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Transgenerational Plasticity is Sex-dependent and Persistent in Yellow Moneyflower (Mimulus guttatus)

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TRANSGENERATIONAL PLASTICITY IS SEX-DEPENDENT AND PERSISTENT IN YELLOW MONKEYFLOWER (*Mimulus guttatus*)

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ABSTRACT

TRANSGENERATIONAL PLASTICITY IS SEX-DEPENDENT AND PERSISTENT IN YELLOW MONKEYFLOWER (*Mimulus guttatus*)

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Transgenerational phenotypic plasticity, whereby environmental cues experienced by parents alter the phenotype of their progeny, has now been documented in diverse organisms. Transmission of environmentally determined responses is known to occur through both maternal and paternal gametes, but the underlying mechanisms have rarely been compared. In addition, the persistence of induction over multiple generations appears to vary widely but has been characterized for relatively few systems. Yellow monkeyflower (*Mimulus guttatus*) is known to exhibit transgenerational induction of increased glandular trichome production in response to simulated insect damage. Here we test for differences between maternal and paternal transmission of this response and examine its persistence over five generations following damage. Maternal and paternal damage resulted in similar and apparently additive increases in progeny trichome production. Treatment of germinating seeds with the genome-wide demethylating agent 5-azacytidine erased the effect of maternal but not paternal damage. The number of glandular trichomes remained elevated for three generations following damage. These results indicate that transgenerational transmission occurs through both maternal and

paternal germ lines, but that they differ in the proximate mechanism of epigenetic inheritance. Our results also indicate that a wounding response can persist for multiple generations in the absence of subsequent damage.

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TABLE OF CONTENTS

LIST OF TABLES

Table

Page

LIST OF FIGURES

Figure

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction to Epigenetics

The term epigenetics has been gaining tremendous traction in the last few decades. More than twenty-five hundred articles were published related to epigenetics in 2006 [1], and a search of scientific journal articles using the word "epigenetic" produces over 26,000 results published in 2018 alone. C.H. Waddington, a British developmental biologist, first used the term "epigenetic landscape" to describe the vast number of developmental pathways a cell can take to differentiation (a cell becoming specialized i.e. kidney cell vs blood cell) [2,3]. Today, epigenetics is defined as modifications that affect gene expression without any change in the DNA sequence [4,5]. Such modifications can result in a change of the organism's appearance (phenotype), even though the genotype remains unchanged.

Epigenetic modifications, which are fundamentally important for development, maintenance of homeostasis, and response to the environment, were until recently thought to be erased in the process of producing germline cells [6]. However, it is now recognized that at least some epigenetic modifications can be passed down to offspring [7,8]. The ability to pass along these stable, albeit reversible, changes to offspring is termed epigenetic inheritance. Heritable adjustments to gene expression result in more phenotypic variation within the species, which may persist for multiple generations [9]. However, environmentally induced epigenetic traits are unique in that they are triggered by an environmental stress placed on an organism instead of through alterations to the DNA sequence. This phenomenon has generated interest in how environmentally

induced, transgenerational epigenetic inheritance might influence a species' ability to adapt to its environment [10-12]. Environmentally induced epigenetic traits may be an important component for speciation and for the success of invasive species [13]. In addition, it is important to elucidate the molecular mechanisms required for these traits to become expressed and passed on to offspring. Knowing the molecular mechanisms for these epigenetic traits grants a complete understanding behind the entire evolutionary process of sessile organisms [14].

Epigenetics and Inheritance

The French naturalist Jean-Baptiste Lamarck is credited as one of the first scientists of his time to propose that traits are not fixed and instead may change over generations [15,16]. His theories were predicated on the idea that species develop traits based on their interaction with the environment. These observations culminated in his theory of Inheritance of Acquired Characteristics. According to his theory, traits are accumulated through the efforts of the previous generations and increase in complexity over time [17]. To illustrate his point, he postulated that giraffes must have adjusted their behavior and began to stretch their necks in order to reach the leaves near the tops of the trees. He hypothesized that long necks are useful for this task, so the trait was then passed to offspring, eventually resulting in giraffes with long necks [17]. The inheritance of acquired traits was never a popular theory [18] and the theory Lamarck put forth has largely been discredited.

The theory of Inheritance through Acquired Characteristics, in addition to other theories, was eventually replaced due to developments in the field of genetics [16] which

were initiated by Gregor Mendel. When Mendel completed his eight-year experiment on the common pea plant (*Pisum sativum*), he introduced three laws of inheritance: the law of segregation, the law of independent assortment, and the law of dominance [19]. His laws explained the pattern of phenotypes inherited by offspring and were later shown to be congruent with and driven by transmission of information via DNA. At the time, Mendel's work was widely overlooked by the scientific community, which favored the popular theory of blended inheritance [20, 21]. However, Mendel's observations were eventually re-discovered, and his laws now serve as the basis of the field of modern genetics [22]. The integration of Mendel's theories with Darwin's theory of natural selection, whereby species evolve through the selection of traits that best help the species survive, shaped the Modern Synthesis [23, 24], which serves as the basis of our current theory of evolution by natural selection. According to this theory, an organism's environment cannot directly induce beneficial changes to the DNA sequence; instead, the environment may indirectly influence the frequency of random mutations over generations, whenever these mutations affect the fitness of individuals [25].

