Unveiling the evolutionary and ecological effects of transgenerational plasticity in monoclonal giant duckweeds

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Alexandra Mireya Chávez Argandoña

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Abstract

Recent studies have demonstrated that stress experienced by previous generations can influence the phenotype of their descendants, yet its impact on fitness and adaptability remains unclear. To investigate this, we exposed multiple genotypes of the clonal giant duckweed (*Spirodela polyrhiza*) to copper excess and herbivory by the waterlily aphid (*Rhopalosiphum nymphaeae*) over five generations. After another five generations without stress, we reintroduced both abiotic and biotic stressors under laboratory and natural conditions. Through ancestral exposure to copper excess in 56 globally distributed genotypes grown under axenic conditions and minimized natural selection, we found consistent evidence that transgenerational plasticity is heritable, reproducible, and dependent on both genotype and environment. The trade-offs of this plasticity could be predicted by the initial resistance of the plants to stress, likely due to resource allocation. Physiological analysis revealed that ancestral copper excess increased cyanidin concentrations, likely protecting photosystem II efficiency. These findings suggest that transgenerational plasticity is regulated by genes involved in plant reproduction and defence. Additionally, plants exposed to ancestral copper excess boosted aphid fitness. Unexpectedly, copper-induced transgenerational jasmonates, when supplemented to plants, benefitted the waterlily aphid, demonstrating that copper-induced transgenerational plasticity can affect interacting species. Similarly, ancestral aphid herbivory led to maladaptive effects under recurring herbivory, further increasing aphid reproduction. Metabolomic and transcriptomic analyses revealed that, in addition to increased jasmonates, aphid herbivory transgenerationally induced the accumulation of tyramine through regulation of its biosynthetic machinery. Supplementation of tyramine to plants and aphids confirmed its adaptive role against herbivory. Under natural conditions, tyramine, but not jasmonates, exhibited transgenerational plasticity over two consecutive years, and accordingly, aphid fitness tended to decline outdoors, highlighting the relevance of executing experiments under both laboratory and natural conditions. Together, these findings demonstrate that transgenerational plasticity is heritable and plays a crucial role in organismal and community evolutionary change, as it not only shapes the fitness and phenotype of the affected organisms but also impacts coexisting species under both controlled and natural conditions.

Zusammenfassung

Jüngste Studien haben gezeigt, dass Stress, dem frühere Generationen ausgesetzt waren, den Phänotyp ihrer Nachkommen beeinflussen kann. Die Auswirkungen auf Fitness und Anpassungsfähigkeit sind jedoch noch unklar. Um dies zu untersuchen, setzten wir mehrere Genotypen der klonalen Riesenwasserlinse (*Spirodela polyrhiza*) über fünf Generationen hinweg einem Kupferüberschuss und Herbivorie durch die Wasserlilienblattlaus (*Rhopalosiphum nymphaeae*) aus. Nach weiteren fünf Generationen ohne Stress führten wir sowohl abiotische als auch biotische Stressoren unter Labor- und natürlichen Bedingungen wieder ein. Durch die Exposition der Vorfahren gegenüber Kupferüberschuss in 56 global verbreiteten Genotypen, die unter axenischen Bedingungen und mit minimierter natürlicher Selektion gewachsen waren, fanden wir konsistente Hinweise darauf, dass transgenerationale Plastizität vererbbar, reproduzierbar und sowohl vom Genotyp als auch von der Umwelt abhängig ist. Die Kompromisse dieser Plastizität ließen sich anhand der anfänglichen Widerstandsfähigkeit der Pflanzen gegenüber Stress vorhersagen, was wahrscheinlich auf die Ressourcenallokation zurückzuführen ist. Physiologische Analysen zeigten, dass der Kupferüberschuss der Vorfahren die Cyanidin-Konzentration erhöhte, was wahrscheinlich die Effizienz des Photosystems II schützte. Diese Ergebnisse deuten darauf hin, dass transgenerationale Plastizität durch Gene reguliert wird, die an der Fortpflanzung und Abwehr von Pflanzen beteiligt sind. Zudem steigerten Pflanzen, die einem Kupferüberschuss der Vorfahren ausgesetzt waren, die Fitness der Blattläuse. Unerwarteterweise hatten kupferinduzierte transgenerationale Jasmonate, die den Pflanzen zugesetzt wurden, einen positiven Effekt auf die Wasserlilienblattlaus, was zeigt, dass kupferinduzierte transgenerationale Plastizität auch interagierende Arten beeinflussen kann. In ähnlicher Weise führte die Herbivorie durch die Vorfahren bei wiederholter Herbivorie zu Fehlanpassungen, was die Reproduktion der Blattläuse weiter steigerte. Metabolomische und transkriptomische Analysen zeigten, dass die Herbivorie durch Blattläuse, neben erhöhten Jasmonaten, auch die Ansammlung von Tyramin transgenerational induzierte, indem sie den Biosynthesemechanismus regulierte. Die Gabe von Tyramin an Pflanzen und Blattläuse bestätigte dessen adaptive Rolle im Kampf gegen Herbivorie. Unter natürlichen Bedingungen zeigte Tyramin, im Gegensatz zu Jasmonaten, über zwei aufeinanderfolgende Jahre transgenerationale Plastizität, während die Fitness der Blattläuse im Freien tendenziell abnahm. Dies unterstreicht die Relevanz der Durchführung von Experimenten sowohl unter Labor- als auch unter natürlichen Bedingungen. Zusammengefasst zeigen diese Ergebnisse, dass transgenerationale Plastizität vererbbar ist und eine entscheidende Rolle bei der evolutionären Veränderung von Organismen und Lebensgemeinschaften spielt, da sie nicht nur die Fitness und den Phänotyp der betroffenen Organismen prägt, sondern auch koexistierende Arten sowohl unter kontrollierten als auch natürlichen Bedingungen beeinflusst.

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Chapter I: Introduction

i. Transgenerational plasticity and its role in evolution

Humans have long questioned the origins and changes of living forms, and in the 19th century, a scientific theory of evolution began to take shape. Lamarck proposed that species evolve through the inheritance of acquired traits (Lamarck, 1809). This idea was later overshadowed by Wallace and Darwin's theory of natural selection (Darwin, 1859; Wallace, 1855), which gained prominence with the integration of Mendel's findings on heredity (Dobzhansky, 1937; Huxley, 1942). However, this Modern Synthesis fell short in explaining all evolutionary phenomena, renewing interest in environmental influences on gene expression and the inheritance of non-genetic changes (Waddington, 1953).

Non-genetic inheritance encompasses both epigenetic mechanisms and external factors that can indirectly influence gene regulation. Epigenetic mechanisms, such as DNA and histone methylation, histone acetylation, and non-coding RNAs, regulate gene expression beyond the DNA sequence. These modifications can affect not only single genetic regions but also chromatin structure (Jablonka & Lamb, 2005). However, non-genetic inheritance also includes vertically transmitted elements like prions, nutrients, hormones, and microbes, which shape cellular phenotypes and influence population evolution (Bell & Hellmann, 2019; Jablonka & Lamb, 2005; Liberman et al., 2019). In this thesis, these inherited mechanisms collectively contribute to what is termed transgenerational plasticity.

Transgenerational plasticity remains controversial due to the challenges of isolating its pure effects (Grossniklaus et al., 2013; Heard & Martienssen, 2014). Demonstrating transgenerational plasticity requires careful control of confounding factors. First, researchers must prevent selection of survival or reproduction variants by taking a random individual per generation under single-descendant lineages (Baugh & Day, 2020). Second, to reduce genetic noise from mutations, experiments must track their accumulation or use highly inbred or clonal organisms with low mutation rates (Johannes et al., 2009; Richards et al., 2017). Third, maternal effects must also be accounted for, as stress exposure affect even two subsequent generations due to the developmental overlap of germline cells (Agrawal et al., 1999; Galloway & Etterson, 2007; Grossniklaus et al., 2013; Mousseau & Fox, 1998). Fourth, to distinguish true transgenerational plasticity from maternal or multigenerational effects, plasticity must persist beyond the third generation, as only long-lasting effects have the potential to shape evolutionary trajectories (Anastasiadi et al., 2021). Additionally, excluding vertically transmitted microbes through surface sterilization allows focus on epigenetic mechanisms. Addressing these conditions allows researchers to identify and interpret true cases of transgenerational plasticity.

While studies on transgenerational plasticity have focused on phenotypic changes, they rarely assess its impact on fitness, essential for understanding the evolutionary relevance of transgenerational plasticity. Measuring phenotypes in a single offspring generation captures only the current performance resulting from ancestral conditions, but such performance can vary across generations (Alvarez et al., 2020; Latzel et al., 2016; Lin et al., 2024). For example, in *Arabidopsis thaliana*, ancestral stress altered traits like silique and fruit number over one to four generations, yet plasticity on these traits had no consistent pattern, making transgenerational plasticity difficult to predict (Lin et al., 2024). Therefore, single-generation assessments risk missing the broader evolutionary role of transgenerational plasticity. In evolutionary biology, fitness–defined as an organism's survival and reproductive output in a given environment–is the primary measure of both adaptive and maladaptive outcomes (Eriksson et al., 2023; Futuyma, 2009; Orr, 2009). Fitness data in transgenerational experiments help determine whether inherited changes confer a selective advantage. However, tracking the phenotype across multiple generations is essential to identify trait changes that persist upon ancestral stress. Only by measuring both fitness and phenotypic traits across successive generations can the full evolutionary implications of transgenerational plasticity be understood.

Transgenerational plasticity may offer evolutionary potential to organisms when its effects are influenced by genetic variation, yet research into its genetic basis remains limited. Adaptive transgenerational plasticity can benefit populations by shielding alleles (Herman & Sultan, 2011). However, if transgenerational plasticity varies among individuals, selection could act on this variation, driving evolutionary change on populations (Nettle & Bateson, 2015). While differences among individuals could rise from diversified bet-hedging (Seger & Brockman, 1987)–referring to increased variation within a genotype–transgenerational plasticity may also depend on the genotype. To determine whether transgenerational plasticity has a genetic basis, researchers must first assess the proportion of transgenerational variance explained by genetic differences. Therefore, plasticity is considered heritable across generations when its variation correlates with genetic differences among individuals.

Heritability is quantified through broad- and narrow-sense heritability estimates (Dudley & Moll, 1969). Broad-sense heritability (H^2) captures all genetic contributions to phenotypic variance, making it applicable to both sexually and asexually reproducing organisms. In contrast, narrow-sense heritability (h^2) focusses on additive genetic effects-i.e. the cumulative influence of individual alleles-which is particularly relevant for sexually reproducing species. However, obtaining reliable heritability estimates requires a large number of genotypes to minimise environmental variance (Schmidt et al., 2019), which has limited such analyses in the context of transgenerational plasticity. Beyond estimating heritability, identifying specific genetic factors underlying transgenerational plasticity is also essential. In *A. thaliana*, for

example, variation in flowering-related traits and transcriptomic profiles across generations was linked to differences in transposon abundance (Lin et al., 2024). Similarly, research on mutagenized *Pristionchus pacificus* nematodes revealed two ubiquitin ligases underlying transgenerational plasticity in mouth morphology (Quiobe et al., 2025). These data suggest that understanding the genetic basis of transgenerational plasticity requires not only rigorous experimental design but also the quantification of both phenotype and fitness across multiple genetic variants.

Characterizing the evolution of genes underlying transgenerational plasticity is crucial for understanding its impact on genome evolution. Transgenerational plasticity can induce gradual genetic changes–either through use or disuse–modulating the plastic response, a process known as genetic accommodation (Jablonka, 2017; Sommer, 2020; Waddington, 1953). Identifying this phenomenon requires examining gene families, orthogroups, copy number variation, and splicing structures. One critical genomic change that facilitates this process is gene duplication, which can lead to evolutionary novelties through whole genome or gene-specific duplication (Panchy et al., 2016). Paralogues–gene duplicates that evolve independently–can be found in the same region or on different chromosomes. Retroduplication, where mature mRNA is retrotranscribed and inserted into the genome, is one mechanism leading to the formation of paralogues (Panchy et al., 2016). While once considered inactive, retrogenes are now known to have functional roles and are subject to selection (Abdelsamad & Pecinka, 2014; Hu et al., 2022; Wang et al., 2006; Xiao et al., 2008). Investigating genes involved in biosynthetic enzymes associated with transgenerational plasticity is essential for understanding how this phenomenon shapes genetic evolution and influences population adaptability.

The adaptiveness of a trait depends on the environmental context and stress interactions. Alleles that promote adaptation in one environment may become maladaptive in a different or mismatched environment, leading to trade-offs (Bono et al., 2017; Hereford, 2009; Via & Lande, 1985). In transgenerational plasticity, abiotic or biotic stress activates various molecular response pathways (Herman & Sultan, 2011), which may be antagonistic, either among different stresses or within the same stress, resulting in further trade-offs (Chang et al., 2020; Gallusci et al., 2023). For example, in *A. thaliana*, exposure to the spider mite *Tetranychus urticae* increased resistance in offspring against recurrent herbivory but made them susceptible to the bacterial pathogen *Pseudomonas syringae* (Singh et al., 2017). Similarly, parental infection with *P. syringae* conferred resistance in the offspring to *Hyaloperonospora arabidopsidis*, but increased susceptibility to the fungi *Alternaria brassicicola*, *Plectosphaerella cucumerina*, and to salt stress (López Sánchez et al., 2021; Luna et al., 2011). In *Spirodela polyrhiza*, exposure to copper enhanced plant fitness under recurrent stress but reduced performance in the absence of stress (Huber et al., 2021). These

examples highlight that the adaptive value of transgenerational plasticity is context-dependent and must be evaluated in relation to environmental predictability and stress interactions.

ii. Characterizing transgenerational plasticity

Selection that balances competing demands for limited resources is thought to underlie trade-offs in plasticity. Coley & Barone (1996) proposed that organisms face a resource ceiling, where energy spent on defence reduces resources available for growth and reproduction. In this view, low-defence individuals may pass on these limitations to offspring, regardless of environment, a phenomenon called the silver-spoon effect (Grafen, 1998). Conversely, mild stress in the parental generation can reallocate resources, improving offspring performance under recurring stress, a phenomenon known as hormesis (Calabrese et al., 2007; Mattson, 2008). Transgenerational hormesis has been observed in species such as *Caenorhabditis elegans*, where parental exposure to abiotic stress enhanced offspring resistance to oxidative stress (Kishimoto et al., 2017), and *Drosophila melanogaster*, where exposure to fungal spores boosted pathogen resistance in offspring lacking antifungal immunity (McClure et al., 2014). As an environment rich in resources does not eliminate plasticity trade-offs (Campos et al., 2016), Monson et al. (2022) proposed the coordinated resource allocation model, considering both environmental resource supply and internal regulation. They suggested that trade-offs may arise from mechanisms such as hormonal crosstalk, transcriptional control, and metabolic coordination (Monson et al., 2022). As such, resource allocation could vary between genotypes depending on their defence capacity, potentially explaining why transgenerational plasticity leads to divergent fitness outcomes across different environmental contexts.

Transgenerational plasticity can occur through two non-exclusive pathways, providing insight into its underlying mechanisms. The first, transgenerational retention, refers to the sustained level of a trait, such as defensive metabolites, across generations without continuous stress exposure (Holeski et al., 2012). This pathway is identified by a significant effect of the ancestral treatment. The second pathway, transgenerational priming, involves plasticity that is either retained or returns to baseline levels under non-stress conditions, but shows stronger induction effects under recurring stress compared to individuals experiencing stress for the first time. This pathway is identified by a significant interaction effect between the ancestral and recurrent treatments (Bell & Hellmann, 2019; Holeski et al., 2012). In clonal plants, where parents and offspring are physically connected through stolon or stipes, direct transfer of nutrients or hormones complicates the distinction between vertical transmission and epigenetic changes. Changes that are transgenerationally retained may arise from either pathway, but traits that are primed under recurrent stress are more likely to involve epigenetic mechanisms (Rasmann et al., 2012). Furthermore, in non-axenic conditions, where microbial transmission is possible, it is important to distinguish between inherited epigenetic regulation and parental

provisioning, such as the transfer of molecules or microbes. Disentangling whether plasticity's persistence in a trait is due to inherited epigenetic regulation or to parental provisioning is essential to understand how transgenerational plasticity operates and evolves.

iii. Ecological implications

Changes in an organism can affect the performance of coexisting individuals, altering ecological interactions such as competition, predation, and herbivory (Agrawal, 2001; Miner et al., 2005). While research on maternal effects has shown this interactions, the impacts of transgenerational plasticity on co-evolving organisms remains scarce (Holeski et al., 2012; Tariel et al., 2020). For example, parental exposure to predators in fish altered egg or offspring body size, affecting their predation risk (Monteforte et al., 2020; Sharda et al., 2021). In plants, herbivory on parents can alter the nutritional quality or chemical defences of their offspring, impacting herbivore resistance, pollinator interactions, and defensive traits. For instance, pollen from heat-stressed plants reduced survival of *Osmia lignaria* bees (Walters et al., 2024). Similarly, *Pieris rapae* caterpillars feeding on the offspring of damaged *Raphanus raphanistrum* radish exhibited 15% slower growth and 25% higher mortality (Agrawal, 2002), while *P. rapae* feeding on *A. thaliana*, and *Helicoverpa zea* feeding on *Solanum lycopersicum*, showed up to 50% reductions in biomass gain when feeding on offspring of herbivore-exposed plants (Rasmann et al., 2012). However, maternal effects do not always benefit plants. In *Populus* species, herbivory by the beetle *Chrysomela scripta* reduced 11–35% of the total phenolic glycosides in the offspring, which likely increased palatability for herbivores (Holeski et al., 2013). These examples highlight the importance of assessing how transgenerational plasticity influences both individual fitness and ecological interactions, which can lead to broader evolutionary consequences.

Biotic stress in plants arises from dynamic interactions between herbivores and their plant hosts, potentially affecting future cohorts. The evolutionary arms race between herbivores and plants has highlighted herbivores' ability to manipulate plant defences, while plants develop strategies to counter these tactics (Wang et al., 2023). Herbivores, such as caterpillars or aphids, suppress plant immunity by introducing salivary enzymes or effector molecules (Chen et al., 2019; Chen et al., 2023; Han et al., 2025; Wang et al., 2020). In response, plants deploy various defensive mechanisms to deter herbivory, such as producing secondary metabolites (Erb, 2018; Erb & Kliebenstein, 2020). These responses are controlled by complex metabolic feedback loops, where the concentration of specific metabolites influences the production of defence compounds (Erb & Kliebenstein, 2020; Li et al., 2024c). Herbivore-induced metabolites in parental plants can also pass to the offspring, affecting their performance (Herman & Sultan, 2011; Rasmann et al., 2012). However, whether these physiological changes occur in transgenerational plasticity remain poorly studied (Huber et al.,

2021). The ability of herbivores to influence the phenotype and fitness of future plant generations highlights the importance of exploring transgenerational effects within a co-evolutionary framework.

The ecological significance of transgenerational plasticity can only be fully understood in natural or outdoor conditions, where organisms face the complexities of their environment. Laboratory experiments are vital for uncovering underlying mechanisms, but they often oversimplify the interactions that occur in natural ecosystems (Gibson, 2008; Uller et al., 2013). In natural settings, organisms are exposed to fluctuating abiotic factors, diverse microbial communities, competition, herbivory, and other biotic interactions, all of which can influence the expression of transgenerational plasticity (Herman & Sultan, 2011). These dynamic and unpredictable conditions may reveal trade-offs and context-dependent effects undetected in the controlled setting of a laboratory. Studying transgenerational plasticity in natural environments allows researchers to assess its adaptive value in conditions where natural selection is at play (Bonduriansky & Day, 2009). This approach also provides insights into how inherited responses influence ecological interactions and fitness across generations. Without considering the ecological context, conclusions about the evolutionary significance of transgenerational plasticity may be incomplete or misleading.

iv. Model organisms

To investigate transgenerational changes in plant fitness, we selected the flowering plant *Spirodela polyrhiza* (giant duckweed) as an organism of study. This globally distributed aquatic plant produces a new generation every two to three days (Ziegler et al., 2015), and primarily reproduces asexually by budding offspring fronds that remain connected to the parent for three to six days through a short stolon, called stipe (Landolt, 1986; Zhang et al., 2020) (**Figure 1A**). Its fast generation time, small size, and simple body structure–roots and a flat thallus-like shoot, known as the frond–allow precise fitness assessments via frond surface area and number, making it ideal for transgenerational studies in both laboratory and natural environments. The plant's clonal reproduction ensures high genetic uniformity, with one point mutation every 80 generations (Xu et al., 2019). In response to low nutrient availability or temperature decreases, *S. polyrhiza* can produce turions, dormant, starch-rich fronds that sink to the bottom of water bodies and survive under unfavourable conditions. These specialised propagules allow the plant to persist across seasons and resume growth when conditions improve, effectively acting as a resource storage mechanism across generations (Appenroth & Nickel, 2010; Landolt, 1986; Ziegler, 2024). Its ability for vegetative reproduction and ecological adaptability make *S. polyrhiza* particularly suited for exploring the mechanisms, persistence, and fitness consequences of transgenerational plasticity in plants.

S. polyrhiza is commonly found in small water bodies, often near human-influenced environments where both abiotic and biotic stressors, such as fluctuating copper levels and herbivory, are prevalent. Copper, a significant aquatic pollutant (Li & Xiong, 2004; Ruas et al., 2022), induces oxidative stress in plants, leading to lipid peroxidation and increased hydrogen peroxide production, which can damage plant cells (Xing et al., 2010). In addition to abiotic stress, *S. polyrhiza* also faces herbivory from the waterlily aphid *Rhopalosiphum nymphaeae*, a generalist herbivore with a heteroecious life cycle (**Figure 1B**). This aphid alternates between sexual reproduction on woody hosts in late fall and parthenogenetic reproduction on aquatic hosts, including duckweeds, during the summer (Center et al., 2002). Under favourable conditions, *R. nymphaeae* reproduces rapidly, with a new generation emerging every 6 to 10 days (Hance et al., 1994). The high reproductive rate of both *S. polyrhiza* and *R. nymphaeae* facilitates the assessment of transgenerational plasticity's effects on both plant and herbivore fitness.

Like other members of the Lemnaceae family, *S. polyrhiza* defends against stress primarily through the production of antioxidative flavonoids, which are diverse within this plant family (McClure & Alston, 1966). Notable among these flavonoids are the glycosylated flavones apigenin and luteolin, as well as two major anthocyanins, cyanidin-3-malonylglucoside and cyanidin-3-glucoside (Böttner et al., 2021; Lee et al., 2022; Liu et al., 2022). While these metabolites play a key role in mitigating oxidative stress, *S. polyrhiza* also activates the phytohormones jasmonic acid in response to stress, albeit to a lesser extent (Upadhyay et al., 2020). This phytohormone derives from the 12-oxo-phytodienoic acid (OPDA) and likely triggers the production of transcription factors that modulate flavonoid metabolism (Ni et al., 2020). On the other hand, though limited in scope, research into the defensive metabolites of *S. polyrhiza* under herbivory suggests that additional defensive responses may occur. For example, herbivory by the freshwater snail *Lymnaea stagnalis* has showed to consistently enhance the amine tyramine over two consecutive seasons of herbivory (Malacrinò et al., 2024). Tyramine, a compound associated with plant defence responses, is thought to influence herbivore nervous systems and may play a role in suppressing herbivore reproduction and altering herbivore behaviour (Figon et al., 2021; Hagel & Facchini, 2005; Kim et al., 2011). In addition to its effects on herbivores, tyramine could also reinforce plant cell walls, thereby reducing herbivore digestibility (Facchini et al., 1999; Hagel & Facchini, 2005). Together, these findings highlight the complex and multifaceted defensive strategies employed by *S. polyrhiza*, involving the regulation of compounds that influence both plant and herbivore interactions.

v. Outline of the thesis

In this thesis, I investigate transgenerational plasticity in plants by examining the effects of copper excess and aphid herbivory on the fitness of the giant duckweed *Spirodela polyrhiza* and its interacting species, the waterlily aphid *Rhopalosiphum nymphaeae*. Specifically, I address the following questions: **(1)** Is transgenerational plasticity heritable, adaptive, and predictable? **(2)** Do transgenerational effects on host plants influence the performance of co-occurring species? **(3)** Does herbivory induce transgenerational effects on plant-herbivore interaction? This thesis demonstrates that exposure to both copper excess as an abiotic stress and aphid herbivory as a biotic stress causes transgenerational changes in plant fitness. This transgenerational plasticity is heritable, and its adaptiveness is context dependant. While we were able to predict the transgenerational effects through genotype resistance to stress, analysis on variance revealed that transgenerational plasticity minimizes the cease of reproduction under recurrent stress, challenging the expected diversified bet-hedging. Further analysis into the defensive anthocyanins, jasmonates and tyramine, revealed that these metabolites are transgenerationally regulated, following either transgenerational retention or transgenerational priming across generations. Copper-induced anthocyanins likely benefited plant fitness under recurring copper excess, while jasmonates, despite their usual role in herbivory defence, unexpectedly promoted aphid fitness. Aphid herbivory also triggered the accumulation of tyramine through the transgenerational regulation of its biosynthetic machinery, which benefited plants by acting as a toxic compound to aphids. Furthermore, field experiments showed that the transgenerationally plastic patterns observed in the laboratory differed in natural environments, where ecological interactions and evolutionary pressures played significant roles. Together, these findings highlight the complexity of transgenerational plasticity and its intra- and interspecific impacts on evolution and ecosystem dynamics.

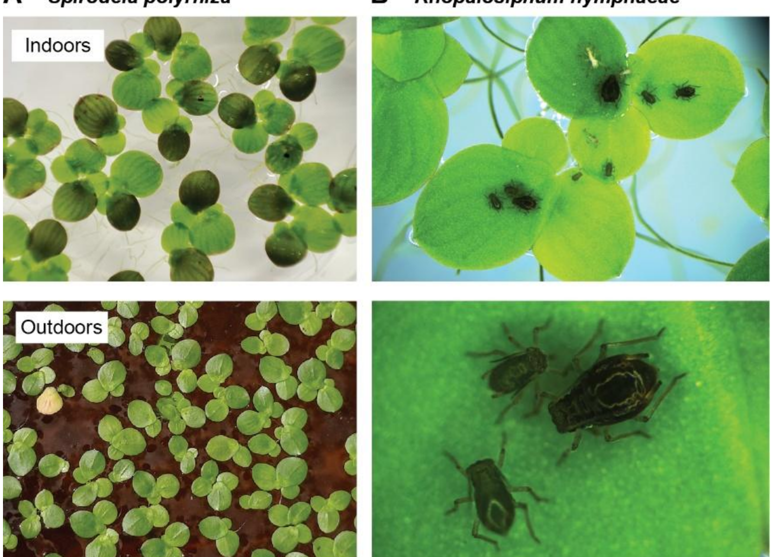

FIGURE 1. MODEL ORGANISMS.

A The giant duckweed *Spirodela polyrhiza* growing free in laboratory conditions (indoors) and in ponds set for outdoor experiments. Pictures: Alexandra Chávez. **B** The waterlily aphid *Rhopalosiphum nymphaeae*, introduced as herbivory stress on the giant duckweed. Pictures: Marieke Theiner using a MZ10 F Modular Stereo Microscope (Leica Microsystems, Wetzlar, Germany).

Chapter II: Manuscript I

Copper resistance predicts heritable transgenerational fitness variation in the clonal duckweed *Spirodela polyrhiza*

Alexandra Chávez[1,2,3], Martin Schäfer[1], Iris Finkemeier[2], Shuqing Xu[1,3], Meret Huber[1,2,4]

1 Institute of Organismic and Molecular Evolution, University of Mainz, Johann-JoachimBecher-Weg 7, 55128 Mainz, Germany; 2 Institute of Plant Biology and Biotechnology, University of Münster, Schlossplatz 7, 48143 Münster, Germany; 3 Institute for Evolution and Biodiversity, University of Münster, Schlossplatz 7, 48143 Münster, Germany; 4 Institute for Quantitative and Computational Biosciences, Johannes Gutenberg University of Mainz, 55128 Mainz, Germany

ABSTRACT

Transgenerational plasticity in the absence of genetic change can alter organismal fitness, yet we know little about the genetic basis of transgenerational fitness effects. Here, we explored whether transgenerational fitness effects can be predicted by the rate of vegetative reproduction, plant resistance and defence. Thereto, we exposed monoclonal, single-descendant lineages from 56 globally distributed genotypes of the clonal duckweed *Spirodela polyrhiza* to five generations of copper excess, followed by five generations without stress, and then measured plant fitness and phenotypes in both environments. Previous copper excess reduced fitness variation within each genotype and elicited heritable and reproducible fitness effects that depended on both the genotype and environment: genotypes that benefited from previous stress under recurring conditions suffered when the stress was absent, and vice versa. These transgenerational fitness effects were predicted best by copper resistance, explaining 30% of the intraspecific variation: the more susceptible a genotype, the more its offspring suffered from ancestral stress in the absence of recurrent stress; however, these offspring became more resistant to copper excess, likely by protecting the photosystem II. Together, these findings show that transgenerational fitness effects are heritable and variable and likely controlled by genes that regulate plant reproduction and defence.

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Copper resistance predicts heritable transgenerational fitness variation in the clonal duckweed Spirodela polyrhiza

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Alexandra Chávez[1,2,3], Martin Schäfer[1], Iris Finkemeier[2], Shuqing Xu[1,3], Meret Huber[1,2,4]

[1] Institute of Organismic and Molecular Evolution, University of Mainz, Johann-Joachim-Becher-Weg 7, 55128 Mainz, Germany; [2] Institute of Plant Biology and Biotechnology, University of Münster, Schlossplatz 7, 48143 Münster, Germany; [3] Institute for Evolution and Biodiversity, University of Münster, Schlossplatz 7, 48143 Münster, Germany; [4] Institute for Quantitative and Computational Biosciences, Johannes Gutenberg University of Mainz, 55128 Mainz, Germany

*Correspondence: Meret Huber, Johann-Joachim-Becher-Weg 7, 55128 Mainz, Germany; meret.huber@uni-mainz.de

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AUTHOR CONTRIBUTION

MH and AC conceptualized the study; AC and MH designed the experiments; MS developed the LC-MS/MS and HPLC-PDA methods; AC collected the data; AC and MH analysed and interpreted the data; SX, IF, MH provided the laboratory and resources to execute the experiments; AC and MH drafted the manuscript; AC, MH and MS contributed to the final version of the manuscript.

INTRODUCTION

Global change accelerates environmental fluctuations. Thus, the ability to retain stress memory and pass environmental cues or traits to offspring may boost species resilience. Indeed, phenotypes can change across generations in the absence of genetic alterations [1]. Most of these changes appear only in immediate offspring –these changes are therefore referred to as maternal effects [1, 2]. More rarely, environmental cues trigger variations that persist for two or more generations, the latter referred to as transgenerational plasticity [3-8]. Notably, transgenerational plasticity can vary among genotypes, suggesting a genetic basis [3, 4, 9].

Elucidating the genetic basis of transgenerational plasticity has proven to be challenging. A recent study showed that across *Arabidopsis thaliana* genotypes, transgenerational effects in gene expression correlates with the abundance of several transposon classes [3]. However, such correlations remain rare, likely because of two hurdles: First, one must confirm that observed phenotypes stem from transgenerational plasticity rather than genetic change. This task requires following single descendants of clonal or highly inbred lineages over several generations [10]. Second, one must quantify phenotypes and organismal fitness in multigenerational experiments using dozens of genotypes. Such large-scale and long-term experiments are not feasible in most higher plants.

The first step in identifying the genetic basis of transgenerational plasticity is to quantify the proportion of variance in these transgenerational effects attributed to genetic differences. Although an increasing number of studies shows that species can be transgenerationally plastic [3-8], heritability estimates of transgenerational plasticity are scarce [11]. Heritability of a trait can be estimated as broad-sense heritability, which explains which fraction of the phenotypic variance is explained by all genetic effects [12]. Such broad-sense heritability are relevant for species reproducing asexually [13]. Heritability can also be estimated as narrow-sense heritability, in which only the additive genetic effects are considered [12]. Narrow-sense heritability is especially important in sexual reproduction because it represents the proportion of phenotypic variance attributable to additive genetic effects, the components most reliably transmitted from parents to offspring [13]. Estimating heritability, either broad- or narrow-sense, is key to infer whether the genetic basis of a trait can be identified.

Although often assumed to be adaptive, transgenerational plasticity is not necessarily beneficial. For example, if stress-induced transgenerational plasticity reduces offspring provisioning, then offspring fitness would decrease regardless of the environment, a phenomenon known as the silver-spoon effect [14]. Conversely, if stress-induced transgenerational plasticity triggers defences that are transmitted to offspring, these defences might incur costs under benign conditions due to the expense of maintaining vertically

transmitted defences, but they could be advantageous under recurring stress. The phenomenon where mild stress benefits organisms, particularly when the stress recurs, is known as hormesis [15, 16]. Although hormesis is often studied within generations [17], its effects can persist across generations. For instance, *Caenorhabditis elegans* exposed to different abiotic stressors increased the lifespan and reproductive traits of non-exposed offspring even three generations after the ancestral stress when compared to the offspring of unstressed parents [9]. Additionally, hormesis may depend on the genetic background: for example, *Drosophila melanogaster* benefited from the application of dead fungal spores only in the absence of antifungal immunity [18]. These observations raise the questions whether transgenerational plasticity is ultimately beneficial or costly, and whether these outcomes may depend on the genotype and environment in which offspring develop.

Apart from altering a genotype's average fitness, transgenerational plasticity may also affect the variability of plant fitness within a genotype. Often, transgenerational plasticity is assumed to increase phenotypic variation within a genotype, enabling certain offspring to succeed, a strategy known as diversified bet-hedging [19]. Diversified bed-hedging is supported from studies on maternal effects: for example, in several vertebrates and invertebrates, previous stress or fluctuating conditions increases egg size variation [20, 21]. However, it remains unclear whether transgenerational effects follow a diversified bet-hedging strategy or instead favour conservative bet-hedging [19], which reduces offspring fitness variation at the cost of maximal fitness.

One of the few flowering plants in which it is feasible to assess whether stress alters both the variance and mean of fitness across multiple generations–without genetic change–is the globally distributed aquatic giant duckweed, *Spirodela polyrhiza*. The plant consists of a flat, thallus-like shoot, the so-called frond, which float on the water surface. Under optimal conditions, the species reproduces very rapidly and exclusively vegetatively through budding every two days [22], thereby allowing precise, real-time measurements across many generations and genotypes. *S. polyrhiza* inhabits small, nutrient-rich water bodies and often endures fluctuating environmental stresses, such as copper excess [23]. Copper excess poses a major threat in aquatic ecosystems [24]; it primarily inhibits photosystem II, thereby triggering oxidative stress much like several herbicides, salt, and UV-light. To defend against oxidative stresses, *S. polyrhiza* produces glycosylated flavones, mainly apigenin and luteolin glucosides, as well as glycosylated and malonylated anthocyanins [4, 25, 26].

Using 56 world-wide sampled *S. polyrhiza* genotypes, we tested whether copper excess triggers transgenerational plasticity, thereby altering both the mean and variance in plant fitness across generations. Notably, we found that the magnitude and direction of these transgenerational fitness effects could be predicted by the copper resistance of each genotype,

suggesting that genes controlling plant reproduction and defence govern transgenerational plasticity.

MATERIALS AND METHODS

Plant growth conditions

Plants were grown inside growth cabinets (GroBank, CLF PlantClimatics, Wertingen, Germany) at 150 µmol photons m^{-2} s^{-1}, 28°C and 16:8 hrs of light and dark cycle using sterilized N-medium, which supports optimal growth [27]. All experiments were conducted under axenic conditions. During single descendant propagation, we minimized biases of environmental variation within the growth cabinets by randomizing the rack order within each shelf and moving the racks from right to left every propagation day (every two to three days). To differentiate mother and offspring, we marked the mother fronds using a permanent marker (Stabilo OHPen Universal, Heroldsberg, Germany).

Statistical analysis

All data was analysed in R version 4.4.0 [28]. To upload, organize and summarize data we used the packages readxl v1.4.3 [29], dplyr v1.1.4 [30], data.table v1.16.4 [31], tidyr v1.3.1 [32]. For mixed effects models, we used the package glmmTMB v1.1.10 [33] and DHARMa v0.4.7 [34] to verify the adjustment of the fitted models. We plotted all data with ggplot2 v3.5.1 [35].

Transgenerational experiment with 56* S. polyrhiza *genotypes

Pre-treatment, recovery, fitness assay

To understand the genetic basis of transgenerational plasticity, we selected 56 worldwide-distributed *S. polyrhiza* genotypes, representing the four genetic clusters of this species (table S1). After surface sterilization with sodium hypochlorite and cefotaxime (methods S1), we acclimated the plants by growing them in 150 mL of N-medium within 250 mL Erlenmeyer flasks (Fisher Scientific, Schwerte, Germany). After one month of acclimation, we placed six fronds per genotype into separate 30 mL polypropylene tubes (Fisher Scientific, Waltham, USA) filled with 25 mL of N-medium and closed with sterilized foam plugs (CarlRoth, Karlsruhe, Germany) (figure S1a, table S2). Once the next generation fully developed, we initiated the "pre-treatment" by transferring the offspring into tubes containing 25 mL N-medium with or without copper excess (20 µM $CuSO_4$) (N = 3 per genotype, figure 1a, figure S1b). Then, the plants were propagated as single descendants for five consecutive generations. Subsequently, the offspring of the fifth generation were moved to control conditions and propagated as single descendants for an additional five generations ("recovery", figure 1a), reaching generation 10.

Finally, we conducted fitness and phenotype assays: We placed the first offspring of the 10th generation in 250 mL transparent polypropylene beakers filled with 150 mL N-medium containing copper excess (20 µM $CuSO_4$), and the second offspring in beakers filled with N-medium without copper excess ("treatment"). The beakers were covered with perforated transparent plastic lids (figure 1a, figure S1c). Plants were grown freely for eight days, corresponding to three to five generations, after which plants were harvested. All plant material, except the plants used to initiate the assay ("starting frond"), were briefly dried with a tissue paper, flash-frozen in liquid nitrogen, and stored at -80°C until metabolite extraction.

To assess plant fitness, we measured the increase in plant surface area across the eight days. Thereto, we photographed the plants at the beginning and end of the assays prior harvest using a camera box equipped with a webcam (HD Pro Webcam C270, Logitech, Lausanne, Switzerland; webcam software 2.12.8). Surface area was estimated using ImageJ 64 v5 [36].

To measure the maximal photosystem II quantum yield of dark-adapted plants, here defined as photosystem II efficiency (F_v/F_m ratios), we collected the starting fronds after the eight-day fitness assay and placed each frond individually into 48-well plates (Sarstedt AG & Co. KG, Nümbrecht, Germany) filled with 1.2 mL of N-medium per well. Once the next generation had emerged under 110 µmol photons m^{-2} s^{-1}, we removed the starting fronds, incubated the plates in darkness for 10 minutes within the climate cabinets and then transferred the plates to an IMAGING-PAM M-Series MAXI version (Heinz Walz, Amtsgericht Bamberg, Germany) to obtain the F_v/F_m ratios (methods S2). The MAXI version included an IMAG-MAX/L measuring head, IMAG-K7 camera and mounting stand IMAG-MAX/GS.

Metabolite quantification

To assess the effect of copper pre-treatment on the accumulation of flavones and anthocyanins, we ground the plant material from all replicates to a fine powder using a MM301 Mixer Mill (Retch, Haan, Germany) and then pooled all samples to obtain mean values per genotype, pre-treatment and treatment (N = 56). 30 mg of the pooled plant material was extracted using acidified methanol. In these extracts, we subsequently quantified the flavones apigenin-7-O-glucoside, apigenin-8-C-glucoside, luteolin-7-O-glucoside and luteolin-8-C-glucoside by LC-MS, and the anthocyanins cyanidin-3-O-(6-O-malonyl-beta-glucoside) and cyanidin-3-O-glucoside–here referred as cyanidin-3-malony-glucoside and cyanidin-3-glucoside–by HPLC-PDA (methods S3). The peaks from all flavones, the LC-MS/MS data, were integrated with LabSolution Insight Version 4.0 SP6 (Shimadzu) and the peaks from anthocyanins, the HPLC-PDA data, with LabSolution Version 5.123 (Shimadzu, Duisburg, Germany).

Estimating fitness and pre-treatment ratios

We estimated daily population growth rates ("fitness") by taking the natural logarithm of the ratio of the final plant surface area to the initial plant surface area and then dividing that value by the number of days the assay lasted (Formula S1) [37]. To assess whether genotypes differ in their transgenerational response, we calculated fitness pre-treatment ratios for each environment. These ratios represent the population growth rates of copper pre-treated plants relative to the mean population growth rates of control pre-treated plants in the respective environment (Formula S2) [5]. Finally, to assess genotype resistance, we calculated the ratio of population growth rates under copper excess to the genotype´s average population growth rates under control condition, using control pre-treated plants (Formula S3).

Effects of transgenerational plasticity on fitness variance

To assess whether copper pre-treatment alters the variance in population growth rates, we first determined whether the pre-treatment increased the overall variance and then examined if this change was due to increased variance within or across genotypes. To compare the homogeneity of variance between the two pre-treatments, we performed Levene tests with the package car v3.1.3 [38] for each environment separately. Similarly, to test whether the pre-treatment altered the variance among genotypes, we calculate mean values per genotype and performed Levene tests. To test whether the pre-treatment altered the variance within genotypes, we compared the variance between the two pre-treatments in each environment using Kruskal-Wallis rank sum tests implemented in R [28].

Furthermore, we counted the number of genotypes that contained one replicate with strongly reduced growth, defined as a reduction in population growth rates of at least 70%. We then assessed whether the pre-treatment altered the frequency of such genotypes using a test of equal or given proportions with the function prop.test of the package stats v4.4.0 implemented in R [28]. To determine whether copper pre-treatment affects the skewness of the population growth rate distributions relative to a normal distribution, we used the function test.skew from statpsych v1.7.0 [39] which employs Monte Carlo p-values. Finally, we compared distribution differences between pre-treatments using the Kolmogorov-Smirnov KS test with the function ks.test of the package stats v4.4.0.

Effects of transgenerational plasticity on fitness means

To assess the effect of the pre-treatment and treatment on the average of population growth rates, we used a linear mixed effect model defined as Fitness ~Pre-treatment *Treatment +(1|Genotype). Similarly, to assess the effect of the treatment and genotype on the pre-treatment ratios, we used the model Fitness pre-treatment ratios ~ Genotype *Treatment +(1|Rack), where "Rack" represents the physical structure of the replicates.

To estimate broad-sense heritability of the fitness pre-treatment ratios, we modified the function Cullis_H2 of the package remotes v0.1 (https://github.com/etnite/bwardr/) to incorporate glmmTMB models and validated the results using the package inti v0.6.6 [40]. To estimate narrow-sense heritability, we generated a kinship SNP matrix for 42 sequenced genotypes [41, 42] using Tassel v5 [43] and obtained heritability estimates with the package sommer v4.3.7 [44]. Finally, to assess whether transgenerational plasticity incurs environment-dependent fitness costs and benefits, we averaged pre-treatment ratios per genotype and environment and correlated these mean pre-treatment ratios across the two environments using the model Fitness pre-treatment ratios in control ~ in copper +(1|Population).

Predicting fitness effects of transgenerational plasticity and inferring its physiological basis

To infer the genetic basis of the transgenerational plasticity, we first calculated the fitness and F_v/F_m mean values per genotype considering naïve plants (plants under control pre-treatment), as well as the fitness and F_v/F_m mean pre-treatment ratios per genotype ("genotype-averaged"). The genotype-averaged values of flavonoids were obtained when pooling the samples before the extractions. Second, we correlated the genotype-averaged fitness pre-treatment ratios for each environment separately with the genotype-averaged population growth rates under control, plant resistance, and the accumulation of flavones and anthocyanins of naïve plants, using linear models. To determine whether genotypes that benefit from ancestral stress under recurring conditions protect their photosystem II, we correlated the genotype-averaged fitness pre-treatment ratios with the genotype-averaged pre-treatment ratios of photosystem II efficiency (F_v/F_m) using the mixed effect model Pre-treatment ratio of fitness ~ Pre-treatment ratio of F_v/F_m * Treatment +(1|Genotype). In addition, we correlated the genotype-averaged pre-treatment ratios of fitness and F_v/F_m for each environment separately using linear models.

To infer whether flavonoids protect photosystem II and thereby benefit plant fitness, we correlated genotype-averaged F_v/F_m ratios and genotype-averaged population growth rates with the concentrations of the major *S. polyrhiza* flavones and anthocyanins, all in naïve plants, using the mixed effect model F_v/F_m ~ Metabolite concentration * Treatment + (1|Plate), as well as Population growth rates ~ Metabolite concentration * Treatment +(1|Plate), where "Plate" denotes the batch of samples from which metabolites were extracted. Additionally, to assess the effect of the pre-treatment and treatment on anthocyanin concentrations, we used the mixed effect model Anthocyanin concentration ~ Pre-treatment *Treatment +(1|Genotype).

Repetition experiment with 16* S. polyrhiza *genotypes

To assess the reproducibility of the transgenerational fitness effects, we repeated the above-described experiment using 16 genotypes that spanned the entire range of fitness pre-

treatment ratios (table S1). First, we confirmed that the genotypes were sterile at the beginning of the experiment by sugar tests (methods S1). Next, we pre-cultivated the plants in 250 mL Erlenmeyer flasks (Fisher Scientific) filled with 150 mL N-medium. The plants were then acclimated to the conditions of the tubes by placing eight single fronds per genotype into 30 mL polypropylene tubes (table S3) and propagating them as single descendants for three generations.

To initiate the pre-treatment, we dived the first and second offspring equally between copper excess (20 µM $CuSO_4$) or control conditions (pre-treatment, figure 1a, table S4). We then propagated the plants for five generations in the respective pre-treatment environment, followed by five generations under control conditions (recovery, figure 1a). Finally, we transferred the first and second offspring (table S4) into 250 mL transparent polypropylene beakers filled with 150 mL N-medium to conduct eight-day fitness and phenotype assays, as described above. Thereto, half of the first and second offspring were placed in beakers containing copper excess (20 µM $CuSO_4$), while the other half were placed in beakers under control conditions ("treatment", N = 8, figure 1a).

To measure plant fitness and pre-treatment ratios, we followed the same procedure as described for the 56 genotypes. To obtain the metabolite profiles for the 16 genotypes, we collected the offspring plant material from the fitness and phenotype assays, excluding the starting fronds. We removed the roots, gently dried the shoot material with tissue paper, and flash-froze the samples in liquid nitrogen. All shoot material was stored at -80°C prior to metabolite extraction. We used 30 mg of plant material per replicate and extracted the flavonoids as described before (methods S3).

To assess whether the genotype-specific transgenerational fitness effects are reproducible, we correlated the standardized beta coefficients of the 16 genotypes from the first and second experiment. We extracted the regression coefficients from the model pre-treatment ratios of fitness ~Genotype *Treatment +(1|Replicate per genotype) +(1|Rack) for each experiment and then standardized the regression coefficients by multiplying by the sample standard deviation of X and dividing by the sample standard deviation of Y, making the coefficients comparable across predictors. To extract the standardized beta coefficients, we modified the stdCoef.merMod function (https://github.com/jebyrnes/ext-meta) to support glmmTMB models.

