td>
<td></td>
<td><strong>0.079</strong></td>
<td>0.013^{ns}</td>
<td><strong>0.055</strong></td>
<td>0.125^{*}</td>
<td><strong>0.121</strong></td>
</tr>
</tbody>
</table>

^{ns} non-significant
Table S1. ANOVA testing the effects of genetic line, environment, genetic line × environment, and spatial block on phenotypic expression for relevant functional traits for each environmental factor (soil moisture, soil nutrients, light conditions). \( F \)-ratios are shown, with significance levels indicated as \( \dagger P < 0.10 \), \( * P < 0.05 \), \( ** P < 0.01 \), \( *** P < 0.001 \), ns, not significant. Sample sizes are provided in parentheses (see *Methods* for details).

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>Genetic line</th>
<th>Environment</th>
<th>Genetic line × Environment</th>
<th>Block</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>High resource vs. Drought</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root: shoot ratio (n=60)</td>
<td>1.70</td>
<td>143.22***</td>
<td>0.36</td>
<td>0.97</td>
</tr>
<tr>
<td>Root length ratio (n=40)</td>
<td>0.33</td>
<td>105.89***</td>
<td>2.91*</td>
<td>0.65</td>
</tr>
<tr>
<td>Root mass: TELA (n=60)</td>
<td>0.85</td>
<td>42.24***</td>
<td>0.38</td>
<td>4.14*</td>
</tr>
<tr>
<td><em>High resource vs. Low nutrients</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root:shoot ratio (n=60)</td>
<td>2.31(\dagger)</td>
<td>44.07***</td>
<td>0.84</td>
<td>0.33</td>
</tr>
<tr>
<td>Root length ratio (n=40)</td>
<td>3.32*</td>
<td>10.32**</td>
<td>0.69</td>
<td>0.53</td>
</tr>
<tr>
<td><em>High resource vs. Shade</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf area ratio (n=57)</td>
<td>6.18***</td>
<td>2002.19***</td>
<td>0.43</td>
<td>9.16**</td>
</tr>
<tr>
<td>Specific leaf area (n=111)</td>
<td>16.16***</td>
<td>2114.79***</td>
<td>1.20</td>
<td>21.30***</td>
</tr>
</tbody>
</table>
Figure 1. Phenotypic responses to soil-moisture environment. Effects of ample moisture vs. drought stress on relevant functional traits: (a) root: shoot biomass ratio, (b) root length ratio, and (c) root mass: total estimated leaf area (TELA) in five *P. persicaria* genetic lines (means are shown). Drought significantly increased all trait values in all genetic lines with the exception of a single, marginally significant case (see Table 1). AMOVA revealed significant drought-induced alterations in MS-AFLP profiles in genetic lines MHF 2 and NAT 2 (solid lines; see Table 2). Dashed lines indicate genetic lines in which the effect of drought on MS-AFLP profiles was not significant. There was no consistent association between the magnitude of phenotypic responses to contrasting moisture environments and the detection of significant effects of those environments on MS-AFLP profile (according to AMOVA).
(a) Genetic line
- MHF 1
- MHF 2
- NAT 1
- NAT 2
- TP 2

(b) Root length ratio (m root/g plant biomass)

(c) Root mass (g) : TELA

Environment
- High resource
- Drought
Figure 2. Phenotypic responses to soil-nutrient environment. Effects of ample NPK vs. low-nutrient stress on relevant functional traits: (a) root: shoot biomass ratio and (b) root length ratio in five *P. persicaria* genetic lines (means are shown). Low-nutrient stress significantly increased root: shoot biomass ratio in four out of five genetic lines and root length ratio in two genetic lines (see Table 1); AMOVA revealed significant alterations in MS-AFLP profiles due to low nutrient environment in only one genetic line (indicated by solid line; see Table 2). The genetic line that showed significant MS-AFLP changes in response to low-nutrient stress did not respond differently with respect to phenotypic traits.
Figure 3. Phenotypic responses to light conditions. Effects of full sun vs. shade on relevant functional traits in five *P. persicaria* genetic lines (means are shown): (a) specific leaf area (SLA) and (b) leaf area ratio (LAR). Shade significantly increased trait values in all genetic lines (see Table 1). AMOVA revealed significant shade-induced alterations in MS-AFLP profiles in genetic lines MHF 1, NAT 2, and TP 2 (solid lines; see Table 2). Genetic lines in which shade did not significantly alter MS-AFLP profiles expressed equally pronounced phenotypic responses as genetic lines that showed significant methylation changes associated with light environment.
(a) SLA (cm² leaf / g leaf)

Genetic line
- MHF 1
- MHF 2
- NAT 1
- NAT 2
- TP 2

(b) LAR (cm² leaf / g plant biomass)

Environment
- High resource
- Shade
Figure 4. Effects of high resource, drought, low nutrient, and simulated understory shade environments on methylation proportion (i.e., the percentage of polymorphic MS-AFLP loci in the methylated state, averaged across replicates within each genotype and a given treatment; means ± SE shown). ANOVA revealed significant genetic line × environment interactions on the percentage of polymorphic loci in the methylated state in response to both drought and shade environments (see Results). Genetic line Nat 2 significantly increased methylation proportion in response to drought stress and simulated understory shade (denoted by asterisks). Dashed lines indicate genetic lines that did not significantly alter methylation percentage in response to stressful environments.
Literature cited


Oosthuizen WC, Bester MN, Altwegg R, McIntyre T, de Bruyn PJN. 2015. Decomposing the variance in southern elephant seal weaning mass: partitioning environmental signals and maternal effects. Ecosphere 6: art139.