Cursorily, epigenetic inheritance appears to be the embodiment of Lamarckian theory, since epigenetically inherited traits can be triggered by environmental stressors on the parental or grandparental generation [8, 9, 26]. In actuality, the field of epigenetics conforms more closely to the concepts of the Modern Synthesis. According to the theory of Inheritance of Acquired Characteristics, acquired traits always add adaptive value [17]. In contrast, traits inherited through epigenetic means can have positive or negative effects [27]. Additionally, according to Lamarck's theory, the phenotype itself is inherited. In

actuality, the epigenetic marker, which often exists on the DNA itself, is passed on from parent to offspring.

Molecular Mechanisms Involved in Epigenetic Inheritance

A great deal of research has been devoted to elucidating the molecular mechanisms involved in the production and inheritance of epigenetic changes [28, 29]. Epigenetic markers are now known to be inherited through a number of mechanisms, including acetylation, methylation, and the use of mediating small RNAs [12, 30]. Both acetylation and methylation are involved in histone modification. Histones are responsible for packaging DNA into nucleosomes and have two ends, the N-terminal and the C-terminal. After DNA has been looped around the histones, N-terminal lysine residues project from the nucleosome [31, 32]. Verdone et al [33] describes the mechanism by which histone tails that undergo posttranslational acetylation are involved in gene expression. The process requires acetyl coenzyme A to act as the acetyl donor. This acetyl donor causes the charge on the histone to be negated, which decreases the contact of the N-termini with the phosphate groups located on DNA, making the chromatin structure more accessible for acetyltransferase enzymes. With more access to the DNA, transcription levels increase, resulting in gene expression [34]. Histone methylation has a similar mechanism, but instead requires methyltransferase as a methyl donor. When a histone tail undergoes methylation, the structure is condensed and transcription levels decrease, resulting in the gene being repressed [35, 36].

In contrast to histone modification, DNA methylation involves the addition of methyl groups occurring at CpG or CpNpG (where N can be any nucleotide) sites of the

DNA. The methyl attachment, catalyzed by DNA methyltransferase, converts cytosine into 5-methylcytosine [37, 38]. The more dense areas of methylation are less transcriptionally active and the gene is therefore less likely to be expressed [12]. In addition, DNA methylation can itself encourage histone modification that condenses chromatin for gene silencing [39, 40]. The agouti mouse model is a well-known example that demonstrates how the degree of methylation can affect the phenotype of an organism. The agouti gene is responsible for the distribution of melanin in mice and plays an important role in the mammals' ability to regulate appetite [41]. Diet can be used to induce different degrees of methylation at the agouti gene in genetically identical mice. Mice that have been hypomethylated are unable to suppress the allele which cause the agouti gene to become overexpressed and are yellow in color and have a higher risk of obesity and cardiovascular disease, while hypermethylated mice can adequately suppress the allele and are brown with no increased health risk [42, 43].

The precise molecular mechanisms utilizing sRNA for epigenetically inherited traits have yet to be deduced. However, a growing number of examples show that small RNA (sRNA) may be able to enter the germline and play a role in transgenerational transmission [44-47]. RNA directed DNA methylation (RdDM) is one such mechanism thought to be involved with the recruitment of epigenetic modifiers to specific loci in order to alter chromatin structure [48]. Mahfouz [49] describes how this process is used to direct methylation of DNA. Small interfering RNA (siRNAs) are produced when the enzyme RNA-dependent RNA polymerase (RDR2) copies single-stranded RNA to produce double stranded RNA. The double stranded RNA is cleaved by the enzyme

DICER into 24 nt siRNAs. An effector complex (AGO4) recruits the siRNA and helps mediate methylation at the target sites of siRNAs.

Environmentally Induced Epigenetic Inheritance

The role of environmental factors in triggering epigenetic modifications is not yet well understood, although it is a focus of intense investigation. A number of examples show that diet may affect the epigenome within a generation. Wang et al. [50] have identified two phytochemicals found in grapes, dihydrocaffeic acid (DHCA) and malvidin-3'-O-glucoside (Mal-gluc), that reduce overall expression of DNA methyltransferase 1 (DMNT1) and histone deacetylases (HDAC2) when included in the diet of mice. DMNT1 is responsible for decreasing the degree of methylation and HDAC2 increases histone acetylation associated with the *Rac1*gene. Both of these epigenetic modifications have been associated with a reduction of symptoms related to depressive disorders [50]. Other examples suggest that diet-induced epigenetic modifications may be passed down to offspring and grand offspring. Methylation of the agouti gene in mice is determined by the diet of the pregnant mother [51]. When the mother is fed a methyl-rich diet, the pups are brown in color and overall in a healthy state compared to pups whose mother was not fed a proper diet [41,43]. Importantly, paternal diet may also lead to transgenerational phenotypic changes, as demonstrated by studies from the Overkalix region in Sweden, which underwent intermittent famine periods in the late 1800s and early 1900s. Results show that mortality rates in men can be partially predicted by their paternal grandfather's access to food during critical periods of development. Males whose paternal grandparents had access to food died on average six

years earlier than men whose grandfathers did not readily have access to nutrition [11]. Interestingly, those grandsons' deaths were usually associated with diabetes [52].