RESULTS

Previous copper excess alters both the mean and the variance in plant fitness

To test whether ancestral copper excess alters plant fitness, we grew single-descendant lineages of 56 world-wide distributed *S. polyrhiza* genotypes (table S1) for five generations

under either control conditions or copper excess (pre-treatment). After five successive generations without stress, we measured population growth rate–using the increase in surface area as a proxy for plant fitness–and assessed plant morphology under both control conditions and recurrent copper excess. (figure 1a). First, we tested whether ancestral copper excess alters the variability of population growth rates. When considering all replicates, control pre-treated plants displayed a greater variance in population growth rates than copper pre-treated plants, particularly under copper excess (control conditions: $P = 0.07$; copper excess: $P = 8x10^{-4}$, Levene test, figure 1b). This difference in variance among the pre-treatments arose from increased variability within genotypes rather than among them: under copper excess but not control conditions, within-genotype variation was higher in control than copper pre-treated plants ($P = 2x10^{-4}$, Kruskal-Wallis rank sum test, figure 1c). In contrast, in either environment, the variance in mean population growth rates per genotype, i.e. the variance between genotypes, did not differ among the pre-treatments ($P > 0.2$, Levene tests, figure S2a-b).

The within genotype-variance was higher in control than copper pre-treated plants because several control pre-treated plants ceased reproduction under copper excess: when pre-treated with control condition, 12 genotypes included a replicate in which population growth rates was reduced by at least 70% compared to the genotype median, whereas when pre-treated with copper excess, only three genotypes showed such a reduction ($P = 0.02$, test of equal or given proportions, figure 1d, figure S2c). Consequently, under copper excess, the growth rate distributions were left-skewed in control but not copper pre-treated plants (copper: $P = 0.03$, Kolmogorov-Smirnov test, figure S2d). Under control conditions, population growth rates of all replicates remained similar. These patterns held even when we adjusted the threshold for reduced growth from 60% to 75% (table S5). Taken together, these data show that ancestral copper exposure narrows fitness differences within genotype under stress by preventing some replicates from ceasing reproduction.

Second, we tested whether ancestral copper excess alters the average fitness of genotypes. For each genotype and environment, we computed fitness pre-treatment ratios, which are the population growth rates of copper pre-treated plants relative to the mean population growth rates of control pre-treated plants. Fitness pre-treatment ratios depended on the genotype and environment: under control conditions, copper pre-treatment caused only marginal changes in fitness, ranging from a reduction of 10% to an increase of 15%, with 90% of the genotypes showing changes within ±8%. Under copper excess, the effects of the copper pre-treatment on fitness were larger, ranging from a reduction of 25% to an increase of 42%, with 90% of the genotypes varying within ±22% (figure 2a, figure S3). Consequently, depending on the genotype, transgenerational plasticity either increased or decreased plant fitness, with particularly strong effects under recurring stress.

To quantify the genetic proportion explaining variance in transgenerational fitness effects, we calculated both broad- (H^2) and narrow-sense (h^2) heritability on the fitness pre-treatment ratios. Under control conditions, heritability of the fitness pre-treatment ratios was moderate with H^2 = 0.44 and h^2 = 0.21. Under copper excess, both estimates increased, reaching H^2 = 0.56 and h^2 = 0.31. These data support the notion that genetic variation of transgenerational plasticity exists within species.

Genetic variation in transgenerational plasticity should evolve when it benefits plant fitness in one environment but reduces plant fitness in another environment. To test this hypothesis, we correlated each genotype's pre-treatment fitness ratio under control conditions with its corresponding ratio under copper excess. The pre-treatment fitness ratios tended to be negatively correlation between the two environments (P = 0.06, mixed effects model, figure 2b). In essence, genotypes in which ancestral stress improved their fitness under recurring stress suffered when the stress did not recur, and vice versa.

Fitness effects of ancestral copper exposure can be predicted by copper resistance

To infer the genetic basis of transgenerational plasticity, we explored which factors predict within-species variation in transgenerational fitness effects by correlating the fitness pre-treatment ratios with population growth rates in control environments, plant resistance, and the production of defensive flavonoids. Plant resistance–defined as the relative reduction in population growth rates upon copper exposure–proved to be the best predictor of fitness pre-treatment ratios. In fact, variation in copper resistance explained roughly 30% of the variation in fitness pre-treatment ratios: the more susceptible a genotype was, the more its offspring suffered from ancestral stress under control conditions ($P = 3x10^{-6}$, R^2 = 0.28) but the better its offspring became protected from copper excess ($P = 7x10^{-8}$, R^2 = 0.33, figure 3a). Because susceptible genotypes reproduced faster in the absence of stress ($P = 6x10^{-6}$, R^2 = 0.27, figure 3b), fitness pre-treatment ratios could also be predicted by population growth rates, particularly under control conditions: the faster a genotype reproduced under control conditions, the more its offspring suffered from ancestral stress under those same conditions ($P = 8x10^{-8}$, R^2 = 0.53), yet its offspring were better protected under recurring stress (P = 0.05, R = 0.05, figure 3c). Individual defensive traits, namely the accumulation of antioxidative anthocyanins and luteolin glucosides, correlated only weakly positively with fitness pre-treatment ratios under control conditions and did not correlate to pre-treatment ratios under copper excess (figure S4). Overall, these findings reveal that intraspecific variation in transgenerational fitness effects can be predicted by differences in plant resistance and population growth rates.

Maintenance of photosystem II efficiency likely mediates transgenerational fitness effects

As copper excess leads to oxidative stress in plants, we examined whether genotypes that benefit from ancestral stress under recurring conditions also protect their photosystem II under stress. Thereto, we correlated the pre-treatment ratios of plant fitness with the pre-treatment ratios of F_v/F_m values, a proxy for photosystem II efficiency. Under copper excess, the pre-treatment ratios of plant fitness correlated positively with those of F_v/F_m, whereas under control conditions, the pre-treatment ratios did not correlate (Control: $P = 0.7$; Copper: $P = 0.02$; $P(F_v/F_m \times Treat) = 9x10^{-5}$, mixed effects models, figure 4a). In other words, offspring benefiting from ancestral stress under recurring conditions also protected their photosystem II from oxidative damage.

We then assessed whether anthocyanins and flavones protect photosystem II and benefit plant fitness under copper excess. Among the 56 genotypes, the level of anthocyanins, but not flavones, correlated positively with F_v/F_m ratios under copper excess but not control conditions (Cyanidin-3-malonyl-glucoside: Control: $P = 0.9$, Copper: $P = 0.002$; Cyanidin-3-glucoside: Control: $P = 0.9$, Copper: $P = 0.01$; mixed effects models, figure 4b, figure S5a). Moreover, the levels of these anthocyanins correlated strongly negatively with population growth rates under control conditions, and only weakly negatively under copper excess, indicating that anthocyanin production is costly under control conditions but beneficial under copper excess ($P(Anthocyanins_x Treat) < 0.03$, mixed effects models, figure 4c, figure S5b). Collectively, these data suggests that the anthocyanins protect photosystem II and thereby benefit plant fitness under copper excess.

Next, we tested whether the genotypes that benefit from previous stress under recurring conditions retain elevated, stress-induced levels of anthocyanins across generations. On average, first-time copper excess doubled the levels of cyanidin-3-malonyl-glucoside and cyanidin-3-glucoside ($P < 8x10^{-10}$, mixed effects model, figure 4d, figure S7a). Moreover, copper pre-treated plants retained 5% higher anthocyanin levels under both control conditions and copper excess (Cyanidin-3-malonyl-glucoside: P(Pre-treatment) = 0.03; Cyanidin-3-glucoside: P(Pre-treatment) = 0.06; mixed effects models, figure 4d, figure S7b). However, the pre-treatment ratios of both cyanidins did not correlate with the pre-treatment ratios of F_v/F_m values or those of plant fitness (figure S7c-d). These findings suggest that either our metabolite analysis occurred at an unsuitable time or that other physiological mechanisms underlie the protection of photosystem II under recurring conditions.

Transgenerational fitness effects are reproducible

To test whether our transgenerational effects in plant fitness are reproducible, we repeated the transgenerational experiment (figure 1a) with a subset of 16 genotypes that spanned the entire range of pre-treatment ratios observed under control conditions and copper excess (figure S8). To assess the reproducibility of our experiments, we compare the effect sizes of the

explanatory variables across the two experiments. Thereto, we calculated the standardized beta coefficients for the pre-treatment ratios of each genotype. The beta coefficients from the two experiments correlated positively under both control and copper excess (*P*(beta coefficients) = 0.02, linear model, figure S6), showing that genotype-specific pre-treatment ratios of plant fitness are reproducible.

DISCUSSION

Transgenerational plasticity can influence organismal fitness in a genotype-dependent manner, yet its genetic basis remains largely unknown. In our study, copper excess produced genotype- and environment-specific effects on offspring fitness after five clonal generations without stress. Notably, this intraspecific variation could be predicted by population growth rates and plant resistance, suggesting that genes governing plant reproduction and defence underlie transgenerational plasticity in *S. polyrhiza*.

Transgenerational plasticity varies among genotypes in several species, including *S. polyrhiza*, *Trifolium repens*, and *A. thaliana* [3, 4, 6], suggesting a genetic basis. Yet, identifying the genetic basis has proven to be challenging. One notable example is the work of Lin X, Yin J, Wang Y, Yao J, Li QQ, Latzel Vet al. [3], which showed that across *A. thaliana* genotypes, transgenerational variation in gene expression correlates with the abundance of several transposon classes. Here, by screening 56 *S. polyrhiza* genotypes, we found that transgenerational fitness effects can be predicted by copper resistance, explaining 30% of the intraspecific variation: more susceptible genotypes produced offspring that suffered from the ancestral stress under control conditions; however, these offspring became more resistant to copper excess. These strong genetic correlations suggests that genes involved in reproduction and defence underlie the fitness effects of transgenerational plasticity.

Two non-exclusive mechanisms could explain why susceptible genotypes produced offspring that suffered from ancestral stress under control conditions but became better protected to recurring copper excess. First, under stress, resistant genotypes might supply more resources to their offspring than susceptible genotypes, boosting offspring performance regardless the environment, a phenomenon known as the silver-spoon effect [14]. However, if this mechanism dominated, we would expect the offspring of resistant genotypes to be better prepared for recurring stress as well, which was not the case. Instead, our data support a second mechanism, referred to as hormesis [15, 16]: in susceptible genotypes, stress may trigger a stronger induction of stress signals or defensive substances than in the resistant genotypes. If the concentrations of these molecules are transgenerationally elevated, the offspring may perform worse under control conditions, due to the cost of defence [45], but better under

recurring stress. To distinguish between these hypotheses, the silver-spoon effect and hormesis, one could increase the stress level in resistant genotypes and assess whether their offspring show enhanced protection against recurring stress.

To identify traits that are transgenerationally plastic and protect susceptible genotypes under recurring stress, we screened candidate defences and photosystem II efficiency. Genotypes that benefited from previous stress under recurring conditions also maintained superior photosystem II efficiency under copper excess, suggesting that photosystem II protection is a key factor mediating transgenerational fitness effects. However, the underlying physiological mechanisms remain largely unclear. We hypothesized that anthocyanins might play a role. Indeed, our data indicate that anthocyanins protect plant fitness and photosystem II efficiency under copper excess, consistent with their well-known role as antioxidants [46-49] and protectors of the photosystem II [50-52]. Furthermore, copper-induced anthocyanin levels were passed on to the next generation. However, intraspecific variation in the transgenerational inheritance of anthocyanins did not correlate with variation in photosystem II efficiency or plant fitness. These patterns suggest that either our metabolite analysis was conducted at an unsuitable time point or other physiological mechanisms contribute to the protection of photosystem II under recurring stress. Future experiments should manipulate anthocyanin levels genetically to determine whether elevated levels protect photosystem II under recurring copper excess. Additionally, testing other recurring stresses such as herbicides, salt or UV-stress could reveal whether antioxidants and photosystem II protection contribute to transgenerational cross-resistance to oxidative stress, thereby clarifying the ecological relevance of transgenerational plasticity.

In our study, we found that previous stress altered not only the average plant fitness but also the variation in fitness within genotypes. Specifically, ancestral copper excess reduced the variance in population growth rates within genotypes under recurrent stress by preventing some replicates from ceasing reproduction, a phenomenon that aligns with conservative bet-hedging [19]. These results contrast with the proposed strategy of asexual and genetically depleted organisms to overcome environmental fluctuations by transgenerationally diversifying, rather than homogenizing, phenotypes and fitness [53-55], a process described as diversified bet-hedging [19]. However, modelling the evolution of maternal effects showed that reduced phenotypic variance in the offspring can potentially stabilize population sizes by minimizing extreme maladaptive phenotypes [56]. Because variation in fitness can influence long-term population growth rates [57] and rates of evolution [58, 59], our findings underscore the importance of assessing not only average fitness but also within-genotype variation when evaluating the evolutionary consequences of transgenerational plasticity.

Although our study focused on an aquatic species reproducing exclusively asexually during the experiments, we argue that the insights gained extend beyond this specific reproductive mode and plant system. First, asexual reproduction is common among flowering plants and lower animal phyla [60-65], and many invasive weeds, crops, and keystone species, including *S. polyrhiza*, reproduce asexually [66, 67]. Therefore, even if our findings primarily apply to mostly asexually reproducing organisms, the data show that transgenerational fitness changes can substantially affect the population dynamics of key stone species. Second, we uncovered a likely genetic basis for transgenerational plasticity by leveraging the experimental advantages of our system, particularly the rapid asexual reproduction. Only with this study system we were able to measure fitness across dozens of genotypes over multiple generations. These mechanistic insights are crucial for understanding the genetic bases of transgenerational plasticity and provide the starting point to test whether transgenerational fitness effects are governed by genes controlling reproduction and defence also in other species, including those that reproduce sexually.

Taken together, our data show that transgenerational plasticity affects both the average and the variability of plant fitness across generations. Importantly, we can now predict a genotype's transgenerational fitness effects from its resistance, suggesting that genes controlling plant reproduction and defence govern transgenerational fitness effects.

DATA AVAILABILITY STATEMENT

All raw data and R scripts for the analyses and plots within this study are deposited in https://github.com/Plant-Evolutionary-Ecology-Lab/Genetic-Base-Transgenerational-Plasticty.

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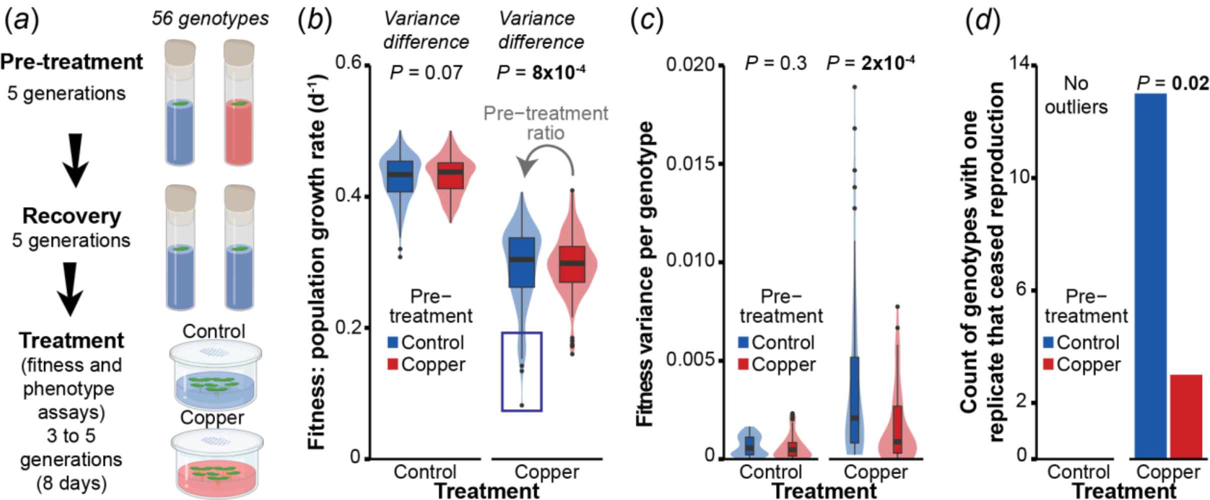

Figure 1. Ancestral copper excess in *Spirodela polyrhiza* reduces within-genotype variation in plant fitness under copper excess. (*a*) Experimental setup. Blue liquids refer to control condition and red liquids refer to copper excess. (*b*) Copper pre-treatment decreased overall variance in population growth rates under copper excess. Variance difference *P*-values, to compare homogeneity of variance between pre-treatments, refer to Levene tests for homogeneity of variance. N = 165-168. Each data point represents an individual sample. (*c*) Copper pre-treatment decreased the variance in population growth rates within genotypes. *P*-values to compare the variance between pre-treatments refer to Kruskal-Wallis rank sum tests. N = 56. Each data point represents one genotype. (*d*) Copper pre-treatment decreased the number of genotypes in which one replicate ceased reproduction (lower than 70% of the genotype´s median). No outliers refer to the absence of genotypes with one replicate ceasing reproduction. The *P*-value refers to a test of equal or given proportions.

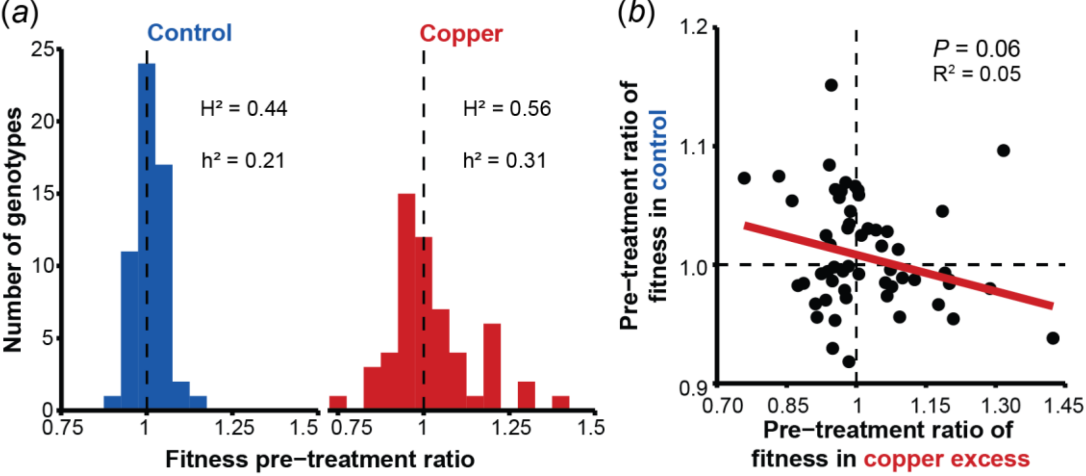

Figure 2. Copper-induced transgenerational fitness effects are heritable, variable across genotypes and environment-dependent, revealing a trade-off between enhanced performance under stress and reduced fitness under control conditions. (*a*) Histogram of the fitness pre-treatment ratios and their estimates of broad-sense (H^2) and narrow-sense (h^2) heritability. Fitness pre-treatment ratios represent the population growth rates of copper pre-treated plants relative to the mean population growth rates of control pre-treated plants, estimated over the 8-day fitness assay. N = 42-56. A pre-treatment ratio of 1, indicated by the dashed vertical line, signifies that ancestral stress had no effect on a genotype's mean fitness. (*b*) Scatterplot illustrating the trade-off in transgenerational fitness effects between environments: genotypes that benefited from copper pre-treatment under recurring stress exhibited reduced fitness when the stress was absent. *P*-value refers to a mixed effects model. Each data point represents the average fitness pre-treatment ratio per genotype, estimated over the 8-day fitness assay. N = 56.

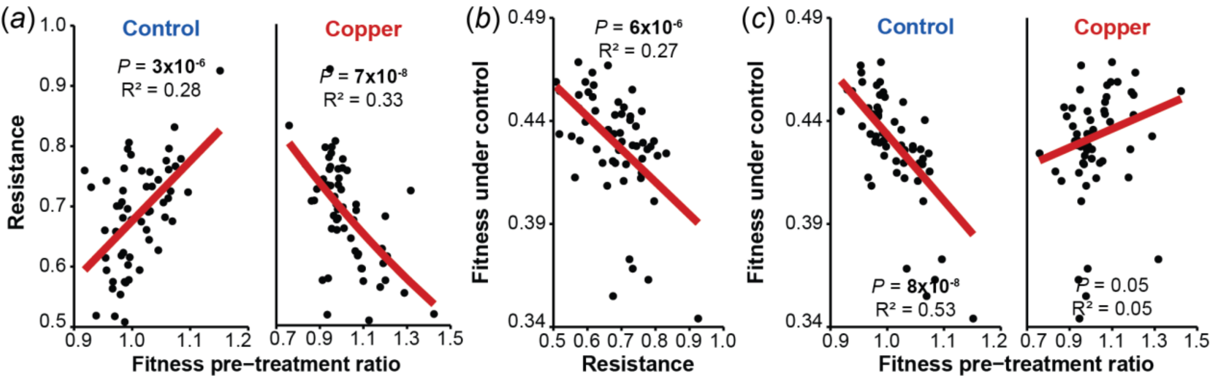

Figure 3. Within-species variation in transgenerational fitness effects were predicted best by copper resistance. (*a*) The fitness pre-treatment ratios (population growth rates of copper pre-treated plants relative to the mean population growth rates of control pre-treated plants) correlated positively with copper resistance under control conditions and negatively under copper excess. Copper resistance is defined as the relative reduction in population growth rates of control pre-treated plants under copper excess. (*b*) Genotypes with high population growth rates ("fitness") under control conditions were susceptible to copper excess. (*c*) Genotypes with high reproductive rates ("fitness") under control conditions suffered from ancestral stress if the stress did not recur; however, their offspring became more resistant to copper excess. *P*-values in a-c refer to linear models. Each data point represents a genotype´s mean values, estimated over the 8-day fitness assay. N = 56.

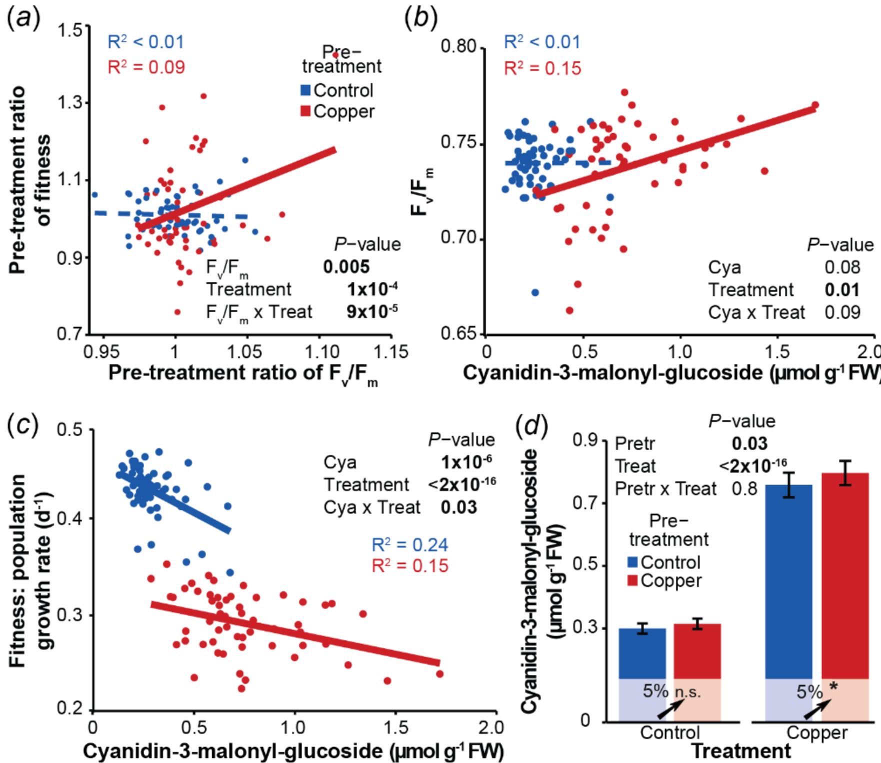

Figure 4. Genotypes that benefit from copper pre-treatment under recurring stress protect their photosystem II from oxidative damage, potentially through transgenerationally inherited anthocyanins. (*a*) The pre-treatment ratios of the photosystem II efficiency (pre-treatment ratio of F_v/F_m) correlate positively with the pre-treatment ratio of fitness under copper excess but not under control conditions. Pre-treatment ratios refer to the ratio in F_v/F_m or plant fitness of copper pre-treated plants relative to the mean values of control pre-treated plants. Plant fitness is defined as daily population growth rates. (*b*) Genotypes with higher concentrations of cyanidin-3-malonyl-glucoside

had higher photosystem II efficiency (F_v/F_m) under copper excess but not under control conditions. (*c*) Cyanidin-3-malonyl-glucoside levels correlated less negatively with population growth rates under copper excess than under control conditions. For (*a*)-(*c*): Each data point represents a genotype´s mean value. *P*-values refer to mixed effects models. Solid lines indicate correlations with $P < 0.05$, whereas dotted lines indicate correlations with $P > 0.05$. Population growth rates and metabolite levels were measured after eight days of fitness assay. F_v/F_m values were measured from an offspring that emerged immediately after the 8-day fitness assay from the plant used to initiate the fitness assay. (*d*) Copper pre-treatment transgenerationally enhanced cyanidin-3-malonyl-glucoside concentrations regardless of the subsequent treatment. Error bars denote standard errors. *P*-values on top of the panel refer to a mixed effect model and *P*-value results within bars refer to mixed effects models, with n.s. for $P > 0.05$, and * for $P < 0.05$. Treat = treatment, Cya = cyanidin-3-malonyl-glucoside, Pretr = pre-treatment. N = 56.

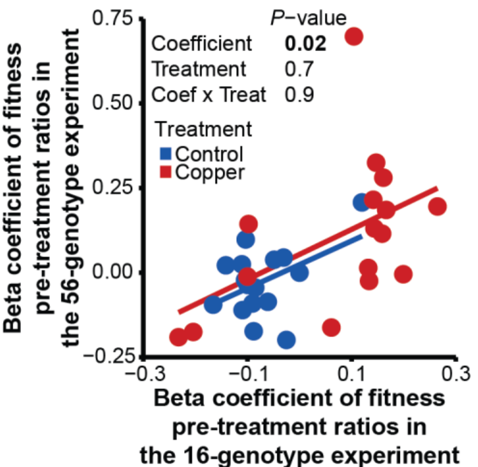

Figure 5. Genotype-specific transgenerational fitness effects are reproducible. Standardized beta coefficients, which quantify the effect of each genotype on the fitness pre-treatment ratio, correlated positively across two independent experiments. Each data point represents a genotype. *P*-value refers to a regression model. Coefficient = beta coefficient of fitness pre-treatment ratios, Coef = coefficient, Treat = treatment. N = 16.

Supplemental Material

Copper resistance predicts heritable transgenerational fitness variation in the clonal duckweed *Spirodela polyrhiza*

Figures

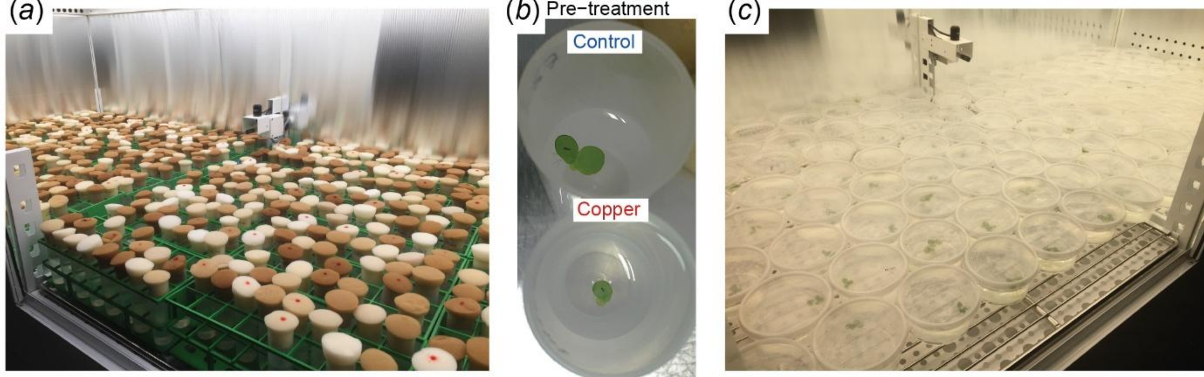

Figure S1. Distribution of samples within polypropylene tubes and beakers. (*a*) Plants within polypropylene tubes were distributed in racks, with maximum two generations per strain to homogenise the light incidence within the racks. Genotypes = 56, pre-treatments = 2, replicates = 3, generations = 2; 672 tubes. (*b*) Single descendant frond in control and copper excess during the pre-treatment phase. (*c*) Plants within polypropylene beakers during fitness and phenotype assays. Genotypes = 56, pre-treatments 2, treatments = 2, replicates = 3; 672 beakers.

1 Supplemental Material

2

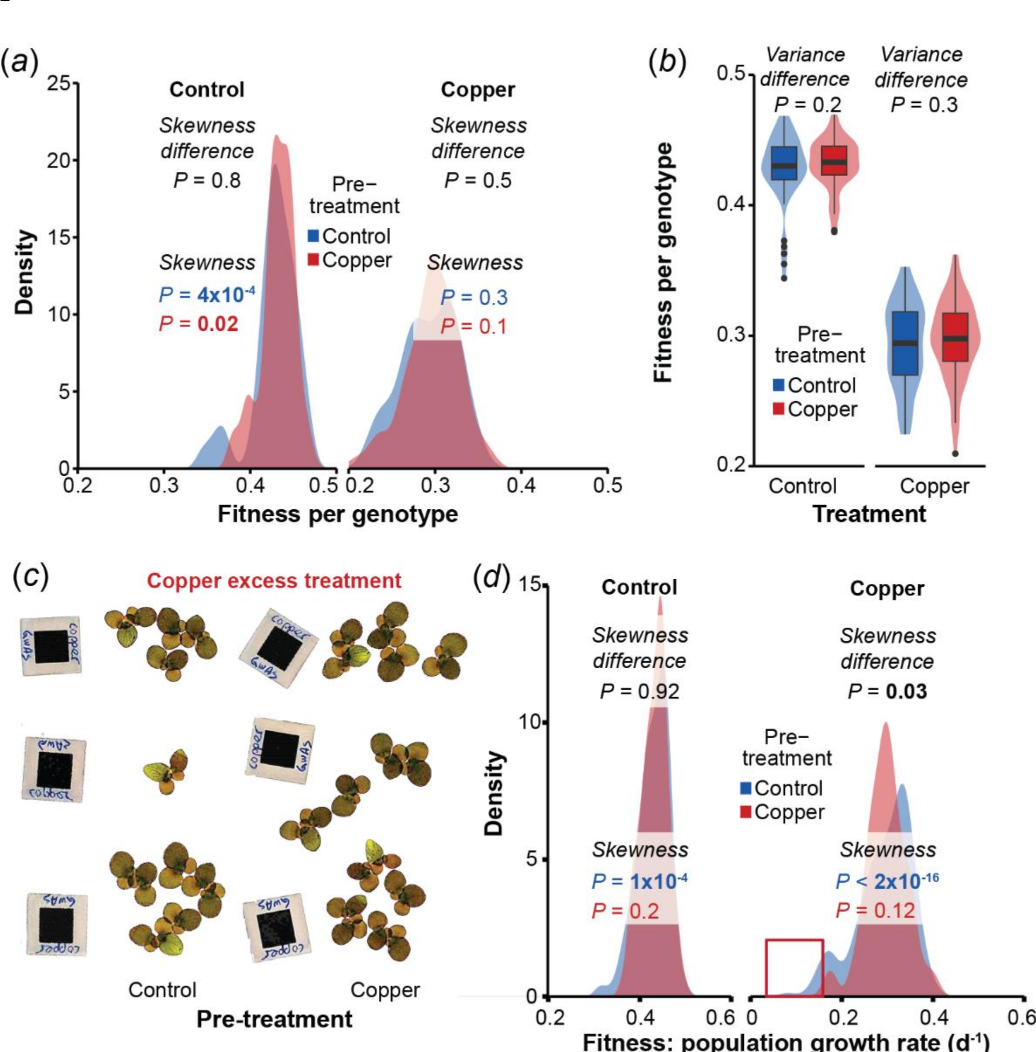

Figure S2. Under recurrent copper excess, ancestral stress exposure affects the distribution of samples within genotypes but not among genotypes. (*a*) Samples of genotypes exposed to copper excess for the first time showed stronger fitness decrease than samples from copper pre-treated genotypes. Skewness *P* values refer to the comparison of the data distribution to a normal distribution using Monte Carlo *p*-values. Skewness difference *P* values on top refer to Kolmogorov-Smirnov tests to compare the distribution between pre-treatments. Control and Copper headers refer to the treatment. Blue and red colour of the *P* values refer to control and copper pre-treatment, respectively. N = 165-168. (*b*) Variance between mean fitness per genotype of control and copper pre-treated plants remained similar under control and copper excess. Variance difference *P* values, to compare homogeneity of variances between pre-treatments, refer to Levene test for homogeneity of variance N = 56. (*c*) Genotype SP223, genotype with the strongest outlier under first time copper excess. (*d*) Mean fitness per genotype did not differ in their distributions when comparing control to copper pre-treated plants. Skewness *P* values refer to the comparison of the data distribution to a normal distribution using Monte Carlo *p*-values. Skewness difference *P* values on top refer to Kolmogorov-Smirnov tests to compare the distribution between pre-treatments. N = 56.

2 Supplemental Material

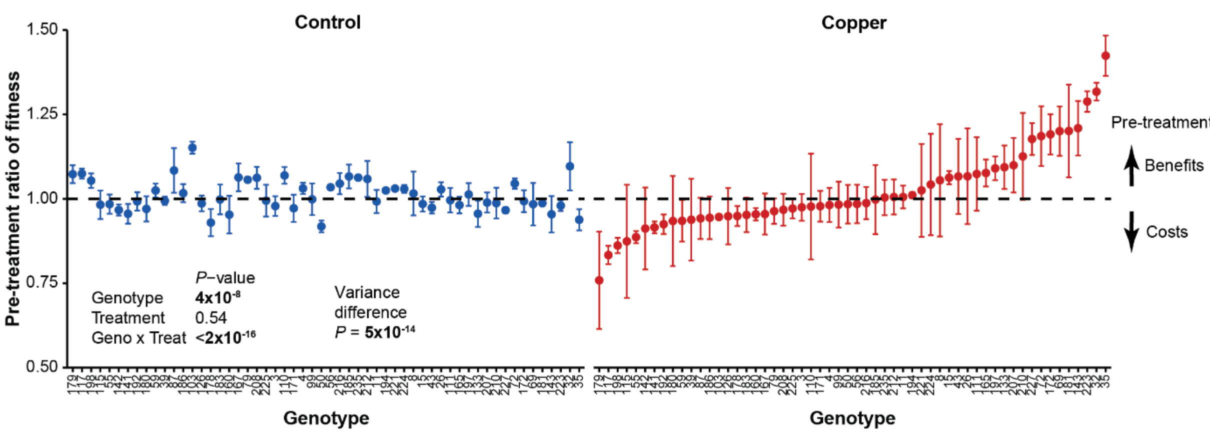

Figure S3. Pre-treatment ratios dependent on the treatment environment and genotype. *P* values on the left refer to a mix effects model; N = 3. Variance difference *P* value, to compare data homogeneity of variances between treatments, refers to Levene test for homogeneity of variance; N = 168. Control and Copper headers refer to the treatment.

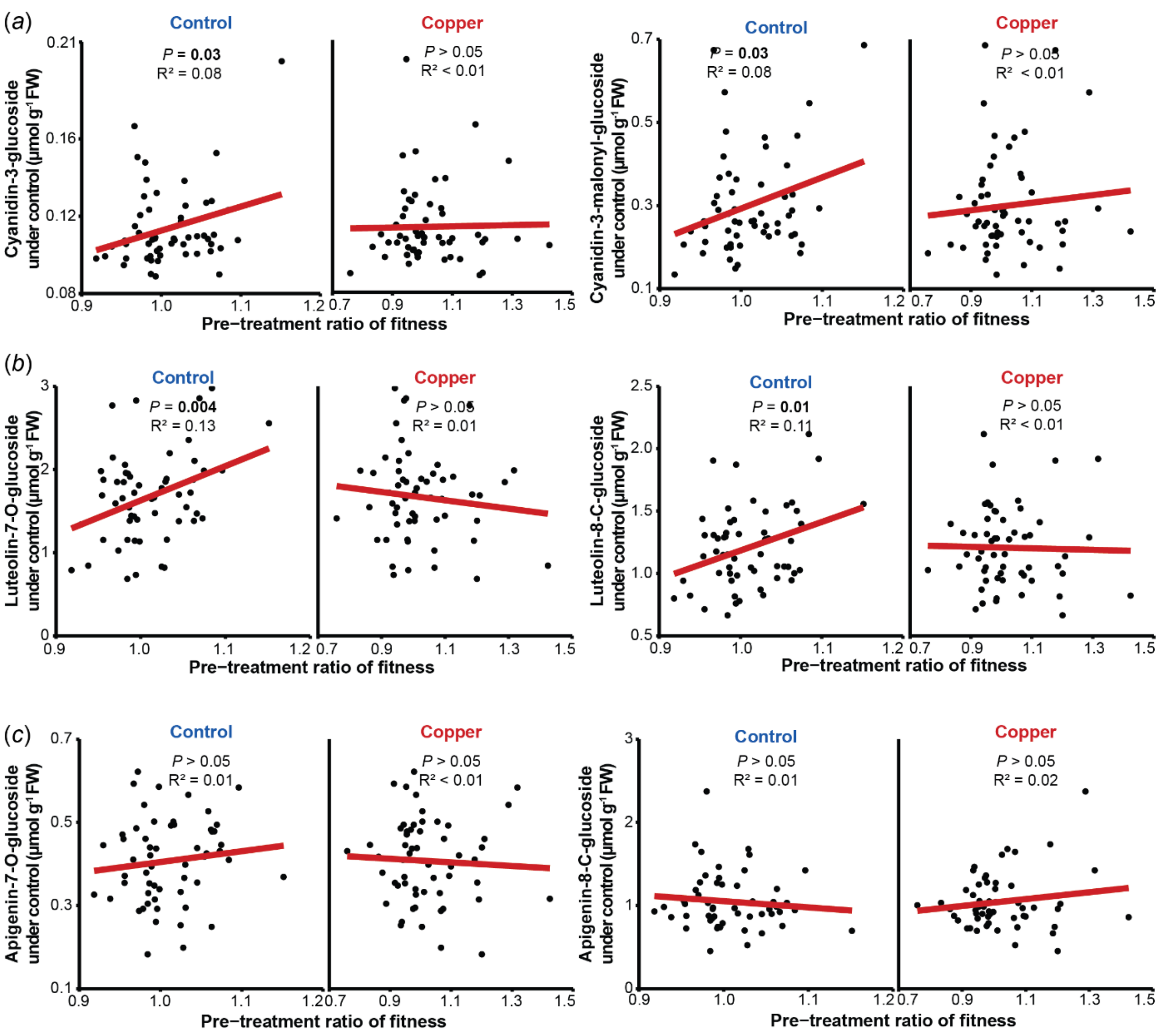

Figure S4. The offspring of genotypes with higher flavonoid concentrations do not have benefits or costs under recurrent copper excess. Genotype concentrations of (*a*) anthocyanins, (*b*) luteolins and (*c*) apigenins under control conditions did not predict the pre-treatment ratios of plant fitness (fitness of copper pre-treated plants relative to the mean fitness of control pre-treated plants). Mean flavone concentration per genotype was obtained through pooling of the genotype replicates before extraction of the metabolites. *P* values per treatment were calculated with simple linear regressions. Control and Copper headers refer to the treatment. N = 56.

4 Supplemental Material

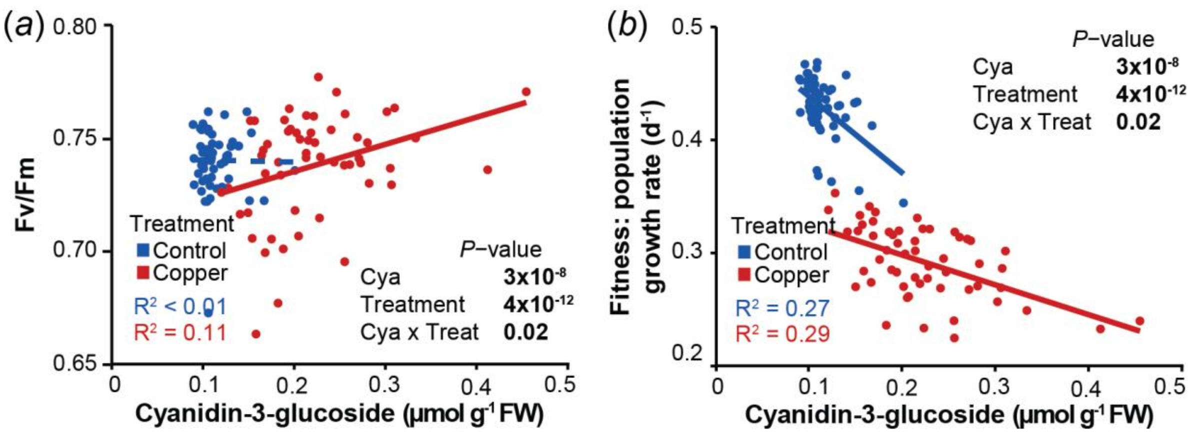

Figure S5. Genotypes with higher concentrations of cyanidin-3-glucoside show better photosystem II activity and less fitness costs. (*a*) Genotypes with higher concentrations of cyanidin-3-glucoside had higher photosystem II activity under copper excess, but no correlation was observed under control conditions. (*b*) The costs of cyanidin-3-glucoside in plant fitness were overcome under copper excess. Fv/Fm represents mean values per genotype. Mean cyanidin concentration per genotype was obtained through pooling of the genotype replicates before extraction of metabolites. Continuous lines refer to correlations with $P < 0.05$, and dotted lines refer to correlations with $P > 0.05$ from mixed effects models. *P* values refer to mixed effects models. *P* values refer to mixed effect models, where significant interactions ($Cyanidin_x Treatment$) show the correlation difference between treatments. N = 56.

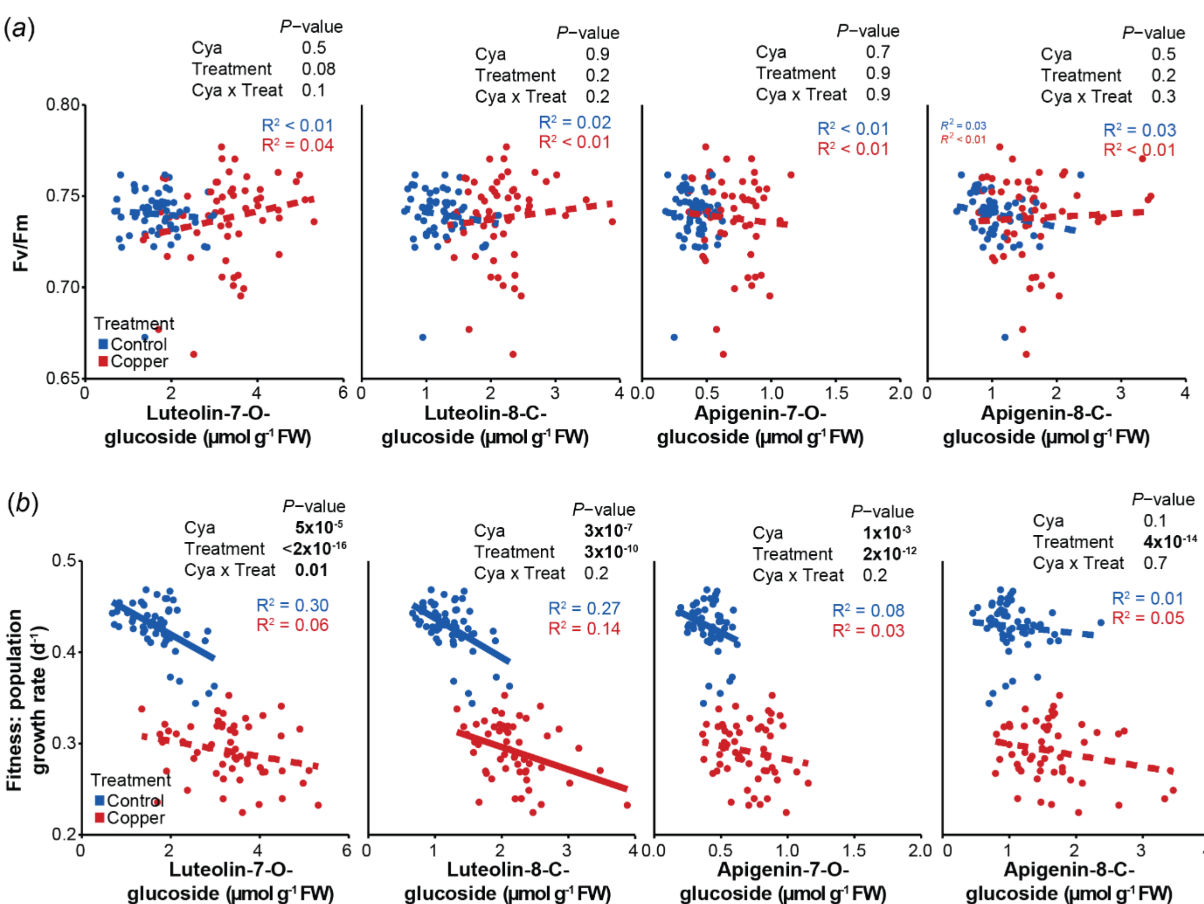

Figure S6. Accumulation of flavones did not represent benefits for plants under first time stress. (*a*) Photosystem II activity (Fv/Fm) did not correlate with flavone concentrations under copper nor control conditions. (*b*) Genotypes with higher flavone concentrations showed costs in plant fitness. Fv/Fm and fitness values represent mean values per genotype. Mean flavone concentration per genotype was obtained through pooling of the genotype replicates before extraction of metabolites. Costs are identified in the absence of significant co-interactions ($Flavones_x$Treatment). Continuous lines refer to correlations with $P < 0.05$, and dotted lines refer to correlations with $P > 0.05$ from mixed effects models. P values on top of each plot refer to mixed effects models. Cya = cyanidin, Treat = treatment. N = 56.