As sessile organisms, plants utilize epigenetic mechanisms to respond to environmental shifts and possibly lead to faster environmental adaptation by increasing variation that natural selection can act upon [53, 54]. For example, Suter and Widmer studied transgenerational epigenetic inheritance in the model species *Arabidopsis thaliana.* Heat stress induced a transgenerational effect (accelerated flowering time) up to the fourth generation while salt stress increased the plants salt tolerance into the fifth generation. These effects were inherited through both the maternal and paternal germline [55]. While Suter and Widmer do not consider the precise mechanism of inheritance, a common mechanism for plants is DNA Methylation. DNA methylation markers have been shown to be passed down faithfully in dandelions [56]. When introduced to different environmental stressors including the application of jasmonic and aalicylic acid, plant hormones responsible for deterring herbivory, increased methylation and were passed on to progeny [56].

Yellow Monkeyflower (*Mimulus guttatus*)

Mimulus guttatus is a well-known annual or perennial angiosperm commonly used for ecological and evolutionary studies due to its rapid growth rate, high fecundity, ease of greenhouse propagation, and phenotypic diversity [57]. *Mimulu*s is found worldwide, but *M. guttatus* primarily grows from Alaska to Southern California [58]. This species can range in size from 10 to 60 cm tall and produces bee-pollinated yellow flowers, usually 20 to 40 mm long. *Mimulus guttatus* has been observed to increase

trichome production when targeted by damage that simulates herbivory [59, 60]. Trichomes are hair like growths on plants that are either non-glandular or glandular. The glandular form can exude chemical metabolites, specifically phenylpropanoid glycosides in *Mimulus guttatus*, that deter predation by repelling predators [61, 62]. The environmentally-triggered alteration to its phenotype makes this an interesting and observable example of epigenetic inheritance [63].

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CHAPTER II

JOURNAL ARTICLE

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Transgenerational plasticity is sex-dependent and persistent in Yellow Monkeyflower (*Mimulus guttatus*)

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Key words: epigenetic inheritance, transgenerational plasticity, induced defense, *Mimulus,* trichome

Abstract

Transgenerational phenotypic plasticity, whereby environmental cues experienced by parents alter the phenotype of their progeny, has now been documented in diverse organisms. Transmission of environmentally determined responses is known to occur through both maternal and paternal gametes, but the underlying mechanisms have rarely been compared. In addition, the persistence of induction over multiple generations appears to vary widely, but has been characterized for relatively few systems. Yellow monkeyflower (*Mimulus guttatus*) is known to exhibit transgenerational induction of increased glandular trichome production in response to simulated insect damage. Here we test for differences between maternal and paternal transmission of this response and examine its persistence over five generations following damage. Maternal and paternal damage resulted in similar and apparently additive increases in progeny trichome production. Treatment of germinating seeds with the genome-wide demethylating agent 5-azacytidine erased the effect of maternal but not paternal damage. The number of glandular trichomes remained elevated for three generations following damage. These results indicate that transgenerational transmission occurs through both maternal and paternal germ lines, but that they differ in the proximate mechanism of epigenetic inheritance. Our results also indicate that a wounding response can persist for multiple generations in the absence of subsequent damage.

Introduction

Transgenerational phenotypic plasticity occurs when environmental cues experienced by parents alter the phenotype of their progeny. This phenomenon has been documented in diverse organisms, including bacteria [1], yeast [2], plants [reviewed by 3,4], and mammals [reviewed by 5,6]. A number of studies provide evidence that transgenerational plasticity can be adaptive [reviewed by 7,8,9]. For example, maternal light [10] and parental soil moisture [11] conditions induce adaptive responses in plant offspring. Parental temperature induces adaptive life history responses in fish [12]. Attack by predators, herbivores, or pathogens can cause transgenerational induction of defenses in both plant and animal species, resulting in progeny that are better defended than offspring from unthreatened parents [reviewed by 13,14,15].

The adaptive potential of transgenerational plasticity depends on the probability that parental environmental cues accurately predict conditions experienced by their descendants [16–19]. Differences in the dispersal of male and female gametes may therefore place different selective pressure on transgenerational inheritance through the male and female germline [20]. In addition, fitness benefits of transgenerational plasticity are expected to be highest when the persistence of an induced effect across generations matches the temporal periodicity of environmental change [18,21–23]. Some authors argue that prediction is likely to be poor over multiple generations, and single-generation inheritance is therefore most apt to produce adaptive effects [18], while others point out that stably inherited states could provide long-term adaptation to changing environmental conditions [24–26]. The precise mechanisms and resultant patterns of transgenerational plasticity may affect the adaptive potential of this phenomenon and

may also be shaped by past selection. Characterizing sex-dependent patterns, proximate mechanisms, and persistence of transgenerational plasticity is thus of prime importance.

The unequal nature of maternal and paternal contributions to zygote cytoplasm, organelles, and offspring provisioning have long prompted investigation into environmentally-determined maternal effects. Most studies have focused on traits such as offspring size, seed size, nutrient provisioning, and accumulation of defensive secondary metabolites [3,19,27,28]. Nevertheless, environmentally-determined paternal effects are now well-documented, even in species without paternal care [reviewed by 29,30,31]. They are often qualitatively different from maternal effects [32,33] and may be transmitted even more effectively than maternal effects over multiple generations [34]. The existence of both maternal and paternal transgenerational effects makes sense in the light of recent evidence that three interrelated epigenetic mechanisms may be involved: DNA methylation, histone modification, and production of small RNA (sRNA), all of which may be stably inherited through meiosis [35–38].

Environmental conditions are associated with changes in DNA methylation [35] and patterns of DNA methylation are often inherited from one generation to the next, particularly in plants [25,39–41]. Environmental cues are also associated with histone modification [reviewed by 42], which can act as a signal integration and storage platform [43,44] and influence transcription by changing the local chromatin structure [45]. In many cases, DNA methylation and histone modification act together to regulate gene expression [46–50]. A variety of biotic and abiotic environmental stressors, such as infection, mechanical stress, cold, heat, salt, and drought have also been linked to expression of sRNA, including small interfering RNA (siRNA) and microRNA (miRNA;

[47,51]. In plants, environmentally induced phytohormones are known to effect changes in expression of sRNA [52–54], which is mobile between cells and throughout the vasculature [55–57]. sRNA molecules are potentially capable of entering the germline [reviewed in 47,58,59] and have been associated with transgenerational transmission [34,47,60,61]. In addition to post-transcriptional regulation, sRNA is involved in recruitment of epigenetic modifiers to specific loci and alteration of chromatin through mechanisms such as RNA directed DNA methylation (RdDM) [62–64,reviewed by 65]. In some cases, sRNA is known to be triggered by stress signaling through phytohormones [52] and involved in transmission of induced states to progeny [54,66]. Small RNAs may thus play a role in transgenerational plasticity by acting to initiate and/or maintain targeted alterations to chromatin in response to environmental conditions [47].

Mimulus guttatus (Phrymacae; [67]) is known to exhibit transgenerational induction of increased glandular trichome density in offspring in response to simulated insect damage administered prior to the development of reproductive tissue [68]. Using a panel of recombinant inbred lines (RILs) derived from a cross between a high-alpine annual population (Iron Mountain) and a perennial coastal population (Point Reyes), Holeski [68] and Scoville *et al.* [69] demonstrated genetic variation in both within-generation and between-generation induction of this response. Studies on one of these RILs showed that transgenerational induction of increased trichome density was associated with reproducible differential expression in over 900 genes. These genes were associated with four functional categories related to trichome formation and clustered into four putative co-regulatory groups, suggesting targeted modification of particular developmental pathways [70]. The putative defensive function of glandular trichomes

[71–74] makes this system a potential example of adaptive transgenerational plasticity. If parental damage correctly predicts the level of herbivory experienced by progeny, transgenerational induction of trichomes can confer a fitness advantage [16,17,19]. However, the adaptive potential of this trait depends on dispersal in seeds and pollen and whether the epigenetic signal is transmitted through the maternal or paternal line, the degree to which this signal persists over multiple generations, and the spatial and temporal dynamics of herbivore populations.

This study represents a first step in comparing the maternal and paternal contributions to transgenerational plasticity, testing for involvement of particular epigenetic mechanisms, and characterizing the persistence of induction across multiple generations in *Mimulus guttatus*. Specifically, we use a single RIL known to exhibit transgenerational induction (RIL 85) to test for sex-dependent differences in the transmission of increased trichome production due to simulated insect damage. In addition, we treat a subset of germinating seeds with the nucleoside analogue 5-azacytidine, which incorporates into the genome of proliferating cells during DNA synthesis and traps DNA methyltransferases, targeting them for degradation and resulting in genome-wide demethylation [75]. This allows us to test for a role of chromatin modification in transgenerational transmission through either the maternal or paternal gamete. Finally, we track the persistence of induction over five generations produced by self-pollination.

Results

Sex-dependent Epigenetic Inheritance

Maternal and paternal damage resulted in significant and comparable increases in the number of glandular trichomes (Figure 1, Supplementary Data 1). The lack of significant interaction between maternal and paternal damage (Table 1), and the magnitude of increase in glandular trichomes among plants receiving both types of ancestral damage (Figure 1) are consistent with an additive effect of maternal and paternal damage. The interaction between maternal damage and treatment with 5-azacytidine was significant, with 5-azacytidine largely erasing effect of maternal damage. In contrast, the interaction between paternal damage and treatment with 5-azacytidine was only marginally significant, with 5-azacytidine increasing the effect of paternal damage. Other effects and interactions were not significantly different from zero. Post-hoc pairwise comparisons of marginal means reveal a significant effect of maternal damage and paternal damage among plants without 5-azacytidine treatment (Table 2). In plants treated with 5-azacytidine, however, the effect of maternal damage is no longer significant while paternal damage remains highly significant.