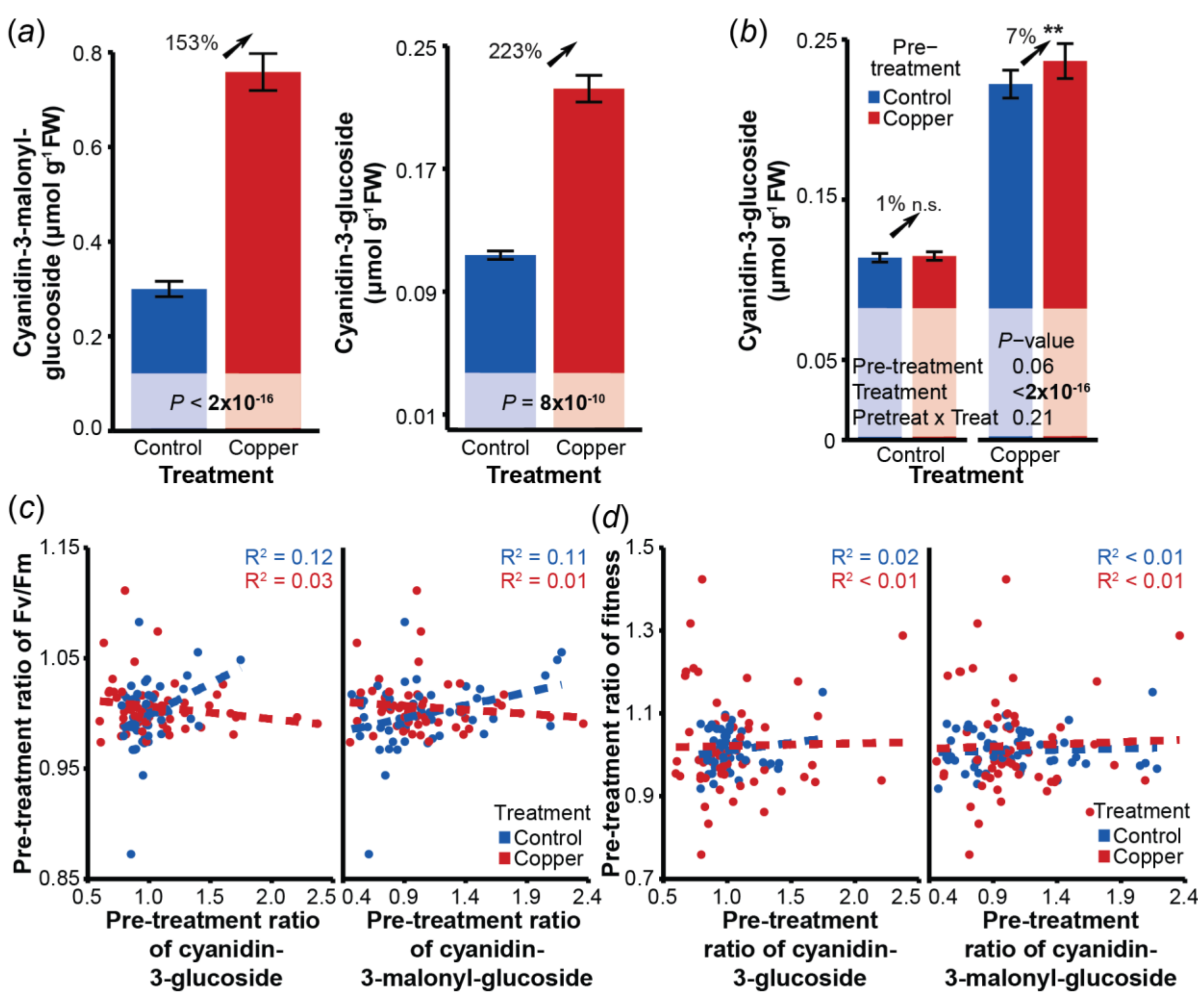

Figure S7. Copper excess induces high cyanidin concentrations that are transgenerationally retained elevated in the offspring, yet its transgenerational effects do not represent fitness nor phenotype advantages. (*a*) Copper excess enhanced cyanidin-3-malonyl-glucoside and cyanidin-3-glucoside concentrations. (*b*) Cyanidin-3-glucoside levels were transgenerationally retained elevated regardless of the presence or absence of recurrent copper excess. *P* values refer to mixed effects models. (*c*) The pre-treatment ratios of both cyanidins did not correlate with the pre-treatment ratios of photosystem II activity. *P* values refer to mixed effects models. For (*a*)-(*c*): Mean cyanidin concentrations per genotype were obtained through pooling of the genotype replicates before extraction of metabolites. (*d*) The pre-treatment ratios of cyanidins did not correlate with the pre-treatment ratios of plant fitness. Pre-treatment ratios of Fv/Fm and fitness refer to mean pre-treatment ratios per genotype. Dotted lines refer to correlations with $P > 0.05$ obtained from simple linear models. Pretreat = pre-treatment, Treat = treatment. N = 56.

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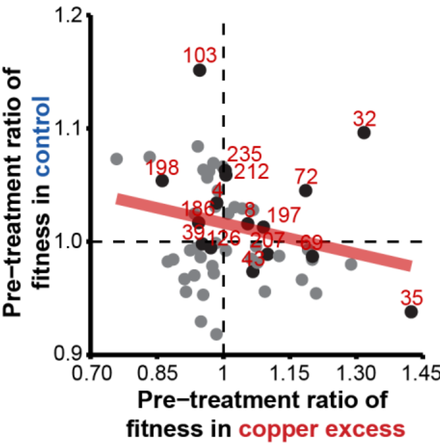

Figure S8. Genotypes in the repetition experiment represent the fitness trade-off effects. 16 genotypes (black dots with red letters) were selected to assess the reproducibility of transgenerational plasticity.

Tables

Table S1. World-wide distributed genotypes of *Spirodela polyrhiza* used for the transgenerational experiments.

Accession ID	Clone ID	Original Library	Population	Continent	Country	16-genotype experiment
SP003	7379	KlausApp	-	Asia	India	
SP004	7498	KlausApp	America	North America	USA	Repeated
SP008	8683	KlausApp	America	Africa	Kenya	Repeated
SP011	9242	KlausApp	America	South America	Ecuador	
SP015	9510	KlausApp	Europe	Africa	Mozambique	
SP026	9633	KlausApp	Europe	Europe	Albania	
SP032	9505	KlausApp	America	North America	Cuba	Repeated
SP035	0109	KlausApp	SE Asia	Asia	China	Repeated
SP039	9650	KlausApp	India	Asia	India	
SP043	9497	KlausApp	India	Asia	India	Repeated
SP050	0090	KlausApp	America	Asia	China	Repeated
SP055	9610	KlausApp	Europe	Europe	Poland	
SP056	9256	KlausApp	Europe	Europe	Finland	
SP059	9506	KlausApp	India	Asia	India	
SP069	6731	KlausApp	-	North America	USA	Repeated
SP072	5522	KlausApp	SE Asia	Asia	China	Repeated
SP079	5513	KlausApp	Europe	Europe	Germany	
SP087	9657	Landolt	America	North America	Canada	
SP099	5524	KlausApp	SE Asia	Asia	Thailand	
SP103		Simon&Martin	Europe	Europe	Switzerland	Repeated
SP110	S21	Simon&Martin	-	Europe	Switzerland	
SP111	Ch1	Sowjanya Sree	-	Asia	China	
SP115	Kor3	Sowjanya Sree	SE Asia	Asia	Korea	
SP117	J2	Sowjanya Sree	SE Asia	Asia	Japan	
SP126	8229	Sowjanya Sree	SE Asia	Asia	Malaysia	Repeated
SP133	1059	Sowjanya Sree	Europe	Asia	China	
SP141		YNQJ1	SE Asia	Asia	China	
SP142		YNKM1	SE Asia	Asia	China	
SP143		GZTR1	SE Asia	Asia	China	
SP160		CSP17	SE Asia	Asia	China	
SP165		CSP22	SE Asia	Asia	China	
SP167		JXYT	SE Asia	Asia	China	
SP171		HNHY	SE Asia	Asia	China	
SP172		HNYZ	SE Asia	Asia	China	
SP178	0245	ZH0245	SE Asia	Asia	China	
SP179	0256	ZH0256	SE Asia	Asia	China	
SP180	0282	ZH0282	SE Asia	Asia	China	
SP181	0289	ZH0289	SE Asia	Asia	China	
SP183	0308	ZH0308	SE Asia	Asia	China	
SP185		SH5	SE Asia	Asia	China	

SP186		SH5	SE Asia	Asia	China	Repeated
SP192	Hgw	17S	Europe	Europe	Germany	
SP194	Wan	18S	Europe	Europe	Germany	
SP197		Weihai5	SE Asia	Asia	China	Repeated
SP198			SE Asia	Asia	China	Repeated
SP207		Jiaonan5	SE Asia	Asia	China	Repeated
SP208			SE Asia	Asia	China	
SP210			SE Asia	Asia	China	
SP212		Jining1	SE Asia	Asia	China	Repeated
SP216		Hefeii	SE Asia	Asia	China	
SP221		UK2_01	India	Asia	India	
SP223		UK2_07	India	Asia	India	
SP224		UK2_10	India	Asia	India	
SP225		NE1	India	Asia	India	
SP227		NE3	India	Asia	India	
SP235		JK5	India	Asia	India	Repeated

Accession ID: Code assigned in our group.

Clone ID: Code assigned in other publications or in the original library from other groups.

Population: Populations were identified from SNP analysis done by Wang Y, Duchen P, Chávez A, Sree KS, Appenroth KJ, Zhao H, Höfer M, Huber MXu S [1] and Xu S, Stapley J, Gablenz S, Boyer J, Appenroth KJ, Sree KS, Gershenzon J, Widmer AHuber M [2].

"-" refers to unknown population as the sample was not sequenced.

Continent and Country: Geographical place were the samples were collected.

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Table S2. Order of the 56 genotypes within the racks for the 56-genotype experiment.

	Genotype			
Rack\Well	1-2	3-4	5-6	7-8
1	11	15	26	32
2	50	69	87	32
3	171	224	181	207
4	172	8	87	225
5	185	186	194	223
6	117	126	133	115
7	178	11	99	227
8	142	143	160	141
9	50	26	110	180
10	35	39	43	50
11	197	198	210	212
12	126	180	143	171
13	35	56	223	115
14	186	210	8	224
15	192	208	3	11
16	99	103	110	111
17	59	194	160	111
18	103	15	179	235
19	43	186	212	126
20	179	180	181	178
21	111	133	160	172
22	165	167	171	172
23	43	192	198	208
24	55	35	99	72
25	59	110	183	142
26	216	221	224	225
27	72	165	3	216
28	227	235	192	208
29	223	216	227	197
30	198	179	185	167
31	72	79	87	183
32	32	55	143	183
33	69	141	197	133
34	55	56	59	69
35	212	181	194	225
36	185	39	117	210
37	4	79	167	221
38	115	141	165	178
39	3	4	8	207
40	142	79	103	117
41	39	15	56	4
42	26	207	235	221

Table S3. Order of the 16 genotypes within the racks for the 16-genotyoe experiment.

	Genotype			
Rack\Well	1-2	3-4	5-6	7-8
1	197	32	207	4
2	32	8	103	207
3	186	212	32	50
4	72	197	35	4
5	69	35	197	198
6	212	186	69	207
7	43	198	197	103
8	35	103	186	32
9	43	72	186	198
10	126	72	212	35
11	103	50	126	72
12	8	212	72	126
13	69	235	186	35
14	235	50	69	186
15	8	103	197	32
16	126	43	72	198
17	235	126	43	72
18	198	43	126	72
19	69	4	50	186
20	212	69	103	50
21	4	32	197	207
22	207	212	197	235
23	207	235	32	197
24	8	4	69	126
25	8	235	69	186
26	43	35	103	4
27	50	4	207	212
28	4	235	35	8
29	198	198	207	8
30	198	43	8	235
31	50	198	35	212
32	126	50	43	103

Table S4. Order in which the offspring of the 16-genotype experiment was placed to start the pre-treatment and treatment phases.

	Pre-treatment phase		**Treatment phase**	
Replicate	Copper	Control	Copper	Control
1	1st offspring	2nd offspring	1st offspring	2nd offspring
2	1st offspring	2nd offspring	2nd offspring	1st offspring
3	1st offspring	2nd offspring	1st offspring	2nd offspring
4	1st offspring	2nd offspring	2nd offspring	1st offspring
5	2nd offspring	1st offspring	1st offspring	2nd offspring
6	2nd offspring	1st offspring	2nd offspring	1st offspring
7	2nd offspring	1st offspring	1st offspring	2nd offspring
8	2nd offspring	1st offspring	2nd offspring	1st offspring

Table S5. Genotypes that showed one replicate with smaller fitness than the 60-75% of the genotype median fitness under copper excess treatment.

Fitness lower than:	**60%**	**65%**	**70%**	**75%**
***P* (proportion test)**	0.003	0.015	0.015	0.0025
	SP15	SP15	SP15	SP15
	SP26	SP26	SP26	SP26
	SP32	SP32	SP32	SP32
		SP72	SP72	SP72
		SP110	SP110	SP110
	SP133	SP133	SP133	SP133
	SP143	SP143	SP143	SP143
Pre-treatment:	SP172	SP172	SP172	SP172
Control		SP181	SP181	SP181
				SP185
	SP194	SP194	SP194	SP194
	SP197	SP197	SP197	SP197
				SP208
				SP221
	SP223	SP223	SP223	SP223
	SP227	SP227	SP227	SP227
Pre-treatment:		SP110	SP110	SP110
Copper			SP142	SP142
		SP179	SP179	SP179

Formulas

Equation S1. Fitness as relative population growth rate [3] based on surface area of fronds.

$$\frac{\ln\text{ (final surface area)} - \ln\text{ (initial surface area)}}{\text{number of days}}$$

Equation S2. Pre-treatment ratios [4].

$$\frac{\text{plant fitness or phenotype of copper pre} - \text{treated plants}}{\text{mean (plant fitness or phenotype of control pre} - \text{treated plants)}}$$

Equation S3. Resistance ratios.

$$\frac{\text{plant fitness under first time stress}}{\text{mean (plant fitness under control conditions)}}$$

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Methods

Methods S1. Sterilization of plants

To surface sterilize the plants, we first treated each *S. polyrhiza* genotype with diluted DanKlorix (sodium hypochlorite containing disinfection solution, CP GABA GmbH, Hamburg, Germany) and subsequently with N-medium containing 100 µg mL^{-1} cefotaxime sodium salt (Duchefa Biochemie, Haarlem, Netherlands) and 50 mM sucrose.

To prepare the media, we added 17.1 g of sucrose to 2L of N-medium and autoclaved the media. We then added 1µl cefotaxime stock (100 mg mL^{-1}) per 1 mL medium under the sterile bench. We dissolve the sodium hypochlorite mix in distilled water in a proportion of 1:10 (5 mL DanKlorix + 45 mL dH_2O).

To sterilize the plants under the sterile bench, we placed 3-5 fronds within a falcon tube containing the dissolved sodium hypochlorite and incubated them for 10-15 min with occasional inversion of the tubes. Afterwards, we transferred the fronds to sterilized 100 mL flasks containing 50 mL of N-medium with cefotaxime and sucrose and let the plants regenerate at 26°C, 120 µmol photons m^2 s^{-1} for minimum eight days. Regenerated plants were transferred into 250 mL flask with 150-180 mL N-medium for pre-cultivation. Recovered plants were placed into sucrose N-medium before experiments to corroborate their sterility.

Methods S2. Parameter within the IMAGING-PAM MAXI version

To measure the photosystem II efficiency, we used the software of IMAGING-PAM M-Series MAXI version, ImagingWinGigE V2.47+, to set all the parameters. The intensity of the saturating pulse to obtain the F_v/F_m ratios was of 3500 µmol m^{-2} s^{-1}, for a width of 720 ms. The setting was:

Set gain == 5
Set damping == 2
Set measuring light == 3
Set measuring frequency == 4
Set saturation light == 10

Once the script was executed, we waited 10 seconds in before and after measuring F_v/F_m ratios.

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Methods S3. Flavonoid analysis

To extract the metabolites, we used 30 mg of flash-frozen and grinded plant material. For the extraction, we applied into the samples acidified methanol (MeOH:water:formic acid 15:4:1 v/v/v). The whole extraction followed similar steps as described in Malacrinò A, Böttner L, Nouere S, Huber M, Schäfer MXu S [5], with focus on the flavonoids.

In details, the flavones were analysed in a 1:100 dilution of the extract in an aqueous mixture of stable isotope labelled amino acid (Algal amino acid mixture-^{13}C,^{15}N, Sigma) by LC-MS. Chromatographic separation was done on a Shimadzu Nexera X3 LC-System. The system was equipped with an Agilent 1290 infinity II inline filter (0.3 µm) and a ZORBAX RRHD Eclipse XDB-C18 column (3x50 mm, 1.8 µm; Agilent Technologies). The mobile phase comprised 0.05% formic acid (Fisher Chemical), 0.1% acetonitrile (Fisher Chemical) in water as Solvent A and methanol (Fisher Chemical) as Solvent B in gradient mode with the elution profile: 0–1.5 min, 2% B; 1.5–3.5 min, 2%–100% B; 3.5–4.5 min, 100% B; 4.5–5 min, 100%-2% B and 5-6 min, 2 % B, with a flow rate of 500 µL/min. The column oven was set to 42 °C. The measurements were performed on a Shimadzu LCMS-8060 equipped with an ESI source, which was operated in multi-reaction-monitoring (MRM) modus. Settings were as follows: Nebulizing Gas Flow: 3 L/min; Heating Gas Flow: 10 L/min; Drying Gas Flow: 10 L/min; Interface Temperature: 300 °C; DL Temperature 250 °C; Heat Block Temperature: 400 °C; CID Gas: 270 kPa; Q1 Resolution: Unit; Q3 Resolution: Unit.

The detector settings (MRM-settings and retention times) were:

Analyte	RT [min]	Q1 [m/z]	Q3 [m/z]	Dwell time [ms]	CE [V]	Q1/Q3 Pre Bias [V]	ISTD
Apigenin-7-O-glucoside	3.83	(+) 431,10	268,10	32	-35	-21 / -17	$^{13}C_9$, $^{15}N_1$-Phe (2.25)
		(+) 431,10	269,20	32	-28	-21 / -17	
Apigenin-8-C-glucoside	3.69	(+) 431,10	311,20	32	-24	-30 / -21	$^{13}C_9$, $^{15}N_1$-Phe (2.37)
		(+) 431,10	283,25	32	-35	-30 / -19	
Luteolin-7-O-glucoside	3.73	(+) 447,09	285,20	32	-26	-22 / -19	$^{13}C_9$, $^{15}N_1$-Phe (1.62)
		(+) 447,09	284,20	32	-37	-22 / -18	
Luteolin-8-C glucoside	3.63	(+) 447,09	327,15	32	-24	-30 / -22	$^{13}C_9$, $^{15}N_1$-Phe (1.26)
		(+) 447,09	357,15	32	-21	-30 / -24	
$^{13}C_9$, $^{15}N_1$-Phe	2.52	(+) 176.11	129.25	247	-15	-20/-20	

RT: retention time.

CE: collision energy.

ISTD: internal standard incl. response factor (nAnalyte = x * nISTD) in brackets.

Qualifiers are highlighted in grey.

On the other hand, anthocyanins were analysed by HPLC-PDA, thereto the chromatographic separation was done on a Shimadzu Nexera XR LC-System equipped with an EC 4/3 Nucleodur Sphinx RP pre-column (5 µm, Macherey-Nagel) and a Nucleodur Sphinx RP column (250x4.6 mm, 5µm, Macherey-Nagel). The mobile phase comprised 0.2% formic acid (Fisher Chemical), 0.1% acetonitrile (Fisher Chemical) in water as Solvent A and acetonitrile (Fisher Chemical) as Solvent B in gradient mode with the elution profile: 0–8 min, 10-21% B; 8-18 min, 21%–49% B; 18–18.1 min, 49-100% B; 18.1–19.0 min, 100% B; 19.0–19.1 min, 100%-10% B and 19.1-24 min, 10 % B, with a flow rate of 1300 µL/min.. The column oven was set to 20 °C. Measurement was performed with a PDA detector.

To quantify the anthocyanins, we used an external dilution curve of the molar quantity of cyanidin-3-O-glucoside. Both cyanidins had the following retention time and absorption wavelength:

Analyte	Retention time [min]	Absortion wave lenght [nm]
Cyanidin-3-O-glucoside	7.055	517
Cyanidin-3-O-(6-O-malonyl-beta-glucoside)	9.41	517

References

1 Wang, Y., Duchen, P., Chávez, A., Sree, K. S., Appenroth, K. J., Zhao, H., Höfer, M., Huber, M., Xu, S. 2024 Population genomics and epigenomics of *Spirodela polyrhiza* provide insights into the evolution of facultative asexuality. *Commun. Biol.* **7**, 581. (https://doi.org/10.1038/s42003-024-06266-7)

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Chapter III: Manuscript II

Copper-induced transgenerational plasticity in plant defence boosts aphid fitness

Alexandra Chávez[1,2,3], Anne Schreyer[1], Pauline Prüsener[2,3], Martin Schäfer[1], Shuqing Xu[1,3], Meret Huber[1,2,4]

1 Institute of Organismic and Molecular Evolution, University of Mainz, Mainz, Rheinland-Pfalz, Germany | 2 Institute of Plant Biology and Biotechnology, University of Münster, Münster, Nordrhein-Westfalen, Germany | 3 Institute for Evolution and Biodiversity, University of Münster, Münster, Nordrhein- Westfalen, Germany | 4 Institute for Quantitative and Computational Biosciences, Johannes Gutenberg University of Mainz, Mainz, Rheinland-Pfalz, Germany

Abstract

Transgenerational plasticity in plants is an increasingly recognized phenomenon, yet it is mostly unclear whether transgenerational plasticity is relevant to both the fitness of the plant and its interacting species. Using monoclonal strains of the giant duckweed (*Spirodela polyrhiza*) and its native herbivore, the waterlily aphid (*Rhopalosiphum nymphaeae*), we assessed whether pre-treating plants with copper excess, both indoors and outdoors, induces transgenerational plasticity in plant defences that alter plant and herbivore fitness. Outdoors, copper pre-treatment tended to increase plant growth rates under recurring copper excess. Indoors, copper pre-treatment either increased or decreased plant growth rates under recurring conditions, depending on the plant genotype. Copper pre-treatment induced anthocyanins that protected plants against copper toxicity, and these elevated levels were transgenerationally retained. Copper pre-treatment also transgenerationally increased the levels of 12-oxo-phytodienoic acid (OPDA), a jasmonate precursor. Nevertheless, aphids grew up to 50% better when the plants were pretreated with copper. The increased aphid growth was likely caused by transgenerationally elevated OPDA levels, as aphids grew better when jasmonates were externally applied to plants. Taken together, this study shows that transgenerational plasticity is relevant to both plant and herbivore fitness, which highlights the role of transgenerational plasticity in plant evolution and species interactions

Plant, Cell & Environment

Copper-Induced Transgenerational Plasticity in Plant Defence Boosts Aphid Fitness

Alexandra Chávez[1,2,3] | Anne Schreyer[1] | Pauline Prüsener[2,3] | Martin Schäfer[1] | Shuqing Xu[1,3] | Meret Huber[1,2,4]

[1]Institute of Organismic and Molecular Evolution, University of Mainz, Mainz, Rheinland-Pfalz, Germany | [2]Institute of Plant Biology and Biotechnology, University of Münster, Münster, Nordrhein-Westfalen, Germany | [3]Institute for Evolution and Biodiversity, University of Münster, Münster, Nordrhein-Westfalen, Germany | [4]Institute for Quantitative and Computational Biosciences, Johannes Gutenberg University of Mainz, Mainz, Rheinland-Pfalz, Germany

Correspondence: Meret Huber (meret.huber@uni-mainz.de)

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Keywords: anthocyanins | aphid herbivory | copper stress | cross-priming | cross-resistance | giant duckweed *Spirodela polyrhiza* | jasmonates | mismatched environments | non-genetic or epigenetic inheritance | transgenerational plasticity

ABSTRACT

Transgenerational plasticity in plants is an increasingly recognized phenomenon, yet it is mostly unclear whether transgenerational plasticity is relevant to both the fitness of the plant and its interacting species. Using monoclonal strains of the giant duckweed (*Spirodela polyrhiza)* and its native herbivore, the waterlily aphid (*Rhopalosiphum nymphaeae*), we assessed whether pre-treating plants with copper excess, both indoors and outdoors, induces transgenerational plasticity in plant defences that alter plant and herbivore fitness. Outdoors, copper pre-treatment tended to increase plant growth rates under recurring copper excess. Indoors, copper pre-treatment either increased or decreased plant growth rates under recurring conditions, depending on the plant genotype. Copper pre-treatment induced anthocyanins that protected plants against copper toxicity, and these elevated levels were transgenerationally retained. Copper pre-treatment also transgenerationally increased the levels of 12-oxo-phytodienoic acid (OPDA), a jasmonate precursor. Nevertheless, aphids grew up to 50% better when the plants were pre-treated with copper. The increased aphid growth was likely caused by transgenerationally elevated OPDA levels, as aphids grew better when jasmonates were externally applied to plants. Taken together, this study shows that transgenerational plasticity is relevant to both plant and herbivore fitness, which highlights the role of transgenerational plasticity in plant evolution and species interactions.

1 | Introduction

Since the discovery of Mendel's work, the inheritance of phenotypes across generations has been attributed to the transmission of DNA sequence variation. However, offspring phenotypes may vary also in the absence of DNA sequence change (Bell and Hellmann 2019). Such variation can be due to maternal effects, in which the environment of the mother alters the phenotypes of the direct offspring (Agrawal, Laforsch, and Tollrian 1999). Offspring phenotypes can also be affected by the environment of grandparents (multi-generational plasticity) or of the great-grandparents and earlier generations (transgenerational plasticity) (Groot et al. 2016; Huber, Gablenz, and Höfer 2021; Lin et al. 2024). It is, however, largely unclear how common transgenerational plasticity is in plants and whether it affects organismal fitness.

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Inferring whether transgenerational plasticity affect organismal fitness is key to assess the evolutionary relevance of this phenomenon. Transgenerational plasticity is not necessarily adaptive: transgenerational plasticity could also be a passive consequence of the previous stress with no or even negative consequences on plant fitness (Baugh and Day 2020; Crisp et al. 2016; Holeski, Jander, and Agrawal 2012; Macartney et al. 2023). Transgenerational plasticity could enhance fitness via at least two non-exclusive ways: first, induced levels of an adaptive phenotype are retained across generations ('transgenerationally retained'), which will benefit offspring fitness if the stress recurs. Second, the induced levels bounce back to basal levels in the absence of stress but the adaptive phenotype is induced stronger or faster once the stress recurs in the offspring ('transgenerationally primed') (Bell and Hellmann 2019; Holeski, Jander, and Agrawal 2012). Transgenerational plasticity could, however, also lead to the emergence of maladaptive traits (Crisp et al. 2016; Macartney et al. 2023). For example, stress might decrease plant growth, which could reduce resource allocation to offspring and thereby impair offspring fitness (Crisp et al. 2016). As only phenotypes with fitness consequences are evolutionary relevant, it is critical not only to measure phenotypes but also fitness, and to infer the adaptive value of a trait by correlating trait expression to fitness or by manipulating the trait genetically or chemically (Huber et al. 2016). To date, it is largely unclear which transgenerational plastic traits affect organismal fitness.

While most studies in transgenerational plasticity only assessed the consequence on the performance and fitness of the focal plant, transgenerational plasticity may also affect the fitness of interacting species. For instance, *Pieris rapae* larvae gained less weight on *Arabidopsis thaliana* plants whose parents or grandparents were exposed to the same herbivore (Rasmann et al. 2012). Furthermore, exposing *A. thaliana* to *Pseudomonas syringae* lowered the performance of the pathogen across two generations (Luna et al. 2011). While these examples highlight that interacting species can be susceptible to multi-generationally plastic defences that are elicited by an initial biotic stress, it is unclear whether interacting species are also sensitive to multi-generationally plastic defences that are induced by an abiotic stress. As different stresses can induce shared but also antagonistic signalling and defence pathways (Chang et al. 2020; Gallusci et al. 2023), exposure to one stress may prime the response of the offspring within another stress, thus causing cross-priming in mismatched environments, and leading to cross-resistance when the priming is beneficial (Lämke and Bäurle 2017; Liu, Able, and Able 2022). Considering that abiotic stresses are prevalent in nature and that herbivores and pathogens often invade plant populations in irregular intervals, studying whether transgenerational plasticity induced by an abiotic stress can alter the fitness of interacting species may reveal unexpected yet common processes in nature. Furthermore, such transgenerational cross-priming could be exploited to generate plants with increased resistance in the field (Vázquez-Hernández et al. 2019; Villagómez Aranda et al. 2022; Yang, Zhi, and Chang 2022).

To infer that transgenerational plasticity is relevant for organismal fitness, the first step is to demonstrate that the traits is truly transgenerationally plastic. However, these demonstrations are rare, as several requirements must be met: first, the phenotypic effects must be independent of DNA sequence variation. DNA sequence variation is minimal in monoclonal or highly inbred lineages, especially in species with low mutation rates. Second, selection of any pre-existing or spontaneously arising genetic and epigenetic variation must be avoided. This can be achieved using single descendant lineages, in which each generation is founded by a single, randomly selected descendant, thereby almost completely avoiding the effect of selection (Baugh and Day 2020). Third, the offspring should not have been exposed to the initial stress: as mother plants already contain the cells that will form the next generation, effects in the first generation can be caused by direct stress exposure, and thus effects in the second generation can be maternal effects (Grossniklaus et al. 2013). Consequently, only effects in the third generation are not confounded by direct stress exposure or maternal effects and are transgenerationally plastic. Fourth, to conclude that a trait is transgenerationally plastic because some epigenetic or other plant-derived factors are inherited, one should perform the experiments under axenic conditions. Otherwise, the initial stress may alter the composition of plant-associated microbes, which may be vertically transmitted and could affect offspring phenotypes (Baldassarre et al. 2022; Gundel, Rudgers, and Whitney 2017).

Transgenerational plasticity seems to be rare in plants reproducing sexually (Bell and Hellmann 2019; Groot et al. 2016; Lin et al. 2024; Sánchez-Tójar et al. 2020; Suter and Widmer 2013), possibly because environment-induced epigenetic marks—which could alter gene expression and phenotypes (Cubas, Vincent, and Coen 1999)—can be erased during meiosis (Crevillén et al. 2014; Iwasaki and Paszkowski 2014; Ono and Kinoshita 2021; Tao et al. 2017). Transgenerational plasticity appears to be more common in asexually reproducing plants, possibly because epigenetic marks can be inherited faithfully through mitosis (He and Li 2018). Evidence of transgenerational plasticity in asexual plants is nevertheless scarce (Huber, Gablenz, and Höfer 2021; Van Antro et al. 2023), particularly under natural conditions outdoors, possibly because transgenerational plasticity has been studied less frequently in asexual than in sexually reproducing plants (Macartney et al. 2023). As most plants can reproduce asexually (Hutchinson 1975; Klimeš et al. 1997; Pyšek 1997; Tiffney and Niklas 1985), and as asexually reproducing plants include many crops, invasive weeds and keystone species (McKey et al. 2010; Pyšek 1997), transgenerational plasticity in asexual plants may play an underappreciated role in agriculture, ecosystem functioning and species evolution.

Two asexually reproducing species that are particularly suitable to study whether traits are transgenerationally plastic and fitness-relevant are the giant duckweed (*Spirodela polyrhiza)* and its native herbivore, the waterlily aphid (*Rhopalosiphum nymphaeae*). *S. polyrhiza* reproduces rapidly and almost exclusively asexually by budding; under ideal conditions, the plant produces a new vegetative generation approximately every 2 days (Ziegler et al. 2015). *S. polyrhiza* has a simple body structure with a flat, thallus-like shoot ('frond'), which allows to

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precisely estimate fitness across several generations by measuring the increase in frond surface area. S. polyrhiza often grows in small water bodies near human-influenced environments, and these water bodies often contain fluctuating levels of anthropogenically derived chemicals such as copper, a major pollutant in aquatic ecosystems (Ruas, Costa, and Bered 2022). *S. polyrhiza* defends against these stresses likely through the production of antioxidative flavonoids, including several flavones and two major anthocyanins, cyanidin-3-malonylglucoside and cyanidin-3-glucoside (Böttner et al. 2021; Liu, Li, et al. 2022). In nature, *S. polyrhiza* is attacked by *R. nymphaeae*, a generalist aphid that, in summer, switches from terrestrial hosts to several aquatic hosts including duckweeds (Center et al. 2002). Under favourable conditions, *R. nymphaeae* reproduces parthenogenetically on duckweeds, giving live birth to a new generation approximately every 6 days (Hance et al. 1994). The rapid asexual reproduction of both *S. polyrhiza* and *R. nymphaeae* allows to assess the consequence of transgenerational plasticity on both plant and herbivore fitness.

In this study, we tested whether exposing *S. polyrhiza* to copper excess outdoors and indoors triggers transgenerational plasticity in plant defences that alter plant and herbivore fitness. By measuring plant and herbivore fitness, and manipulating transgenerationally plastic plant defences, we show that copper-induced transgenerational plasticity can have unexpected consequences on the fitness of interacting species, highlighting the role of transgenerational inheritance in plant evolution and species interactions.

2 | Materials and Methods

2.1 | Plant Material and Growth Conditions

We used six different *S. polyrhiza* genotypes with cosmopolitan distribution (Supporting Information: Table S1), covering three of the four genetic clusters (Wang et al. 2024; Xu et al. 2019). Indoors, plants were cultivated inside growth cabinets (GroBank, CLF PlantClimatics, Wertingen, Germany) operating at either 26°C and 135 µmol photons m^2 s^{-1} for the outdoor experiment, or 28°C and 150 µmol photons m^2 s^{-1} for the indoor experiments, both within a 16:8 h light/dark cycle, and using the optimal growth-supporting N-medium (Appenroth, Teller, and Horn 1996). To measure *S. polyrhiza* growth rates, we took a picture of the plants at the beginning and end of assays using a camera box installation with a webcam (HD Pro Webcam C270, Logitech, Lausanne, Switzerland; webcam software 2.12.8).

2.2 | Statistical Analysis

All data were analysed in R v4.4.0 (R Core Team 2024). As fitness parameter, we calculated relative growth rates, here called 'growth rates', based on *S. polyrhiza* surface area, as well as *R. nymphaeae* number. Growth rates are the difference between the natural logarithm of the final and initial values, divided by the days the plants or aphids grew (Hunt 1982) (Supporting Information: Equation S1). To estimate surface area, we used ImageJ 64 v5 (Schneider, Rasband, and Eliceiri 2012). Indoors, we estimated as morphology traits: surface area per frond (mm^2), fresh weight per frond (mg FW) and surface area per fresh weight (mm^2 mg^{-1} FW). To count the final fronds in the assays we used dotdotGoose v1.5.3 (Ersts). Additionally, we estimated the pre-treatment ratio of the different variables as the rate between the copper pre-treated plants relative to the mean value of the control pre-treated plants within each treatment environment (Huber, Gablenz, and Höfer 2021) (Supporting Information : Equation S2).

Mixed effects models were performed with the package glmmTMB v1.1.9 (Brooks et al. 2017) for all analyses, except for the screening of altered metabolites per environment, conducted with lme4 v1.1.34 (Bates et al. 2015). *p*-values of the fixed-effects models were estimated with analysis of variance (ANOVA), using Wald chi-square of the package car (R Core Team 2024). R^2 values were obtained with ggpmisc v0.5.6 (Aphalo 2024). Post-hoc *p*-values were calculated with emmeans v1.10.2 (Lenth 2024) and letters were obtained with multcomp v1.4.25 (Hothorn, Bretz, and Westfall 2008). We used the package DHARMa v0.4.6 (Hartig 2022) to verify whether the scaled residuals of our fitted models correlated with the simulated values from the same model, and the package effects v4.2.2 (Fox and Weisberg 2018) to extract fit values. To estimate deviation from neutral, we performed Wilcoxon Rank-Sum tests of the pre-treatment ratios per environment against one (mu = 1). We displayed plots with ggplot2 v3.4.3 (Wickham 2016) and organized data with readxl v1.4.2 (Wickham and Bryan 2023), data.table v1.14.8 (Barrett et al. 2024), tidytext v0.4.1 (Silge and Robinson 2016) and dplyr v1.1.2 (Wickham et al. 2023).

2.3 | Effects of Copper Pre-Treatment on Plant Fitness Outdoors

To test whether copper excess under natural conditions alters *S. polyrhiza* fitness under transgenerationally recurring copper excess, we grew genetically uniform *S. polyrhiza* populations of genotype SP004 (Clone 7498, Supporting Information: Table S1) outdoors in the presence and absence of copper excess (Figure 1a). The genotype was chosen because it previously displayed transgenerational plasticity upon copper excess (Huber, Gablenz, and Höfer 2021). Within the populations, we followed monoclonal, single descendant lineages for five generations ('pre-treatment', Supporting Information: Figure S1) and after five additional generations of growth in the absence of stress, we assessed *S. polyrhiza* fitness in the presence and absence of recurring copper excess.

2.3.1 | Pre-Treatment Phase

In May 2021, we set up 20 ponds (80 × 60 × 32 cm, Auer-Packaging, Amerang, Germany) inside an open field site in Münster, Germany (51°57′54.0′N, 7°36′22.4′E). Ponds were buried to the rim with soil and filled with 10 L of pond soil (Floragard, Oldenburg, Germany), 40 mL of organic fruit and vegetable fertilizer (organic NPK 3.1 + 0.5 + 4.1; COMPO, Münster, Germany) and tap water with a total volume of 100 L. The boxes had a draining scape and lids consisting of a stainless-steel mesh (mesh opening 0.63 mm, wire diameter, 0.224 mm; Haver&Boeker, Oelde, Germany). On May 26th, each box received around 3000 *S. polyrhiza* fronds of the

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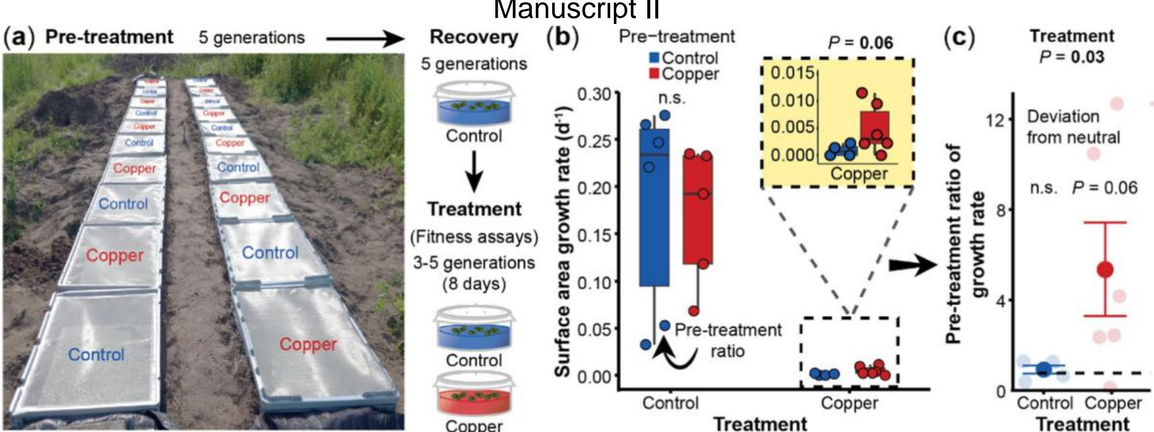

FIGURE 1 | Copper excess outdoors benefits *Spirodela polyrhiza* fitness under recurrent copper stress. (a) Overview of the outdoor experimental setup. Monoclonal *S. polyrhiza* plants of genotype SP004 were grown for five generations as single descendants outdoors in the presence and absence of copper excess, and after five generations of recovery indoors, plant fitness and phenotypes were assessed after 8 days of free growth in each environment. (b) Copper pre-treatment tended to increase surface area growth rates upon recurring copper excess. *p*-values above each boxplot pair refer to mixed effects models per treatment. (c) The pre-treatment ratios of surface area growth rates (growth rates of copper pre-treated plants relative to the mean growth rate of control pre-treated plants) were higher under copper than control conditions. Bold circles represent the mean value and error bars the standard error. Background circles represent individual pre-treatment ratio values. Statistical analysis on the deviations from neutral (horizontal dotted line) refer to Wilcoxon Rank-Sum tests. Variation between treatments corresponds to a linear regression. $N = 4$–6.

genotype SP004 that had been pre-cultivated indoors for 1 month in 10% N-medium at 26°C, 135 µmol photons m^{-2} s^{-1} and 16:8 h light/dark cycle. After 1 week of acclimation, we added 20 µM $CuSO_4$ to half of the ponds ($N = 10$) to initiate the stress pre-treatment (Figure 1a). This $CuSO_4$ concentration reduced plant growth indoors by approximately 40% (Huber, Gablenz, and Höfer 2021). Across the entire experiment, we measured copper concentration weekly and after each rainfall using the HI702 Checker Copper HS (Hanna instruments, Vöhringen, Germany); if needed, we added $CuSO_4$ to maintain 20 µM $CuSO_4$. After adding $CuSO_4$ for the first time, we randomly chose four plants per pond as the founders of the single descendant lineages. To trace the single descendant lineages, we marked the plants with a dot using a permanent marker (Stabilo OHPen Universal, Heroldsberg, Germany) (Supporting Information: Figure S1). The remaining population reproduced freely and covered the surface after 12 days, after which we randomly removed 30% of the plants to avoid crowding. We grew the single descendants within the population to simulate natural conditions, as in nature *S. polyrhiza* usually grows in populations rather than as isolated plants, and isolated plants often stop reproducing because of competing algae (Böttner et al. 2024).

2.3.2 | Recovery Phase

After five generations of pre-treatment, we moved the sixth generation of the single descendants (approx. 24 days of pre-treatment) into separate cavities of polyvinyl chloride disks (7 cm diameter) that were floating inside the control ponds. Due to algae bloom, these plants did not produce any offspring outdoors. We thus transferred the very same plants to the initial indoor conditions (after 38 days of outdoor conditions), within 250 mL polypropylene beakers (Plastikbecher GmbH, Giengen an der Brenz, Germany) filled with 150 mL 10% N-medium and covered with perforated transparent lids. The lineages of each pond were kept in separate beakers. To reduce algae growth, we changed the medium every 2–3 days and wrapped the sides of the beakers with three layers of black plastic foil to decrease light incidence from the sides. Inside the plastic beakers, we propagated each lineage as singles descendants for five generations ('recovery').

2.3.3 | Fitness Assays

To assess *S. polyrhiza* fitness under recurring copper excess, we subsequently placed either the first or the second offspring into separate 250 mL polypropylene beakers containing control conditions (10% N-medium) and copper excess (20 µM $CuSO_4$) ('treatment'), with half of the replicates per treatment using the first offspring, and half of the replicates the second offspring. Subsequently, the plants grew freely for 8 days. Towards the end of the assays, plants died under recurrent stress, likely because outdoor grown plants were weakened and could not resist otherwise sublethal stress levels. We nevertheless took a picture to assess growth rates.

To test whether copper pre-treatment alters *S. polyrhiza* growth rates, we fitted mixed effects models using as random factor the pair of control and copper ponds ('Pair'): Growth ~Pre-treatment*Treatment + (1|Pair). Additionally, we analysed the pre-treatment effect within each treatment using the model Growth ~Pre-treatment + (1|Pair). To assess the effect of the treatment on the pre-treatment ratio (i.e., the trait value of copper pre-treated plants relative to the mean trait value of the control pre-treated plants in the respective environment), we used linear models (Pre-treatment ratio ~Treatment).

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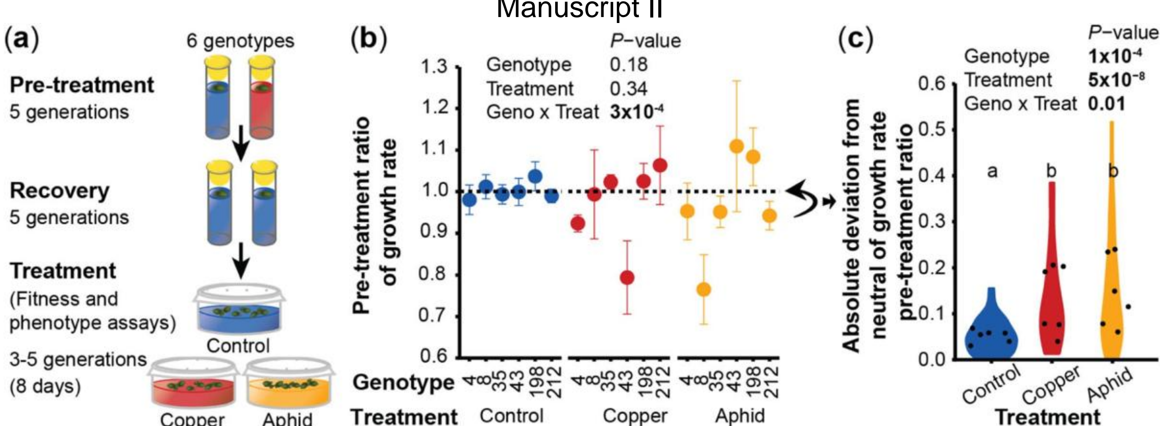

FIGURE 2 | Copper excess indoors alters *Spirodela polyrhiza* fitness in a genotype- and environment-dependent manner. (a) Overview of the indoor experiment. Monoclonal *S. polyrhiza* of six different genotypes were grown as single descendants for five generations indoors in the presence and absence of copper excess, and after five generations of recovery under control conditions, plants fitness and phenotypes were assessed after 8 days of free growth under control conditions, copper excess and aphid herbivory. (b) The pre-treatment ratios of surface area growth rates (growth rates of copper pre-treated plants relative to the mean growth rate of control pre-treated plants) depended on the interaction of the genotype and the treatment. Circles represent the mean value and error bars the standard error. *p*-values refer to a mixed effects model. *N* = 3–6 per genotype (c) Copper pre-treatment ratios of growth rates deviated more strongly from neutral under copper excess and aphid herbivory than under control conditions. Black dots represent the mean value per genotype within a treatment. *p*-values refer to a mixed effects model. Pairwise comparisons were obtained with least-squares means performed on treatments from a mixed effects model. *N* = 28–32. Geno, genotype; Treat, treatment. [Color figure can be viewed at wileyonlinelibrary.com]

2.4 | Effects of Copper Pre-Treatment on Plant and Aphid Fitness and Phenotype Indoors

To assess whether copper pre-treatment alters *S. polyrhiza* fitness and phenotype in different genotypes under axenic conditions, and whether copper pre-treatment alters also herbivore fitness, we pre-treated single descendant lineages of six *S. polyrhiza* genotypes—including the genotype used outdoors (Supporting Information: Table S1)—for five generations with and without copper excess indoors, followed by five generations of recovery under control conditions and finally 8 days of fitness and phenotype assay under control conditions, copper excess or herbivory of the aphid *R. nymphaeae* (Figure 2a).

2.4.1 | Pre-Treatment Phase

To acclimate the plants prior to the experiment, we grew six surface-sterilized plants per genotype for four generations as single descendants inside 30 mL polypropylene tubes (Fisher Scientific, Waltham, USA) filled with 25 mL N-medium and closed with sterilized foam plugs (CarlRoth, Karlsruhe, Germany). The first and second offspring of the fourth generation were placed inside new polypropylene tubes filled with N-medium with and without copper excess (20 µM $CuSO_4$), with half of the replicates per pre-treatment coming from the first offspring and half from the second offspring (Supporting Information: Figure S2.1). Plants were grown as single descendants under these conditions for five generations ('pre-treatment').

2.4.2 | Recovery Phase and Fitness and Phenotype Assays

After five generations of pre-treatment, we grew the plants inside the polypropylene tubes for another five generations under control conditions ('recovery'). Subsequently, to assess the effect of copper pre-treatment on plant fitness and phenotype, we placed the next generations into 250 mL transparent polypropylene beakers filled with 150 mL N-medium and covered with perforated transparent lids, following a structured branching design that harnesses enough plants without introducing biases in the offspring sequence (Supporting Information: Figure S2.1). We allowed the plants to grow freely for 8 days under three treatments: control conditions, copper excess (20 µM $CuSO_4$), and aphid herbivory (three adult aphids per frond) ('treatment', Figure 2a). Subsequently, we measured *S. polyrhiza* growth rates as mentioned above, counted the number of aphids, and harvested plants by briefly drying them with a tissue paper, weighting them and subsequently flash-freezing them in liquid nitrogen. Samples were stored at -80°C until metabolite extraction. Because the aphids contaminated some replicates with algae, we excluded those replicates from the analysis. The experiment was carried out under axenic conditions, except during the 8 days of fitness and phenotype assays within the treatment phase.

2.4.3 | Effects of Copper Pre-Treatment on *Spirodela polyrhiza* Fitness and Morphology

To assess the effect of the pre-treatment on *S. polyrhiza* fitness and morphology of all six genotypes, we used mixed effects models considering as random factors the replicate from which the pre-treated and treated samples derived ('Replicate'), the developmental pockets observed when starting assays ('Offspring'), the rack where plants were propagated during the experiment ('Rack') and the offspring used at the beginning of assays ('Branching') (Supporting Information: Figure S2.1). Details on the models are described in Supporting Information: Methods S1.