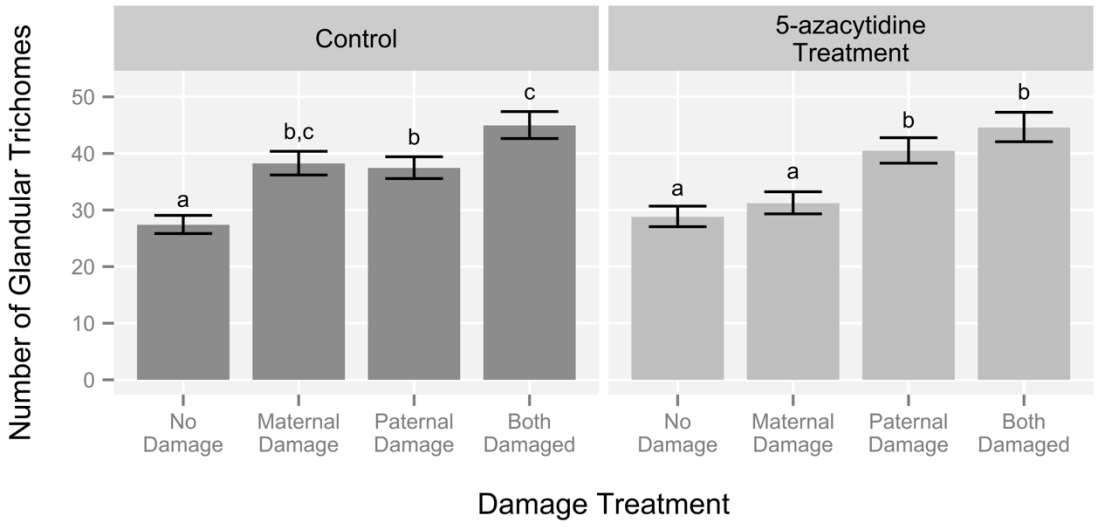

Figure 1: Number of glandular trichomes produced along a mid-leaf transect across the underside of both leaves in the 5th leaf pair. Bars represent marginal means for each combination of maternal and paternal damage, for control plants and plants treated with 5-azacytidine at germination. Letters indicate significant differences measured via pairwise comparisons within control or 5-azacytidine treated plants (□ = 0.05). Error bars show ± 1 SE. N = 1314.

Table 1 Results from generalized linear mixed-model predicting number of glandular trichomes as a function of all 2-way interactions involving maternal damage, paternal damage, and treatment with 5-azacytidine.

Factor	Effect Size (SE)	DF	T	P
Maternal Damage	0.29 (0.08)	21	3.88	**0.0009***
Paternal Damage	0.27 (0.07)	21	3.72	**0.0013***
5-azacytidine Treatment	-0.00 (0.05)	1284	-0.04	0.9687
Maternal Damage * 5-azacytidine Interaction	-0.16 (0.05)	1284	-2.92	**0.0036***
Paternal Damage * 5-azacytidine Interaction	0.12 (0.05)	1284	2.12	**0.0338**
Maternal Damage * Paternal Damage Interaction	-0.08 (0.10)	21	-0.81	0.4279

Effect sizes and standard errors are reported on the natural log scale. Significance is denoted by bold type (P<0.05) and *(P<0.005). N = 1314.

Table 2 Results for post-hoc pairwise comparisons isolating the effects of maternal and paternal damage on the number of glandular trichomes under control conditions and after treatment with 5-azacytidine.

Treatment	Factor	Effect Size (SE)	T	P
Control	Maternal Damage	0.25 (0.05)	-4.67	**0.0001***
	Paternal Damage	0.23 (0.05)	-4.31	**0.0003***
5-azacytidine	Maternal Damage	0.10 (0.06)	-1.67	0.1107
	Paternal Damage	0.35 (0.06)	-5.97	**0.0000***

Effect sizes and standard errors are reported on the natural log scale, and P-values are adjusted using the Tukey method. Significance is denoted by bold type (P<0.05) and *(P<0.005). Degrees of freedom = 21 for each comparison. N = 1314.

Persistence of Transgenerational Induction

The number of glandular trichomes remained elevated for at least three generations following damage, demonstrated by non-overlapping 95% credible intervals for control and damaged lineages (Figure 2, Table S1, Supplementary Data 2). Generation 4 showed no evidence of increased trichome production in response to ancestral damage. The results from Generation 5 are inconclusive: damaged lineages produced a higher mean number of trichomes, but there is no clear separation between credible intervals. Residual variance (i.e., overdispersion) varied among combinations of generation and damage treatment, although no clear pattern was evident with respect to generation or treatment (Table S2). Generation 2 plants grown after 6 months of seed

storage (during the production of Generation 3 seeds) showed a similar response to damage compared to plants grown after 31 months of seed storage (Block A*treatment interaction = 0.40; 95% credible interval = -1.81 – 2.82), or 56 months of storage (Block B*treatment interaction = -0.40; 95% credible interval = -2.31 – 1.63; Supplementary Data 3).

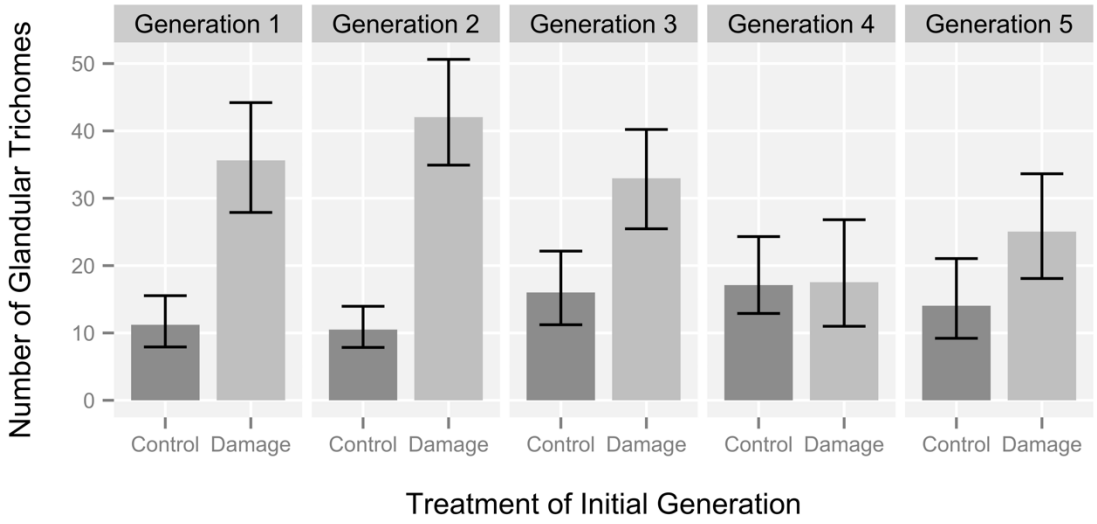

Figure 2: Number of glandular trichomes along a mid-leaf transect for 5 generations of plants originating from either control or damaged Generation 0 ancestors and produced by self-pollination. Bars represent marginal means for each combination of generation and ancestral damage treatment. Error bars show 95% credible intervals. N = 670.

Discussion

Maternal versus Paternal Effects

Although the existence of maternal [3,19,27,28], paternal [29–31], and biparental [e.g., 12,53] transgenerational plasticity is well-established, very few studies to date

explicitly compare maternal and paternal contributions within a single system [but see 32]. Our results indicate that transgenerational transmission of increased glandular trichome production occurs through both the maternal and paternal gamete. The effects of maternal and paternal damage are similar in magnitude and apparently additive. This is consistent with a scenario in which both parents transmit the same type of epigenetic change that contributes to a continuous, rather than a threshold, response. Alternatively, maternal and paternal transmission could be accomplished through different but complementary modes of action.

Data from Arabidopsis show that patterns of DNA methylation can be stably inherited for many generations and are associated with changes in gene expression and phenotype [25]. DNA methyltranferases are active during plant gametogenesis and embryogenesis [reviewed by 47] and functional activity of gametophytic cytosine-DNA-metyltransferase 1 (MET1), which maintains CG methylation, is necessary for epigenetic inheritance during gametogenesis [41]. These results lend support to the notion that faithful reproduction of DNA methylation patterns through meiosis is the causal mechanism for transgenerational epigenetic inheritance [reviewed by 40]. Treatment with 5-azacytidine results in genome-wide demethylation via destruction of methyltransferases [75,76]. Recently, 5-azacytidine has also been shown to affect the integrity of histone methylation complexes and change genomic histone patterns in complex ways, such as erasing repressive histone marks from promotors but increasing them in other parts of genome, or switching histone variants [77]. Importantly, treatment with 5-azacytidine erased most if not all of the maternal contribution but none of the paternal contribution to transgenerational induction of increased trichome production.

The marginally significant paternal damage*5-azacytidine interaction indicates that the 5-azacytidine treatment may actually have increased the effect of paternal damage, although these results should be interpreted with caution, given the approximate nature of *P*-values obtained from generalized linear mixed-models.

Potential Mechanisms

Persistence of the effect of paternal damage but not maternal damage after treatment with 5-azacytidine indicates that the two germ lines differ in the proximate mechanism of epigenetic inheritance through meiosis. Erasure of the effect of maternal damage via treatment with 5-azacytidine is consistent with maternal epigenetic inheritance via faithful reproduction of methylation patterns. This pattern may also be consistent with epigenetic inheritance via persistence of histone modifications rather than methylation changes.

In contrast, paternal inheritance in this system is accomplished via a mechanism that is apparently resistant to 5-azacytidine treatment of seeds during germination. Because each seed contains a multicellular plant embryo resulting from multiple rounds of mitosis, maternal and paternal DNA should be equally susceptible to the effects of this treatment. Histone modifications could thus be responsible for paternal inheritance [78], depending on their susceptibility to alteration by 5-azacytidine. However, these data are also consistent with involvement of sRNA, a prime candidate for transgenerational epigenetic inheritance [38,65,79]. Critical components of sRNA pathways, including those mediating miRNA and siRNA, show microsporophyte-specific expression patterns throughout pollen development and in the sperm [31,80–82]. Developing pollen shows

accumulation of mature miRNAs [81], and there is evidence that sRNAs derived in the vegetative nucleus migrate to sperm cells as the pollen matures, coinciding with silencing of transposable elements [59,82]. Data on compromised pollen tube growth in dicer mutants indicates that the transcriptional activity of mature pollen may be regulated by siRNAs [82]. In Arabidopsis, some methylation states that are erased in the absence of functional DNA (cytosine-5)-methyltransferase 1 (MET1) are restored in later generations, once *met1* mutations are complemented with wild-type alleles [83]. This indicates that methylation at a subset of sites can be re-initiated by another mechanism, such as the continued production of sRNAs [47]. These lines of evidence suggest that male-specific sRNA might be produced in the microspore or microgametophyte, packaged with sperm and inherited by the zygote [81,84] where it could initiate de novo DNA methylation in the developing embryo and thus contribute to transgenerational inheritance of DNA methylation patterns [84].