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2.4.4 | Effects of Copper Pre-Treatment on *Rhopalosiphum nymphaeae* Fitness

To identify the effects of copper pre-treatment on growth rates of *R. nymphaeae*, we used the mixed effects model Growth ~Pre-treatment + (1|Replicate) + (1|Genotype) + (1|Branching), including the replicates of all six *S. polyrhiza* genotypes. Additionally, to estimate the effect of these genotypes, we used the model Growth ~Pre-treatment*Genotype + (1|Replicate) + (1|Branching). To assess the effects of the six *S. polyrhiza* genotypes on *R. nymphaeae* growth rate pre-treatment ratios, we used the model Pre-treatment ratio ~Genotype + (1|Branching). To correlate the pre-treatment ratios of both *S. polyrhiza* fitness and morphology to the pre-treatment ratio of aphid fitness, we used the model Pre-treatment ratios of duckweed ~Pre-treatment ratios of aphid + (1|Genotype) + (1|Branching), considering the replicates of all six *S. polyrhiza* genotypes.

2.4.5 | Effects of Copper Pre-Treatment on *Spirodela polyrhiza* Metabolites

To identify whether copper pre-treatment alters the concentrations of metabolites upon recurring stresses, we screened the levels of phytohormones, free amino acids, amines and specialized metabolites of the phenylpropanoid pathway using all replicates from the six genotypes collected in the fitness and phenotype assays (Supporting Information: Table S1). Thereto, we used the target screening method of Schäfer et al. (2016), with the modifications described in Malacrinò et al. (2024). In short, we applied into the samples acidified methanol (MeOH:water:formic acid 15:4:1 v/v/v) containing the internal standards for the phytohormones and phenylpropanoid quantification. An aliquot of the extract was set apart to quantify flavonoids and chlorogenic acid. To quantify free amino acids, another aliquot of the extract was diluted 1:100 in an aqueous mix of isotope-labelled amino acids (algal amino acid mixture-13C-15N; Sigma-Aldrich). Finally, phytohormones and several phenylpropanoids were purified and partially concentrated from the remaining extract through two solid-phase extraction steps utilising Chromabond HR-X and HR-XC columns (Macherey-Nagel, Düren, Germany).

To quantify flavonoids and the chlorogenic acid, we used the Nexera XR HPLC System (Shimadzu, Duisburg, Germany) which was equipped with a 5 µM EC 4/3 Nucleodur Sphinx RP precolumn (Macherey-Nagel), a 250 × 4.6 mm 5 µM Nucleodur Sphinx RP column (Macherey-Nagel) and a PDA detector (Shimadzu). The mobile phase comprised of an aqueous solution of 0.2% formic acid (Fisher Scientific) with 0.1% acetonitrile (Fisher Scientific) as solvent A and acetonitrile as solvent B in gradient mode. We used the gradient programs, the column oven settings for the chromatographic separation, and the detector settings described in Malacrinò et al. (2024). The absolute concentrations of the flavones, anthocyanins and chlorogenic acid were calculated based on external standard curves of identical compounds, except for cyanidin-3-malonylglucoside that was quantified based on the cyanidin-3-glucoside standard.

The analysis of amino acids, amines, and phytohormones was performed on a Nexera X3 UHPLC-System (Shimadzu) connected to a LCMS 8060 mass spectrometer (Shimadzu). The LC-System was equipped with a 0.3 µM 1290 infinity II precolumn (Agilent Technologies, Frankfurt am Main, Germany) and a 50 × 3 mm, 1.83 µM Zorbax Eclipse XDB-C18 column (Agilent Technologies, Frankfurt am Main, Germany). The mobile phase comprised of an aqueous solution of 0.05% formic acid with 0.1% acetonitrile, as solvent A and methanol (Fisher Scientific) as solvent B in gradient mode. The mass spectrometer was equipped with an electrospray ionization (ESI) source and operated in multi-reaction monitoring (MRM) modus. The MRM-settings, ESI-settings, the gradient program and column oven settings were used as described in Malacrinò et al. (2024), with the addition of a putative chlorogenic acid isomer to method 1A (Supporting Information: Table S2). Analytes were quantified based on internal standards as described in Malacrinò et al. (2024) and Supporting Information: Table S2. The peaks from all metabolites were integrated with LabSolution Version 5.123 (Shimadzu, Duisburg, Germany) for the HPLC-PDA data and with LabSolution Insight Version 4.0 SP6 (Shimadzu) for the LC-MS/MS data.

To screen for metabolites that were affected by the pre-treatment within each treatment, we used mixed effects models, considering as additional random factors the plate used for the extraction ('Plate') and the batch of samples extracted and read at one time by the machines ('Batch'). Thereto, we used the model Concentration ~Pre-treatment + (1|Genotype) + (1|Replicate) + (1|Plate) + (1|Batch), with slight variations depending on the specific questions (Supporting Information: Methods S2). All metabolite analyses included the replicates of all six genotypes.

2.4.6 | Benefits of Transgenerationally Plastic Metabolites Under Stress

To infer whether anthocyanins and tryptamine—which were transgenerationally retained—protect plants against copper excess and aphid herbivory, we correlated the concentrations of these metabolites with *S. polyrhiza* fitness in each environment using the control pre-treated plants of all genotypes. Thereto, we used the model Pre-treatment ratio of growth rates ~pre-treatment ratio of metabolites + (1|Genotype) + (1|Replicate), considering the replicates of all six genotypes.

To test whether the level of 12-oxo-phytodienoic acid (OPDA)—which was transgenerationally plastic—were positively correlated with the levels of the defence hormones jasmonic acid and jasmonic acid-isoleucine, we used the model Pre-treatment ratios of jasmonates ~pre-treatment ratios of OPDA + (1|Genotype), considering the replicates of all six genotypes.

2.5 | External Application of Methyl Jasmonate on Plants

To assess whether transgenerational plasticity in OPDA affects *R. nymphaeae* fitness, we externally applied methyl jasmonate to *S. polyrhiza* and subsequently assessed *R. nymphaeae* growth rates. Thereto, we placed three individual fronds of genotype SP050 (Supporting Information: Table S1), a standard genotypes

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in our laboratory, into 50 mL falcon tubes filled with 3 mL of either N-medium or 100 µM methyl jasmonate in N-medium ('Induction', $N = 12$ per treatment). To effectively dissolve the methyl jasmonate, 0.5% DMSO was added to both the control and methyl jasmonate-containing N-medium. We split the experiment into three batches, performed one after the other. After 72 h in the presence and absence of methyl jasmonate, each colony formed from the three fronds was moved into individual 250 mL transparent polypropylene beakers filled with 150 mL N-medium and subjected to either control conditions or aphid herbivory (three adult aphids per plant). After 2 days, we counted the number of aphids. The experiment was repeated once in a similar way to confirm the findings and to assess whether methyl jasmonate induces OPDA, jasmonic acid and jasmonic acid-isoleucine (Supporting Information: Methods S3).

To assess whether externally applied jasmonates alter *R. nymphaeae* growth rates, we used mixed effects models considering as an additional random factor the batch of samples worked per day ('Group'). The model was Growth ~Induction*Treatment + (1|Replicate) + (1|Group) (Supporting Information: Methods S4). Two samples per treatment were excluded from the growth rate analysis as the aphids did not produce any offspring.

3 | Results

3.1 | Copper Pre-Treatment Outdoors Tends to Benefit Plant Fitness Under Transgenerationally Recurring Copper Stress

To assess whether copper excess alters *S. polyrhiza* fitness under transgenerationally recurring copper excess in natural conditions, we pre-treated plants with copper excess outdoors, and after five generations of growth under control conditions, we assessed plant fitness in the presence and absence of recurring copper excess (Figure 1a). While copper pre-treatment did not affect *S. polyrhiza* growth rates under control conditions, copper pre-treatment tended to increase plant growth rates by approximately 400% under copper excess ($p = 0.06$, mixed effects model, Figure 1b, Supporting Information: Table S3). Similarly, the pre-treatment ratios, i.e. the ratio between plant growth rates of copper compared to control pre-treated plants, differed between control and copper conditions ($p = 0.03$, mixed effects model, Figure 1c): under control conditions, copper pre-treatment ratios were close to neutral, whereas under copper excess, the pre-treatment ratios tended to be positive (deviation from neutral in control: $p > 0.1$; in copper: $p = 0.06$, Wilcoxon rank sum test, Figure 1c).

3.2 | Copper Pre-Treatment Indoors Alters Plant Fitness and Morphology in an Environment- and Genotype Dependent Manner

To assess whether copper pre-treatment alters *S. polyrhiza* fitness not only under copper excess but also under herbivory of the waterlily aphid *R. nymphaeae*, and to test whether these transgenerational effects are genotype-specific, we grew six world-wide sampled genotypes (Supporting Information: Table S1) for five generations as single descendants indoors under control and copper excess, followed by five generations under control conditions prior to fitness assays under control conditions, copper excess and aphid herbivory (Figure 2a). While copper pre-treatment did not affect plant surface area growth rates when expressed in raw values (Supporting Information: Figure S2.2), copper pre-treatment altered the growth rates in a genotype and environment-dependent manner when expressed as pre-treatment ratios (p [genotype × treatment] = 3×10^{-4}, mixed effects model, Figure 2b): under control conditions, copper pre-treatment did not affect *S. polyrhiza* growth rates, whereas under copper excess and aphid herbivory, copper pre-treatment either increased or decreased *S. polyrhiza* growth rates, depending on the genotype. These pre-treatment effects were not marginal: in some genotypes, copper pre-treatment reduced *S. polyrhiza* growth rates by up to 25%, whereas in other genotypes, copper-pretreatment increased growth rates by 10%. Across all genotypes, growth rate pre-treatment ratios were close to neutral under control conditions but deviated from neutral, both positively and negatively, under copper excess and aphid herbivory (absolute deviation from neutral among treatments: $p = 5\times 10^{-8}$, mixed effects model, Figure 2c). The genotypes that suffered from copper pre-treatment under recurring copper excess were not the same genotypes that suffered from copper pre-treatment under aphid herbivory, since the pre-treatment ratios on surface area growth rates did not correlate among the environments (Supporting Information: Figure S2.3). These data show that depending on the genotype, copper pre-treatment can either benefit or harm plant fitness when a stress recurs, with no evidence for transgenerational cross-resistance under mismatched environments.

We next assessed whether copper excess alleviated stress-induced morphological changes by analysing area per frond, biomass per frond and area per biomass, three morphological traits that closely correlated with each other ($R^2 > 0.3$; $p < 3\times 10^{-7}$, mixed effects model, Supporting Information: Figure S2.4). Similar to the patterns observed for *S. polyrhiza* fitness, copper pre-treatment had the weakest effects on all three morphological parameters when plants were grown under control conditions (Supporting Information: Figure S2.5). Under copper excess and aphid herbivory, copper pre-treatment either alleviated or increased the stress-induced morphological changes, depending on the genotype (Supporting Information: Figure S2.5). Taken together, these data show that copper pre-treatment alters *S. polyrhiza* morphology in a genotype and environment dependent manner, with particularly strong effects of the pre-treatment under recurring stress.

To assess whether the pre-treatment induced alterations in *S. polyrhiza* morphology are predictive for plant fitness, we correlated pre-treatment ratios of the different morphological parameters to the pre-treatment ratios of *S. polyrhiza* fitness. Under copper excess and aphid herbivory, the stronger the stress-induced morphological changes were, the more the plant growth rates suffered from copper pre-treatment ($R^2 > 0.35$, $p > 1\times 10^{-4}$, mixed effects model, Supporting Information: Figure S2.6). These patterns were absent or even reversed under control conditions (Supporting Information: Figure S2.6). Taken together, these data show that plants that show stronger

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stress-induced morphological changes under copper excess and aphid herbivory also suffer more from the copper pre-treatment in these environments.

3.3 | Copper Pre-Treatment Benefits Aphid Fitness

We next assessed whether copper pre-treatment not only altered *S. polyrhiza* but also *R. nymphaeae* fitness in six different *S. polyrhiza* genotypes (Supporting Information: Table S1). Surprisingly, copper pre-treatment increased *R. nymphaeae* growth rates in average by 9% (p [pre-treatment] = 0.008, mixed effects model, Figure 3a, Supporting Information: Figure S3.1). These effects were genotype-specific (p [pre-treatment × genotype] = 0.055, mixed effects model, Figure 3a): while copper pre-treatment had no effects on *R. nymphaeae* fitness of most plant genotypes, one genotype, SP008, supported more than 50% higher aphid growth rates when plants were pre-treated with copper (deviation from neutral in genotype SP008: p = 0.03, Wilcoxon rank sum test; variation among genotypes: $p = 6\times 10^{-4}$, Figure 3b).

To assess whether the increased growth of *R. nymphaeae* due to the copper pre-treatment reduces *S. polyrhiza* fitness, we correlated the pre-treatment ratio of aphid fitness with the pre-treatment ratios of plant surface area growth rate and morphology. The better the aphids grew, the worse the plants performed, and stronger the stress-induced morphological changes became (*S. polyrhiza* growth rates: $R^2 = 0.32$, $p = 3\times 10^{-4}$, mixed effects model, Figure 3c; *S. polyrhiza* morphologies: $R^2 > 0.24$, $p < 0.003$, mixed effects model, Supporting Information: Figure S3.2). These data show that copper pre-treatment benefitted aphid fitness on some but not all genotypes, and that the increase in aphid growth reduced plant fitness.

3.4 | Copper Pre-Treatment Enhances the Accumulation of Anthocyanins Under Control Conditions and of Jasmonates Under Stress

To infer which copper-induced transgenerationally plastic metabolites altered the fitness of *S. polyrhiza* and *R. nymphaeae*, we assessed whether copper pre-treatment affected the accumulation of different phytohormones, free amino acids and their amines, as well as specialized metabolites derived from the phenylpropanoid pathway using the plant material from the fitness assay of six different genotypes (Supporting Information: Table S1). We first assessed whether copper pre-treatment altered the accumulation of metabolites under control conditions. Copper pre-treated plants accumulated 13% and 5% higher levels of the anthocyanins cyanidin-3-malonylglucoside and cyanidin-3-glucoside, respectively, 7% higher levels of auxin, and 6% lower levels of the free amino acids L-aspartic acid and L-valine compared to control pre-treated plants ($p < 0.05$, mixed effects models, Figure 4).

We next assessed whether copper pre-treatment enhances the accumulation of metabolites under stress. Under copper excess, copper pre-treated plants accumulated 16% and 13% higher levels of the anthocyanins cyanidin-3-malonylglucoside and cyanidin-3-glucoside, respectively, and 111% higher levels of 12-oxo-phytodienoic acid (OPDA) than control pre-treated plants ($p < 0.05$, mixed effects models, Figure 4). The levels of OPDA were also elevated by the copper pre-treatment under aphid herbivory, where it enhanced OPDA levels in average by 90% ($p < 0.05$, mixed effects model, Figure 4). The strongest increase in OPDA levels upon copper pre-treatment under aphid herbivory were found in genotype SP008 (Supporting Information: Figure S4.1), the genotype in which the copper pre-treatment increased aphid growth the most. The OPDA-derived defence hormones jasmonic acid and jasmonic acid-isoleucine were not affected by the pre-treatment in

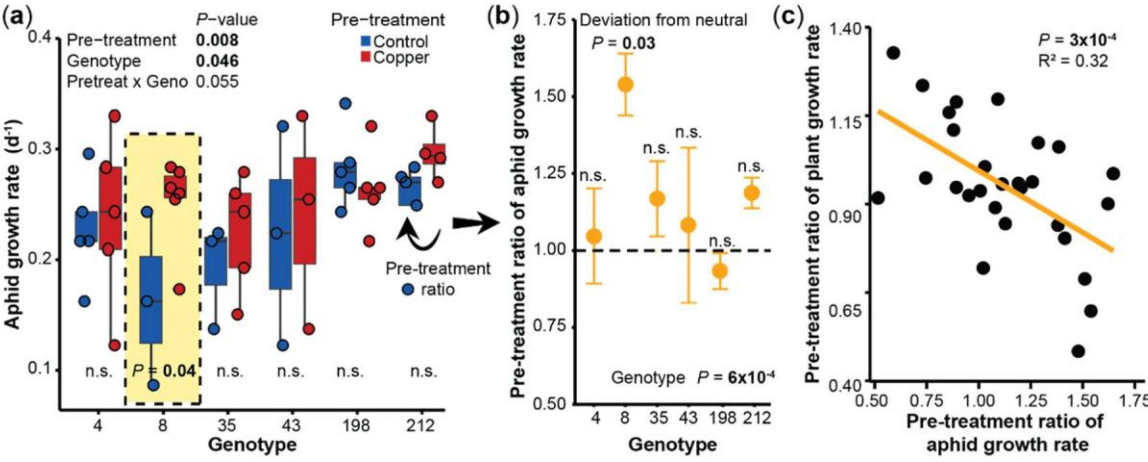

FIGURE 3 | Copper pre-treatment enhances the fitness of the aphid *Rhopalosiphum nymphaeae*, thereby reducing plant fitness. (a) *R. nymphaeae* reproduced faster on copper than control pre-treated plants. p-values refer to mixed effects models. Circles display individual data points (b) The pre-treatment ratios of *R. nymphaeae* growth rates (growth rates on copper pre-treated plants relative to the mean growth rate on control pre-treated plants) varied among genotypes. Horizontal dotted line indicates neutral effect of the pre-treatment. Differences among genotypes refer to mixed effects models. Significant deviations from neutral were analysed with Wilcoxon Rank-Sum tests. Circles represent the mean value and error bars the standard error. N = 3–6. (c) The pre-treatment ratios of *R. nymphaeae* fitness negatively correlated with the pre-treatment ratios of plant surface area growth rates. The circles display the replicates of all six genotypes. p-value refers to a mixed effects model. N = 28. Geno, genotype; Pretreat, pre-treatment. [Color figure can be viewed at wileyonlinelibrary.com]

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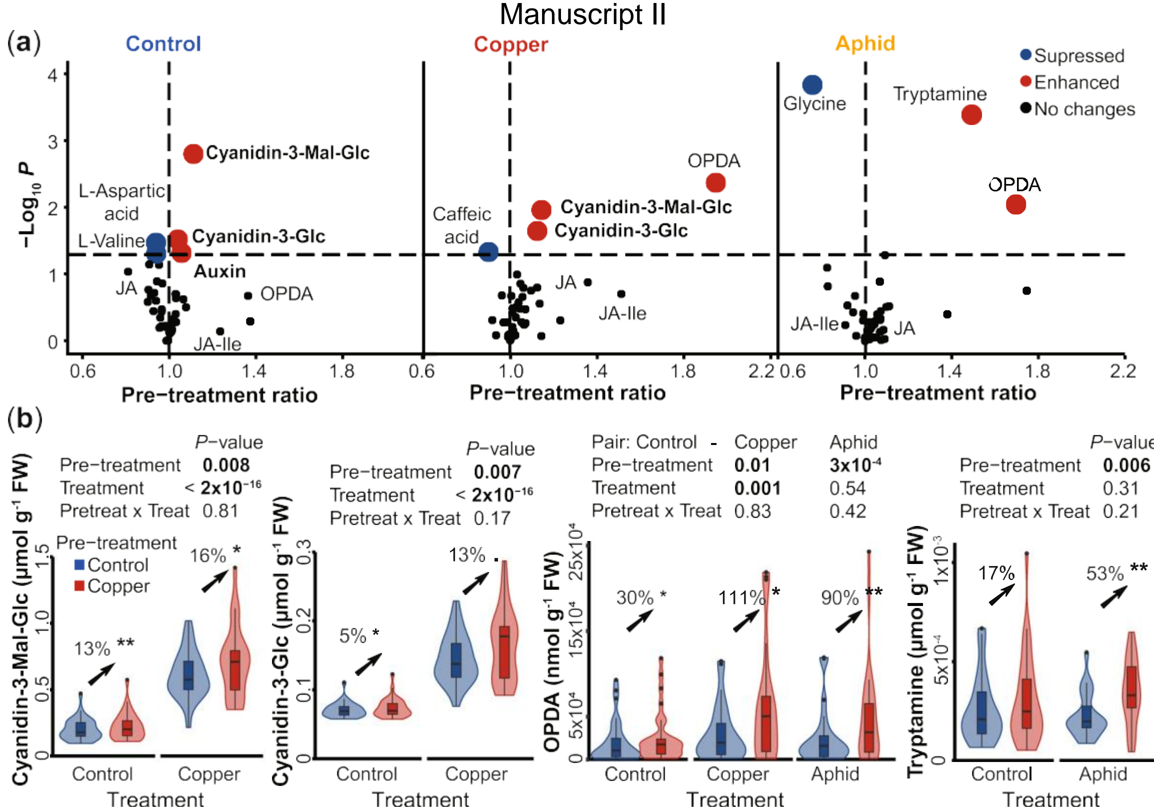

FIGURE 4 | Copper pre-treatment in *Spirodela polyrhiza* transgenerationally enhances the accumulation of anthocyanins and jasmonate precursors. (a) Copper pre-treatment transgenerationally elevated the levels of anthocyanins, auxin, tryptamine and the jasmonate precursor 12-oxo-phytodienoic acid (OPDA) under copper excess and aphid herbivory. p-values (y-axis) refer to ANOVA test on mixed effects models. Each circle represents the mean value from the replicates of six genotypes per environment. (b) The transgenerationally plastic metabolites were transgenerationally retained rather than primed. Transgenerationally retained refers to significant effects of the pre-treatment, whereas transgenerationally primed refers to significant interactions of the treatment and pre-treatment. The data display the replicates from all six genotypes. p-values refer to an ANOVA test on mixed effects models. Asterisks next to the percentages refer to p-values from mixed effects models. < 0.06; * < 0.05; ** < 0.01. $N = 23–33$. Cyanidin-3-Glc, cyanidin-3-glucoside; Cyanidin-3-Mal-Glc, cyanidin-3-malonylglucoside; FW, fresh weight; JA, jasmonic acid; JA-Ile, jasmonic acid-isoleucine; OPDA, 12-oxo-phytodienoic acid. [Color figure can be viewed at wileyonlinelibrary.com]

any environment (Figure 4a, Supporting Information: Figure S4.1). Under aphid herbivory, copper pre-treated plants also accumulated 53% higher levels of the putative defence metabolite tryptamine compared to control pre-treated plants ($p < 0.05$, mixed effects model, Figure 4). Most of these pre-treatment-induced changes in metabolite levels observed under aphid herbivory and copper excess were also seen under control conditions, but to a lower extent: indeed, transgenerational priming (significant interaction of the treatment and pre-treatment) was not present in any of these metabolites (Figure 4b). To further assess whether these metabolites are primarily transgenerationally retained or primed, we analysed the concentration of these metabolites individually considering genotype as a fixed rather than random effect. This analysis showed that while genotype-specific transgenerational priming contributes to the elevated levels of these metabolite upon copper pre-treatment, these elevated levels are transgenerationally retained rather than primed (Supporting Information: Figure S4.2).

We next assessed whether these metabolites were induced by first-time exposure to copper excess and whether the metabolites were regulated by the copper pre-treatment in the same direction as under the respective stress. The two cyanidins, whose levels were elevated by about 10% by the copper pre-treatment under both control conditions and copper excess, were induced by copper excess by approximately 150% ($p < 2\times 10^{-16}$, Supporting Information: Figure S4.3). OPDA, whose level was doubled by copper pre-treatment under copper excess and aphid herbivory, was induced by 50% upon copper excess but not upon aphid herbivory ($p = 0.002$, Supporting Information: Figure S4.3). Tryptamine, whose level was elevated by 50% by the copper pre-treatment under aphid herbivory, was induced by copper excess by almost 3000%, but was not affected by aphid herbivory ($p < 2\times 10^{-16}$, Supporting Information: Figure S4.3). Together, these data show that all transgenerationally plastic metabolites were plastic upon first-time exposure to copper excess, and that copper exposure mostly reinforces the induction of these metabolites when another stress recurs.

3.5 | Anthocyanins Likely Protect Plants Against Copper Excess

To infer whether the transgenerational plasticity of the specialized metabolites is adaptive, we correlated anthocyanin and tryptamine concentrations with *S. polyrhiza* fitness of the six

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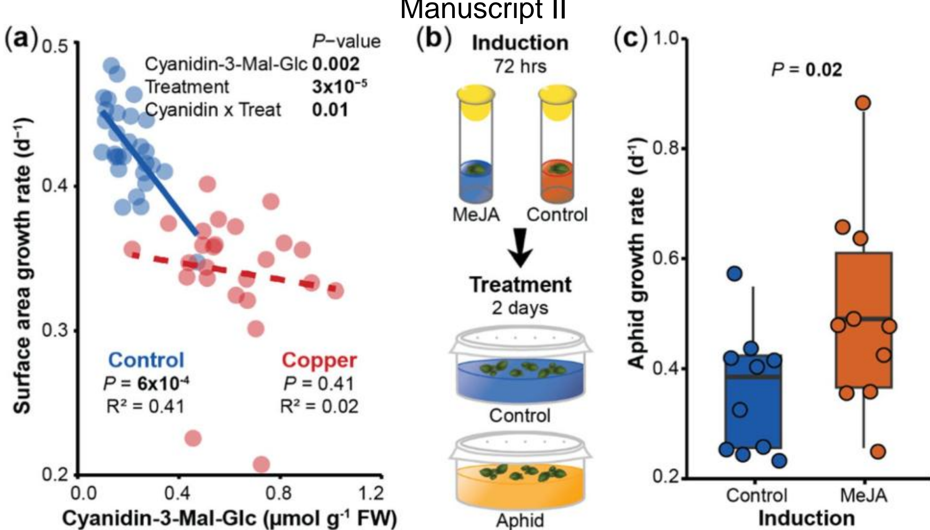

FIGURE 5 | Transgenerationally plastic metabolites benefit *Spirodela polyrhiza* fitness under copper excess but improve *Rhopalosiphum nymphaeae* growth. (a) The levels of cyanidin-3-malonylglucoside correlated negatively with plant fitness under control conditions but not under copper excess. Circles display the values of all replicates from the six genotypes from the transgenerational experiment indoors upon 8 days of growth under control conditions or copper excess. *p*-values refer to mixed effects models. $N = 27–32$. (b) Overview of the induction experiment to test the effect of jasmonates on *R. nymphaeae* growth rates. (c) *R. nymphaeae* produced more offspring when methyl jasmonate was externally applied on plants of genotype SP050. Circles display values of individual samples upon 2 days of growth. *p*-value refers to a mixed effects model. $N = 10$. Cyanidin-3-Mal-Glc, cyanidin-3-malonylglucoside; FW, fresh weight; MeJA, methyl jasmonate; Treat, treatment. [Color figure can be viewed at wileyonlinelibrary.com]

different genotypes (Supporting Information: Table S1) under control conditions and under the environment in which the enhanced metabolite was inherited. The concentrations of both cyanidins negatively correlated with *S. polyrhiza* growth rates under control conditions but not under copper excess (cyanidin-3-malonyglucoside: $p = 6 \times 10^{-4}$ in control and $p = 0.41$ in copper, interaction: $p = 0.01$, mixed effects models, Figure 5a; cyanidin-3-glucoside: Supporting Information: Figure S5.1). Thus, the production of cyanidins is likely costly under control conditions but these costs are alleviated under copper excess. The concentrations of tryptamine did not correlate with plant fitness under control conditions or aphid herbivory (p [tryptamine × treatment] = 0.91, mixed effects model, Supporting Information: Figure S5.1).

3.6 | External Application of Methyl Jasmonate in Plants Increases OPDA Concentrations and Benefits Aphid Growth Rates

We next tested the effect of jasmonates on *R. nymphaeae* growth rates, as the jasmonate precursor OPDA was elevated by the copper pre-treatment under aphid herbivory, and as pre-treatment ratios of OPDA and jasmonates were positively correlated ($R > 0.14$, $p \leq 0.05$, Supporting Information: Figure S5.2). Thereto, we measured the growth rates of *R. nymphaeae* when *S. polyrhiza* genotype SP050 was pre-treated for 3 days with either a mock solution or methyl jasmonate (Figure 5b), the latter of which induced the levels of OPDA, jasmonic acid and jasmonic acid-isoleucine by 640%, 200% and 240%, respectively (Supporting Information: Figure S5.3). Surprisingly, *R. nymphaeae* had 39% higher growth rates within 48 h when plants were pre-treated with methyl jasmonate ($p = 0.02$, mixed effects model, Figure 5c). To confirm this unexpected finding, we repeated the experiment, yielding similar results ($p = 0.06$, mixed effects model, Supporting Information: Figure S5.4). Taken together, these data show that transgenerational plasticity of anthocyanins is likely adaptive, whereas transgenerational plasticity of jasmonates may be maladaptive.

4 | Discussion

In this study, we showed that previous exposure of *S. polyrhiza* to copper excess can alter the fitness of both the plant and its native herbivore. On one hand, previous exposure to copper excess benefitted *S. polyrhiza* fitness in some genotypes under recurring stress conditions, likely because copper excess induced defensive anthocyanins, and these elevated levels were transgenerationally retained. On the other hand, previous exposure to copper also benefitted the fitness of a native aphid herbivore and thereby harmed *S. polyrhiza* fitness, a phenomenon that was likely mediated by transgenerationally elevated levels of OPDA, a jasmonate precursor. Thus, these data reveal that pollutants that elicit transgenerational plasticity in plants can have unexpected consequences on both the fitness of plants and their herbivores.

Inheritance of traits across generations is an increasingly recognized phenomenon in plants. While trait inheritance is common across one unexposed generation (maternal effects), and can persist across two generations (Macartney et al. 2023; Yin et al. 2019), there is little evidence that traits are transgenerationally inherited (Anastasiadi et al. 2021), meaning being plastic for three or more generations (Groot et al. 2017; Huber, Gablenz, and Höfer 2021; Lin et al. 2024; Van Antro et al. 2023). Here, we demonstrate such transgenerational plasticity. First,

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copper pre-treatment altered plant morphologies in an environment- and genotype dependent manner. Second, copper excess induced the accumulation of anthocyanins, and these elevated levels were transgenerationally retained. Third, copper excess elevated the levels of OPDA, and these elevated levels were retained under recurring copper excess and aphid herbivory. Together, these data show that in *S. polyrhiza*, morphological and metabolic traits can be plastic for more than three generations.

We did not attempt to identify the molecular mechanisms underlying trait inheritance. It is very likely that the transgenerational variation was due to plasticity rather than underlying genetic variation, because *S. polyrhiza* reproduces clonally and accumulates only one point mutation every 80 generations (Xu et al. 2019). Plasticity that persists for three or more generations is thought to rely on the inheritance of epigenetic marks or microbes rather than the transmission of substances (Jablonka and Raz 2009; Perez and Lehner 2019). To reduce the effect of microbes, we performed our indoor experiments under axenic conditions. Under axenic conditions, copper pre-treatment no longer protected genotype SP004 under recurring stress as observed outdoors, possibly because of vertical transmission of microbes outdoors. In general, the transgenerational effects on fitness were weaker under axenic conditions indoors compared to the ones outdoors, and also weaker than previous transgenerational studies with *S. polyrhiza* under non-axenic environments indoors (Huber, Gablenz, and Höfer 2021); nevertheless, transgenerational plasticity under axenic conditions indoors was present. Thus, it is possible that epigenetic inheritance mediates the observed transgenerational responses. Indeed, the closely related duckweed *Lemna minor* transmits stress-induced variation in DNA methylation across multiple generations (Van Antro et al. 2023), similar to other clonally reproducing plants whose methylome is altered by stress across several generations (Latzel, Rendina González, and Rosenthal 2016). Future experiments that investigate transgenerational variation in epigenetic marks, and that genetically manipulate the epigenetic machinery or epigenetically regulated genes are required to elucidate the molecular mechanisms of transgenerational plasticity.

Transgenerational plasticity is hypothesized to benefit plant fitness under recurring stress by either retaining elevated levels of defences or priming of the defences. In our study, we found that copper-induced levels of anthocyanins were transgenerationally retained rather than transgenerationally primed. The anthocyanins likely protected *S. polyrhiza* under copper excess, because anthocyanin concentrations were negatively correlated with plant fitness under control conditions but not under copper excess. The observed protective function is in line with previous studies showing that anthocyanins are induced under abiotic stresses and increase plant stress tolerance (Ai et al. 2018; Naing and Kim 2021). Taken together, these data show that transgenerational plasticity can be adaptive under matching environments by retaining stress-induced levels of defensive metabolites.

While our data show that transgenerational plasticity can benefit plant fitness under matched environments, we also obtained evidence that transgenerational plasticity can reduce plant fitness under unmatched environments. First, pre-treatment ratios of *S. polyrhiza* growth rates did not correlate across the tested environments, suggesting that transgenerational cross-resistance is not common. This is in line with previous multi-generational studies, in which infection of the pathogens *P. syringae* and *Plectosphaerella cucumerina* increased the disease resistance in *A. thaliana* offspring in matched environments, but increased susceptibility in unmatched environments (López Sánchez et al. 2021). Together with our data, these patterns suggest that transgenerational plasticity may involve trade-offs, by increasing fitness in one environment but decreasing fitness in another environment. Such trade-offs could also explain why in our study transgenerational plasticity varied among genotypes.

Second, we found that copper pre-treatment benefitted *R. nymphaeae* fitness—in some genotypes up to 50%—and increased aphid growth was associated with reduced *S. polyrhiza* fitness. Aphids likely grew better because OPDA, a jasmonate precursor, was induced by copper excess and these elevated levels were transgenerationally retained. Although boosting of the jasmonate pathway is hypothesized to reduce aphid growth (Aslam et al. 2022; Züst and Agrawal 2016), *R. nymphaeae* grew 40% better when *S. polyrhiza* was pre-treated with methyl jasmonate. Although we used different genotypes for the external methyl jasmonate application and the transgenerational experiments, it is likely that methyl jasmonate has similar effects on plant resistance and aphid growth across genotypes, as the jasmonate pathway is conserved (Katsir et al. 2008). Similarly, in *Sorghum bicolor*, external application of jasmonates promoted aphid feeding and colonization, and plants with impaired jasmonate biosynthesis supported lower aphid population growth (Grover et al. 2022). In our study, methyl jasmonate induced not only jasmonic acid and jasmonic acid-isoleucine but also OPDA, the latter of which can have jasmonate-independent functions (Jimenez Aleman et al. 2022). Thus, we do not know whether jasmonate-dependent or -independent mechanisms of OPDA accounted the increased aphid growth upon copper pre-treatment and methyl jasmonate application. Nevertheless, our study highlights that not all aphids are susceptible to jasmonate-induced defences, and that jasmonates may be a key hormone in orchestrating transgenerational plasticity (Rasmann et al. 2012). Transgenerational plasticity in jasmonates can, however, be maladaptive in unmatched environments, by promoting aphid growth and reducing plant fitness.

The increased aphid growth due to the previous copper exposure could have far-reaching consequences on species composition and ecosystem functioning: duckweeds can quickly cover water surfaces, causing dark and anoxic conditions (Scheffer et al. 2003). Increased growth of *R. nymphaeae* could counteract these conditions, and thereby alter species composition in these aquatic ecosystems, which in turn, could alter ecological and evolutionary processes on land. Such interactions of aquatic and terrestrial ecosystems may be common: for instance, terrestrial flowering plants received more pollinators

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and were less pollen limited when the ponds nearby contained fishes that prey on dragon fly larvae, as mature dragon flies consume insect pollinators (Knight et al. 2005). Thus, transgenerational plasticity of an aquatic, clonally reproducing key stone species, such as duckweeds, may have unexpected consequences on species interactions and eco-evolutionary dynamics both in water and on land.

Taken together, our study shows that a common aquatic pollutant induces defence hormones and metabolites, and that elevated levels of these defences are retained across multiple generations. Transgenerational plastic defences can benefit plant fitness if the same stress recurs but can also harm plant fitness in the presence of herbivores by promoting herbivore population growth. Our results thus provide evidence that a pollutant early in the growing season can boost herbivore growth later in the season, thus revealing unexpected consequences of transgenerational plasticity on species interactions.

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Data Availability Statement

All raw data and R scripts for the analyses and plots within this study are deposited in https://github.com/Plant-Evolutionary-Ecology-Lab/Transgenerational-Cross-Resistance.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

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Copper-induced transgenerational plasticity in plant defence boosts aphid fitness

Alexandra Chávez, Anne Schreyer, Pauline Prüsener, Martin Schäfer, Shuqing Xu, Meret Huber

Supporting figures

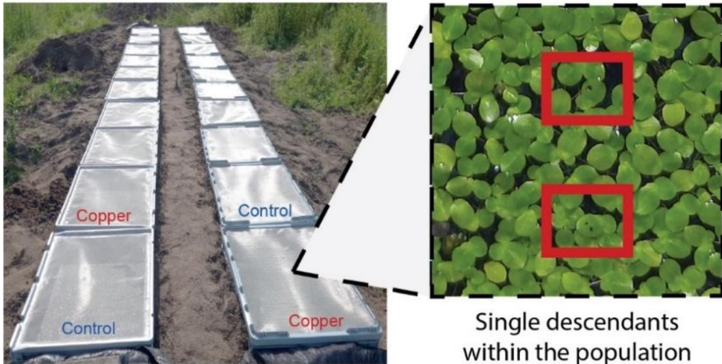

Figure S1. Pre-treatment phase in outdoor conditions. Outdoors, *S. polyrhiza* genotype SP004 was grown under copper and control conditions as part of the pre-treatment phase. Within the plant population single descendant lineages were marked and followed for five generations. Marking was done as: first generations, one dot; second generation, two dots; third generation, three dots; fourth generation one dot; fifth generation two dots; sixth generation starting fitness assays, three dots. Once the new generation naturally detached from the previous generation, the older generation was taken out of the experiment. N = 10.

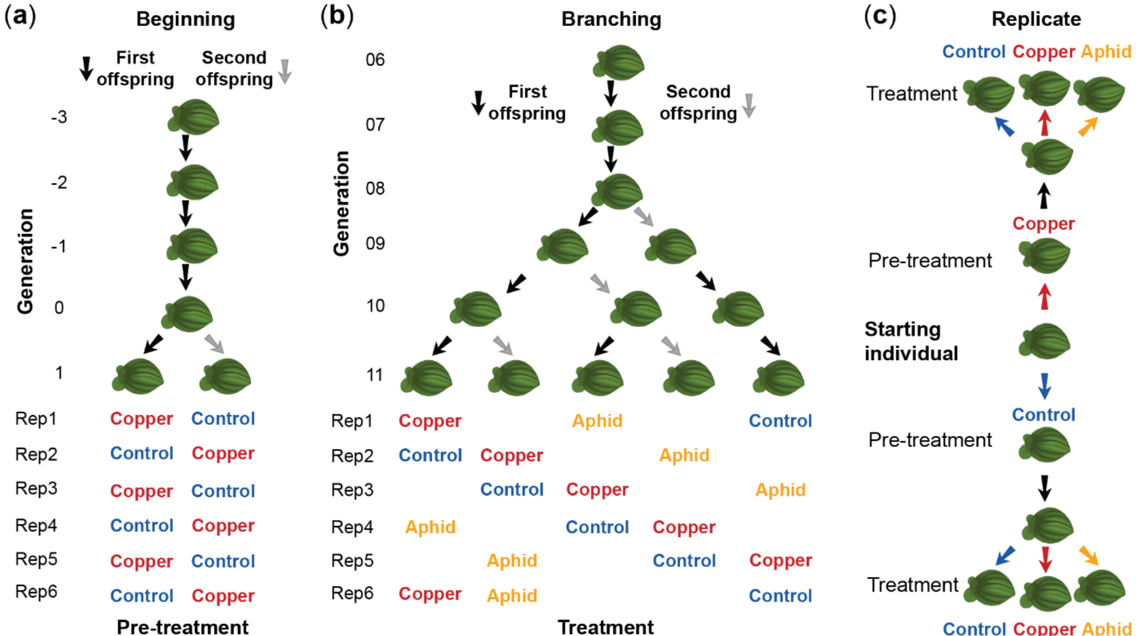

Figure S2.1. Propagation diagrams of six different genotypes of *Spirodela polyrhiza* under axenic conditions. (**a**) The first and second daughters of single descendants were placed into copper and control condition to start the pre-treatment. (**b**) Single descendants indoors were branched after copper pre-treatment using the first and the second offspring and placed into determined treatments depending on the replicate number. (**c**) Each of the six starting individuals were branched into the different pre-treatment and treatment environments. Rep = Replicate. .

1 Supporting Information

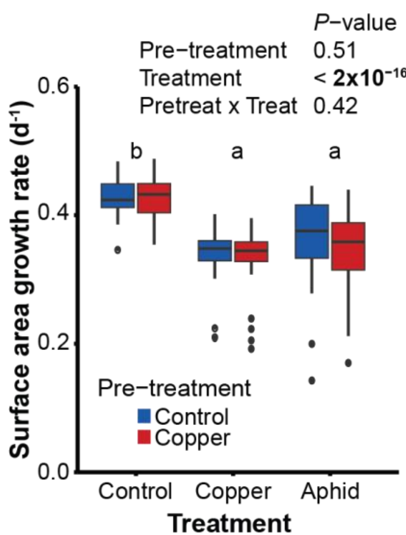

Figure S2.2. Surface area growth rates of *Spirodela polyrhiza* varied among the different stress treatments. The replicates from all six different *S. polyrhiza* genotypes showed fitness differences due to the treatment, but not the pre-treatment environments. *P*-values refer to an ANOVA test on a mixed effects model. Pairwise comparisons among treatments come from least-squares means on treatments of the mixed effects model. N = 24-32.

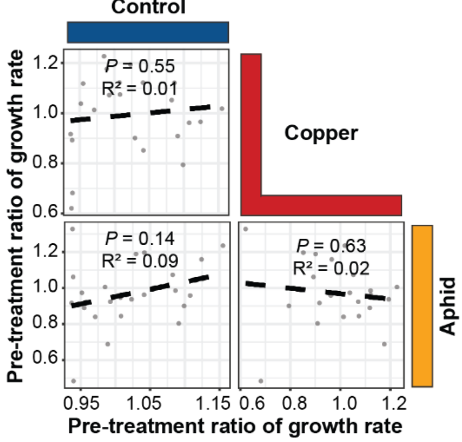

Figure S2.3. Copper pre-treatment in *Spirodela polyrhiza* does not lead to cross-resistance. The pre-treatment ratios on surface area growth rates (growth rates of copper pre-treated plants relative to the mean growth rate of control pre-treated plants per treatment) did not correlate among treatments when considering the replicates of all six genotypes. Dots represent individual values. *P*-values refer to mixed effects models. N = 23.

2 Supporting Information

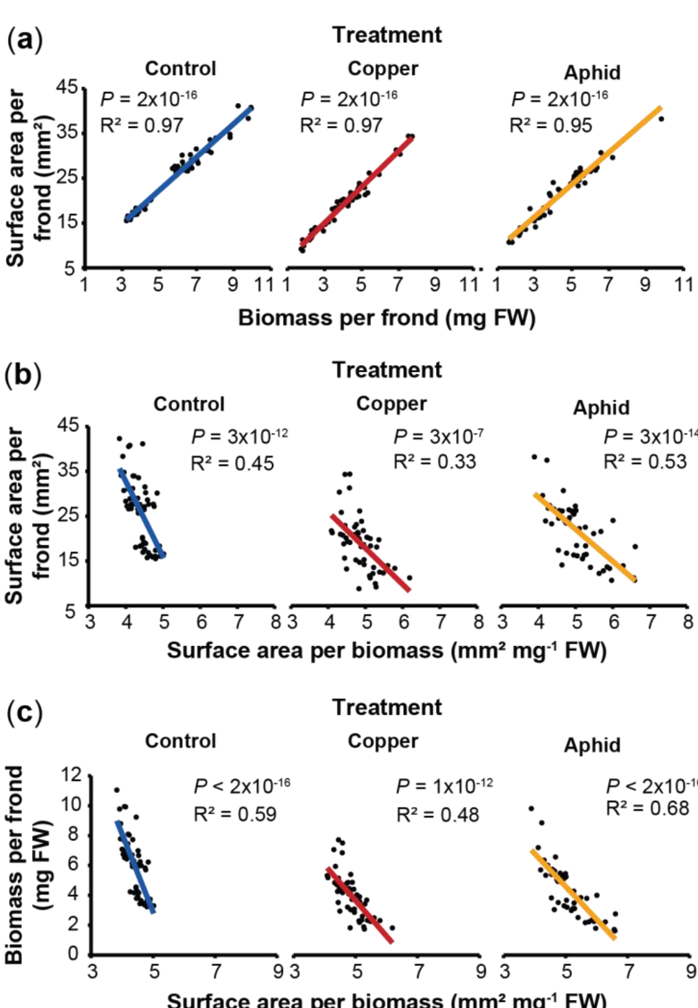

Figure S2.4. Morphological traits of *Spirodela polyrhiza* correlate among each other. (**a**) Biomass per frond positively correlated with surface area per frond. (**b**) Surface area per biomass negatively correlated with surface area per frond in all environments. (**c**) Surface area per biomass negatively correlated with biomass per frond in all environments. Dots represent individual replicates of all six genotypes. Correlation lines are only shown for significant correlations. *P*-values on right of the panels refer to ANOVA tests on mixed effects models. N = 52-60. Morph = morphology, Treat = treatment, FW = fresh weight.

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3 Supporting Information

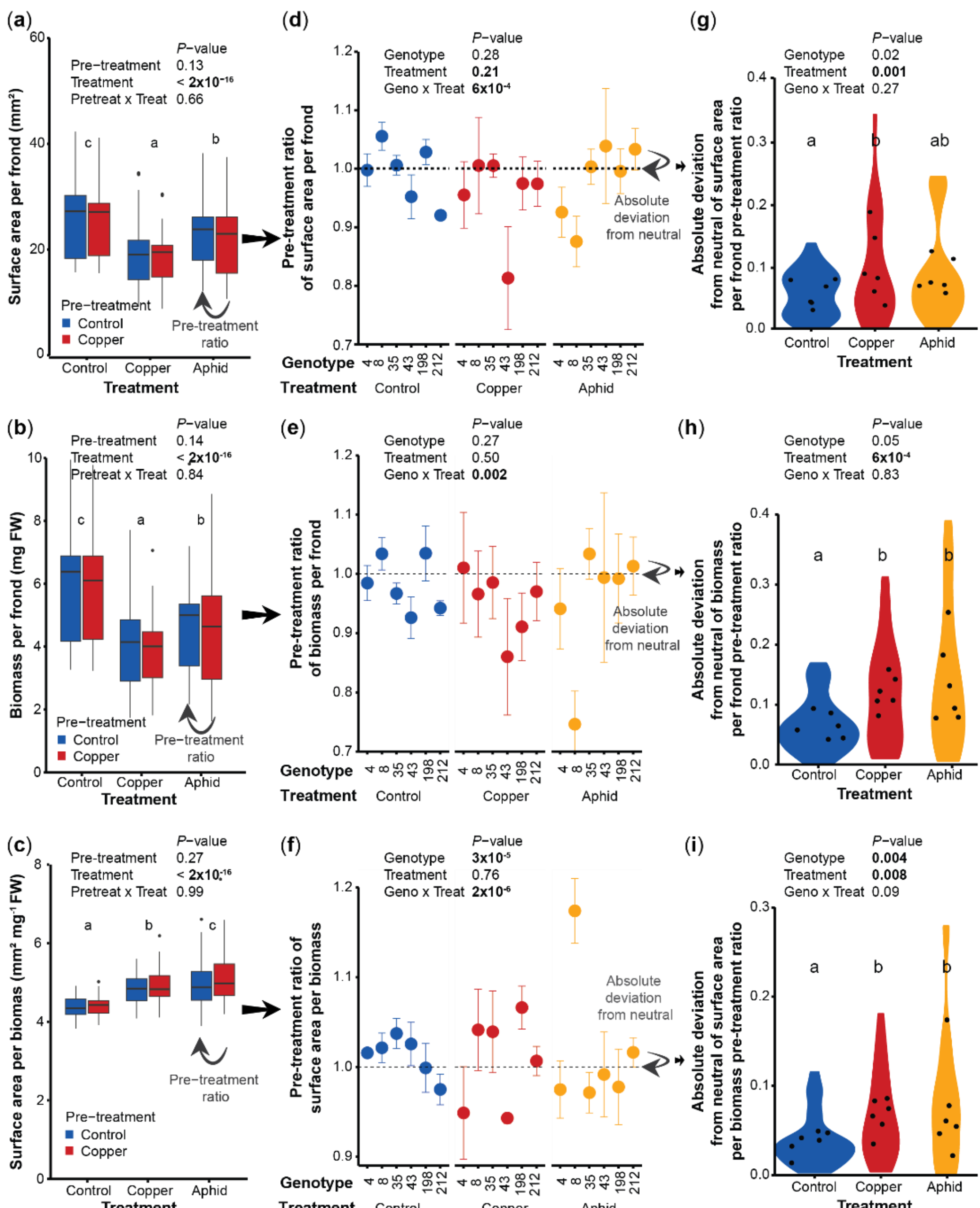

Figure S2.5. Copper excess indoors alters *Spirodela polyrhiza* morphology in a genotype and treatment dependent manner. (**a**) Surface area per frond, (**b**) biomass per frond and (**c**) surface area per biomass varied among treatments when considering the replicates of all six genotypes. Pairwise comparisons among treatments refer to least-squares means. *P*-values refer to ANOVA tests on mixed effects models. N = 24-32. (**d**) Surface area per frond, (**e**) biomass per frond and (**f**) surface area per biomass pre-treatment ratios (phenotype of copper pre-treated plants relative to the mean phenotype of control pre-treated plants per treatment) depended on the genotype and treatment, and their interaction. Dots represent the mean value and error bars the standard error per genotype and treatment. *P*-values refer to ANOVA tests on mixed effects models. N = 4-6. (**g**) Surface area per frond, (**h**) biomass per frond and (**i**) surface area per biomass pre-treatment ratios of replicates from all six genotypes deviated more from neutral under copper excess and aphid herbivory than under control conditions. N = 24-32. *P*-values refer to ANOVA tests on mixed effects models. Pretreat = Pre-treatment, Treat = Treatment, Geno = Genotype.

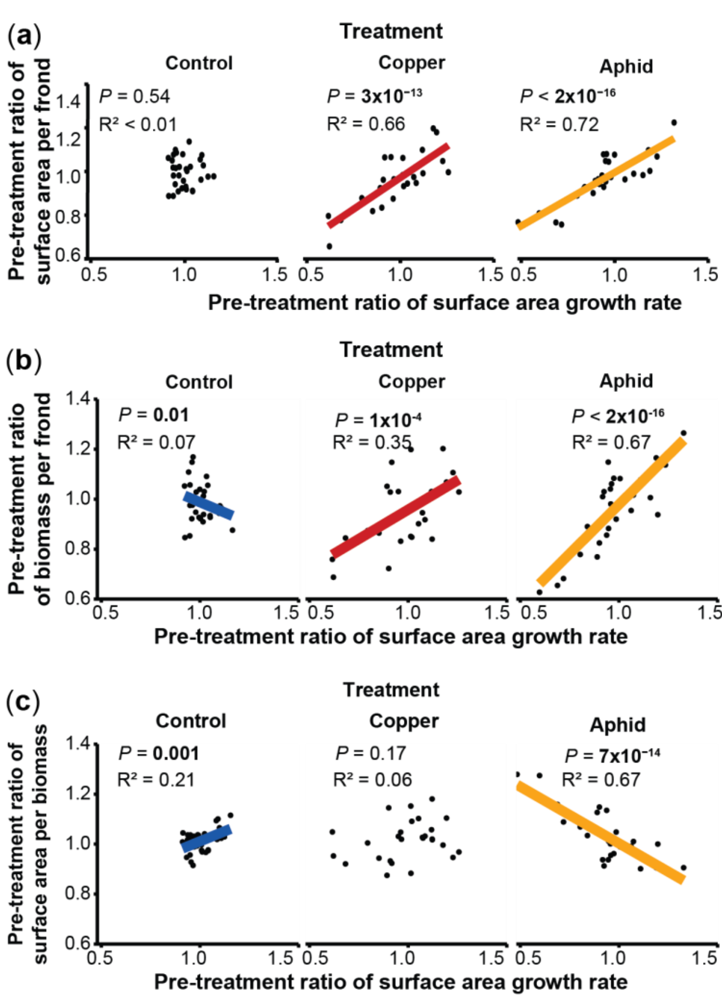

Figure S2.6. Pre-treatment effects on plant morphology were predictive for plant fitness, particularly under copper excess and aphid herbivory. Pre-treatment ratios on surface area growth rates correlated to the pre-treatment ratios on (**a**) surface area per frond, (**b**) biomass per frond, and (**c**) surface area per biomass. Dots represent individual replicates of all six genotypes. Correlation lines are only shown for significant correlations. *P*-values within a treatment refer to ANOVA tests on mixed effects models. mixed effects models. N = 27-32.

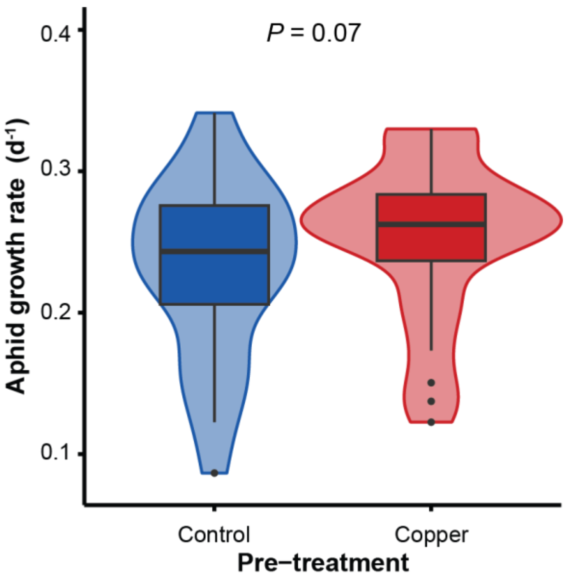

Figure S3.1. Copper pre-treatment on *Spirodela polyrhiza* tends to increase the growth rate of the aphid *Rhopalosiphum nymphaeae*. Copper pre-treatment on *S. polyrhiza* increased aphid growth on the replicates of all six genotypes. *P*-value refers to an ANOVA performed on a mixed effects model. N = 24-28.

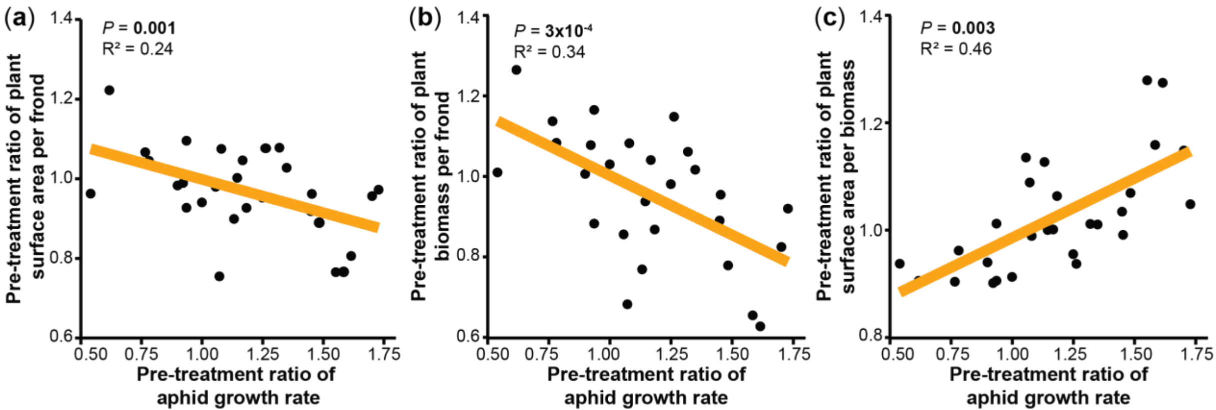

Figure S3.2. The more *Rhopalosiphum nymphaeae* benefitted from the copper pre-treatment, the stronger the stress-induced morphological changes became in *Spirodela polyrhiza*. Correlation between the pre-treatment ratios of *R. nymphaeae* growth rates (growth rates on copper pre-treated plants relative to the mean growth rate on control pre-treated plants) and the pre-treatment ratios of (**a**) surface area per frond, (**b**) biomass per frond, and (**c**) surface area per biomass. Dots represent individual replicates of all six genotypes. *P*-values refers to ANOVA tests performed on mixed effects models. N = 28.

6 Supporting Information

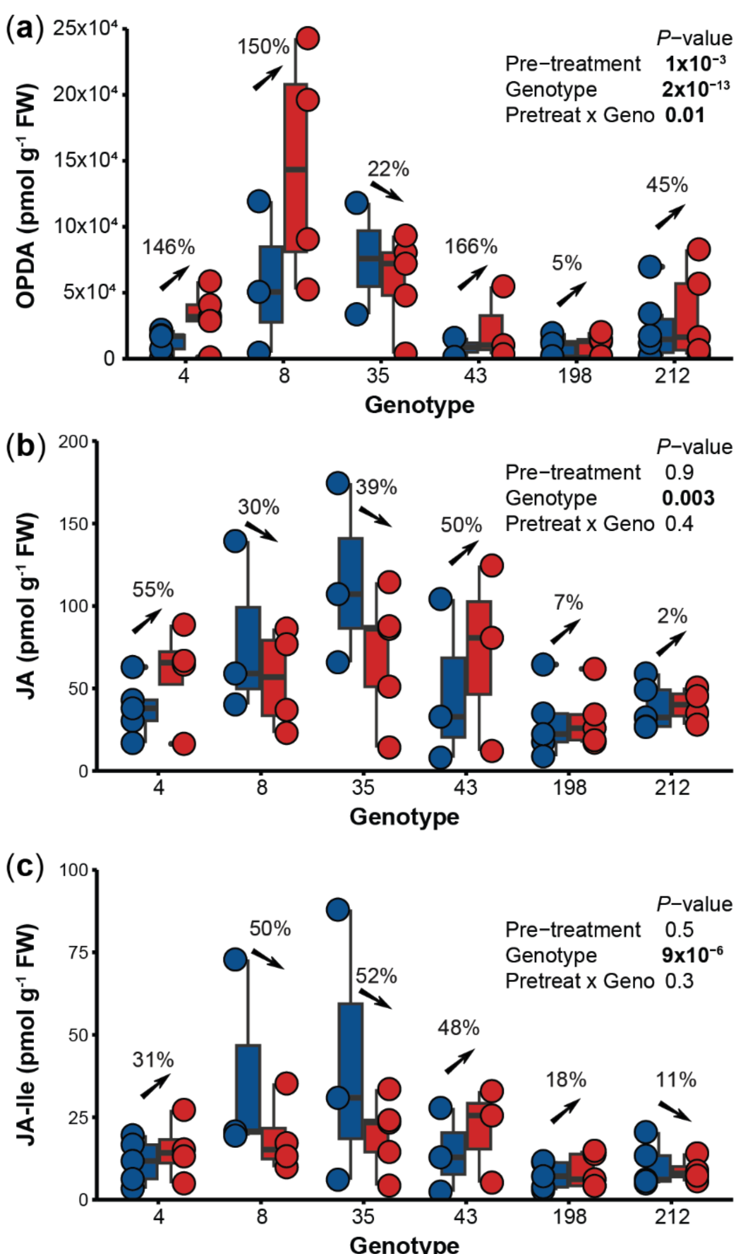

Figure S4.1. Copper pre-treatment enhances the concentrations of 12-oxo-phytodienoic acid in *Spirodela polyrhiza*, but not jasmonates under aphid herbivory. (**a**) Under aphid herbivory, **c**opper pre-treatment enhanced 12-oxo-phytodienoic acid in a genotype dependant manner, with the highest concentrations observed in SP8, genotype that carried the greatest aphid growth rates. (**b**) Copper pre-treatment did not affect jasmonic acid concentrations, nor (**c**) jasmonic acid-isoleucine concentrations. Dots represent individual replicates per genotype. *P*-values refer to ANOVA on mixed effects models. N = 23-32. OPDA = 12-oxo-phytodienoic acid, JA = jasmonic acid, JA-**I**le = jasmonic acid-isoleucine, FW = fresh weight.

7 Supporting Information

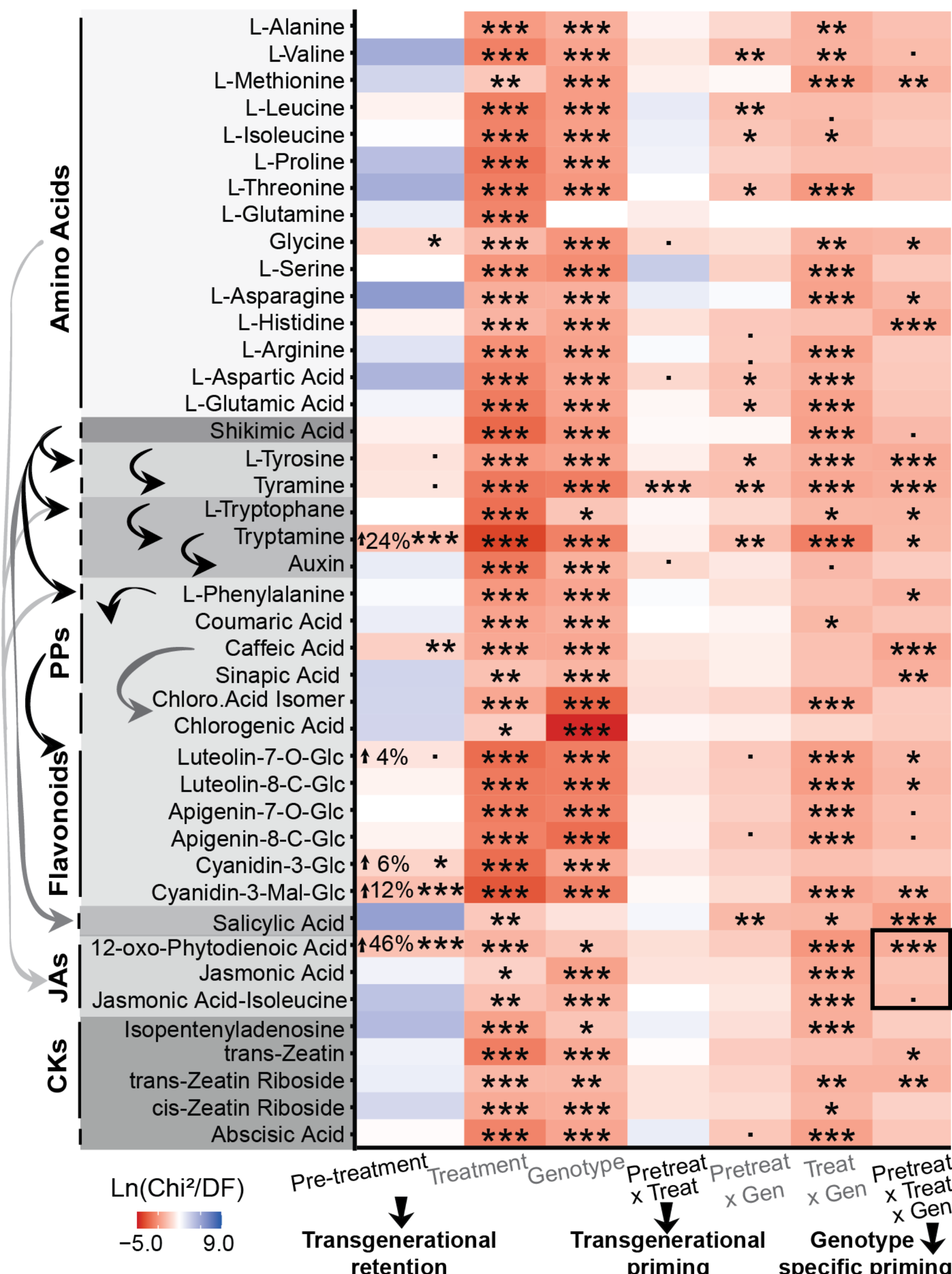

Figure S4.2. Copper excess transgenerationally retains copper-induced levels of anthocyanins and jasmonates. Heatmap displays the Chi² values relative to their degrees of freedom (DF) obtained from ANOVA tests performed on mixed effects models of the pre-treatment, treatment, genotype effects on metabolite concentrations. Numbers within the heat map refer to the fit mean metabolite levels between the copper to control pre-treated plant. Upward pointing arrow refers to increased levels upon copper pre-treatment. *P*-values refer to ANOVA tests on mixed effects models. The different shades of grey cluster metabolites that have a shared biosynthetic pathway, and arrows connect precursors and derivates within and between the assessed pathways. Grey arrows connect indirect precursors and derivates. *P*-values significance: **-** < 0.1, * < 0.05. ** < 0.01, *** < 0.001. N = 3-6 per each of the six genotypes. Cyanidin-3-Glc = cyanidin-3-glucoside, Cyanidin-3-Mal-Glc = cyanidin-3-malonylglucoside, Glc = glucoside, PPs = phenylpropanoids, JAs = jasmonates, CKs = cytokines.

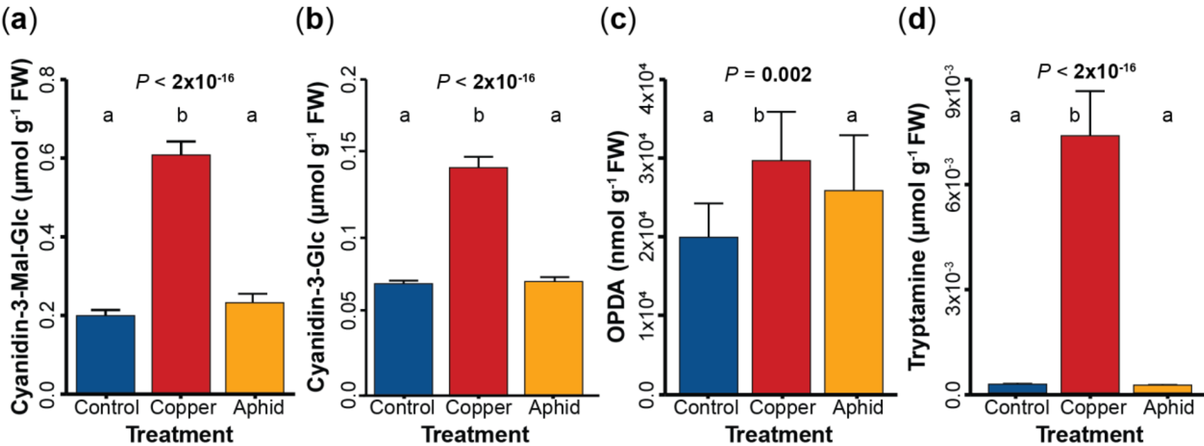

Figure S4.3. First time exposure to copper excess enhances the levels of transgenerational plastic metabolites in *Spirodela polyrhiza*. Copper excess, but not aphid herbivory elevated the levels of (**a**) cyanidin-3-malonylglucoside, (**b**) cyanidin-3-glucoside, (**c**) 12-oxo-phytodienoic acid and (**d**) tryptamine. *P*-values refer to ANOVA on mixed effects models. Pairwise comparisons were obtained with least-squares means. The data display the mean and standard errors of the replicates of all six genotypes. N = 23-32. Cyanidin-3-Mal-Glc = cyanidin-3-malonylglucoside, cyanidin-3-Glc = cyanidin-3-glucoside, OPDA = 12-oxo-phytodienoic acid, FW = fresh weight.

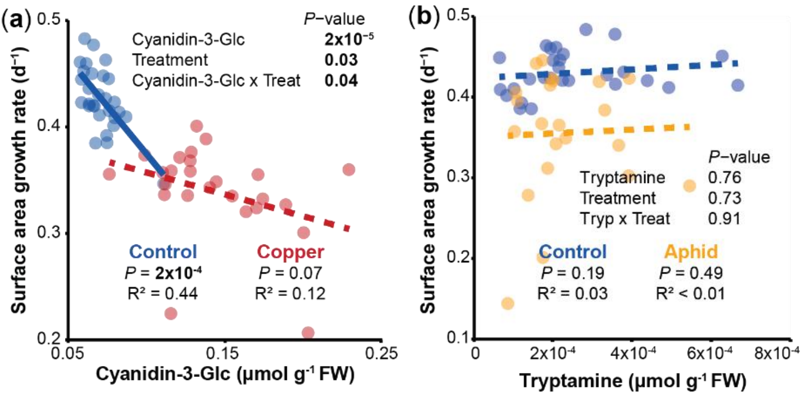

Figure S5.1. Transgenerationally plastic cyanidins have an adaptive role in plants against copper, but not tryptamine against aphid herbivory. (**a**) The levels of cyanidin-3-glucoside negatively correlated with plant fitness under control but not copper conditions. (**b**) Tryptamine levels did not correlate with plant fitness neither under control conditions nor herbivory of the aphid *R. nymphaeae*. Circles display individual replicates of all six genotypes. *P*-values refer to ANOVA tests on mixed effects models. N = 27-32. Cyanidin-3-Glc and Cya3Glc = cyanidin-3-glucoside, Tryp = Tryptamine, Treat = Treatment.

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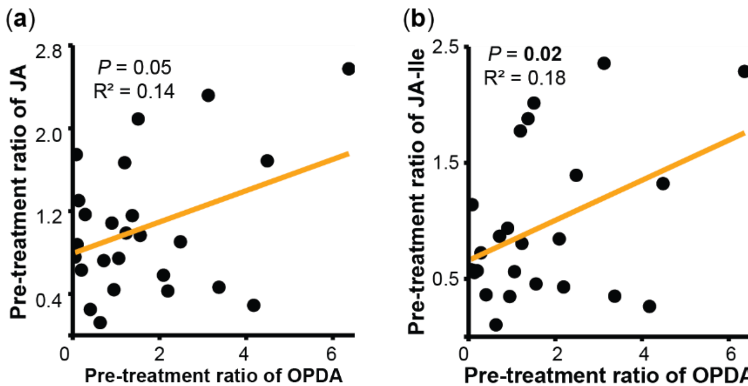

Figure S5.2. Pre-treatment ratios of jasmonates and its precursor 12-oxo-phytodienoic acid (OPDA) correlate among each other. Pre-treatment ratios (metabolite concentrations of copper pre-treated plants relative to the mean metabolite concentration of control pre-treated plants) of OPDA positively correlated with the pre-treatment ratios of (**a**) jasmonic acid and (**b**) jasmonic acid isoleucine under aphid herbivory treatment. Circles display individual replicates of all six genotypes. *P*-values refer to ANOVA tests on mixed effects models. N = 25. JA = jasmonic acid, JA-Ile = jasmonic acid isoleucine, OPDA =12-oxo-phytodienoic acid.

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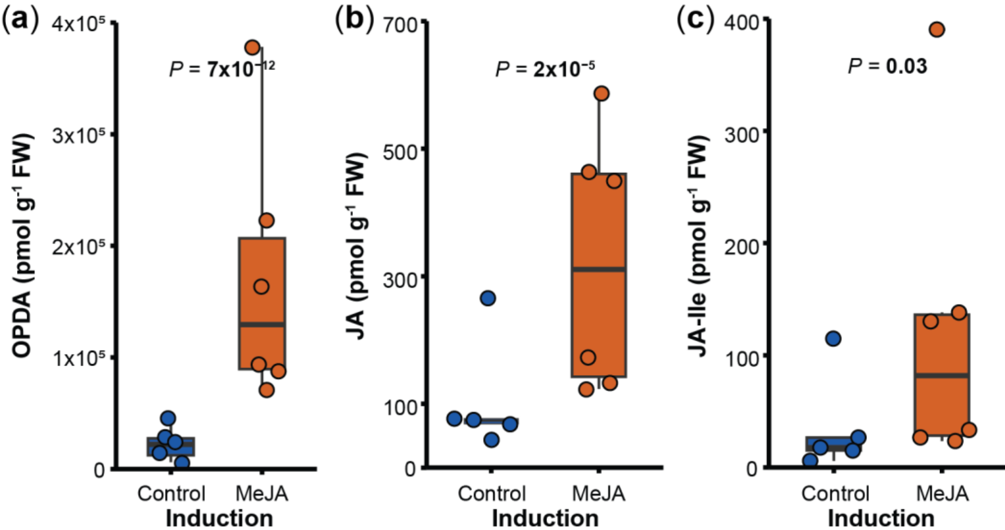

Figure S5.3. Exogenous application of methyl jasmonate in *Spirodela polyrhiza* elevates the concentrations of jasmonates and their precursor. Application of methyl jasmonate on *S. polyrhiza* genotype SP050 enhanced the concentrations of (a) 12-oxo-phytodienoic acid, (b) jasmonic acid and (c) jasmonic acid-isoleucine. *P*-values refer to ANOVA tests on mixed effects models. Circles display individual data points. N = 5-6. JA = jasmonic acid, JA-Ile = jasmonic acid isoleucine, OPDA =12-oxo-phytodienoic acid.

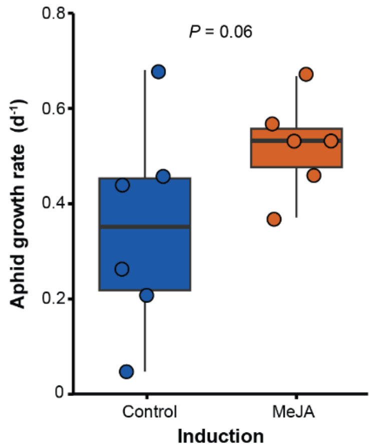

Figure S5.4. External application of methyl jasmonate to *Spirodela polyrhiza* tends to increase the growth rate of the aphid *Rhopalosiphum nymphaeae*. Repeating the application of methyl jasmonate on *S. polyrhiza* genotype SP050 tended to favour aphid growth after two days of assays. *P*-value refers to an ANOVA test on a mixed effects model. Circles display individual data points. N= 6. MeJA = methyl jasmonate, 12-oxo-phytodienoic acid = OPDA.

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Supporting tables

Table S1. Genotypes of *Spirodela polyrhiza* used in the experiments.

Clone ID	Accession ID	Genetic cluster	Continent	Country	Latitude	Longitude	NCBI accession ID	Experiment
7498	SP004	America	North America	USA	35.994033	-78.898619	SAMN09429369	Outdoor/Indoor
8683	SP008	America	Africa	Kenya	0.0880828	38.189978	SAMN09429399	Indoor
0109	SP035	SE Asia	Asia	China	30.196789	103.5065	SAMN09429365	Indoor
9497	SP043	India	Asia	India	9.9252007	78.119775	SAMN09429373	Indoor
0090	SP050	SE Asia	Asia	China	30.410755	104.54677	SAMN09429379	Induction
NA	SP198	SE Asia	Asia	China	39.428484	118.90827	SAMN17885320	Indoor
NA	SP212	SE Asia	Asia	China	35.40409	116.59243	SAMN17885333	Indoor

SE Asia: Southeast Asia.

Note: Modified table from Wang et al. (2024).

Table S2. MRM-settings and retention times of a putative chlorogenic acid isomer.

	RT [min]	Q1 [m/z]	Q3 [m/z]	Dwell time [ms]	CE [V]	Q1/Q3 Pre Bias [V]
Putative chlorogenic acid isomer	3,240	(+)355,10	163,15	32	-15	-23 / -17
		(+)355,10	135,10	32	-35	-23 / -14

RT: retention time.

CE: collision energy.

Qualifier is highlighted in grey.

Note: The analyte was added to the protocol in Method 1A of Malacrinò et al. (2024). Quantification was done relative to the isotope labelled phenylalanine standard in the original method (Schäfer et al., 2016). Due to the lack of the correct standard no correction factor was applied, and the data represent arbitrary units.

Table S3. Copper excess treatment effects *Spirodela polyrhiza* surface area growth rate independently of the outdoor pre-treatment.

	Chisq	Df	Pr(>Chisq)
Pre-treatment	0.4753	1	0.86
Treatment	159.7881	3	4×10^{-11}
Pre-treatment x Treatment	3.7389	3	0.74

Note: ANOVA test performed on a mixed effects model.

Spirodela polyrhiza genotype SP004.

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Supporting methods

Additional information in experimental setup and statistical analyses.

Methods S1. Variables and models used to characterize the effects that the pre-treatment has on *Spirodela polyrhiza* fitness and morphology.

To assess the effects of the pre-treatment, the genotype and the treatment on plant fitness and morphology of six different genotypes, we included in the statistical analysis the following random variables:

Replicate Lineages started with a single individual (N = 6 per genotype) which offspring was divided into the copper and control pre-treatment, and afterwards, into control, copper, and aphid treatments (Figure **S2.1c**). Therefore, individuals coming from the same ancestor (Replicate in 2_SterileExp.xlsx) were not independent and were clustered with this variable.

Genotype Genetic cluster to which the samples belonged to (Genotype in 2_SterileExp.xlsx).

Offspring Despite starting fitness assays with plants at similar developmental stages, some plants showed no, one or two developmental pockets. As the presence of developmental pockets could create bias in fitness and morphology measurements based on surface area, but mostly on frond number, we included this information (offsringStart in 2_SterileExp.xlsx) into the analyses.

Rack Each rack allowed eight tubes to stay together during the whole propagation term, which was approximately 2.5 months. This means that four different genotypes with their two corresponding pre-treatments were always one next to the other. Therefore, this physical structure (Rack in 2_SterileExp.xlsx) was considered.

Branching The giant duckweed produces one offspring after the other, and the effects of using the first instead of the second offspring for fitness assays are unknown. Therefore, the information of being the first and second offspring when starting fitness assays (Figure **S2.1b**, G9:G10:G11 in 2_SterileExp.xlsx) was considered.

We excluded some of the random variables for specific questions when: first, the random factors did not explain any variance for most of the analysed response variables; second, when the fitted values deviated from the observed values – revised through plots; third, the random factor lost biological meaning. Thus, when using the pre-treatment ratios, the random variable *Offspring* lost biological meaning and was excluded from the statistical analyses.

Considering these random variables and the replicates from all six genotypes, we performed six different analyses:

1. To assess the effect of the pre-treatment on fitness and morphology ("Response" variable) under different environments, we used the model:
   Response ~Pre-treatment*Treatment
   +(1|Replicate)+(1|Genotype)+(1|Offspring)+(1|Rack)+(1|Branching).
2. To analyse the effect of the genotype on the pre-treatment ratios of fitness and morphology ("Pre-treatment ratio") under different treatments, we used the model:
   Pre-treatment ratio ~Genotype*Treatment+(1|Replicate)+(1|Branching).
3. To test the effect of the genotype on the absolute deviation of the pre-treatment ratios from null under different treatments, we used the model:
   Absolute deviation ~Genotype*Treatment+(1|Replicate).
4. To correlate the pre-treatment ratio of surface area growth rates among the three treatments, we first merged the data, keeping only samples present in all treatments. With this dataset, we applied the model
   pretreatment ratios in the treatment1 ~ pretreatment ratios in treatment2+(1|Genotype), and verified the R^2 values with simple linear models.
5. To correlate the different morphologies, we used the model:
   Morphology1 ~Morphology2*Treatment+(1|Pre-treatment)+(1|Genotype).
6. To correlate among the pre-treatment ratios of the morphologies we used the model
   Pre-treatment ratio1 ~Pre-treatment ratio2+(1|Genotype). We used the same model to correlate the pre-treatment ratios of morphology with the pre-treatment ratios of fitness within each treatment.

Methods S2. Variables and models used to characterize the effect of the pre-treatment on *Spirodela polyrhiza* metabolites.

To assess the effects of the pre-treatment and the treatment on the metabolite concentration of six genotypes of *S. polyrhiza*, we included in the statistical analysis the following random variables:

Replicate Lineages started with a single individual (N = 6 per genotype) which offspring was divided into the copper and control pre-treatment, and afterwards, into control, copper, and aphid treatments (Figure **S2.1**). Therefore, individuals coming from the same ancestor (Replicate in 2_SterileExp.xlsx) were not independent and thus clustered with this variable.

Genotype Genetic cluster to which the samples belonged to (Genotype in 2_SterileExp.xlsx).

Plate The samples were divided into four 96-well-plates, which were extracted and measure in different days. To avoid structuring, we organized samples by the number of replicate (one

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to six). Thus, plate one started with Replicate 1 of all genotypes and each of them with their pre-treatments and treatments. To consider the pure effect of the plate number, we introduced this variable (Platte in 2_SterileExp.xlsx) into the analysis.

Batch Within each plate, we worked with eight vertical wells per time, which included the addition of buffers during the extraction, as well as the reading of the metabolite concentrations. Therefore, we included the batch of 8 wells (Platte:ColumnInPlatte in 2_SterileExp.xlsx) into the analysis.

We excluded some of the random variables for specific models when: first, the random factors did not explain any variance for most of the analysed metabolites; second, when the fitted values visually deviated from the observed values.

Besides the screening of transgenerationally plastic metabolites within each treatment, we performed four additional analyses considering the replicates of all six *S. polyrhiza* genotypes:

1. To assess the effect of both, the pre-treatment and the treatment on those transgenerationally plastic metabolites (i.e. cyanidins, OPDA and tryptamine), we used the model:
   Concentration ~Pre-treatment*Treatment+(1|Genotype)+(1|Replicate)+(1|Plate)+(1|Batch).
   Furthermore, to assess the metabolite concentration differences within each treatment, we used the model:
   Concentration ~Pre-treatment+(1|Genotype)+(1|Replicate)+(1|Plate)+(1|Batch).
2. To assess the effects of the pre-treatment and genotype on the concentrations of jasmonates within the aphid herbivory treatment, we used the model:
   Concentration ~Pre-treatment*Genotype+(1|Replicate)+(1|Plate)+(1|Batch).
3. To additionally identify the effect of the genotype on all metabolites, we used the model:
   Concentration ~Pre-treatment*Treatment*Genotype+(1|Replicate)+(1|Batch).
4. To assess whether the metabolites that accumulated higher concentrations upon copper pre-treatment – namely anthocyanins, tryptamine and jasmonates – were induced by first-time copper exposure, we focused on control pre-treated plants that were challenged to control, copper and aphid treatments. Thereto, to analyse the induction changes under first time stress exposure we used the model:
   Concentration ~Treatment+(1|Genotype)+(1|Replicate)+(1|Plate)+(1|Batch).

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Methods S3. Assessing the effects of *Spirodela polyrhiza* induced jasmonates in the growth rates of *Rhopalosiphum nymphaeae*.

To assess the reproducibility of aphid growth rates in plants pre-treated with methyl jasmonate, we cultivated six plant replicates of *S. polyrhiza* genotype SP050, each consisting of two pseudo-replicates which included 10 fronds in different developmental stages – three to four fronds per colony. We placed the pseudo-replicates in 7.5 mL of 100 µM methyl jasmonate and control conditions within 6-well-plates ("Induction"). Methyl jasmonate was dissolved in N-medium by shaking at 1200 rpm for 2 hours until no globules of fat were visible. Plates were sealed with parafilm for 96 hrs, after which, a total of 20 fronds per replicate were moved into 150 mL N-medium with and without 10 aphids per replicate. All remaining fronds from the induction phase were flash-frozen and stored in -80°C for posterior metabolite extraction. This experiment was performed under 26°C, 135 µM photons m^2 s^{-1}, 16:8 hours light/dark.

To assess whether the application of methyl jasmonate alters OPDA and jasmonates, we extracted and measured these metabolites as described in "*Effects of copper pre-treatment on* Spirodela polyrhiza *metabolites*" within Materials and Methods. Afterwards, to test whether methyl jasmonate induced the levels of these metabolites, we used the model OPDA ~Pretreat+(1|Plate).

Besides the effects of methyl jasmonates on jasmonate induction, we measured the effects of methyl jasmonate on aphid growth rates. Thereto, we used the model
Aphid growth rate ~Induction+(1|Plate)).

Within this experiment, *Plate* considered the physical structure during the induction phase.

Methods S4. Variables to assess the effects of methyl jasmonate on *Rhopalosiphum nymphaeae* growth rates.

To assess the effects of methyl jasmonate on the aphid *R. nymphaeae* growth rates, we included in the statistical analysis the following random variables:

Replicate During the pre-treatment phase ("induction"), replicates contained three fronds per tube which were placed into the different treatments. Therefore, these fronds were not independent (Replicate in 4_InductionExp.xlsx) and thus clustered with this variable.

Group Due to the total number of samples (N = 72), we divided the experiment into three batches of four replicates, each performed one day after the other. Therefore, we introduced this variable (DateStart in 4_InductionExp.xlsx) to consider the structure of time.

Equations used for the transformation of data

Equation S1. Relative growth rate (Hunt, 1982).

$$\frac{\ln(\text{final measurement}) - \ln(\text{initial measurement})}{\text{number of days}}$$

Equation S2. Pre-treatment ratio (Huber et al., 2021).

$$\frac{\text{plant fitness or phenotype of copper pre} - \text{treated plants}}{\text{mean (plant fitness or phenotype of control pre} - \text{treated plants)}}$$

and

$$\frac{\text{aphid fitness of individuals growing on copper pre} - \text{treated plants}}{\text{mean (aphid fitness of individuals growing on control pre} - \text{treated plants)}}$$

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Chapter IV: Manuscript III

Maladaptive aphid-induced transgenerational plasticity is overcome in nature

Alexandra Chávez[1,2,3], Martin Lohr[1], Lisa-Marie Laik[1], Martin Schäfer[1,3], Shuqing Xu[1,3], Meret Huber[1,2,4]

[1] Institute of Organismic and Molecular Evolution, University of Mainz, Johann-Joachim-Becher-Weg 7, 55128 Mainz, Germany; [2] Institute of Plant Biology and Biotechnology, University of Münster, Schlossplatz 7, 48143 Münster, Germany; [3] Institute for Evolution and Biodiversity, University of Münster, Schlossplatz 7, 48143 Münster, Germany; [4] Institute for Quantitative and Computational Biosciences, Johannes Gutenberg University of Mainz, 55128 Mainz, Germany

Abstract

Transgenerational plasticity is widely presumed adaptive, yet empirical evidence is scarce, especially in natural conditions. Using the clonally reproducing giant duckweed (*Spirodela polyrhiza*) and its native herbivore, the waterlily aphid (*Rhopalosiphum nymphaeae*), we show that herbivores benefit from plant transgenerational plasticity. In monoclonal, single-descendant lineages indoors, duckweed supported higher aphid reproduction and suffered decreased fitness when their ancestors encountered aphids five generations earlier. Gene expression and metabolite profiling suggest transgenerational priming as underlying mechanism: duplicated L-tyrosine decarboxylase genes elevated tyramine levels, an adaptive response as tyramine supplementation suppressed aphid reproduction. Contrary, transgenerational jasmonate priming was maladaptive, as methyl-jasmonate application enhanced aphid reproduction. Remarkably, in a two-year outdoor mesocosm experiment with monoclonal *S. polyrhiza* populations of up to one million individuals, transgenerational tyramine priming persisted, whereas transgenerational jasmonate priming and its costs disappeared. These findings indicate that herbivores can hijack plant transgenerational plasticity, but that natural environments may counteract such changes.

Manuscript for Nature Communications

Maladaptive aphid-induced transgenerational plasticity is overcome in nature

Alexandra Chávez[1,2,3], Martin Lohr[1], Lisa-Marie Laik[1], Martin Schäfer[1,3], Shuqing Xu[1,3], Meret Huber[1,2,4]

[1] Institute of Organismic and Molecular Evolution, University of Mainz, Johann-Joachim-Becher-Weg 7, 55128 Mainz, Germany; [2] Institute of Plant Biology and Biotechnology, University of Münster, Schlossplatz 7, 48143 Münster, Germany; [3] Institute for Evolution and Biodiversity, University of Münster, Schlossplatz 7, 48143 Münster, Germany; [4] Institute for Quantitative and Computational Biosciences, Johannes Gutenberg University of Mainz, 55128 Mainz, Germany

Keywords:

transgenerational plasticity, aphid herbivory, jasmonates, tyramine, non-genetic epigenetic inheritance, priming, plant defence, herbivory, duckweed *Spirodela polyrhiza*, waterlily aphid *Rhopalosiphum nymphaeae*

Introduction

Transgenerational plasticity, the inheritance of environmentally induced traits, is often assumed to be adaptive. This assumption rests on the idea that stress triggers a phenotype that improves offspring fitness under similar conditions but may reduce fitness when conditions differ, partly due to the costs of defence[1, 2]. Indeed, mounting evidence from both plants and animals supports this view[3, 4, 5, 6, 7]. However, not all transgenerational plastic traits are necessarily adaptive[7, 8, 9, 10, 11]. For instance, stress experienced by parents may reduce resource provisioning to their offspring, thereby lowering offspring fitness regardless of environmental conditions, a phenomenon known as the silver spoon effect[12]. In addition, some herbivores and pathogens can manipulate host physiology to their own benefit[13, 14]. Whether such antagonist-induced changes–those that benefit the attacker while harming the host–are inherited across generations remains unclear, particularly under natural outdoor conditions.

Testing the adaptive value of transgenerational plasticity poses several challenges. First, it is essential to ensure that observed phenotypes result from the inheritance of environment-induced traits, rather than from genetic differences or early-life exposure effects[15]. This requires single-descendant lineages, ideally from clonally reproducing or highly inbred lines, tracked across multiple generations[16, 17]. Second, determining the fitness consequences of a given phenotype is not trivial, particularly when dealing with traits such as hormones or specialized metabolites, which may have multiple or context-dependent functions[18]. For instance, jasmonic acid is generally considered to reduce herbivore performance, yet in *Spirodela polyrhiza*, external application of methyl jasmonate increased, rather than decreased, aphid reproduction[11]. Similarly, aphids reproduced better rather than worse on *Sorghum bicolor* genetically impaired in jasmonate biosynthesis[19]. These examples highlight the importance of evaluating the adaptive value of metabolites in a system-specific context.

Another metabolite that exemplifies such multi-functionality is tyramine, which acts as a central hub in the metabolism of the conditionally essential amino acid L-tyrosine. L-tyrosine can be decarboxylated to tyramine by L-tyrosine decarboxylases, partially followed by hydroxylation to octopamine[20]; alternatively, L-tyrosine can be hydroxylated to L-DOPA and then decarboxylated to dopamine— key precursors of melanin, the dark pigment often measured by reductions in exoskeletal redness[21, 22] (Fig **S1.1**). As such, tyramine can have dual functions in plant-herbivore interactions: on the one hand, tyramine can act as plant defence compound by reinforcing cell walls or exerting toxicity, especially when channelled into phenolamides or alkaloids[23, 24]. Furthermore, plant-derived tyramine and its derivative octopamine may perturb aphid signalling pathways via their activity as neurotransmitters, neurohormones, and neuromodulators [25, 26]. On the other hand, tyramine may serve as an insect nutrient, as plant-derived tyramine may feed the L-DOPA/dopamine branch driving cuticle melanisation. Feeding

the L-DOPA/dopamine pathway may occur by sparing L-tyrosine from decarboxylation—thereby boosting the precursor pool for melanin synthesis—rather than by direct conversion of tyramine, since insects appear to lack the enzymes needed to convert tyramine into dopamine or to recycle it back to L-tyrosine[27, 28]. Thus, the same molecule could function as a plant defence metabolite or an insect resource, and its net impact on herbivore fitness is uncertain.

Molecules like tyramine can exhibit transgenerational plasticity either by being directly transferred to offspring—thereby elevating baseline levels—or by priming the biosynthetic machinery across generations, leading to enhanced synthesis upon recurrent stress[10, 11]. For instance, in *Arabidopsis thaliana*, herbivory did not increase jasmonic acid levels in seeds; however, the progeny's expression of JA-biosynthetic genes was primed across generations[10]. Distinguishing between these scenarios requires measuring metabolite levels under both control and stress conditions, alongside expression analyses of the biosynthetic genes.

Identifying the relevant biosynthetic genes and characterizing how they evolved–including their associated gene families, orthogroups, and copy number variation–is essential for assessing whether plasticity may contribute to genome evolution. One proposed mechanism is retroposition, in which mRNAs are reverse transcribed and reinserted into the genome, often yielding intron-free copies at new chromosomal locations[29]. While many such retrogenes are non-functional due to the lack of regulatory elements, some retain or acquire new functions[29]: for example, a retrotransposed gene influences fruit shape in tomato[30]. Although direct evidence linking environmentally induced gene expression to increased retroposition remains limited [31], particularly in plants, transcriptional plasticity may increase the likelihood that highly expressed genes become retrotransposed over evolutionary time[32, 33, 34]. This possibility aligns with the broader concept of genetic accommodation, whereby the plasticity of traits is refined, stabilized, or modified, either through genetic or heritable epigenetic change[35, 36].

To investigate whether transgenerational plasticity exists, affects fitness, and may contribute to genome evolution, a genetically stable and experimentally tractable model system is essential. A strong candidate for such a system is the plant–herbivore interaction between the giant duckweed (*Spirodela polyrhiza*) and its native herbivore, the waterlily aphid (*Rhopalosiphum nymphaeae*), both of which have high-quality reference genomes [37, 38, 39]. Under optimal conditions, *S. polyrhiza* reproduces exclusively asexually by budding approximately every two days. Since *S. polyrhiza* also has the lowest known mutation rate among multicellular eukaryotes–about one mutation per 80 generations[40]–mother and offspring can be considered genetically identical. The plant's flat, thallus-like shoot, known as a frond, allows accurate estimation of fitness via surface area growth. Mother and daughter fronds remain connected for several days by a stolon-like stipe containing vascular tissue, potentially enabling intergenerational exchange of signals or molecules. In winter, *S. polyrhiza*

forms dormant turions that overwinter at the pond bottom[41, 42, 43]. *R. nymphaeae* leaves its winter host in early summer to colonize duckweeds and other aquatic plants, on which the aphids reproduce exclusively via parthenogenesis[44]. Because aphids are mobile, *S. polyrhiza* populations often experience fluctuating levels of herbivory.

Using this experimental system, we tested the hypothesis that transgenerational plasticity occurs and is adaptive. Unexpectedly, we found that while some transgenerational responses enhanced plant fitness, others favoured aphid reproduction. Strikingly, only the adaptive responses were maintained under natural outdoor conditions.

Results

Indoors, aphids alter plants to their own benefit

To test whether ancestral aphid herbivory alters plant fitness and phenotypes, as well as aphid fitness, we grew eight *S. polyrhiza* genotypes for five generations as monoclonal, single descendant lineages in the presence and absence of the aphid herbivore *R. nymphaeae* (pre-treatment), followed by five generations under control conditions ("recovery"). Subsequently, we measured plant and aphid population growth rates ("fitness") and phenotypes under control conditions and recurrent herbivory (treatment) across eight days of free growth (Fig **1a**). Unexpectedly, aphid pre-treatment benefitted plant fitness under control conditions but decreased plant fitness under aphid herbivory (P(Pre-treatment$_x$Treatment) = 0.002, mixed effects model, Fig **1b**), albeit in a genotype-dependent manner (P(Treatment$_x$Genotype) = $5x10^{-4}$, mixed effects model, Fig **1c**): under control conditions, three genotypes benefitted from aphid pre-treatment, with a maximal fitness increase of 5%. Under aphid herbivory, the fitness effects were stronger, reducing plant fitness in average by 7%. Some genotypes even suffered fitness loss of 15% due to the aphid pre-treatment.