Persistence of Transgenerational Effects

Current studies document a wide range of persistence patterns for transgenerational epigenetic inheritance [18,25,34,50,85] and the reason for differences in persistence remain unclear [86]. Most examples of adaptive transgenerational plasticity involve just a single generation, although many studies do not explicitly test for persistence beyond that [reviewed by 7]. Here, we show that a significant effect of parental damage on trichome production persists for at least three generations. Persistence beyond the first generation demonstrates that this phenomenon is truly an example of transgenerational inheritance to offspring whose cells were not exposed to the

initial environmental cue [42,87], or even the somatic response to that cue. In addition, the damage response remained similar for generation 2 plants, whether they were grown during production of generation 3 seeds, one year later (Block A), or two years later (Block B), indicating no detectable change due to storage of seeds at 4°C.

Future Studies

The use of a single RIL in this study allowed us to isolate epigenetic transmission of an environmentally induced signal within a uniform genetic background. This was critical, as it is often difficult to disentangle epigenetic and genetic variation [25,88]. However, numerous studies indicate the existence of genetic variation in transgenerational effects [e.g., 50,89–91], which is necessary for evolution of this phenomenon. Other lines from our panel of RILs show greater or lesser amounts of transgenerational induction [68,69], and may exhibit different patterns of maternal transmission, paternal transmission, or persistence over generations. Studies of additional RILs will help elucidate the nature of genetic variation in patterns of epigenetic inheritance in our panel. In addition, our panel of RILs was derived from a cross between two populations from disparate ecological settings and does not, therefore, represent a natural population. Expanding this investigation to natural populations of *M. guttatus* and its predators will be a next step in evaluating whether or not the induction of increased trichome production is a case of adaptive transgenerational plasticity, shaped by natural selection, and understanding the ecological and evolutionary consequences of sex-specific patterns and persistence of this response. Finally, the effects of treatment

with 5-azacytidine are complex, and have been associated with both increases and decreases in gene expression, as well as changes in both DNA methylation and histone modification [77]. Tissue and developmental stage-specific studies of chromatin structure and sRNA production will be needed to reveal the molecular mechanisms underlying differences in maternal and paternal transmission, as well as trait-specific patterns of persistence, indicated by our results.

Methods

Experiment 1: Sex-dependence

Fifty plants were grown from a single recombinant inbred line (RIL 85; [69]). Half were randomly assigned to a damage treatment that involved punching two holes of roughly 3mm diameter in each leaf of the 2^{nd} to 5^{th} leaf pair as soon as the subsequent leaf pair expanded [modified from 68]. Plants were then randomly paired and intercrossed to create a full factorial experiment involving maternal and paternal damage. Each combination of treatments, including no damage, only maternal damage, only paternal damage, and damage of both parents, was represented by 6-7 independent pairs of plants that were unilaterally crossed to produce seeds that were stored at 4ºC until germination. Progeny germinated from these seeds were raised together in standard greenhouse conditions in three successive blocks. Plants were grown in 4 inch pots that were placed randomly into flats. Flats were bottom watered and rotated daily on the greenhouse bench. Natural light was supplemented with a 16h light/8 hour dark cycle with Sylvania Lumalux LU1000 high pressure sodium bulbs. Plants received fertilizer

(2.6 ml Jack's Professional® 10-30-20 Blossom Booster Water-Soluble Fertilizer/1 L water) every week, plus Marathon® II Liquid Insecticide and Subdue Maxx® Fungicide (2 ml/L water each) every other week.

The first block of plants included 8 replicate progeny per parent pair, totaling 200 plants. In order to test for a role of DNA methylation, the second and third blocks included 12 replicate progeny per parent pair and an additional 12 replicate progeny per parent pair that were treated with 5-azacytidine, totaling 576 plants per block. For these blocks, seeds were soaked in ultra-purified water in the dark for 48 hours (control plants) or for 24 hours, followed by 24 hours in a 1mM solution of 5-azacytidine (treatment plants). This concentration was chosen to equal or exceed treatments shown to result in measurable genome-wide demethylation in other plants [92,76,93] without causing increased mortality in preliminary experiments. All seeds were then rinsed with ultra-purified water, transplanted into pots, and raised in standard greenhouse conditions. When progeny reached expansion of the 6^{th} leaf pair, we measured trichome production on the underside of the 5^{th} leaf pair by folding the tip of the leaf to the base and counting the total number of trichomes visible above the fold across both leaves together.

Experiment 2: Persistence

Eight plants were grown from the same recombinant inbred line (RIL 85; [69]). Half were randomly assigned to the same damage treatment described above. Each plant was used to establish an independent lineage that was propagated by self-pollination each generation for 5 subsequent generations. Seeds were pooled from multiple plants within

each generation of each lineage and stored at 4°C prior to germination. Finally, seeds from all generations and lineages were grown together in two replicate blocks (generations 1-4 in block A and generations 1-5 in block B) and measured for trichome production on the underside of the 5th leaf pair, as described above. A total of 365 plants were measured in block A and 305 in block B. In each generation and each block, plants were grown together in standard greenhouse conditions, randomized in location, and rotated around the bench daily. By growing plants from multiple generations together, we controlled for variation due to block-level effects. However, seeds from earlier generations experienced a longer time at 4°C prior to germination, compared to seeds from later generations. In order to test for an effect of storage time on transgenerational transmission, we also grew and phenotyped a subset of generation 2 plants during production of generation 3 seeds (planted January 2013), and compared these with generation 2 plants grown in Block A (planted February 2014) and Block B (planted March 2015).