Apart from reducing plant fitness, aphid pre-treatment amplified stress-induced morphological changes: aphid herbivory reduced surface area per frond and biomass per frond, as well as increased surface area per biomass (Fig **S1.2**). These effects were amplified when the plants were pre-treated with aphids: under aphid herbivory, aphid pre-treated plants had 11% lower surface area per frond, 20% lower biomass per frond, and 11% higher surface area per biomass compared to control pre-treated plants (P(Pre-treatment$_x$Treatment) < 0.03, mixed effects models, Fig **S1.2**). Like plant fitness, these effects were genotype-specific (Fig **S1.2**). Taken together, ancestral aphid herbivory harmed plant fitness and inflicted stronger stress-induced morphological changes when the aphids recurred.

Since plants suffered from ancestral herbivory under recurring conditions, we suspected that the aphids manipulate the plants to their own benefit. Indeed, aphid pre-treatment increased

aphid fitness, in average by 5% (*P* = 0.02, mixed effects model, Fig **1d**). These effects were particularly strong in the genotypes Sp50, Sp59 and Sp102 (deviation from neutral: *P* < 0.05, Wilcoxon rank sum test, Fig. **1e**), with a fitness increase of more than 10%. These genotypes were also the ones that suffered most from the aphid pre-treatment under recurrent aphid herbivory (Fig **1c**). Consequently, across the eight genotypes, the pre-treatment ratios of aphid fitness correlated negatively with the pre-treatment ratios of plant fitness under aphid herbivory (*P* = 0.002, linear model, Fig **1f**), showing that the more the aphids benefited from previous stress, the more the plants suffered.

Within species variation in transgenerational fitness effects, as observed here, should evolve if transgenerational plasticity has environment-dependent fitness cost and benefits. Indeed, the more a genotype suffered from ancestral herbivory under recurring aphid herbivory, the better it performed when the aphids did not recur (*P* = 0.05, linear model, Fig **1g**). Taken together, these data show that the fitness consequence of transgenerational plasticity is environment dependent, but contrary to expectation, can be maladaptive under recurring stress.

Tyramine and jasmonate are transgenerationally primed

To elucidate which metabolites may have accounted for the increased aphid fitness and associated decrease in plant fitness, we screened the levels of amino acids, amines, phytohormones, and phenylpropanoids in genotype 102, which supported higher aphid fitness when pre-treated with the aphids (Fig **1e**, Fig **S1.3**). Under control conditions, ancestral herbivory enhanced the accumulation of four metabolites: the amine tyramine, chlorogenic acid, a putative isomer of chlorogenic acid, and the neurotransmitter GABA. These metabolites increased by 20-300% in response to ancestral herbivory (Fig **2a**, left panel). Under aphid herbivory, the effects of ancestral aphid herbivory on metabolite accumulation were stronger: 14 out of 46 metabolites accumulated to higher levels when plants were previously exposed to aphid herbivory, with some metabolite levels increasing up to three-fold. Interestingly, not a single metabolite was significantly suppressed by the aphid pre-treatment (Fig **2a**, right panel). The metabolites that accumulated to higher levels due to the pre-treatment were mostly phenylpropanoids, including chlorogenic acids, as well as free and glycosylated forms of the flavones apigenin and luteolin (Fig **S2.1**). Additionally, the insect neuromodulator tyramine and the plant defence hormone jasmonic acid accumulated to higher levels when the plants were pre-treated with aphids (Fig **2b**). Taken together, these data show that under control conditions and particularly under aphid herbivory, metabolites accumulate to higher levels when their ancestors five generations ago were exposed to aphid herbivory.

To assess whether the transgenerationally plastic metabolites may account for increased aphid fitness and reduced plant fitness upon recurrent aphid herbivory, we first studied tyramine, as it may be an insect nutrient. Tyramine levels were transgenerationally retained as well as primed (Fig **2b**): upon first-time aphid exposure, tyramine levels increased nine-fold (P = 0.001, mixed effects model, Fig **S2.2**). These induced levels were retained to a lower extent in the absence of stress until the end of the fitness assay, with aphid pre-treated plants accumulating 60% more tyramine than control pre-treated plants (P(Pre-treatment) = $7x10^{-6}$, mixed effects model, Fig **2b**). In addition, tyramine levels were transgenerationally primed: under aphid herbivory, tyramine levels tripled when plants were pre-treated with aphids (P(Pre-treatment$_x$Treatment) = 0.06, Fig **2b**). The transgenerational retention and priming of tyramine were not only observed in Sp102, but also in half of the other genotypes (Fig **S2.3**). In essence, tyramine is induced upon aphid herbivory and the elevated levels are transgenerationally retained, as well as transgenerationally primed.

Transgenerational priming suggests that not only is tyramine itself vertically transmitted, but its biosynthetic machinery transgenerationally regulated. Tyramine is synthesized by tyrosine decarboxylases (TYDCs), which decarboxylate L-tyrosine into the amine tyramine and CO_2 (Fig **3a**). Based on the annotated *S. polyrhiza* genome[80], we identified three putative *TYDCs*: *TYDC1*, *TYDC2*, and *TYDC3*. To confirm that the encoded enzymes decarboxylase tyramine, we constructed a phylogenetic tree based on amino acid sequence similarity with characterized aromatic amino acid decarboxylases from other species, as well as the annotated *S. polyrhiza* L-tryptophan decarboxylase (TDC), a closely related enzyme (Table **S2**-**S4**). All three putative *S. polyrhiza* TYDCs clustered together with other tyramine decarboxylases (Fig **3b,** Fig **S3.1**); however, these clusters also contained other enzymes: phenylacetaldehyde synthases, which decarboxylate and deaminate L-phenylalanine, and aromatic L-amino acid decarboxylases (AADC), versatile enzymes that decarboxylates aromatic amino acids to amines like serotonin, dopamine, histamine and tyramine. Notably, the cluster containing SpTYDC1 and SpTYDC2 contained almost exclusively L-tyrosine decarboxylases, including the monocotyledon *Oryza sativa* TYDC. In contrast, the L-tryptophan decarboxylases built a distinct monophyletic cluster with only that catalytic function (Fig **3b**). Taken together, although all three SpTYDCs may decarboxylate L-tyrosine, as well as other aromatic amino acids, TYDC1 and TYDC2 are the most likely candidates to function primarily as L-tyramine decarboxylases.

To reconstruct the evolutionary history of these genes, we compared their amino acid and DNA sequence similarities, as well as chromosomal positions. *TYDC1* and *TYDC2*, both lacking introns, are the most closely related genes, sharing 82% amino acid and 80% nucleotide sequence similarity. However, the two genes are located on different chromosomes: *TYDC1* on chromosome 15, and *TYDC2* on chromosome 3. Notably, within a region spanning 18 kb,

the chromosomal segment containing *TYDC1* harbours three tandem copies of the gene along with one *TYDC1* pseudogene (*TYDC1-1*, *TYDC1-2*, *TYDC1-3*, Fig **3c**, Table **S4**). Similarly, *TYDC2* is accompanied by a *TYDC2* pseudogene (Fig **3c**, Table **S4**). TYDC3, sharing 53-56% amino acid and nucleotide sequence similarity with TYDC1, was located on chromosome nine, contained 12 introns and 6 tandem copies (Fig **3c**, Table **S4**). Taken together, the expansion of the *TYDC* gene family suggests an important function for tyramine.

We next evaluated whether the transcript levels of these three putative tyrosine decarboxylases match the tyramine levels, using RNAseq data generated from the plant material used for the metabolite quantification. The expression levels of two genes, *TYDC1* and *TYDC2*, correlated positively with tyramine levels: *TYDC1* was expressed only under aphid herbivory, and under these conditions, its expression doubled in plants with an aphid pre-treatment (P < 0.05, generalized model, Fig **3d**). *TYDC2* showed a similar pattern, although at lower levels, with aphid pre-treatment increasing its expression under control conditions rather than under aphid herbivory (Fig **3d**). In contrast, *TYDC3* was expressed at high levels uniformly across all conditions (Fig **3d**). Thus, *TYDC1* was transgenerationally primed and likely the primary enzyme responsible for the elevated tyramine levels observed across generations.

We next tested whether tyramine alters plant and aphid fitness, since tyramine–a potential toxin, plant cell wall reinforcer, insect neurotransmitter, and semi-essential amino acid derivative (Fig **S1.1**)–could either boost or hamper aphid reproduction. First, we measured plant and aphid fitness over eight days of aphid feeding on Sp102 plants supplemented with tyramine at concentrations that produced internal levels of approximately 0.01 µmol g^{-1} fresh weight (≈10 µM), matching those observed in our transgenerational experiment (Fig **4a**; Fig **S4.1**). While tyramine application slightly reduced plant fitness under control conditions, this correlation was reversed under aphid herbivory (P(Tyramine$_x$Treatment) = 0.08, linear model, Fig **4a**) (P(Tyramine$_x$Treatment) = 0.08, linear model, Fig **4a**). Furthermore, tyramine decrease aphid reproduction in a dose-dependent manner, a pattern that was consistent after two days (P = 0.03, linear model, Fig **S4.2**) as well as eight days of feeding (P = 0.004, generalized linear model, Fig **4a**). These data suggests that plants produce tyramine to supress aphid load.

Second, to confirm that tyramine hampers aphid fitness rather than serving as a precursor for the dark pigment melanin, we measured coloration of aphids when feeding on the plants supplemented with tyramine. After eight days of feeding, aphids became redder rather than darker when tyramine was supplemented to the plants (P = 0.05, generalized linear model, Fig **4a**). Thus, tyramine is unlikely used by the aphids as a melanin precursor.

Third, as tyramine supplementation to plants altered also the levels of dopamine inside the plants (Fig **S4.1**), we fed aphids with sugar water supplemented with tyramine (Fig **4b**) to test whether tyramine is sufficient to reduce aphid fitness and increase aphid redness. As expected, tyramine reduced aphid reproduction across the five days of feeding in a dose-dependent manner: already 2 µM of tyramine, corresponding to 20% of the in-planta tyramine levels (Fig **S4.1**), reduced aphid reproduction (P = 9x10^{-5}, linear model, Fig **4b**). Similarly, tyramine addition increased aphid redness at a concentration as low as 2 µM (P = 0.02, linear model, Fig **4b**). Taken together, these data support the notion that induction, transgenerational retention and priming of tyramine protects plants against its aphid herbivore across generations.

As in our experiment aphids benefitted rather than suffered from ancestral herbivory, we assessed the contribution of other metabolites that are putatively involved in plant defence against aphids: jasmonic acid and its isoleucine derivative jasmonic acid isoleucine. These jasmonates were not induced upon first time exposure to aphid herbivory (P > 0.05, mixed effects model, Fig **S5.1**), and consequently, under control conditions, their concentrations were not affected by ancestral herbivory (JA: P(pre-treatment) = 0.21, Fig **2b**; JA-Ile: P(pre-treatment) = 0.24, Fig **S5.2**). However, under recurrent aphid herbivory, aphid pre-treated plants accumulated 70% higher levels of jasmonic acid compared to control pre-treated plants (P(Pre-treatment$_x$Treatment) = 0.009, Fig **2b**). Similar patterns and magnitudes were observed for jasmonic acid-isoleucine (P(Pre-treatment$_x$Treatment) = 0.04, Fig **S5.2**). These data show that jasmonates are transgenerationally primed under recurrent aphid herbivory.

As the biosynthetic pathway of jasmonates involves multiple enzymes with several homologues whose functions and relative contributions are unclear in *S. polyrhiza*, we did not attempt to identify differentially expressed genes in the biosynthetic pathway of jasmonates. Instead, we assessed whether jasmonic acid protects plants against aphids by externally applying methyl-jasmonate to the *S. polyrhiza* genotype SP102 (Fig **5**). Unexpectedly, plants that were continuously exposed to methyl-jasmonate supported 17% higher aphid population growth rates after eight days compared to control plants (P = 0.05, mixed effects model, Fig **5**). These data suggest that aphids can transgenerationally prime jasmonates in their host offspring for their own benefit.

Plants overcome maladaptive plasticity in natural conditions

To test whether tyramine and jasmonates are not only transgenerationally plastic indoors but also outdoors—and thereby affect plant and aphid fitness, as well as plant morphology—we performed reciprocal transplant experiments in outdoor mesocosms. In these mesocosms,

Sp102 was grown for two consecutive years in large populations—up to a million individuals—under both low and high herbivory of the waterlily aphid (Fig **6a**, Fig **S6.1**)[45].

First, we tested whether tyramine and jasmonates are plastic in the outdoor mesocosms. In July 2021, when aphid intensities peaked (Fig **6b**), tyramine levels were 50% higher in the aphid exposed plants compared to controls ($P = 0.004$, mixed effects model, Fig **S6.2**). Even in August 2021, when aphid densities levelled between the two treatments, tyramine levels remained 76% higher in aphid exposed plants than controls ($P = 1x10^{-9}$, mixed effects model, Fig **S6.2**). Furthermore, tyramine levels were 88% higher in the turions —the overwintering stage —of aphid exposed plants than controls when collected in October 2021 (Fig **S6.2**). In contrast, the levels of jasmonates were not affected by aphid herbivory at any time point ($P > 0.05$, mixed effects model, Fig **S6.2**).

Second, we assessed whether elevated levels of tyramine are transmitted across years, and thereby affect plant and aphid fitness, as well as stress-induced morphological changes. Thereto, we collected the turions that emerged in spring 2022, germinated them and grew the resulting plants for three weeks under control conditions in boxes floating in the ponds ("recovery"). Subsequently, we grew plants for another three weeks with and without the aphids ("treatment", Fig **6c-d**, Fig **S6.3**). Neither tyramine nor jasmonate levels were affected by the previous year's herbivory- regardless of the presence of recurrent aphid herbivory-in either the plants or the spring-collected turions (Fig **S6.4**). Consequently, aphid herbivory in the previous year did not affect aphid or plant fitness, nor plant morphology (*P*(Pre-treatment) > 0.5, mixed effects model, Fig **S6.5)**. These data show, first, that elevated tyramine levels under aphid herbivory resulted from plasticity rather than genetic change and, second, that outdoors, aphid-induced tyramine levels are not transmitted through the resting stage across years.

Third, we tested whether tyramine and jasmonates are transgenerationally plastic within a year. Thereto, in July 2022—when aphid densities peaked—we grew randomly selected plants in floating boxes under control conditions for three weeks, followed by three weeks under either continued control conditions or aphid herbivory (Fig **6c-d**). Similar to our indoor experiment, ancestral aphid herbivory boosted tyramine levels up to threefold regardless of recurrent stress (*P*(Pre-treatment) = 0.03, mixed effects model, Fig **6e**). In contrast, neither initial herbivory nor ancestral herbivory altered jasmonate concentrations (JA: *P*(Pre-treatment) = 0.6, mixed effects model, Fig **6f**; JA-Ile: *P*(Pre-treatment) = 0.5, mixed effects model, Fig **S6.6**). Consequently, aphid tended to reproduce worse rather than better on aphid pre-treated plants ($P = 0.08$, mixed effects models, Fig **6g**). Nevertheless, similar to indoor conditions, ancestral aphid herbivory still inflicted stronger stress-induced morphological changes (*P*(Pre-treatment) = 0.003, mixed effects model, Fig **S6.7**), but did not alter plant fitness (*P*(Pre-treatment) = 0.5,

mixed effects models, Fig **6h**). Taken together, these data indicate that plants are transgenerationally plastic outdoors within but not across years, and that plants can overcome aphid-induced priming of jasmonates and the resulting boost in aphid fitness when grown in large populations under natural conditions.

Discussion

Transgenerational plasticity is typically considered adaptive under recurrent environmental conditions. However, experimental evidence—especially from natural settings—remains scarce. Here, we show that aphids, unexpectedly, alter their host plant across generations to their own benefit. Yet, these maladaptive, aphid-induced transgenerational effects were mitigated when the plants were grown under natural outdoor conditions.

Transgenerational plasticity is often assumed to be adaptive. However, this is not always the case, for several reasons. First, stressed parents may provision less to their offspring, thereby reducing offspring performance regardless of the environment they experience, a phenomenon often referred to as the "silver spoon effect"[12]. Second, herbivores and pathogens can manipulate host physiology for their own benefit[13, 46], and such manipulations may be inherited across generations. Whether these host-plant manipulations can persist for multiple generations, however, remains largely unclear. In fact, herbivores and pathogens often enhance rather than reduce plant resistance in subsequent generations. For example, the chewing herbivore *Pieris rapae* induced resistance in *A. thaliana* that persisted for two generations[10]. Similarly, exposure of *A. thaliana* to the bacterial pathogen *Pseudomonas syringae* increased resistance to the oomycete *Hyaloperonospora arabidopsidis* in the offspring, even after one stress-free generation [3]. Here, we show that herbivore-induced manipulations of host plants can extend across generations and, depending on the plant genotype, reduce plant fitness while enhancing herbivore reproduction.

Aphids likely benefited from increased fitness due to a boost in jasmonate levels. Although jasmonates are often considered to reduce aphid performance[47], this is not always the case. For example, elevated levels of the jasmonate precursor OPDA were associated with higher aphid reproduction in *S. polyrhiza*[11]. Furthermore, exogenous application of jasmonates increased aphid reproduction in other *S. polyrhiza* genotypes as well[11]. Likewise, in *S. bicolor*, exogenous jasmonate application enhanced aphid feeding and colonization, and plants genetically impaired in jasmonate biosynthesis supported lower aphid population growth compared to wild types[19]. In our study, the transgenerational priming of jasmonates–and the associated increase in aphid fitness–was overcome under natural outdoor conditions, possibly because jasmonate levels were ten-fold higher outdoors than indoors. Together, these findings

underscore the importance of examining metabolite function within the specific context of each experimental system and highlight the need for ecologically realistic outdoor experiments.

While transgenerational priming of jasmonates was likely maladaptive for the plant, tyramine priming, which occurred both indoors and outdoors, appeared adaptive. Tyramine–which accumulates in different cell types including the phloem[48, 49]–may serve multiple roles in plant–aphid interactions. It could act as a defence metabolite, either through direct toxicity, cell wall strengthening, or conversion into toxic phenolamides or other alkaloids[23, 24, 50]. Alternatively, it may increase melanisation through its hormonal activity[27] or serve as a nutritional compound for aphids by acting as a precursor of octopamine[25, 51], thereby sparing L-tyrosine from decarboxylation. Additionally, plant-derived tyramine or octopamine may influence aphid behaviour and fitness as insect neurotransmitters, neuromodulators and neurohormones[26, 51]. In our study, tyramine reduced aphid reproduction and increased aphid redness at ecologically relevant concentrations when applied both via the plant and a sucrose solution, showing that tyramine alone is sufficient for these effects. The red coloration may stem from altered synthesis or transport of pigments such as aphins or carotenoids[52, 53], rather than altered melanisation. Together, these findings suggest that tyramine, and its transgenerational plasticity observed both indoors and outdoors, is likely adaptive to plants when attacked by the aphids.

Enhanced tyramine levels under recurring aphid herbivory were likely due to the transgenerational priming of the tyrosine decarboxylase gene *TYDC1*. Interestingly, *TYDC1* occurs as three tandem copies, along with an additional pseudogene. Moreover, *TYDC1* has a closely related paralog, *TYDC2*, which shares 80% DNA sequence identity but is located on a different chromosome. In our experiment, *TYDC1* was expressed at roughly three times the level of *TYDC2*, possibly due to its higher copy number.

Expansion of gene families underlying transgenerationally plastic traits have been previously reported in the nematode *Pristionchus pacificus*[54]. One possible explanation is that such genes, due to their elevated transcriptional activity, are more prone to retroposition—a process in which mRNA is reverse transcribed and inserted into the genome, typically yielding intronless copies at new chromosomal locations[29]. This pattern is consistent with the genomic features observed for both *TYDC1* and *TYDC2*. Thus, *TYDC1* may be a retrogene derived from *TYDC2*, or vice versa, or alternatively, both may descend from a common retrogene ancestor derived from the intron-rich *TYDC3*. In contrast, the tandem repeats of *TYDC1* were likely generated through a different mechanism, most plausibly tandem duplication via unequal crossing-over[29]. Interestingly, retrogenes themselves appear to be prone to tandem duplication: in *Thlaspi arvense*, orthogroups that had significantly expanded and included retrogenes contained more tandem duplicates than significantly expanded orthogroups

composed solely of non-retrotransposed genes[55]. To test whether the *TYDC* copies originated via retroposition, one could examine the presence of poly-A remnants at the 3′ end and flanking direct repeats—two hallmark features of retroduplication[29]. The identification of these features would support the hypothesis that *TYDC1* or *TYDC2* arose through retroposition. More broadly, retroposition of transgenerationally plastic genes would suggest that retroduplication acts as a mechanism by which the plasticity of a responses becomes refined, stabilized or modified through genetic change, a process known as genetic accommodation[36, 56, 57].

While our study revealed multigenerational transgenerational plasticity in both metabolite levels and gene expression, we did not attempt to dissect the underlying epigenetic mechanisms of inheritance. In principle, plastic responses that persist across multiple generations—such as those observed here—are considered to have an epigenetic basis[15]. Due to its vegetative reproduction, epigenetic marks could be transmitted in *S. polyrhiza* via several mechanisms, including small RNAs and DNA methylation. For instance, small RNAs can move through plasmodesmata and the phloem[58, 59], the latter connecting mother and daughter fronds for few days, and can be amplified in each generation by RNA-dependent RNA polymerases—enzymes that are present in *S. polyrhiza*[60]. Thus, vertical transmission of plant- or aphid-derived small RNAs could potentially mediate the observed responses. Alternatively, aphid-induced changes in DNA methylation could be inherited, as vegetative reproduction involves only mitotic divisions, allowing stress-induced methylation patterns to persist across generations[61, 62]. In addition to these mechanisms, the inheritance of histone modifications or the vertical transmission of metabolites, microbes or effector proteins cannot be excluded. To elucidate the underlying mechanism, future studies should profile epigenetic marks in a non-targeted manner, manipulate components of the epigenetic machinery genetically, or perform forward genetic screens using random mutagenesis, as in Quiobe, Kalirad [63], to directly assess the epigenetic basis of the observed transgenerational effects.

Taken together, our data demonstrate that herbivores can alter plant physiology across generations to enhance their own fitness. However, we also show that plants can counteract such transgenerational changes under natural conditions, likely through mechanisms that buffer or override stress memory. These findings challenge the common assumption that transgenerational plasticity is inherently adaptive. Our work further suggests that transgenerational plasticity may play an important role in the evolutionary dynamics of plant–herbivore interactions. We speculate that transgenerational plastic responses might become genetically encoded through processes such as gene duplication or retroposition. This perspective aligns with the concept of genetic accommodation and supports the view that phenotypic plasticity within and across generations not only enables rapid responses to environmental change but also creates substrates for long-term evolutionary innovation.

Materials and methods

Plant and aphid growth conditions

For the indoor experiments, we grew plants and aphids inside growth cabinets (L4Z-TDL+, poly klima GmbH, Freising, Germany) operating at 28°C, 150 µmol photons m^{-2} s^{-1}, with a 16:8 hrs light/dark cycle. Plants were cultivated in N-medium[64], which supports optimal growth. To minimize microbial contamination, we surfaced-sterilized all plants at the beginning of experiments, after which the plants were grown for four weeks in 250 mL Erlenmeyer flasks (Fisher Scientific GmbH, Schwerte, Germany) before starting the experiment. Aphid nymphs of the strain RN001[39] were cleared of surface fungi and algae by rearing them on sterile plants for at least five consecutive generations; during this time, the insects were sprayed every week with a copper-based antifungal solution commonly used in aquaria (Sera mycopur, Heinsberg, Germany). The resulting aphids were then acclimated for one month on sterile plants of genotype Sp050, grown in autoclaved 2.6 L boxes (Lock & Lock, Seul, South Korea) filled with 1 L N-medium. The boxes were covered with lids in which an area of 25 cm^2 was excised and covered with a metal mesh (mesh opening 0.3 mm, wire diameter 0.2 mm; Haver & Boeker, Oelde, Germany) for gas exchange.

For outdoor experiments, we pre-cultivated plants and aphids in 2.6 L boxes (Lock & Lock) filled with 1 L N-medium within walking chamber maintaining 26°C, 125 µmol photons m^{-2} s^{-1} and a 16:8 hrs light/dark cycle. In spring 2021 plant and aphid populations were stablished as described in Schäfer, Malacrinò[45].

Statistical analysis

All data were analysed in R v4.4.0[65]. As fitness metric, we calculated relative growth rates[66] (Equation **S1**), hereafter "population growth rates", based on plant surface area, and aphid number. For short assays with aphids, we calculated the daily per capita reproduction rate, defined as the number of nymphs produced per mother per day[67] (Equation **S2**). To assess plant morphology, we measured surface area per frond (mm^2), fresh weight per frond (mg) and surface area per fresh weight (mm^2 mg^{-1}). Additionally, we calculated the pre-treatment ratios by dividing each aphid pre-treated plant measurement by the mean value of the control pre-treated plants within each treatment environment[68] (Equation **S3**).

Mixed effect models were performed with the package glmmTMB v1.1.9[69]. In the absence of random factors, we performed simple linear models and generalized models with lm and glm, respectively[65]. *P*-values of the fixed-effects models were estimated with analysis of variance (Anova), using Wald chi-square of the package car[65]. R^2 values were obtained with ggpmisc v0.5.6[70]. Post-hoc *P*-values were calculated with emmeans v1.10.2[71] and letters were obtained with multcomp v1.4.25[72]. To assess the fitted models, we used the package DHARMa v0.4.6[73]

and effects v4.2.2[74]. To estimate deviation from neutral, we performed Wilcoxon Rank-Sum tests of the pre-treatment ratios per environment against one (mu=1). We displayed plots with ggplot2 v3.4.3[75] and organized data with readxl v1.4.2[76], data.table v1.14.8[77], tidytext v0.4.1[78] and dplyr v1.1.2[79].

Transgenerational experiment with eight S. polyrhiza genotypes

Pre-treatment, recovery, and fitness and phenotype assays

To assess whether ancestral aphid herbivory alters duckweed fitness, phenotype, and aphid reproduction, we exposed single-descendant lineages of *S. polyrhiza* to five generations of *R. nymphaeae* herbivory ("pre-treatment"), followed by five generations under control conditions ("recovery"). Subsequently, we measured plant and aphid fitness, as well as plant morphology, both in the presence and absence of aphid herbivory across eight days of free growth ("treatment") (Fig **1a**). We used eight range-wide sampled *S. polyrhiza* genotypes (Table **S1**)– two from each of its four genetic clusters[40, 80]–and the German *R. nymphaeae* RN001 strain[39], with eight replicates per genotype and treatment.

Prior to the experiment, we acclimated the plants by propagating individual fronds as single descendant lineages for four generations. We then began the pre-treatment phase by allocating the first and second offspring of each plant equally to a control and an herbivory treatment, the latter consisting of three adult aphids per plant. Lineages were propagated for five generations under these conditions ("pre-treatment"), then for an additional five generations under control conditions ("recovery"), resulting in generation 10. Throughout the acclimation, pre-treatment and recovery phase, each plant was grown individually in a 30 mL polypropylene tube (Fisher Scientific, Waltham, USA) filled with 25 mL N-medium and closed with a sterilized foam plug (CarlRoth, Karlsruhe, Germany). At each generation, as soon as an offspring frond had fully expanded, we transferred that offspring into a fresh tube. To distinguish mother and offspring, we marked the mothers with a permanent marker pen (Stabilo OHPen Universal, Heroldsberg, Germany).

To assess plant fitness and phenotype, as well as aphid fitness, we allocated the first and second offspring of generation 10 equally to control conditions or aphid herbivory, with three adult aphids per plant ("treatment" in the "phenotype and fitness assay"). These offspring were grown in 250 mL transparent polypropylene beakers filled with 150 mL N-medium and covered with perforated transparent lids (Fig **1a**). Medium was replaced every 3-5 days to overcome any contamination caused by the aphids. After eight days, we counted the number of aphids and subsequently removed them. We then harvested the plants by briefly drying them with tissue paper, weighting them and immediately flash-freezing them in liquid nitrogen. To quantify surface area and frond number, we captured images at the beginning and end of each

assay using a camera box installation with a webcam (HD Pro Webcam C270, Logitech, Lausanne, Switzerland; webcam software 2.12.8). We analysed surface area of plants with the package pliman v2.1.0[81] and counted fronds with dotdotGoose v1.5.3[82]. All samples were stored at -80°C until metabolite and RNA extractions.

To assess whether the pre-treatment affects plant and aphid fitness, as well as plant morphology, across the eight genotypes, we fitted linear mixed effect models with pre-treatment, treatment and their interaction as fixed effects, and replicate, genotype, number of offspring for assays and the rack as random effects: Variable ~Pre-treatment*Treatment +(1|Replicate)+(1|Genotype)+(1|Offspring)+(1|Rack). As variables, we used plant and aphid population growth rates, surface area per frond, biomass per frond, and surface area per biomass. To analyse whether the pre-treatment ratios per genotype were affected by the treatment, we used a simpler mixed-effects model with treatment as a fixed effect and replicate as a random effect: Variable ~Genotype*Treatment +(1|Replicate). When analysing aphid populations growth rates and their pre-treatment ratios, we dropped the treatment fixed effect. To identify genotypes in which the pre-treatment ratios of plant and aphid fitness differed from neutral, we used Wilcoxon Rank Sum tests.

Using genotype means, we fitted linear models to examine two relationships: first, whether the plant fitness pre-treatment ratios under aphid herbivory correlated with the corresponding aphid fitness pre-treatment ratios; and second, whether the plant fitness pre-treatment ratios measured under control conditions correlated with those measured under aphid herbivory.

Metabolite quantification

Amino acids, amines, phenylpropanoids and phytohormones were analysed via LC-MS following Malacrinò, Böttner[83]. In short, we ground the flash-frozen plant material of each replicate to a fine powder using a MM301 Mixer Mill (Retch, Haan, Germany). Subsequently, we extracted 20 mg ground samples using acidified methanol (MeOH:water:formic acid 15:4:1 v/v/v) containing the internal standards for the quantification of phytohormones and phenylpropanoids. We then diluted an aliquot of this extract 1:100 in an aqueous solution of isotope-labelled amino acids (algal amino acid mixture-13C-15N; Sigma-Aldrich) to quantify free amino acids and amines. The remaining extract was purified and partially concentrated via two solid-phase extraction steps using Chromabond HR-X and HR-XC columns (Macherey-Nagel, Düren, Germany) to quantify phytohormones and phenylpropanoids. The MRM-settings, ESI-settings, the gradient program and column oven settings were used as described in Malacrinò, Böttner[83] and [11], with the addition of kynurenine, kynurenic acid, serotonin, L-DOPA, dopamine, GABA and melatonin to method 1A (Table **S2**). Analytes were quantified

based on internal standards. The peaks from all metabolites were integrated with LabSolution Insight Version 4.0 SP6 (Shimadzu).

To determine whether the pre-treatment altered metabolites accumulation, we fitted linear mixed-effects models separately within control and aphid treatments, using the pre-treatment as a fixed effect and the replicate and genotype as random effect: Metabolite ~Pre-treatment +(1|Replicate)+(1|Genotype). Similarly, to assess whether metabolites accumulation is transgenerationally primed (interaction of pre-treatment and treatment), we fitted a model with the full factorial design: Metabolite ~Pre-treatment *Treatment +(1|Replicate)+(1|Genotype). When analysing Sp102, we dropped the genotype random effect in both mixed effect models.

Identification of L-tyrosine decarboxylases in S. polyrhiza

To identify the genes responsible for tyramine biosynthesis, we first mined the latest *S. polyrhiza* genome annotation[80]–which is based on the clone 7498 genome (Sp004)–for loci annotated as L-tyrosine decarboxylase (*TYDC*). This search returned three candidates: *TYDC2* (*SpGA2022_004481*), *TYDC1* (*SpGA2022_016537*), and *TYDC3* (*SpGA2022_012613*). Because tyrosine decarboxylases are sometimes mis-annotated as tryptophane decarboxylases, we also retrieved the genes annotated as tryptophane decarboxylases (*TDCs*): *SpGA2022_000740*, *SpGA2022_000739*, and *SpGA2022_000738*. To confirm that the open reading frames of these genes are complete and to identify any tandem duplicates, we searched for homologs in two additional *S. polyrhiza* genome assemblies–clone 7498[37] and clone 9512[38]–using amino-acid sequence homology (BLASTP). Gaps in the *TYDC* region of the 7498 assembly prompted us to focus subsequent analyses on clone 9512. Finally, we aligned each predicted coding sequence to the clone 9512 assembly with ClustalW in BioEdit Sequence Alignment Editor v7.0.9.0[84].

To corroborate the function of the annotated L-tyrosine and L-tryptophan decaboxylases, we constructed a phylogenetic tree with characterized L-tyrosine and L-tryptophan decarboxylases from other species. We constructed two phylogenies of nucleotide sequences with RAXML v8.2.10 for multi-threading, using advanced vector extensions; one phylogeny included the main annotated genes and a the second included all the identified copies of each gene. We applied a rapid bootstrap analysis and searched for best-scoring maximum likelihood trees, using the Whelan and Goldman substitution model with gamma-distributed rate variation and 100 bootstrap replicates. Finally, to identify relatedness among the genes and gene copies, we built an identity matrix using the default similarity matrix BLOSUM62 of BioEdit.

Transcriptome sequencing

To assess whether the identified tyrosine decarboxylases are transcriptionally regulated by the pre-treatment, we sequenced the transcriptomes of genotype Sp102 using the plant material from five replicates from each treatment and pre-treatment combination from the fitness and phenotype assay. Thereto, we extracted whole RNA from 10 mg of ground plant material with the NucleoSpin RNA Plant Mini kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Then, library preparation with polyA enrichment and sequencing were performed by Novogene GmbH (Germany). After quality control, the libraries were sequenced on Illumina NovaSeq X Plus with 150 base pair paired end reads. From all 24 libraries, we obtained 3.8-9.9G; after filtering low-quality reads, these data resulted in an average of 38 Mio reads per library (Table **S8**). To quantify gene expression, Novogene perfomed a bioinformatic analysis of the data, which included quality control, mapping to the 7498-clone genome reference[80], gene expression quantification and differential expression between pre-treatments within control and aphid herbivory treatments. An average of $4x10^7±8x10^6$ clean reads per library were obtained (Table **S8**) and mapped with HISAT2[85] to the reference gene, obtaining an average mapping rate of 80%. For differential gene expression, abundance of transcripts–standardized in fragments per kilobase of transcript sequence per millions base pairs sequences (FPKM)–was used for hypothesis testing with DESeq2[86], considering a negative binomial distribution and Wald test to obtain *P* values. To test whether *TYDCs* are differentially expressed, we considered *P*-values prior FDR corrections, as we assessed the expression values of only five genes.

External application of tyramine to plants

To test whether tyramine alters plant and aphid fitness, as well as aphid melanisation, we externally applied tyramine to the plants and measured its effects on aphid population growth rates and colouration. First, we determined at which external tyramine concentrations the plant tyramine levels raised to similar extent as observed in the indoor transgenerational experiment. Thereto, we dissolved tyramine (2-(4-Hydroxyphenyl)-ethylamine; Carl Roth, Karlsruhe, Deutschland) in DMSO and diluted it in N-medium to reach 0 µM, 100 µM, 200 µM and 400 µM tyramine and 0.05% v/v DMSO in N-medium. We then placed a single frond of genotype Sp102 into 150 mL of these tyramine-supplemented media, with five replicates per concentration. After eight days of free growth, we determined plant population growth rates as described above. Furthermore, we washed the plants by dipping them inside distilled water and subsequently determined shoot tyramine and dopamine concentrations as described above.

Second, we examined whether supplementing plants with tyramine benefitted plant fitness under herbivory and reduced aphid population growth rates. Tyramine was dissolved in DMSO and added to N-medium to final concentrations of 0, 50, 100, 200, and 400 µM tyramine and 0.05% v/v DMSO in N-medium. To synchronize plant age, we grew plants of genotype Sp102 as single descendants for two generations inside polypropylene tubes. We then transferred a single frond frond-the first offspring of generation two-to 250 mL plastic beakers containing the tyramine-supplemented media. After five days of growth, we infested half of the beakers at each concentration with three similarly aged adult aphids, approximately six days old, yielding 12 infested and 12 un-infested replicates per concentration. After days two and eight days, we counted the number of aphids to calculate daily per-capita reproduction rates and population growth rates. Furthermore, we captured an image of the beakers at the beginning and end of the assay to estimate plant population growth rates. To assess whether tyramine supplementation altered plant fitness in the presence and absence of aphid herbivory, we fitted linear models with the mean plant population growth rates as a response variable and log-transformed tyramine concentration as well as treatment as explanatory variables. Log-transforming tyramine concentration improved model fit. To assess whether tyramine supplementation reduced aphid fitness, we correlated the mean daily per-capita reproduction rate with the external tyramine concentration using linear models.

To assess whether tyramine affected aphid melanisation, we collected five wingless adult aphids per replicate, mounted them on sticky flea traps, and photographed with an MZ10 F Modular Stereo Microscope (Leica Microsystems, Wetzlar, Germany) using Leica Application Suite X v3.10.0.28982. Cuticle colour intensity was quantified from the images with the "Histogram" function in ImageJ 64 v5[87]. To test whether tyramine reduces aphid redness—corresponding to increased melanisation—we first averaged the pseudo-replicates within each replicate, then averaged across replicates, and finally correlated the resulting treatment means with tyramine concentration in a linear model.

Artificial diet supplemented with tyramine

We evaluated whether tyramine alone reduces aphid fitness and enhances cuticle redness by measuring per-capita growth rates and colour of aphids reared on sucrose diets supplemented with ecologically relevant tyramine levels. A 35 % (w/v) sucrose solution in distilled water served as the base medium; tyramine was first dissolved in 100% DMSO and then diluted to produce the final concentrations of 0, 2, 5, 10, 20, 50, 100, 200, and 400 µM tyramine and 0.5% v/v DMSO in an aqueous sugar solution. We dispensed 8 mL of each solution into 60x15 mm petri dishes (Sarstedt, Nümbrecht, Germany), with five replicates per concentration. We stretched Parafilm (Paul Marienfeld, Lauda Königshofen, Germany), and cut it into 1 cm²

squares. We then floated three squares on the surface of the solution in each beaker, transferred eight similarly aged aphids-approximately 4 days old-onto the squares, and sealed the plates with Parafilm. Five days later, we counted the aphids to calculate per-capita growth rates. We then collected five wingless adults per replicate for colour analysis as described above. After averaging per-capita growth rates and aphid redness for each treatment, we used linear models to correlate these means with the natural logarithm of tyramine concentration. The log transformation improved model fit.

External application of methyl jasmonate to plants

To evaluate the impact of jasmonates on aphid fitness, we monitored aphid population growth on Sp102 plants continuously treated with methyl-jasmonate, following Chávez, Schreyer [11] but applying the hormone throughout the entire experiment. To homogenize plant age, we propagated plants as single descendants for three generations and transferred the first offspring of generation three into 150 mL of N-medium containing 100 µM methyl-jasmonate solution and 0.5 % v/v DMSO; control plants received N-medium with 0.5 % v/v DMSO only. After two days of induction, we added three adult aphids to half of the vessels, resulting in 10 replicates per treatment. After eight days of free growth, we counted the number of aphids to calculate population growth rates. We excluded in total four replicates because winged morphs appeared by day two, strongly reducing reproduction. The experiment was performed in two batches that were separated by one day. To test whether methyl jasmonate reduced aphid population growth, we fitted a mixed-effects model with treatment as a fixed effect and batch as a random effect: aphid population growth rate ~Treatment +(1|Batch).

Transgenerational experiments in natural conditions

To assess whether plants are transgenerationally plastic also outdoors, we used an outdoor mesocosm experiment at the Swiss Federal Institute of Aquatic Science and Technology (Eawag) in Dübendorf, Switzerland in which genotype Sp102 had been grown for two consecutive years with and without herbivory of the *R. nymphaeae* genotype RN001 (pre-treatment or pond treatment) in 2021 and 2022[45]. Ponds had a size of 4 m^2, depth of 1.5 m and volume of 15 m^3, and at the peak of duckweed population growth, contained approximately 1 million duckweed individuals.

Phenotypic plasticity within the year

To test whether tyramine and jasmonates are phenotypically plastic outdoors, we randomly selected plants of the ponds in July and August 2021. Plant material of whole fronds was

cleared of aphids, rinsed in tap water, briefly dried with a tissue paper, flash-frozen in liquid nitrogen and subsequently stored at -80 degree until metabolite analysis, see below. To measure the levels of these metabolites in turions, we collected detached turions from the bottom of the ponds in the shallow water zones in October 2021. Turions were briefly dried with tissue paper, flash-frozen in liquid nitrogen and stored at -80°C until metabolite analysis.

For metabolite quantification, we freeze-dried shoot and turion material, ground it to a fine powder, and extracted approximately 10 mg for tyramine and jasmonate analysis following the protocol used in the indoor transgenerational experiment. To test whether the pond treatment alters metabolite accumulation, we fitted mixed effect models with pond treatment as a fixed effect and group of ponds as a random effect: Metabolite ~Pond treatment +(1|Group of ponds), where "Group of ponds" represented the physical four-pond block in the field (Fig **6a**).

Transgenerational plasticity across years

To assess whether tyramine and jasmonates are transgenerationally plastic across years, and thereby alter plant and aphid fitness, as well as plant morphology, we performed in-situ transplant experiments. During spring of 2022 (calendar week 17, Fig **6b**), we collected 20 *S. polyrhiza* surface-floating turions per pond and transferred them to boxes (17 cm width × 12 cm deep × 11.5 cm high, Eurobox, Auer Packaging) that were floating within the aphid herbivory ponds. Thus, each herbivory pond contained two floating boxes: one held its own turions, and the other held turions collected from a neighbouring control pond. We used only the herbivory ponds to avoid contaminating the control ponds. After three weeks of free growth ("recovery"), we transferred 20 newly emerged fronds from each box into fresh floating boxes and grew the plants freely for another three weeks under control conditions or *R. nymphaeae* herbivory, with 5 aphids per box ("treatment"). We then harvested the plants: we counted the aphids, gently dried the plants with tissue paper and flash-froze the plant material in liquid nitrogen. All plant material was stored at -80°C until metabolite extraction. We quantified plant surface area and frond number from photographs taken at the start and end of the treatment phase with a Nikon D5300 (Nikon, Tokyo, Japan) mounted in a camera box. Surface area was measured in ImageJ 64 v5[87] and fronds counted with dotdotGoose v1.5.3[82].

To quantify tyramine and jasmonate concentrations, we ground the frozen plant and turions material, aliquoted 20 mg ground material and subsequently measure tyramine and jasmonate levels as described above. To assess whether the pre-treatment altered metabolite accumulation in turions, we fitted mixed effects models with the ancestral treatment as fixed effect and the group of four ponds as random factor. Therefore, the model was Metabolite ~Pre-treatment +(1|Receiving pond). Meanwhile, to assess whether the pre-treatment altered metabolite accumulation in plants, we fitted mixed effect models with the pre-treatment and

treatment as fixed effects and the receiving pond ("Receiving pond") as random effect. The model was: Metabolite ~Pre-treatment *Treatment +(1|Receiving pond). Plant fitness and morphology were analysed in a similar way, using the model Variable ~Pre-treatment *Treatment +(1|Receiving pond), whereas we used the model Variable ~Pre-treatment +(1|Receiving pond) for aphid fitness.

Transgenerational plasticity within years

To test whether plants are transgenerationally plastic within a year, we performed a similar reciprocal transplant experiment. In July (calendar week 30), when aphid intensities almost peaked (Fig **6b**), we collected 40 adult fronds carrying a daughter frond from each pond. We brushed off all aphids, marked both the mother and its daughter with a permanent marker (Stabilo OHPen Universal, Heroldsberg, Germany), and placed 20 marked mothers carrying one daughter into floating boxes positioned either in their home pond or in a neighbouring pond of the opposite treatment. Thus, each pond contained floating boxes with plants from both treatments.

After three weeks of free growth without aphids ("recovery"), we took 40 unmarked fronds from each box, split them equally between two new boxes floating in the same pond, and introduced five adult aphids to one of the boxes; the other served as a control. After a further three-week free growth period ("treatment"), we counted aphids and determined surface area growth rates and plant morphology as described above. Shoots were flash-frozen in liquid nitrogen and stored at −80°C until metabolite extraction.

To quantify metabolites, we ground the frozen plant material and used 20 mg ground material to determine tyramine and jasmonate levels as described above. To assess whether the pre-treatment altered metabolite accumulation, we fitted mixed effect models with the pre-treatment and treatment as fixed effects and the initial pond and receiving pond as random effects. "Initial pond" is the pond from which the plants were taken before the recovery phase; "Receiving pond" is the ponds in which the recovery and treatment were performed: Metabolite ~Pre-treatment *Treatment +(1|Initial pond)+(1|Receiving pond). For plant fitness and morphology traits, we accounted for the herbivory or control conditions the ponds had at the start of the fitness and phenotype assays, yielding: Variable ~Pre-treatment *Treatment +(1|Initial pond)+(1|Receiving pond)+(1|Pond treatment). For aphid fitness, we included as random factor the number of adults fronds (11 to 15 adult fronds) when starting fitness and phenotype assays, as the developmental stage of the plant may affect aphid performance, using the model Variable ~Pre-treatment +(1|Initial pond)+(1|Receiving pond)+(1|Pond treatment)+(1|Adult fronds). For all analysis, we excluded replicates in which the control treatment got contaminated with aphids or were aphids died due to fungi infection.

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Author contribution

MH and AC conceptualized the study; AC and MH designed the experiments; AC, ML, MS collected the data; AC, MH and ML analysed and interpreted the data; SX and MH provided the laboratory and resources to execute the experiments; AC and MH drafted the manuscript; AC, MH and MS contributed to the final version of the manuscript.

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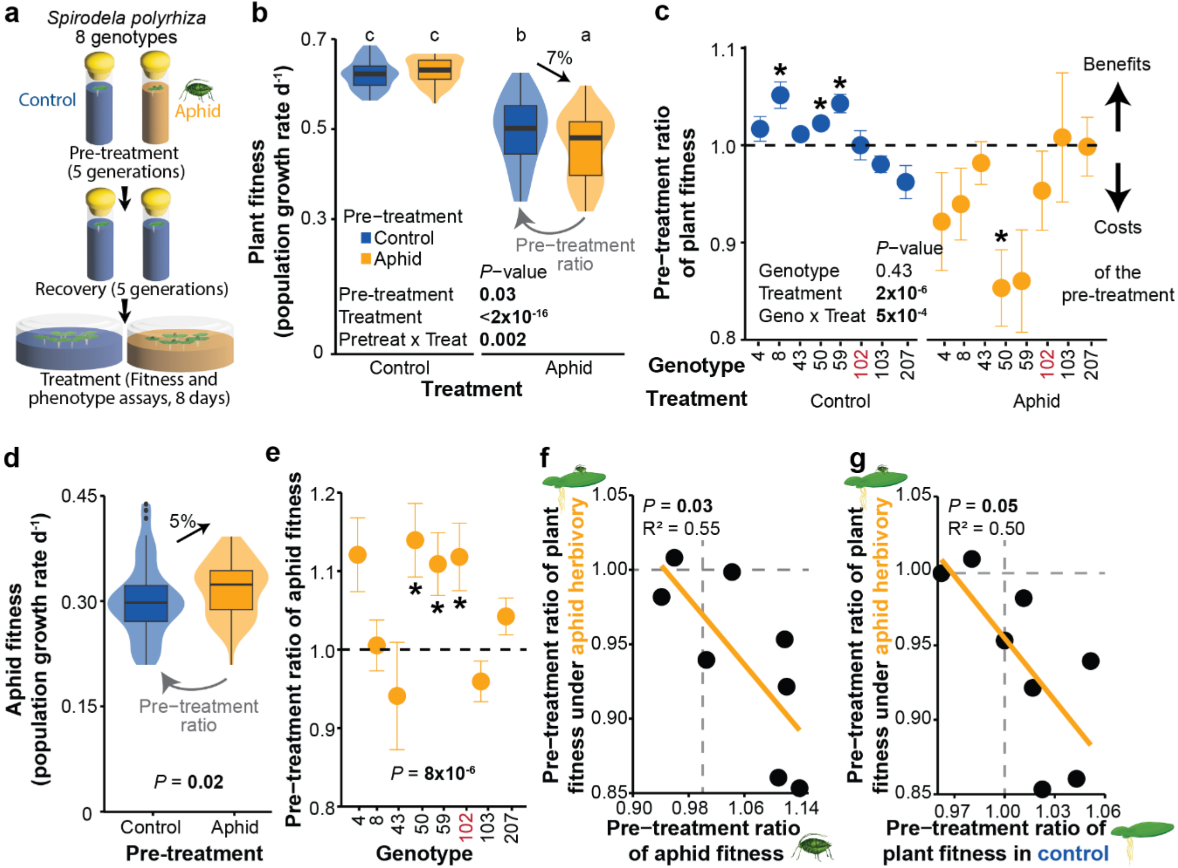


Fig 1: Indoors, ancestral herbivory by the waterlily aphid *Rhopalosiphum nymphaeae* boosts aphid reproduction and thereby decreases the fitness of the duckweed *Spirodela polyrhiza*. a Schematic of the indoor experiment with eight *S. polyrhiza* genotypes with and without recurring aphid herbivory. **b** Across all *S. polyrhiza* genotypes, ancestral aphid herbivory reduced plant fitness (daily population growth rates) under recurrent stress. *P*-values refer to a mixed effects model, and letters above the violins indicate significant groupings in the least squares means post hoc test. N = 58-64 (N ~ 8 per genotype). **c** Pre-treatment ratios of plant fitness (fitness of aphid pre-treated plants relative to the mean fitness of control pre-treated plants) varied among genotypes. Error bars denote ± standard errors. Dashed line at ratio = 1 marks neutrality; asterisks denote significant deviation from neutrality (Wilcoxon rank-sum test, $P < 0.05$). *P*-values refer to the mixed-effects model. N = 5-8. **d** Across all *S. polyrhiza* genotypes, ancestral aphid herbivory boosted aphid fitness (daily population growth rates). *P*-values refer to the mixed-effects model. N = 58. **e** Aphids benefitted from ancestral aphid herbivory only in a subset of *S. polyrhiza* genotypes. Error bars denote ± standard errors. Asterisks denote significant deviations from neutrality (Wilcoxon Rank Sum tests). *P*-value refer to a mixed effects model. N = 5-8. **f** The more the aphids benefited from ancestral stress, the more the plants suffered. Each circle represents a genotype mean. *P*-value refers to a linear regression. N = 8. **g** The more a genotype suffered from ancestral herbivory under recurring herbivory, the better it performed when the aphids did not recur. Each circle represents a genotype mean. *P*-value refers to a linear regression. N = 8. Geno = genotype, Pretreat = pre-treatment, Treat = treatment.

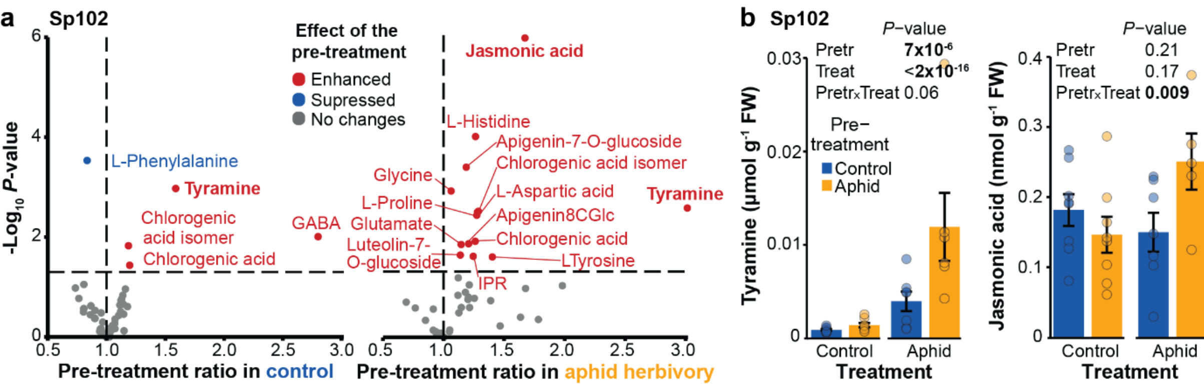


Fig 2: Ancestral aphid herbivory transgenerationally primes *Spirodela polyrhiza* for elevated accumulation of tyramine and jasmonates. a In *S. polyrhiza* genotype Sp102, ancestral aphid herbivory transgenerationally primed plants to accumulate elevated levels of roughly one-third of the metabolites measured, with the most pronounced effects on tyramine and jasmonic acid. In the volcano plot, vertical dotted lines mark the neutral fold change (no pre-treatment effect), and horizontal dotted lines indicate the significance threshold ($P < 0.05$, mixed effects model). Each point represents the mean metabolite level across eight Sp102 replicates, measured at the end of the indoor fitness and phenotype assay. **b** In Sp102, tyramine concentrations were induced by aphid herbivory, modestly retained across generations, and strongly primed upon subsequent attack. In contrast, jasmonic acid levels were induced by aphid feeding only in plants whose ancestors had previously been exposed to aphids. Error bars denote ± standard errors. *P*-values refer to mixed effects models. N = 5-8. GABA = γ-aminobutyric acid, iPR = isopentenyladenine riboside. FW = fresh weight, Pretr = pre-treatment, Treat = treatment.

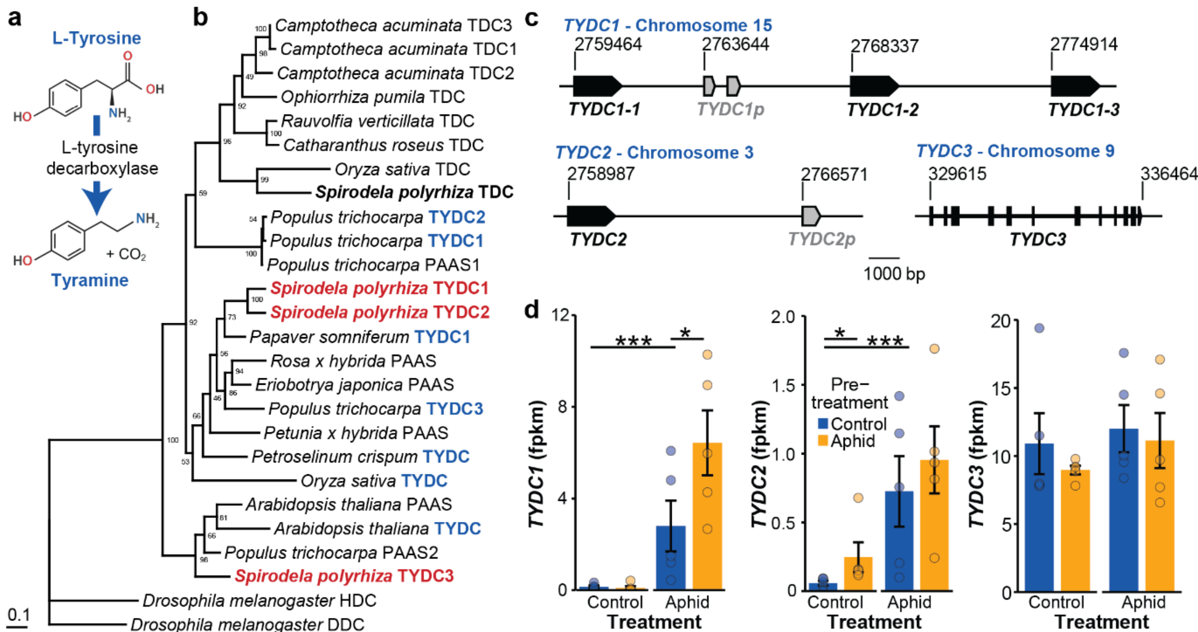
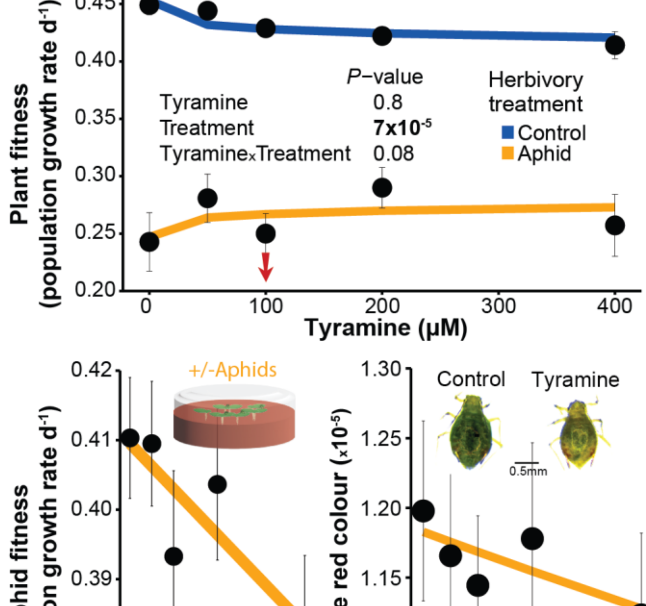


Fig 3: Ancestral aphid herbivory primes *Spirodela polyrhiza* to upregulate its tandemly duplicated L-tyrosine decarboxylase genes under aphid attack. a L-tyrosine decarboxylases (TYDC) catalyse the decarboxylation of the L-tyrosine to tyramine. **b** Phylogenetic tree of characterized aromatic amino acid decarboxylases and their homologs in *S. polyrhiza*. L-tyrosine decarboxylases are highlighted in blue; the three *S. polyrhiza* L-tyrosine decarboxylases are shown in bold red, and the *S. polyrhiza* L-tryptophan decarboxylase in bold black. Numbers at branching points refer to bootstrap values. **C** Genomic organization and gene models of the three *S. polyrhiza TYDC* homologs. Black boxes represent predicted coding exons, grey boxes denote pseudogenes, and connecting lines are introns. Chromosomal coordinates (bp) are indicated above each locus. **d** Ancestral aphid herbivory elevated expression of the *S. polyrhiza* L-tyrosine decarboxylases *TYDC1* and *TYDC2*, both of which are induced by aphid feeding. Asterisks denote significant differences using generalized linear models on transcriptome data derived from Sp102 plants harvested at the end of the fitness and phenotype assay indoors. N = 5. AADC = aromatic L-amino acid decarboxylase, DDC = L-dopa decarboxylase, HDC = histidine decarboxylase, PAAS = phenylacetaldehyde synthase, TDC = tryptophan decarboxylase, TYDC = tyrosine decarboxylase; FPKM = fragments per kilobase of transcript sequence per millions base pairs sequences. * < 0.05; *** < 0.001.



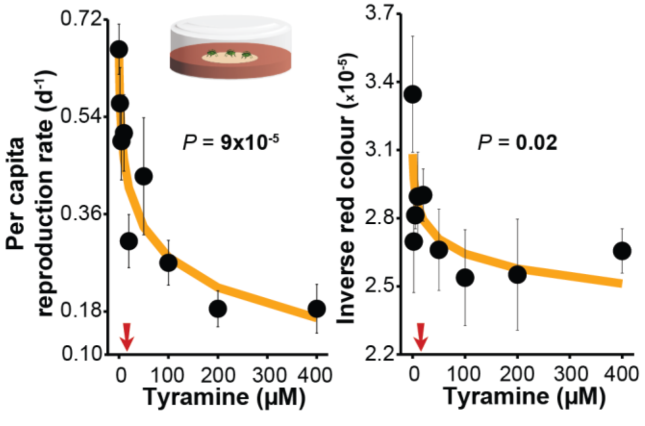


Fig 4: Tyramine protects plants against the aphid *Rhopalosiphum nymphaeae*. **a** Top panel: supplementing plants with tyramine decreased plant fitness (daily population growth rates) under control conditions but tended to improve it under aphid herbivory. Lower panel: supplementing plants with tyramine decreased aphid fitness (daily population growth rates) and increased aphid cuticle redness in a dose-dependent manner. Aphids were confined for eight days on plants grown in medium supplemented with 0-400 μM tyramine. The red arrow denotes the in-planta tyramine concentration equivalent to that measured in Sp102 after recurring indoor aphid herbivory. N = 12 per concentrations. **b** Supplementing a sucrose solution with tyramine was sufficient to reduce daily per-capita reproduction rates and increase cuticle redness. Aphids were confined for five days on 35% sucrose solutions containing 0-400 μM tyramine. The red arrow indicates the tyramine concentration in the sucrose solution that matches levels measured in Sp102 after repeated indoor aphid herbivory. N = 5 per concentration. For **a-b**: Each circle refers to the mean value per concentration. Error bars denote standard errors. *P*-values refer to linear models.

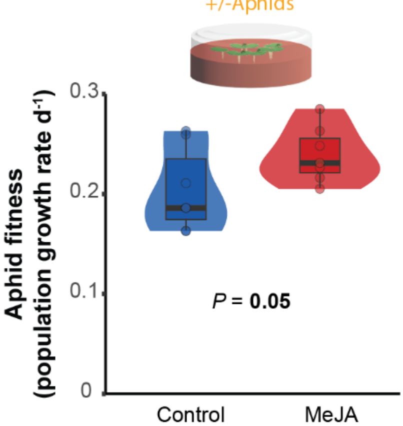


Fig 5: Inducing *Spirodela polyrhiza* with methyl jasmonate boosts aphid fitness. Aphid fitness (daily population growth rates) was tracked over eight days on Sp102 plants growing on 0 or 100 μM methyl-jasmonate. *P*-value refers to a mixed effects model. N = 7.

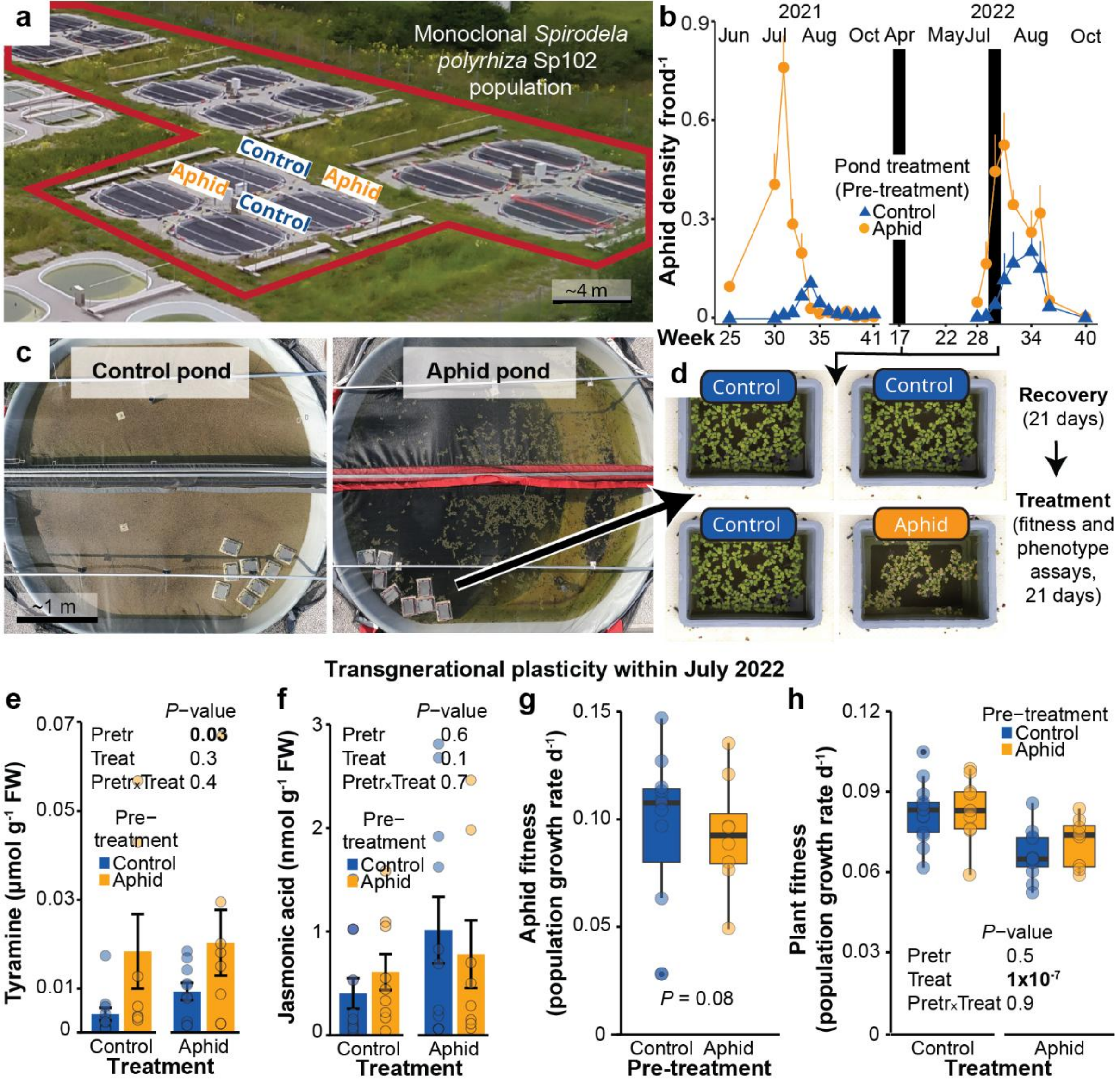


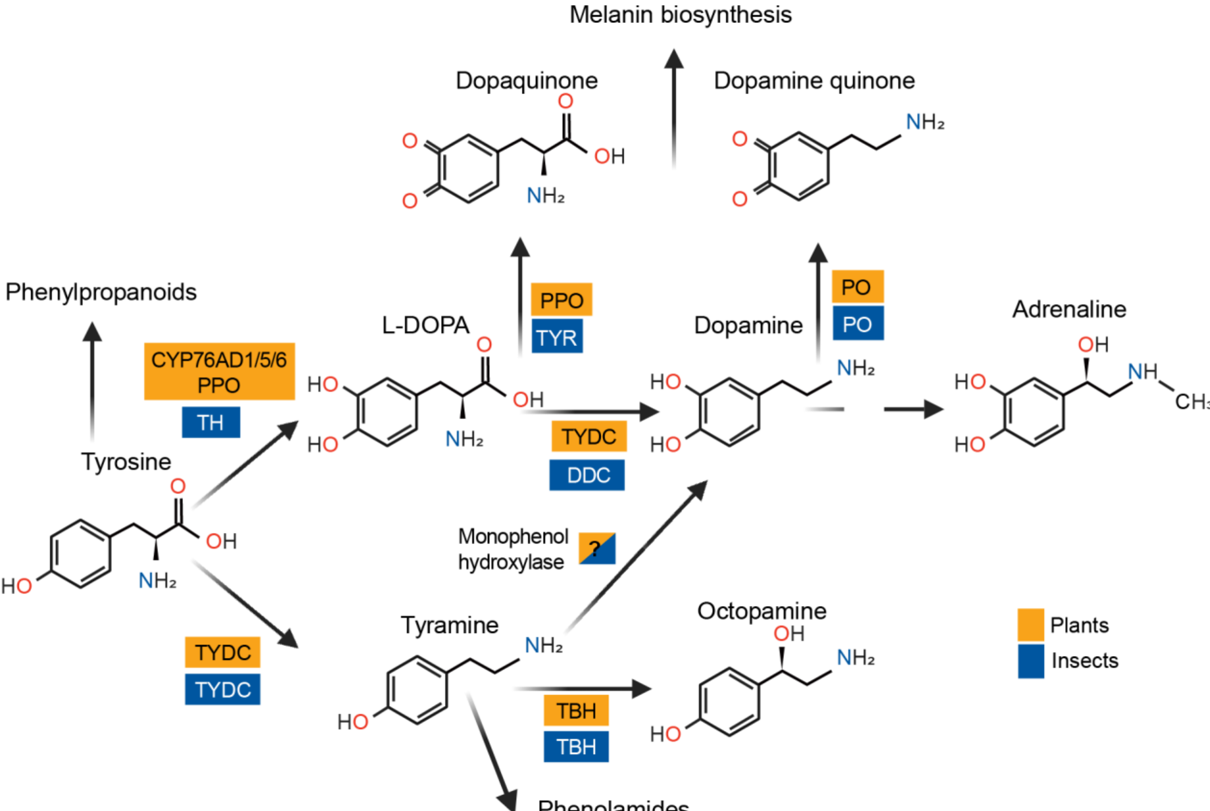
Fig 6: Outdoors, transgenerational tyramine priming persisted, whereas transgenerational jasmonate priming and its costs disappeared. a Experimental setup of outdoors mesocosms with *Spirodela polyrhiza* genotype Sp102 growing over two consecutive years in the presence and absence of the aphid *Rhopalosiphum nymphaeae*. N = 8 mesocosms per treatment. Experimental ponds are outlined in red. **b** Aphids density across the two years. Figured based on Schäfer et al. (2024). Vertical black bars indicate the calendar weeks when plants were collected for the recovery phase. **c** Overhead views of representative control and aphid ponds, each containing floating plant boxes from the fitness and phenotype assay. **d** Schematic of the recovery phase, and fitness and phenotype assay, conducted in boxes floating within the mesocosms. **e-h** Within-year transgenerational plasticity, inferred from fitness and phenotype assay in summer 2022, in metabolites (e-f), aphid fitness (daily population growth rates) (g), and plant fitness (daily population growth rates) (h). Ancestral aphid herbivory tended to decrease aphid fitness, likely because transgenerational priming of tyramine but not jasmonate persisted. Error bars refer to standard errors. *P*-values refer to mixed effects models. N = 8-13.

Supplemental material

Maladaptive aphid-induced transgenerational plasticity is overcome in nature

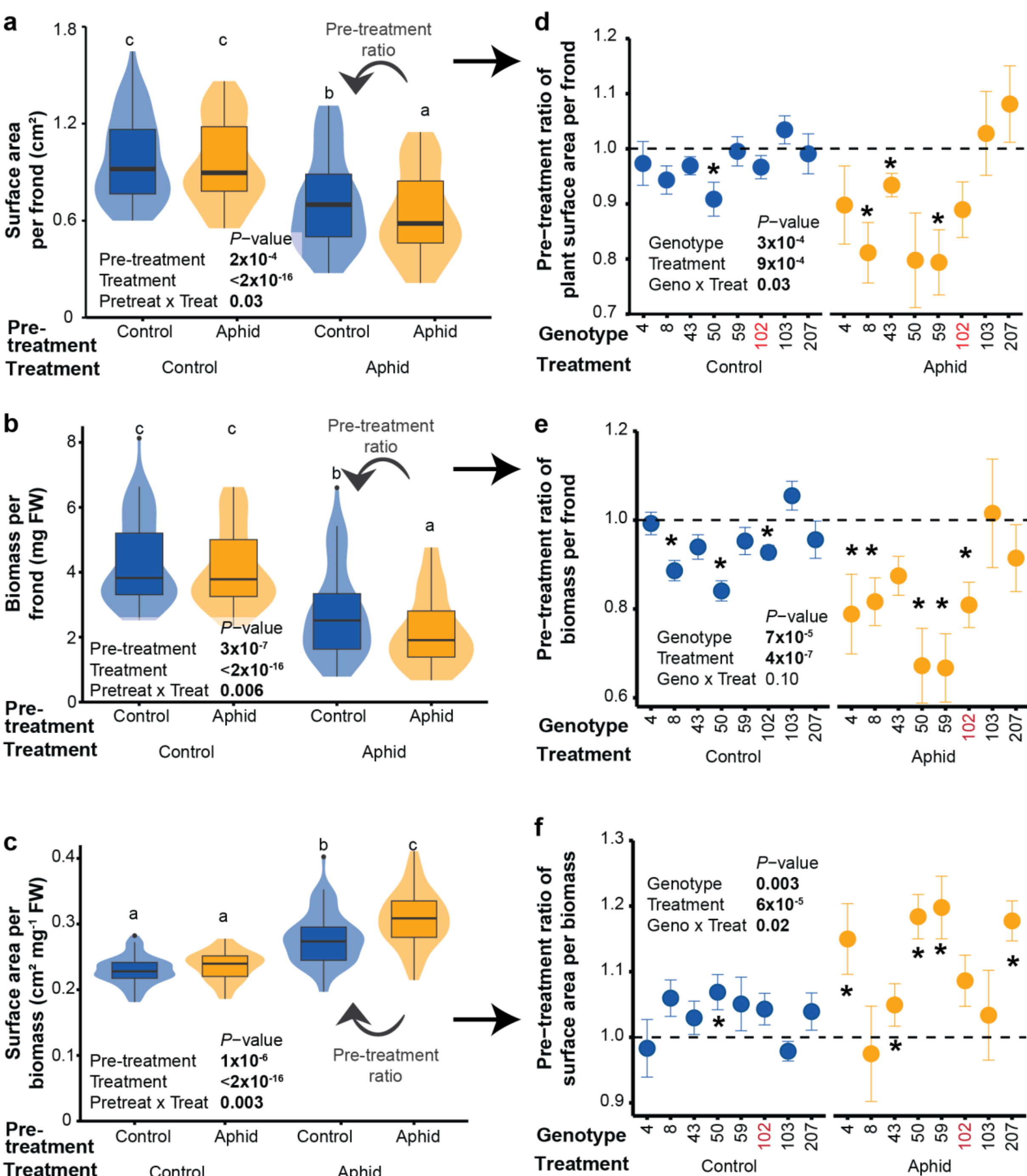
Alexandra Chávez, Martin Lohr, Lisa-Marie Laik, Martin Schäfer, Shuqing Xu, Meret Huber

Figures



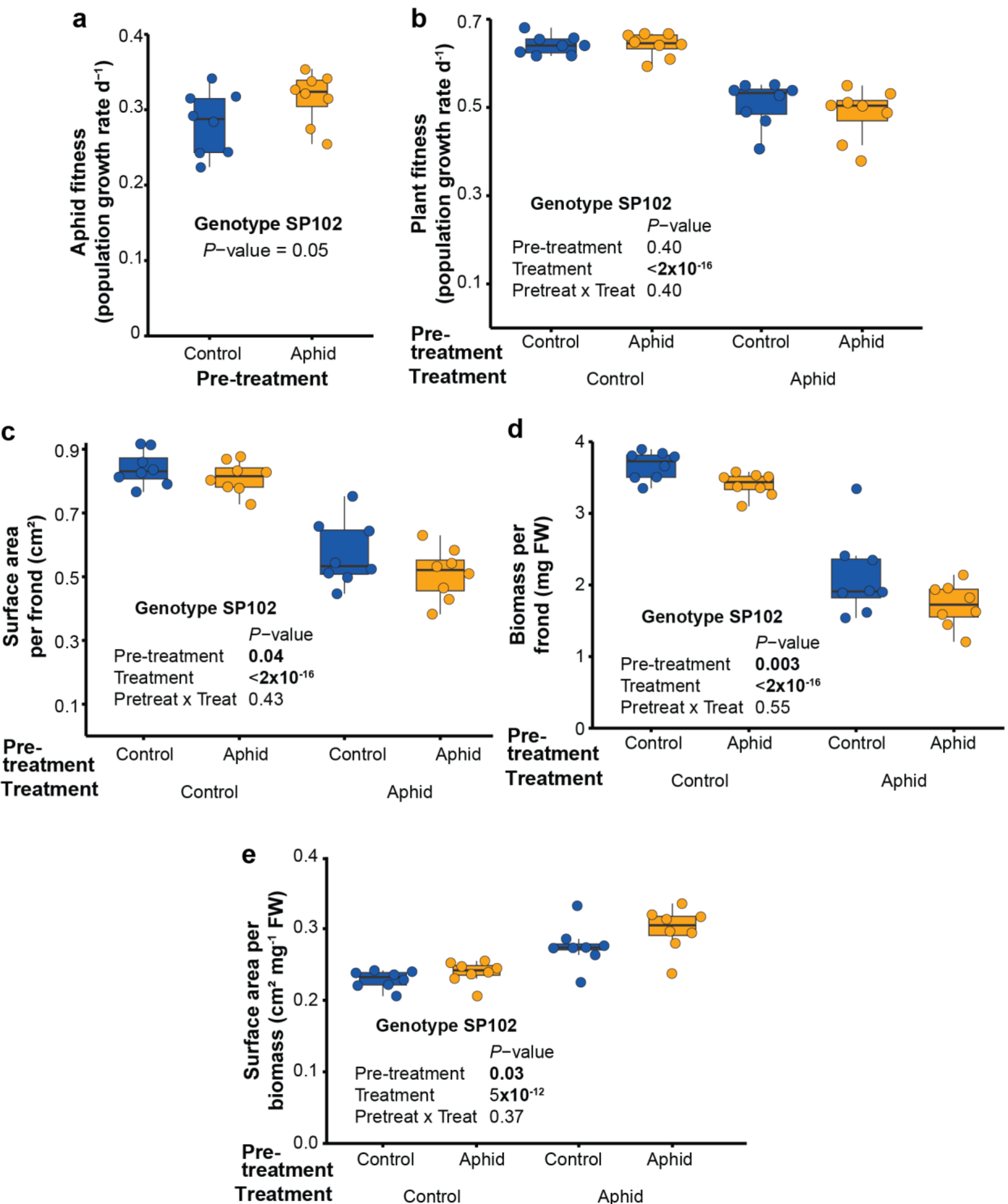
Supplementary Figure 1.1. Biosynthetic routes of tyramine. Tyramine originates from L-tyrosine, and derives into the neurotransmitter octopamine, the phenolamides and even the melanisation pathway. Enzymes synthetizing each metabolite are written on yellow and blue rectangles. Enzyme names were taken from: Xu et al. (2020), Brandau & Axelrod (1972), Soares et al. (2014). DDC = dopa decarboxylase, PO = phenoloxidase or dopamine quinone synthase, PPO = polyphenol oxidase, TBH = tyramine β-hydroxylase, TH = tyrosine hydroxylase, TYDC = tyrosine decarboxylase, TYR = tyrosinase.

1 Supplemental material



Supplementary Figure 1.2. Indoors, ancestral aphid herbivory supresses plant morphology when considering eight genotypes. Ancestral aphid herbivory decreased **a** surface area per frond and **b** biomass per frond, and accordingly increased **c** surface area per biomass. *P*-values refer to mixed effects models and letters on top refer to a post hoc test. N = 58-64 (N ~ 8 per genotype). The pre-treatment ratios (morphology of aphid pre-treated plants relative to the mean morphology of control pre-treated plants) of **d** surface area per frond, **e** biomass per frond and **f** surface area per biomass varied with the genotype. Morphology traits were measured after eight days of reproduction. Error bars denote ± standard errors. Dashed line at ratio = 1 marks neutrality; asterisks denote significant deviation from neutrality (Wilcoxon rank-sum test, $P < 0.05$). *P*-values refer to the mixed-effects model. N = 5-8. Geno = genotype, Pretreat = pre-treatment, Treat = treatment, FW = fresh weight.

2 Supplemental material



Supplementary Figure 1.3. Indoors, ancestral aphid herbivory on genotype Sp102 boost aphid fitness and supresses plant morphology. **a** Ancestral aphid herbivory in genotype Sp102 increased aphid fitness in comparison to plants exposed for first time to stress. **b** Ancestral aphid herbivory did not alter fitness in genotype Sp102, but decreased **c** surface are per frond, **d** biomass per frond, and enhanced **e** surface area per biomass. Fitness and morphology were measured after eight days of reproduction. *P*-values refer to mixed effects models. Pretreat = pre-treatment, Treat = treatment, FW = fresh weight.

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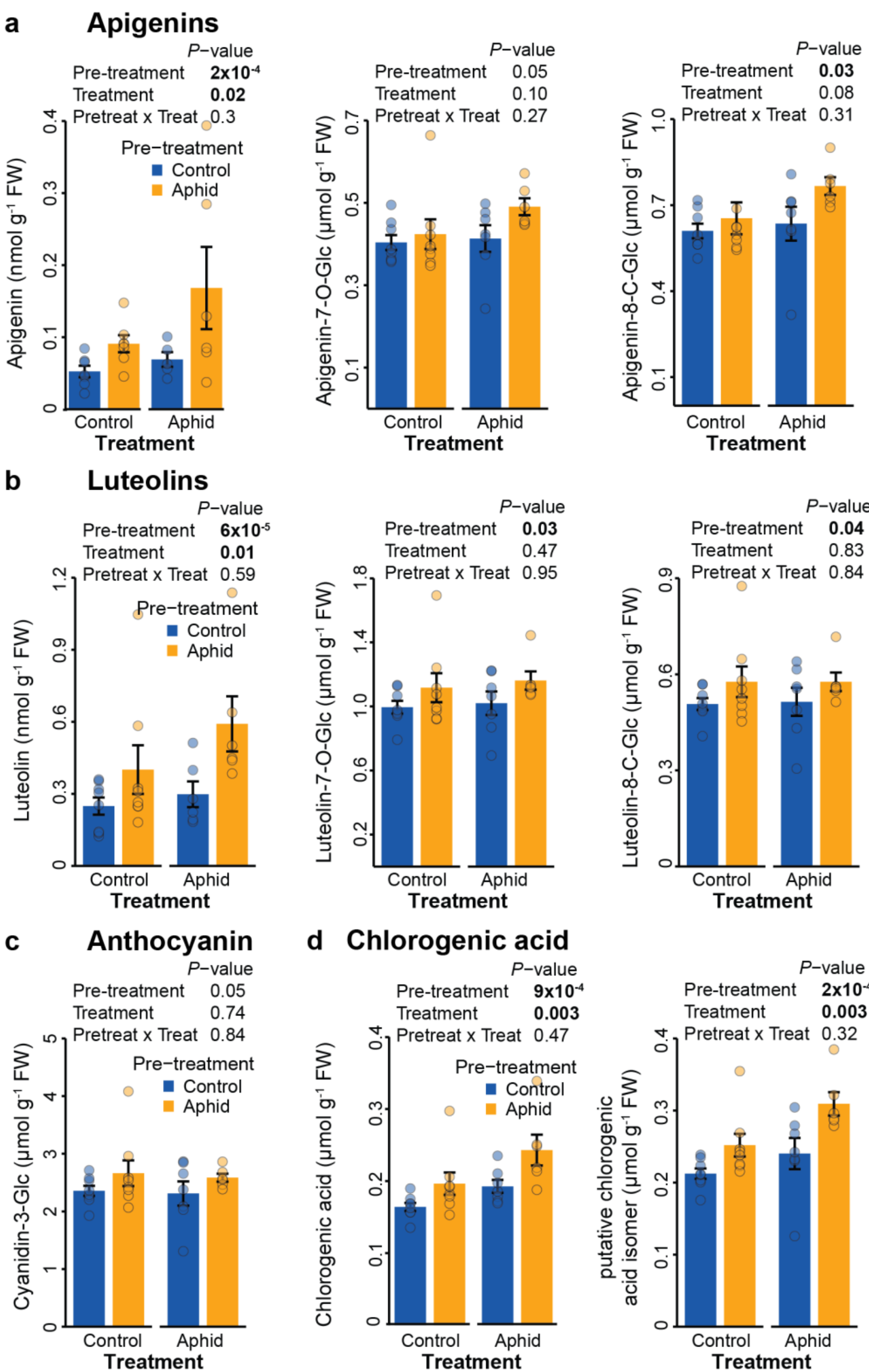
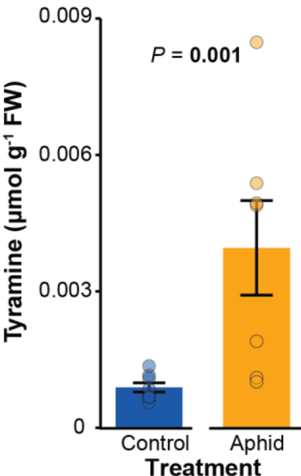
3 Supplemental material

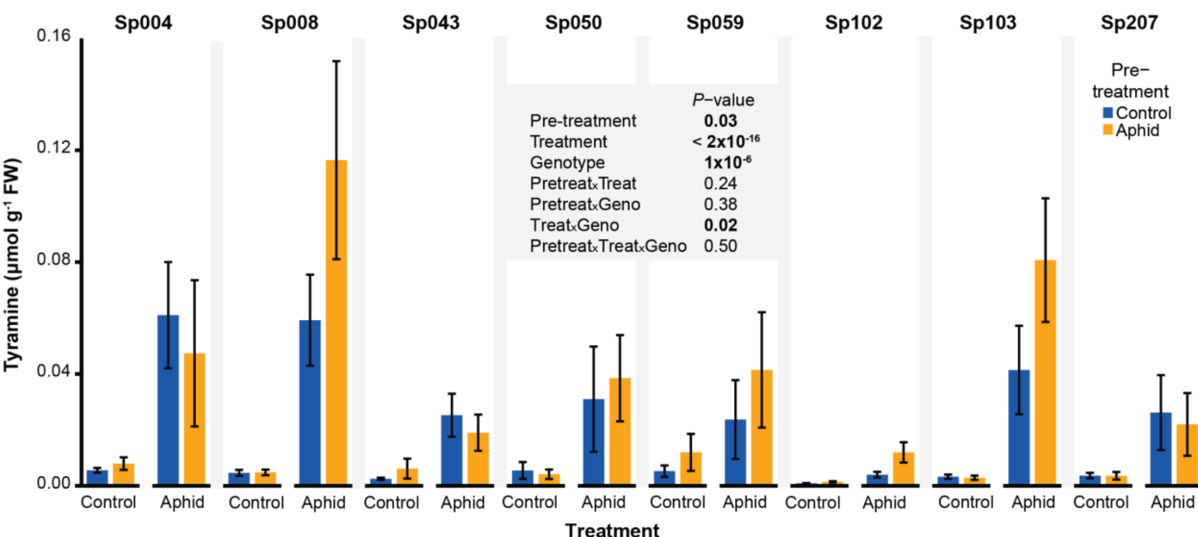


Supplementary Figure 2.1. Indoors, ancestral aphid herbivory on genotype Sp102 transgenerationally enhances flavones and chlorogenic acid concentrations. Ancestral aphid herbivory transgenerationally enhanced **a** apigenins and **b** luteolins in shoots of genotype Sp102 independent of the environment, but not **d** cyanidins. **d** Ancestral aphid herbivory transgenerationally enhanced the concentrations of chlorogenic and its isomers in shoots of genotype Sp102. Metabolites were measured in shoot material of plants that reproduced freely for eight days. Error bars denote ± standard errors. *P*-values refer to mixed effects models. N = 8. Pretreat = pre-treatment, Treat = treatment, FW = fresh weight.

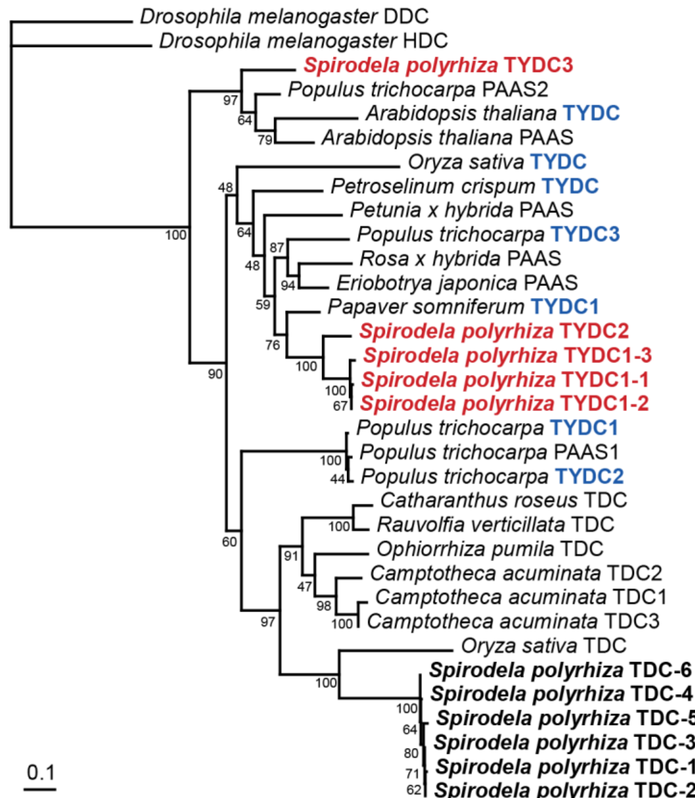
4 Supplemental material



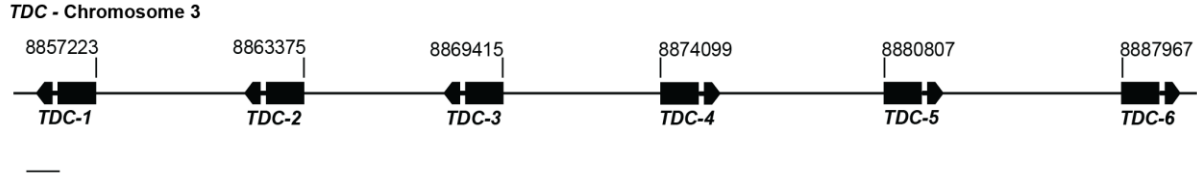
Supplementary Figure 2.2. Indoors, tyramine in genotype Sp102 is induced under first time aphid herbivory. Tyramine was measured in shoot material of plants that reproduced freely for eight days. Error bars denote ± standard errors. *P*-value refers to a mixed effects model. N = 8. FW = fresh weight.



Supplementary Figure 2.3. Indoors, ancestral aphid herbivory transgenerationally enhanced tyramine concentrations in eight genotypes. Tyramine for all genotypes was measured in shoot material of plants that reproduced freely for eight days. Error bars denote ± standard errors. *P*-value refers to a mixed effects model. N = 5-8. FW = fresh weight.

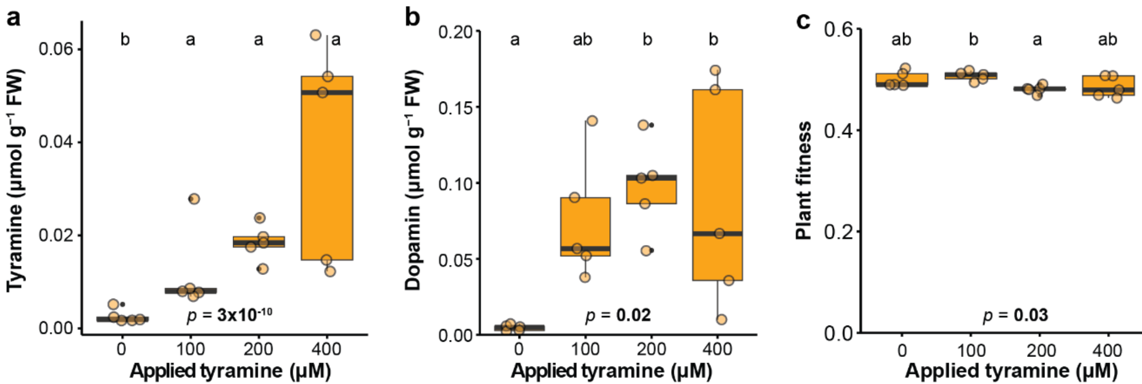


Supplementary Figure 3.1. *S. polyrhiza* L-tyrosine and L-tryptophan decarboxylase homologues cluster together with similar decarboxylases. L-tyrosine decarboxylases are written in blue; the three *S. polyrhiza* L-tyrosine decarboxylases and their tandem copies are shown in bold red, while the *S. polyrhiza* L-tryptophan decarboxylase and the tandem copies are in bold black. AADC = aromatic L-amino acid decarboxylase, DDC = L-dopa decarboxylase, HDC = histidine decarboxylase, PAAS = phenylacetaldehyde synthase, TDC = tryptophan decarboxylase, TYDC = tyrosine decarboxylase.

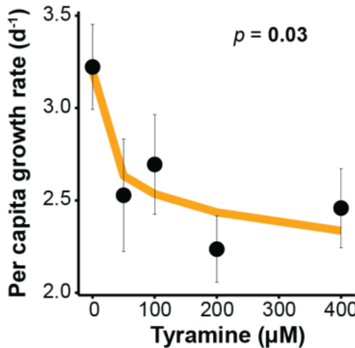


Supplementary Figure 3.2. Physical location of the L-tryptophan decarboxylase copies. Numbers denote the starting position of each gene copy.

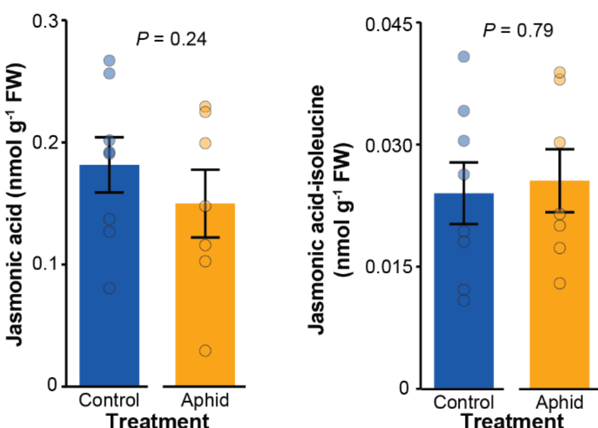
6 Supplemental material



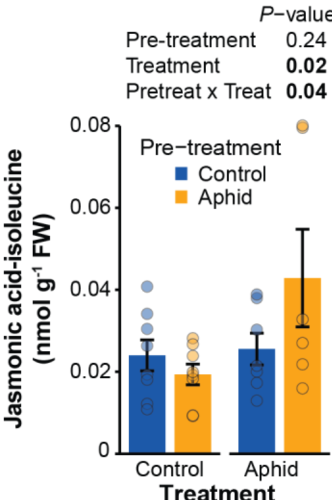
Supplementary Figure 4.1. External supplementation of tyramine in genotype Sp102 increases tyramine and dopamine concentrations. External application of tyramine in *S. polyrhiza* genotype Sp102 enhanced **a** tyramine and **b** dopamine concentrations in shoots. **c** Higher external tyramine concentrations tended to decrease plant fitness (surface area growth rates per day). Metabolites were measured in shoot material of plants that reproduced freely for eight days. *P*-values refer to linear models and letters refer to least squares means post hoc tests. N = 5. FW = fresh weight.