Statistical Analysis

To analyze the data for experiment 1, we applied a generalized linear mixed-model, executed with the glmmPQL function from the MASS package in R [94]. The number of glandular trichomes was modeled as a function of block, all 2-way interactions involving maternal damage, paternal damage, and treatment with 5-azacytidine, and parent pair, with parent pair treated as a random effect. We used a log-link function and a Poisson distribution of error terms, allowing for overdispersion. This model

appropriately represents unique parent pairs, which are nested within each combination of parental treatment, as the unit of independent replication [94,95]. Following "best practices" [96], estimation was performed via penalized quasi-likelihood and hypothesis testing of fixed effects was performed using Wald t statistics, which account for uncertainty in the estimates of overdispersion. We performed specific post-hoc pairwise comparisons using the lsmeans function from the lsmeans package in R [97], with p-values adjusted using the Tukey method and degrees of freedom calculated using the "between-within" rule [98]. To probe the robustness of our results, we fit the same model using maximum likelihood estimation based on Laplace approximation, executed with the glmer function from the lme4 package in R [99], as well as Bayesian Markov chain Monte Carlo simulations, executed with the MCMCglmm function from the MCMCglmm package in R [100]. We confirmed that all three analyses yielded closely matched estimates, confidence/credible intervals, and p/pMCMC-values.

For the second experiment, we again applied generalized linear mixed-models with a log-link function and a Poisson distribution of error terms, allowing for overdispersion. First, we used data from all generations (Block A and B) to model the number of glandular trichomes as a function of block, damage treatment of the initial generation, number of generations since damage, damage treatment*generation interaction, and lineage, with lineage treated as a random effect. Second, we analyzed all generation 2 data, including plants grown in an additional block during production of generation 3 seeds, by modeling the number of glandular trichomes as a function of block, damage treatment of the initial generation, block*damage treatment interaction, and lineage, with lineage treated as a random effect. We used the block*damage

treatment interaction in order to assess the effect of seed storage time on transgenerational induction. Both models appropriately represent lineages founded by unique generation 0 plants, which are nested within damage treatment of the initial generation, as the unit of independent replication [94,95].

In our experiment 2 analyses, residual variance was heterogeneous among combinations of damage treatment and generation (analysis 1) and damage treatment and block (analysis 2). We therefore exploited the flexibility of Baysian MCMC simulations (executed with the MCMCglmm function in R; [100]) to fit models with four different variance structures: 1) our original model, with a single among-line variance; 2) a separate line-level variance within each combination of damage treatment and generation/block; 3) a separate residual variance (i.e., overdispersion) within each combination of damage treatment and generation/block; and 4) separate line-level variances and residual variances within each combination of damage treatment and generation/block. In each case, we used weak proper priors (a multivariate Gaussian distribution with mean=0 and variance = I*1+e10 for fixed effects, and an inverse Wishart with V = 1 and nu = 0.002 for random effects) and a burnin period of 10,000 draws, followed by 500,000 iterations with a thinning interval of 25. We confirmed convergence from different starting values, as well as adequate mixing and absence of autocorrelation in the resultant chains.

For both analyses, we compared model fits based on DIC score, averaged between two runs. For the first analysis, Models 3 and 4 yielded comparable DIC values ($\Delta DIC < 1$), which were superior to model 1 ($\Delta DIC = 67$) and model 2 ($\Delta DIC = 60$). For parsimony, and based on highly overlapping 95% credible intervals for all lineage-level

variances estimated from Model 4, we present results derived from Model 3. We also confirmed that Model 4 yields qualitatively similar results. For analysis of all Generation 2 data, Model 4 yielded a better average DIC score than Model 1 (ΔDIC = 45), Model 2 (ΔDIC = 34), or Model 3 (ΔDIC = 2). Model 4 also yielded non-overlapping 95% credible intervals for both line and residual-level variances, indicating the importance of including this structure in our analysis. We thus present results from Model 4, but also confirmed that Model 3 yields qualitatively similar results.

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Conflict of interest statement. There are no conflicts of interest to report.

Supplementary data

Supplementary data is available at EnvEpig online.

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CHAPTER III

CONCLUSION

Inheritance patterns and the persistence of increased glandular trichome production, a known epigenetically inherited, rapid adaptive response to the environmental stress of herbivory, was studied using Yellow Monkeyflower (*Mimulus guttatus)*. This response was conserved for at least three generations in the recombinant inbred line 85 (RIL 85) of *M. guttatus* showcasing an example of transgenerational plasticity in a short-term defensive strategy. Results of this study indicate that epigenetic inheritance of glandular trichome production in *M. guttatus* is transmitted through both paternal and maternal gametes, Furthermore, the effect of bi-parental transmission of trichome production due herbivory damage in parents, is additive for glandular trichome production in offspring. Maternal and paternal gametes utilized different modes of inheritance and although further studies are required to determine the precise mechanisms for inheritance, this research shows the complexity of epigenetically inherited traits and affirms the need for elucidating the molecular mechanisms for transmission of these traits through paternal and maternal germlines. Results of this study contribute to our understanding of persistent epigenetic transmission of traits and ongoing studies in mechanisms of inheritance in plants.