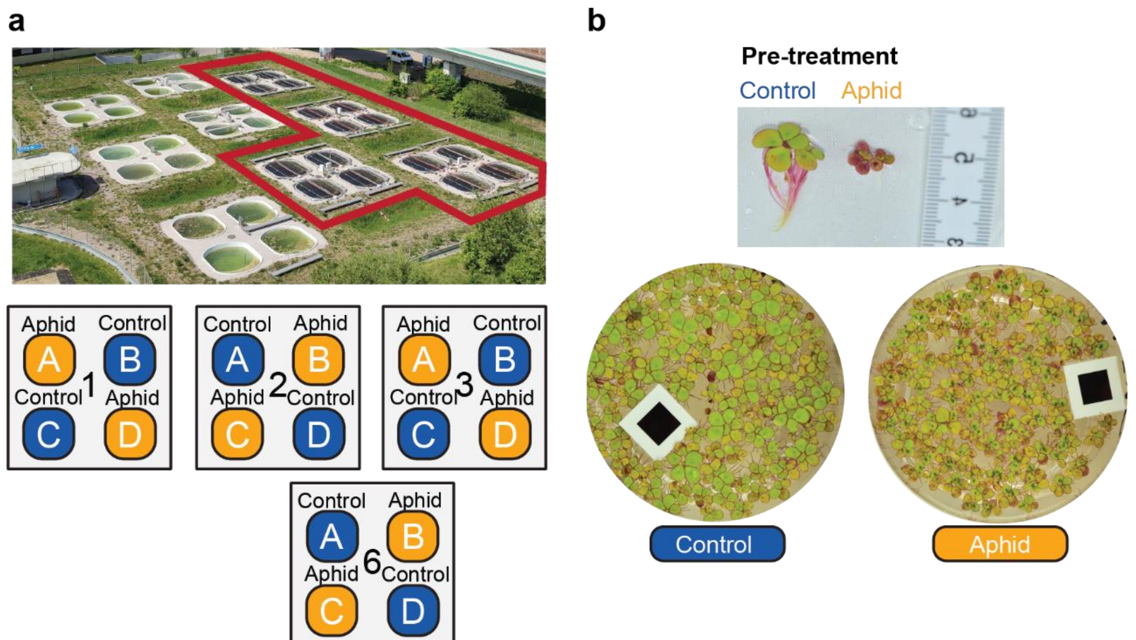
Supplementary Figure 4.2. Artificial supplementation of tyramine in a sucrose solution decreased the number of offspring per aphid after two days of feeding. Each circle refers to the mean value per concentration. Error bars denote ± standard errors. *P*-value refers to a linear regression. N = 5 per tyramine concentration.



Supplementary Figure 5.1. Indoors, aphid herbivory on genotype Sp102 does not alter jasmonate concentrations. Aphid herbivory in genotype Sp102 did not elevate the concentrations of the jasmonates jasmonic acid nor jasmonic acid-isoleucine in shoots. Jasmonates were measured in shoot material of plants that reproduced freely for eight days. Error bars denote standard errors. *P*-values refer to mixed effects models. N = 8. FW = fresh weight.

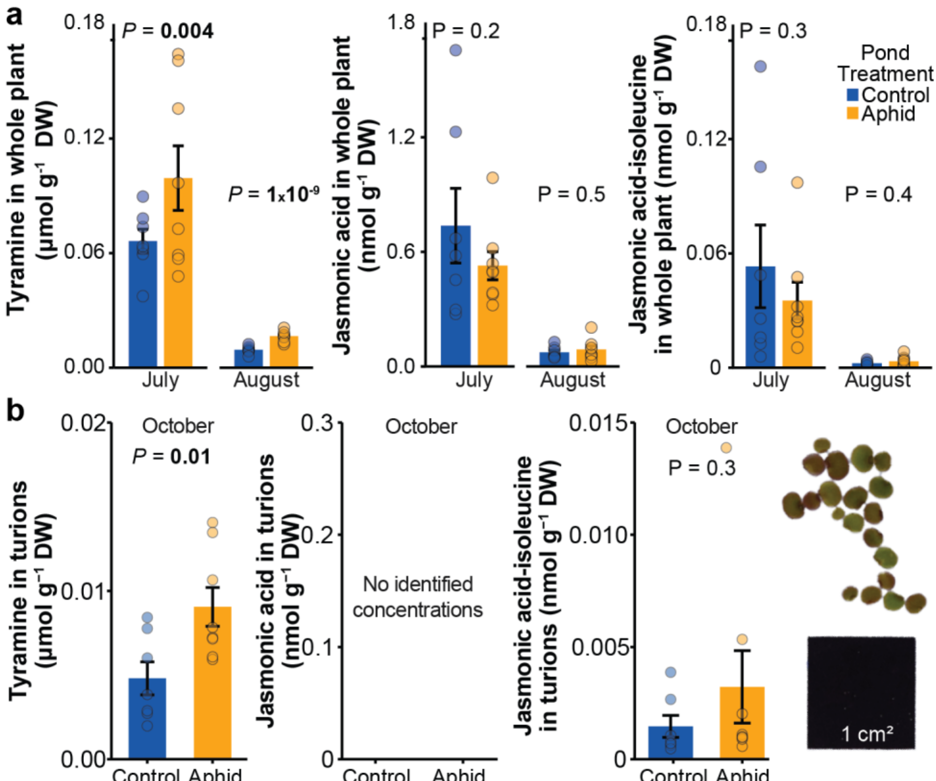


Supplementary Figure 5.2. Indoors, ancestral aphid herbivory on genotype Sp102 transgenerationally primes jasmonic acid-isoleucine under recurrent herbivory. Jasmonic acid-isoleucine was measured in shoot material of plants that reproduced freely for eight days. Error bars denote standard errors. *P*-values refer to a mixed effects model. N = 8. FW = fresh weight.

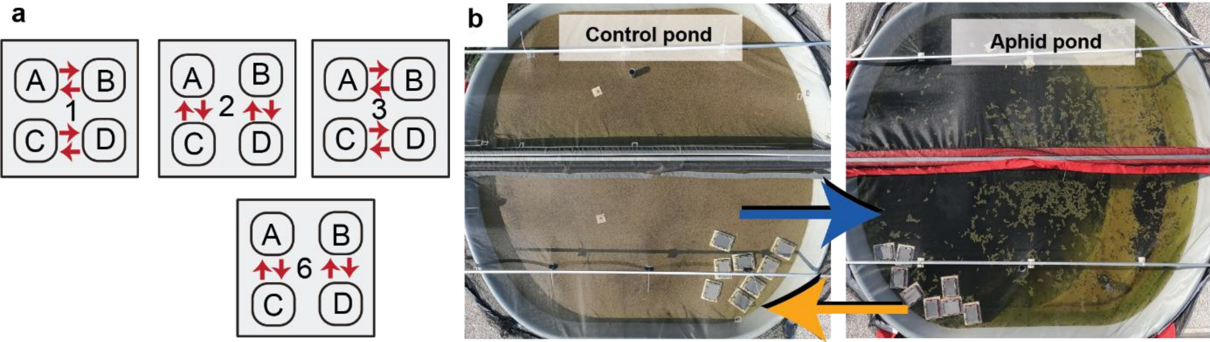


Supplementary Figure 6.1. Outdoors, herbivory stress causes strong phenotypic differences. **a** Distribution of ponds and herbivory pond treatment (pre-treatment) in the field. Red lines delimit the experimental ponds, which included four ponds per group. **b** Aphid herbivory reduced frond and root size and increased red colouration in shoots of the population. The reference stone (black area) is 1cm^2.

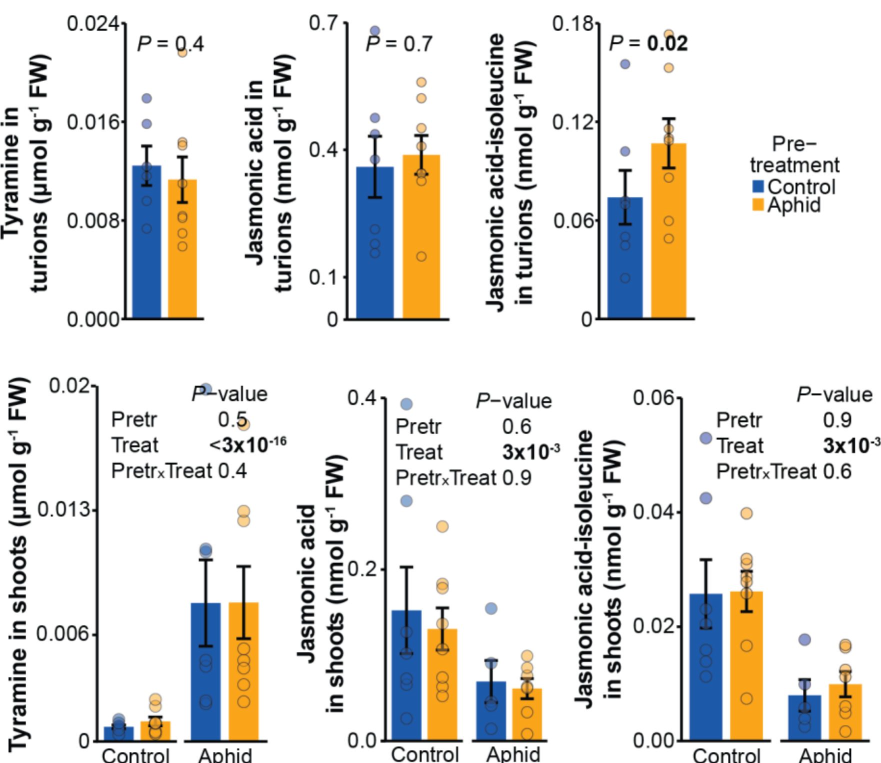
8 Supplemental material



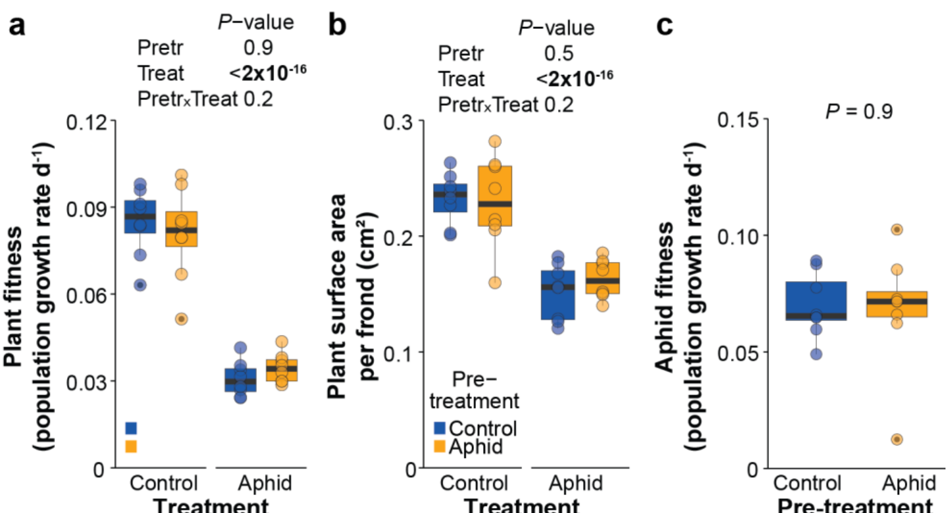
Supplementary Figure 6.2. Outdoors, aphid herbivory on genotype Sp102 enhances tyramine concentrations in whole plant and turions but does not alter jasmonate concentrations. **a** Aphid herbivory increase tyramine but not jasmonates in whole plant material of genotype Sp102 during July and August of 2021. **b** Enhanced tyramine concentrations remained in the turions in October (calendar week 42), 2021. Metabolites were measured in whole plant material of random plants growing in large populations for two to three months. The reference stone (black area) is 1cm^2. Error bars refer to standard errors. *P*-values refer to mixed effects models. N = 8 per pond treatment. DW = dry weight.



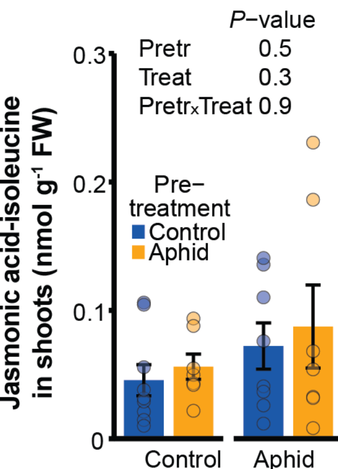
Supplementary Figure 6.3. Overview of the reciprocal transplant experiment outdoors. **a** Pairs created to move plants between ponds. **b** Overview of two ponds where the plants were transplanted within floating boxes.



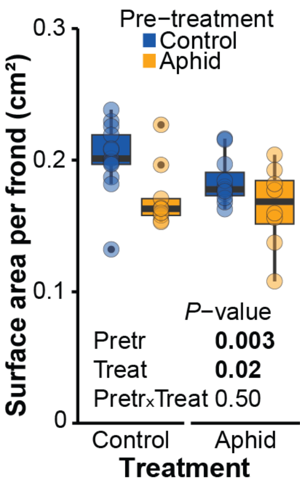
Supplementary Figure 6.4. Outdoors, the physiological effects of ancestral aphid herbivory on genotype Sp102 are erased between years. Plastic tyramine concentrations in 2021 where reset at the beginning of 2022 in shoots of genotype Sp102. Metabolites were measured in floating turions and in shoot material of plants that reproduced freely for three weeks. Error bars refer to standard errors. *P*-values refer to mixed effects models. N = 8. FW = fresh weight.



Supplementary Figure 6.5. Outdoors, ancestral aphid herbivory on genotype Sp102 does not alter plant morphology nor plant and aphid fitness between years. Ancestral aphid herbivory in 2021 did not alter **a** plant fitness (plant population growth rate per day), **b** plant morphology, nor **c** aphid fitness (aphid population growth rate per day) in spring of 2022. Fitness and morphology were measured in plants growing freely for three weeks. P-values refer to mixed effects models. N = 8.



Supplementary Figure 6.6. Outdoors, aphid herbivory on genotype Sp102 does not alter jasmonic acid-isoleucine concentrations within the year. Concentration of jasmonic acid isoleucine in summer of 2022 remained unaffected in genotype Sp102. Jasmonic acid-isoleucine was measured in shoot material of plants that reproduced freely for three weeks. Error bars refer to standard errors. P-values refer to mixed effects models. N = 8-13 per treatment. FW = fresh weight.



Supplementary Figure 6.7. Outdoors, ancestral aphid herbivory on genotype SP102 supresses plant morphology within the year. Morphology was measured in plants that reproduced freely for three weeks. *P*-values refer to mixed effects models. N = 8-13 per treatment.

Tables

Supplemental Table 1. Accession description of the *Spirodela polyrhiza* genotypes used in the indoor and outdoor experiments.

Accession ID	Clone ID	Population	Continent	Country	Latitude	Longitude	NCBI accession ID
SP004	7498	America	North America	USA	35.9940329	-78.898619	SAMN09429369
SP008	8683	America	Africa	Kenya	0.0880828	38.1899782	SAMN09429399
SP043	9497	India	Asia	India	9.9252007	78.1197754	SAMN09429373
SP050	0090	Southeast Asia	Asia	China	30.410755	104.546774	SAMN09429379
SP059	9506	India	Asia	India	17.385044	78.486671	SAMN09429387
SP102	NA	Europe	Europe	Switzerland	47.3768866	8.541694	SAMN17885246
SP103	NA	Europe	Europe	Switzerland	47.3768866	8.541694	SAMN17885247
SP207	NA	Southeast Asia	Asia	China	35.864482	120.029182	SAMN17885329

Accession ID refers to the code given in our group collection.
Clone ID refers to the code given by other authors.
Population refers to the genetic group classified by Wang et al. (2024) and Xu et al. (2019).

12 Supplemental material

Supplemental Table 2. List of species and genes used in the phylogenetic analysis of the aromatic decarboxylases.

Organism	Abbreviation based on activity	Abbreviation in reference	Genbank accession (Gene ID)	Assay	Reference
Arabidopsis thaliana	TYDC		AEE85523 (AT4G28680)	purified recombinant His_6-protein	Lehmann & Pollmann (2009)
Oryza sativa	TYDC		AK065830 / BAG89694	tyramine in overexpressing rice leaves	Lee et al. (2009), Park et al. (2012)
Papaver somniferum	TYDC1		AAC61844	recombinant protein *E. coli* extract	Facchini & De Luca (1994), Facchini & De Luca (1995)
Petroselinum crispum	TYDC		AAA33860	recombinant protein *E. coli* extract	Kawalleck et al. (1993)
Populus trichocarpa	TYDC1	AADC1	PNT06818 (Potri.013G052800)	purified recombinant His_6-protein	Günther et al. (2019)
Populus trichocarpa	TYDC2	AADC2	PNS99079 (Potri.016G114300)	purified recombinant His_6-protein	Günther et al. (2019)
Populus trichocarpa	TYDC3	AADC3	PNT39402 (Potri.004G036200)	purified recombinant His_6-protein	Günther et al. (2019)
Camptotheca acuminata	TDC1		AAB39708	recombinant protein *E. coli* extract	López-Meyer & Nessler (1997)
Camptotheca acuminata	TDC2		AAB39709	recombinant protein *E. coli* extract	López-Meyer & Nessler (1997)
Camptotheca acuminata	TDC3		UZH44855	purified recombinant His_6-protein	Qiao et al. (2022)
Catharanthus roseus	TDC		P17770.1 / TDC_CATRO	recombinant protein *E. coli* extract	(De Luca et al., 1989)
Ophiorrhiza pumila	TDC		BAC41515	purified recombinant His_6-protein	Yamazaki et al. (2003)
Oryza sativa	TDC		BAG91223	purified recombinant His_6-protein	(Kang et al., 2007)
Rauvolfia verticillata	TDC		ADL28270	purified recombinant His_6-protein	Liu et al. (2012)
Arabidopsis thaliana	PAAS		Q8RY79 / PAAS_ARATH (AT2G20340)	purified recombinant His_6-protein	Gutensohn et al. (2011)
Petunia hybrida	PAAS		ABB72475	purified recombinant His_6-protein	Kaminaga et al. (2006)
Rosa hybrida	PAAS		ABB04522	purified recombinant His_6-protein	Kaminaga et al. (2006)
Eriobotrya japonica	PAAS	AADC1	BBE38027	purified recombinant His_9-protein	Koeduka et al. (2017)
Populus trichocarpa	PAAS1	AAS1	PNT06819 (Potri.013G052900)	purified recombinant His_6-protein	Günther et al. (2019)
Populus trichocarpa	PAAS2	AAS2	KAI9400498 (Potri.002G255600)	purified recombinant His_6-protein	Günther et al. (2019)
Drosophila melanogaster	DDC		P05031	DDC = dopa decarboxylase	Sandmeier et al. (1994)
Drosophila melanogaster	HDC		X70644 (CAA49989)	HDC = histidine decarboxylase	Sandmeier et al. (1994)

Supplemental Table 3. Function of genes used in the phylogenetic analysis of the aromatic decarboxylases.

		Decarboxylation				**Decarboxylation + Transamination**			
Organism	**Abbreviation based on activity**	tyrosine => tyramine	tryptophan => tryptamine	phenyl alanine => phenylethylamine	L-Dopa => Dopamine	phenyl alanine => phenylacetaldehyde	tyrosine => 4-OH-phenylacetaldehyde	tryptophan => indole-3-acetaldehyde	L-Dopa => L-Dopaldehyde
Arabidopsis thaliana	TYDC	**yes**	no	no	no				
Oryza sativa	TYDC	**yes**							
Papaver somniferum	TYDC1	**yes**	no	very low	**yes**				
Petroselinum crispum	TYDC	**yes**	no	very low	low				
Populus trichocarpa	TYDC1	**yes**	yes	**yes**					
Populus trichocarpa	TYDC2	**yes**	no	low					
Populus trichocarpa	TYDC3	**yes**	no	yes					
Camptotheca acuminata	TDC1	no	**yes**	no	no				
Camptotheca acuminata	TDC2	no	**yes**	no	no				
Camptotheca acuminata	TDC3		**yes**						
Catharanthus roseus	TDC		**yes**						
Ophiorrhiza pumila	TDC		**yes**						
Oryza sativa	TDC	no	**yes**	no	no				
Rauvolfia verticillata	TDC		**yes**						
Arabidopsis thaliana	PAAS					**yes**	no	no	**yes**
Petunia hybrida	PAAS					**yes**	no	no	no
Rosa hybrida	PAAS					**yes**	no	no	no
Eriobotrya japonica	PAAS					**yes**			
Populus trichocarpa	PAAS1					**yes**	no	no	
Populus trichocarpa	PAAS2					**yes**	yes	yes	
Drosophila melanogaster	DDC								
Drosophila melanogaster	HDC								

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Supplemental Table 4. Description of the L-tyrosine and L-tryptophan decarboxylase genes in *Spirodela polyrhiza*.

Gene	Copy	Chromosome	N° exons	Start position	Ending position	Gene strand	Exon length	Accession 1	Accession 2
SpTYDC1	SpTYDC1	3	1	2758987	2760498	+	1512	CM046531.1	JANEYH010000003.1
	SpTYDC1p1		0	*2766571*	*2767158*	+	*(588)*		
SpTYDC2	SpTYDC2_1	15	1	2759464	2760990	+	1530	CM046543.1	JANEYH010000015.1
	SpTYDC2p_1		0	*2763644*	*2763961*	+	*(858-1167)*		
	SpTYDC2p_2		0	*2764475*	*2764791*	+	*(1197-1515)*		
	SpTYDC2_2		1	2768337	2769863	+	1530		
	SpTYDC2_3		1	2774914	2776443	+	1530		
SpTYDC3	SpTYDC3	9	13	329615	329716	+	1-102	CM046537.1	JANEYH010000009.1
				330087	330159	+	103-175		
				330316	330546	+	176-406		
				331510	331673	+	407-570		
				332102	332242	+	571-711		
				332898	332957	+	712-771		
				334305	334469	+	772-936		
				335088	335144	+	937-993		
				335568	335632	+	994-1058		
				335876	335999	+	1059-1182		
				336120	336257	+	1183-1320		
				336336	336464	+	1321-1449		
SpTDC	SpTYDC3_1	3	2	8857223	8856138	-	1-1086	CM046531.1	JANEYH010000003.1
				8855991	8855566	-	1087-1506		
	SpTYDC3_2		2	8863375	8862290	-	1-1086		
				8862143	8861718	-	1087-1506		
	SpTYDC3_3		2	8869415	8868330	-	1-1086		
				8868183	8867758	-	1087-1506		
	SpTYDC3_4		2	8874099	8875184	+	1-1086		
				8875323	8875742	+	1087-1506		
	SpTYDC3_5		2	8880807	8881892	+	1-1086		
				8882045	8882464	+	1087-1506		
	SpTYDC3_6		2	8887967	8889052	+	1-1086		
				8889205	8889614	+	1087-1496		

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Supplemental Table 5. Gradient and retention times of neurotransmitters.

Analyte	RT [min]	Q1 [m/z]	Q3 [m/z]	Dwell time [ms]	CE [V]	Q1/Q3 Pre Bias [V]	ISTD
Kynurenic acid	3.5	(+) 190.05	144.10	20	-20	-24 / -22	L-Tyrosine
		(+) 190.05	89.05	15	-40	-23 / -16	
		(+) 190.05	116.15	15	-32	-13 / -11	
Kynurenine	2.515	(+) 209.09	192.10	20	-10	-10 / -20	L-Tyrosine
		(+) 209.09	146.10	15	-19	-10 / -15	
Serotonin	2.12 & 3.15	(+) 177.10	160.10	20	-14	-30 / -30	L-Tyrosine
		(+) 177.10	115.10	15	-28	-22 / -23	
Dopamine	0.99	(+) 154.09	91.10	20	-24	-11 / -19	L-Tyrosine
		(+) 154.09	137.20	15	-15	-11 / -14	
L-DOPA	0.93	(+) 198.08	135.15	15	-20	-14 / -25	L-Tyrosine
		(+) 198.08	152.00	20	-14	-13 / -16	
Melatonin	3.88	(+) 233.13	174.10	20	-15	-20 / -20	L-Tyrosine
		(+) 233.13	130.15	15	-43	-11 / -23	
		(+) 233.13	159.20	15	-26	-11 / -30	
GABA	0.535	(+) 104.07	87.10	20	-13	-10 / -16	L-Tyrosine
		(+) 104.07	69.10	15	-16	-22 / -12	

RT: retention time.
CE: collision energy.
Qualifiers are highlighted in grey.

Supplemental Table 6. Distribution of genotype replicates within the 24-well racks.

Rack	Genotype			
1	Sp004	Sp008	Sp043	Sp050
2	Sp059	Sp102	Sp103	Sp207
3	Sp008	Sp043	Sp050	Sp059
4	Sp102	Sp103	Sp207	Sp004
5	Sp043	Sp050	Sp059	Sp102
6	Sp103	Sp207	Sp004	Sp008
7	Sp050	Sp059	Sp102	Sp103
8	Sp207	Sp004	Sp008	Sp043
9	Sp059	Sp102	Sp103	Sp207
10	Sp004	Sp008	Sp043	Sp050
11	Sp102	Sp103	Sp207	Sp004
12	Sp008	Sp043	Sp050	Sp059
13	Sp103	Sp207	Sp004	Sp008
14	Sp043	Sp050	Sp059	Sp102
15	Sp207	Sp004	Sp008	Sp043
16	Sp050	Sp059	Sp102	Sp103

Supplemental Table 7. Distribution of first and second offspring into the start of pre-treatment and bioassays.

	Pre-treatment		Treatment	
Replicate	Control	Aphid	Control	Aphid
1	2nd daughter	1st daughter	1st daughter	2nd daughter
2	1st daughter	2nd daughter	1st daughter	2nd daughter
3	2nd daughter	1st daughter	2nd daughter	1st daughter
4	1st daughter	2nd daughter	2nd daughter	1st daughter
5	2nd daughter	1st daughter	1st daughter	2nd daughter
6	1st daughter	2nd daughter	1st daughter	2nd daughter
7	2nd daughter	1st daughter	2nd daughter	1st daughter
8	1st daughter	2nd daughter	2nd daughter	1st daughter

Supplementary Table 8. Summary of the RNAseq data quality

Treatment	Library	Raw reads	Raw bases	Clean reads	Clean bases	Error rate	Q_{Phred} 20	Q_{Phred} 30	GC_pct
Control	CtrCtr102_1	36439634	5.47G	35332794	5.3G	0.02	97.01	91.52	57.57
Recurrent control	AphCtr102_1	31717432	4.76G	30867196	4.63G	0.02	96.79	91.01	58.47
First time herbivory	CtrAph102_1	30637166	4.6G	29639046	4.45G	0.01	96.97	91.36	57.31
Recurrent herbivory	AphAph102_1	28618342	4.29G	27456896	4.12G	0.02	96.28	89.92	57.35
Control	CtrCtr102_2	25575982	3.84G	24612236	3.69G	0.02	95.91	88.99	58.82
Recurrent control	AphCtr102_2	65998370	9.9G	62854542	9.43G	0.02	96.91	91.31	57.76
First time herbivory	CtrAph102_2	44029604	6.6G	42909840	6.44G	0.02	96.62	90.15	57.54
Recurrent herbivory	AphAph102_2	39795074	5.97G	38445924	5.77G	0.02	96.79	90.94	57.62
Control	CtrCtr102_4	46814044	7.02G	45319562	6.8G	0.02	96.91	91.33	58.38
Recurrent control	AphCtr102_4	42940640	6.44G	41608930	6.24G	0.02	96.88	91.24	57.92
First time herbivory	CtrAph102_4	33545628	5.03G	32429206	4.86G	0.01	97.12	91.58	57.11
Recurrent herbivory	AphAph102_4	34608740	5.19G	33403204	5.01G	0.02	96.7	90.76	57.97
Control	CtrCtr102_5	36237892	5.44G	34761616	5.21G	0.02	96.35	89.82	58.05
Recurrent control	AphCtr102_5	43301092	6.5G	42014790	6.3G	0.02	96.81	90.98	58.12
First time herbivory	CtrAph102_5	32848532	4.93G	31700254	4.76G	0.02	96.68	90.52	58.17
Recurrent herbivory	AphAph102_5	36127624	5.42G	34540868	5.18G	0.01	98.11	94.73	57.78
Control	CtrCtr102_7	41755790	6.26G	40274198	6.04G	0.02	96.78	91.02	58.36
Recurrent control	AphCtr102_7	45568450	6.84G	43675358	6.55G	0.02	96.91	91.27	57.94
First time herbivory	CtrAph102_7	42187840	6.33G	40566184	6.08G	0.02	96.61	90.67	58.5
Recurrent herbivory	AphAph102_7	41362274	6.2G	39576098	5.94G	0.02	96.71	90.71	58.29

Raw reads: Reads count from the raw data, four rows as a unit, with statistics of reads count for every sequencing.
Raw bases: Base number of raw data = number of raw reads * sequence length, converting unit to G.
Clean reads: Base number of raw data after filtering = number of clean reads * sequence length, converting unit to G.
Clean bases: (clean base=clean reads*150bp) number multiply read length, saved in G unit.
Error rate: Average sequencing error rate, which is calculated by Qphred=-10log10(e).
Q_{Phred} 20: The percentage of the bases whose Q_{Phred} values is greater than 20. (Number of bases with Q_{Phred} value > 20) / (Number of total bases) *100.
Q_{Phred} 30: The percentage of the bases whose Q_{Phred} values is greater than 30. (Number of bases with Q_{Phred} value > 30) / (Number of total bases) *100.
GC pct: The percentage of G&C base numbers of total bases.(G&C base number) / (Total base number)*100.

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Equations

Equation S1. Fitness as relative population growth rate (Hunt, 1982) based on surface area of fronds.

$$\frac{\ln(\text{final value}) - \ln(\text{initial value})}{\text{number of days}}$$

Where value can be the surface area of fronds or the number of aphids.

Equation S3. Per capita growth rates (Carey, 1993).

$$\frac{\left(\frac{\text{final number of aphids} - \text{starting number of aphids}}{\text{starting number of aphids}}\right)}{\text{number of days}}$$

Equation S3. Pre-treatment ratios (Huber et al., 2021).

For plants:

$$\frac{\text{plant fitness or phenotype of copper pre} - \text{treated plants}}{\text{mean (plant fitness or phenotype of control pre} - \text{treated plants)}}$$

For aphids:

$$\frac{\text{aphid fitness on herbivory pre} - \text{treated plants}}{\text{mean (aphid fitness on control pre} - \text{treated plants)}}$$

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21 Supplemental material

Chapter V: Discussion

Evidence on the controversial role of transgenerational plasticity has increased over the last years, yet many questions on their effects remain unanswered. In the present thesis I demonstrate that transgenerational plasticity alters the phenotype and fitness of monoclonal *S. polyrhiza* individuals, expanding these effects to their aphid herbivore, *R. nymphaeae*. In this discussion I characterize transgenerational plasticity, by uncovering its genetic base, environment dependant effects and by predicting the adaptiveness of transgenerational plasticity. Then, I go deeper into the pathway of inheritance based on the behaviour of physiological traits and finally show the ecological role of transgenerational plasticity and the relevance of studying it not only under controlled condition in the laboratory, but also in natural environments. Although new questions emerged with this research, the data discussed in this thesis proves robust in defining the base knowledge of transgenerational plasticity and its relevance in population evolution.

i. The adaptive role of transgenerational plasticity

The effects of transgenerational plasticity extend beyond the organismal phenotype to influence organismal fitness. Until recently, most studies on non-genetic inheritance focused primarily on phenotypic changes in the direct offspring or grand-offspring of stressed parents (Anastasiadi et al., 2021; Che et al., 2024; López Sánchez et al., 2021). However, recent studies on genetically constrained organisms, reproduced for more than three generations after stress, have provided evidence of transgenerational plasticity (Groot et al., 2016; Huber et al., 2021; Lin et al., 2024; Quiobe et al., 2025; Rendina González et al., 2018). For instance, stresses such as copper excess in *Trifolium repens* and cadmium in *A. thaliana* resulted in improved plant biomass and flowering, respectively (Lin et al., 2024; Rendina González et al., 2018). Nonetheless, these studies typically measured plasticity in individual generations, often overlooking the long-term effects that transgenerational plasticity can have on organismal evolution, which are crucial for understanding its adaptive significance (but see Huber et al. (2021) and Van Antro et al. (2023)). In the three manuscripts presented in this thesis, I show that when clonal *S. polyrhiza* duckweeds are grown for five generations under stress and five generations without stress, ancestral stress induces changes in both the presence and absence of recurrent stress, compared to naïve plants. Furthermore, repeating the experiment with a subset of 16 genotypes from the original 56 exposed to ancestral copper excess revealed that these transgenerational effects are reproducible (manuscript I). These changes were not limited to fitness but also altered long-term phenotypic traits, such as plant morphology (manuscript II and III), and physiology through metabolite accumulation (manuscript I, II, and III), providing valuable insights into the role of transgenerational plasticity in species evolution.

i.a. Transgenerational plasticity has a genetic base

The presence of transgenerational effects on organismal fitness raises important questions about the evolutionary role of transgenerational plasticity. One of the most pressing questions is whether transgenerational plasticity is adaptive, i.e. grants organisms the ability to respond positively to environmental change (Engqvist & Reinhold, 2016; Herman & Sultan, 2011). To identify this adaptiveness, one must assess the factor that limit it: the link between transgenerational plasticity and the genetic architecture of organisms (Jablonka, 2017). The genetic framework within which transgenerational plasticity acts may either enhance or constrain its potential to drive evolutionary changes.

Genotype-dependant effects of transgenerational plasticity are consistent across various organisms, pointing towards a genetic base in transgenerational plasticity. Due to the fast rate at which epigenetic marks can change compared to genetic mutations, transgenerational plasticity is often expected to uncouple from the genotype, potentially benefiting organisms in rapidly changing environments (Anastasiadi et al., 2021; Bonduriansky & Day, 2009; Jablonka, 2017). However, studies involving single descendant lineages or small populations of both animal and plant organisms, such as the ciliate *Tetrahymena thermophila*, the nematodes *C. elegans* and *P. pacificus*, the clonal plants *Trifolium repens* and *S. polyrhiza*, and *A. thaliana*, have repeatedly shown that transgenerational plasticity is closely linked to the genetic architecture of these organisms (Alvarez et al., 2020; Cayuela et al., 2022; Huber et al., 2021; Lin et al., 2024; Rendina González et al., 2018). In support of this, theoretical models of DNA methylation epialleles demonstrate that transgenerational changes over time occur through genotype-specific methylation pathways, benefiting some genotypes while having little effects or even disadvantageous consequences on others under the same environmental conditions (Romero-Mujalli et al., 2024). Similarly, across all the experiments presented in this thesis, we observed that the abiotic copper excess and the biotic aphid herbivory-induced transgenerational plasticity caused genotype-dependent effects: ancestral copper excess consistently benefited some but not all genotypes in large and small experimental set-ups (manuscript I and II), and, while ancestral herbivory of the waterlily aphid suppressed plant fitness, its effects were not as strong in all genotypes (manuscript III). These results support the idea that the genetic architecture of an organism influences how it responds upon ancestral environmental stress that is passed over multiple generations.

Building on the observed genotype-dependent effects of transgenerational plasticity, we aimed to quantify the genetic contribution in transgenerational plasticity. For this, we estimated broad- and narrow-sense heritability parameters in the pre-treatment ratios of plant fitness, i.e. fitness of ancestrally stressed plants relative to the mean fitness of naïve plants, in 56 different genotypes and copper excess as stress (manuscript I). Surprisingly, broad-sense

heritability explained almost half of the observed variance, while narrow-sense heritability accounted for 30% of the intraspecific variation, indicating that transgenerational plasticity is shaped by the population's allele frequencies. These findings suggest that transgenerational plasticity is not inherently adaptive; rather, it generates variability within populations, providing a substrate for natural selection to act upon.

The genetic basis of transgenerational plasticity suggests that specific genetic regions, including genes, play a key role in determining an organism's ability to adapt to changing environments. Previous research in *A. thaliana* and *P. pacificus* showed that transposons, as well as ubiquitin ligases underlie transgenerational plasticity in these organisms (Lin et al., 2024; Quiobe et al., 2025). To identify the potential genes modulating the transgenerational response in *S. polyrhiza*, first we analysed the physiological changes through metabolite concentrations and resistance of genotypes to copper excess (manuscript I). Through the likely protective role of anthocyanins and the trade-offs between resistance and growth, we concluded that genes involved in growth and defence determined transgenerational plasticity (manuscript I). To further identify these genes, we searched into the biosynthetic machinery of the aphid herbivory-induced, transgenerationally plastic tyramine. Surprisingly, the L-tyrosine decarboxylase gene (*TYDC*) not only presented several copies, but its expression varied accordingly to the tyramine transgenerational plasticity. Although further validation of the gene is required, these data corroborate that defence genes model the transgenerational effects in *S. polyrhiza.*

Genes underlying transgenerational molecular changes may evolve rapidly due to genetic accommodation. In our search for the genetic basis of tyramine's transgenerational plasticity, we found that the three *TYDC* copies likely originated from the same gene (manuscript III). *TYDC1* and *TYDC2*, highly similar and intronless, but located on different chromosomes, were likely retroposed in *S. polyrhiza*. In contrast, *TYDC3*, containing introns, likely gave rise to the paralogues. Additionally, *TYDC2* whose expression was transgenerationally primed rather than retained, showed three copies of itself within an 18 kb region, highlighting a potential increase in the gene transcription (Wang et al., 2006). Although the concept of genetic accommodation (Waddington, 1953) remains controversial (Jablonka, 2017), evidence is not scarce (Sommer, 2020). Heat-shock in the hornworm *Manduca sexta* produced colour phenotypes that were not encoded in the genome but persisted over multiple generations when artificially selected (Suzuki et al., 2025; Suzuki & Nijhout, 2006). Furthermore, recent research on gene families involved in the transgenerational mouth plasticity of *P. pacificus* (Quiobe et al., 2025), revealed gene expansion (Röseler et al., 2022), supporting the notion that genetic accommodation proceeds transgenerational plasticity. These findings suggest that genes driving transgenerational plasticity are evolving rapidly,

although further selection experiments are needed to confirm the genetic assimilation process and fully understand its impact on genomic evolution.

i.b. Transgenerational plasticity is under evolution

If transgenerational plasticity is heritable, it becomes subject to evolutionary forces. Transgenerational plasticity that depends on the allele frequencies provides populations with adaptive flexibility, enabling them to respond to environmental changes across generations (Jablonka, 2017). However, when fluctuation occur between largely different environmental conditions, transgenerational plasticity may vary, which in turn influences its evolutionary trajectory, refining its response to specific challenges (Engqvist & Reinhold, 2016; Nettle & Bateson, 2015). This variability in transgenerational plasticity expression across environments underscores its potential to evolve as a mechanism for adaptation.

Selection on transgenerational plasticity can only occur if its fitness effects are beneficial in one environment but costly in another environment. To identify these trade-offs, first we observed the copper induced transgenerational fitness effects of a single genotype under natural conditions (manuscript II). We found that ancestral copper excess increased fitness of plants by 36% under recurrent copper excess but decreased fitness by 16% in the absence of recurrent stress. Second, we correlated the mean pre-treatment ratios per genotype of analysis under control and recurrent biotic and abiotic stress conditions and found that genotypes with enhanced fitness under recurrent stress had strong costs if the stress did not recur, and vice versa (manuscript I and III). These transgenerational trade-offs, likely arising from incomplete resetting (Uller et al., 2015), are consistent in multigenerational effects in *A. thaliana*. When infected with the pathogen *P. syringae* increased resistance of plants was observed only if the same stressed recurred but stresses such as salt and the fungus *Alternaria brassicicola* enhanced plant susceptibility (López Sánchez et al., 2021). Similarly, ancestral copper excess enhanced fitness by 36% under recurring stress but decreased fitness by 16% in the absence of stress (Huber et al., 2021). In terms of theoretical modelling, transgenerational plasticity was favoured when the ancestral environment strongly correlated with the offspring environment, but unfavoured in weak or non-correlated environments (Colicchio & Herman, 2020). Opposite response under matched and mismatched environments shows that the adaptiveness potential of transgenerational plasticity is context-dependant, indicating that transgenerational plasticity is under evolution.

The evidence presented in this section highlights the potential of transgenerational plasticity to drive evolutionary changes. Our findings demonstrate that transgenerational plasticity extends beyond phenotypic changes to influence organismal fitness. Its effects align with the genetic architecture of organisms, proving that transgenerational plasticity is heritable and may modulate genome evolution. Crucially, we show that transgenerational plasticity is

not inherently adaptive; its effects can be beneficial in some environments and detrimental in others. This variability can potentiate evolutionary dynamics by increasing directional selection pressures (Ghalambor et al., 2015). Thus, transgenerational plasticity itself is subject to evolutionary forces, and its adaptive or maladaptive outcomes can be predicted through the resistance of organisms. In summary, transgenerational plasticity encompasses both adaptive and maladaptive effects, providing a mechanism for organisms to evolve in response to environmental challenges.

ii. Mechanisms underlying the behaviour of transgenerational plasticity

ii.a. Predicting transgenerational inheritance

If transgenerational plasticity has a genetic basis and varies according to the environment, can it be predicted? Previous research in *A. thaliana* struggled to identify the magnitude of transgenerational changes in flowering phenotypes, making it difficult to predict the extent of transgenerational plasticity (Alvarez et al., 2020; Lin et al., 2024). By exposing 56 different genotypes to ancestral copper excess, we identified that susceptible genotypes, which were negatively affected by the ancestral stress when the stress did not recur, benefited largely from recurrent stress. In contrast, resistant genotypes, largely benefited in the absence of recurrent stressed but constrained fitness under recurring copper excess. In doing so, we not only identified a genetic and environment dependency for transgenerational plasticity but were also able to predict its effects. Nevertheless, these results may be limited to the stress concentrations and the number of generations within and after stress (**Figure 2**). Cadmium applied to *A. thaliana* in high and low concentrations showed that transgenerational plasticity has a dose-dependent effect, with higher concentrations leading to stronger transgenerational effects (Lin et al., 2024). Similarly, lower concentrations of cyanobacterium *Microcystis aeruginosa* exudates in the parents of the duckweed *Lemna* minor promoted growth in the offspring, while higher concentrations inhibited growth (Li et al., 2024b). Meanwhile, transgenerational plasticity of mouth forms in *P. pacificus* were only retained if the nematode was exposed to a minimum of five generations of differential diet (Quiobe et al., 2025) and transgenerational benefits and costs varied with the recovery length in *S. polyrhiza* (Huber 2021). Although further experiments are required to test different concentrations and number of generations within and after stress, our data suggest that transgenerational plasticity can be predicted.

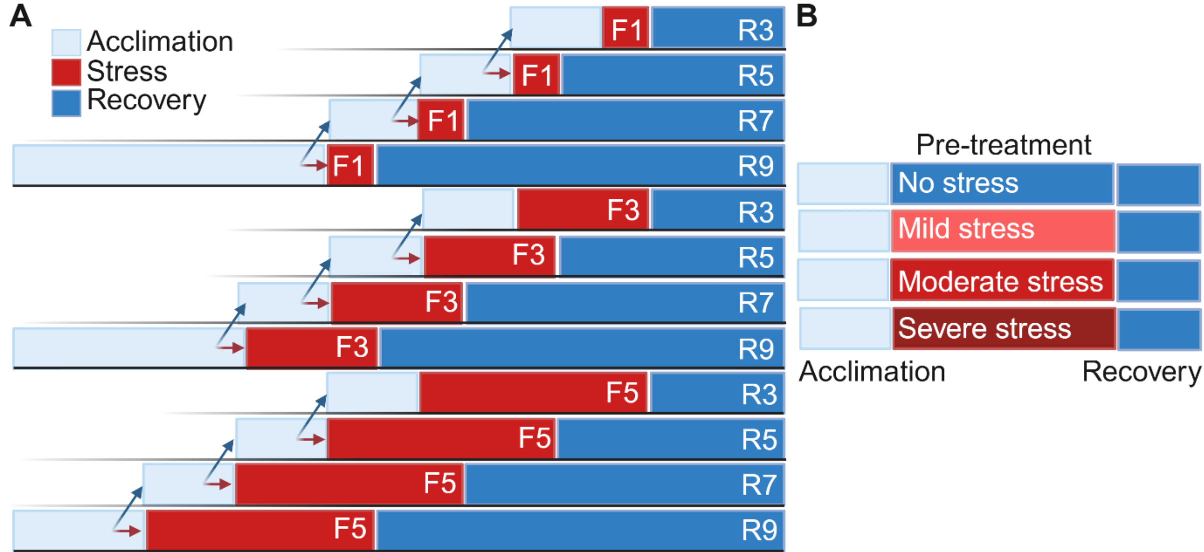

Figure 2. Influence of generational timing within and after stress, and stress intensity on transgenerational plasticity.

A To assess the number of generations required to induce transgenerational plasticity and the duration of this plasticity in *S. polyrhiza*, plants can be exposed to stress for one, three, or five generations–known to produce transgenerational plasticity–(pre-treatment). After exposure, plants are recovered in stress-free conditions for a minimum of three generations All treatments require their corresponding controls per lineage opened for stress and recovery conditions. Red arrows indicate offspring selected for stress pre-treatment, while blue arrows indicate offspring selected to continue acclimation, maintaining the same number of propagated generations during the experiment. F = generations under ancestral stress (pre-treatment), R = generations in the absence of ancestral stress (recovery). **B** To evaluate the strength of transgenerational plasticity, varying stress intensities during ancestral exposure (pre-treatment) should also be considered.

ii.b. Mechanisms responsible for environment dependant transgenerational trade-offs

The mechanisms underlying the environmental trade-offs of transgenerational plasticity remain unclear. Engqvist & Reinhold (2016) and Metcalfe (2024) proposed that organisms with adaptive responses benefit in matched environments, while those with parental advantages, lacking predictive adaptive responses, may fare better in mismatched environments. Under ancestral copper excess, resistant genotypes increased fitness in the offspring once the stress ceased (manuscript I), resembling a silver-spoon effect. This phenomenon benefits the offspring of advantageous parents, regardless of environmental matching (Grafen, 1998), suggesting that the benefits of parental exposure to copper do not rely on environmental prediction and cannot be considered adaptive (Engqvist & Reinhold, 2016). Different from the observed transgenerational non-additive changes, the silver-spoon effect lacks predictive value, as it provides consistent fitness outcomes regardless of the environment (Engqvist & Reinhold, 2016). Alternatively, hormesis effects, where mild stress boosts offspring fitness (Herms & Mattson, 1992), may explain environment-dependent benefits. Copper excess may

trigger stronger defences in susceptible genotypes, reducing the resources for growth and reproduction in the offspring, but enhancing fitness under recurrent stress (Bell & Hellmann, 2019; Moran et al., 2010). Nevertheless, these mechanisms poorly explain the observed trade-offs in both resistant and susceptible genotypes, highlighting the complexity of resource allocation dynamics. This suggests that a better understanding of transgenerational resource allocation is needed to clarify how environmental stresses shape phenotypic and fitness outcomes in matched and mismatched environments (Auld et al., 2009; Bell & Hellmann, 2019; López Sánchez et al., 2021).

Transgenerational resource allocation provides a useful framework to understand the environmental trade-offs between resistance and reproduction in plants. In line with recent studies, I propose a third mechanism in which offspring of resistant genotypes prioritize plant growth and reproduction, while offspring of susceptible genotypes allocate more resources to defence (**Figure 3A**). Although controversial, this mechanism assumes that in the absence of a resource ceiling (He et al., 2022), stress-resistant organisms, rich in defence resources, can mitigate stress effectively and redirect stored growth resources once stress diminishes (Bell & Hellmann, 2019). This allows their offspring to outcompete susceptible genotypes in benign conditions. In contrast, susceptible genotypes may focus on defence, benefiting their offspring under the same type of recurrent stress (López Sánchez et al., 2021). In our experiments, genotypes that benefited from benign conditions following ancestral aphid herbivory not only showed reduced fitness under recurrent herbivory but also boosted aphid fitness (manuscript III). These data suggest that plants stored growth and reproduction energy away during ancestral aphid herbivory and remobilized the energy back in the offspring, potentially benefiting plant feeders like the waterlily aphid (He et al., 2022). Similar prioritization of growth recovery at the expense of resistance has been observed in *Populus angustifolia* and *Populus fremontii*, where offspring showed decreased defence resources but enhanced growth-related traits (Holeski et al., 2013). To differentiate between hormesis effects and transgenerational resource allocation, researchers could first expose resistant genotypes to stress levels that reduce fitness similarly to susceptible genotypes and then assess whether the fitness of offspring reverts under recurrent stress. Additionally, screening metabolites related to defence and growth in resistant and susceptible genotypes under both first-time and recurrent stress (**Figure 3B**) could shed light on the mechanisms underlying plant resource allocation strategies.

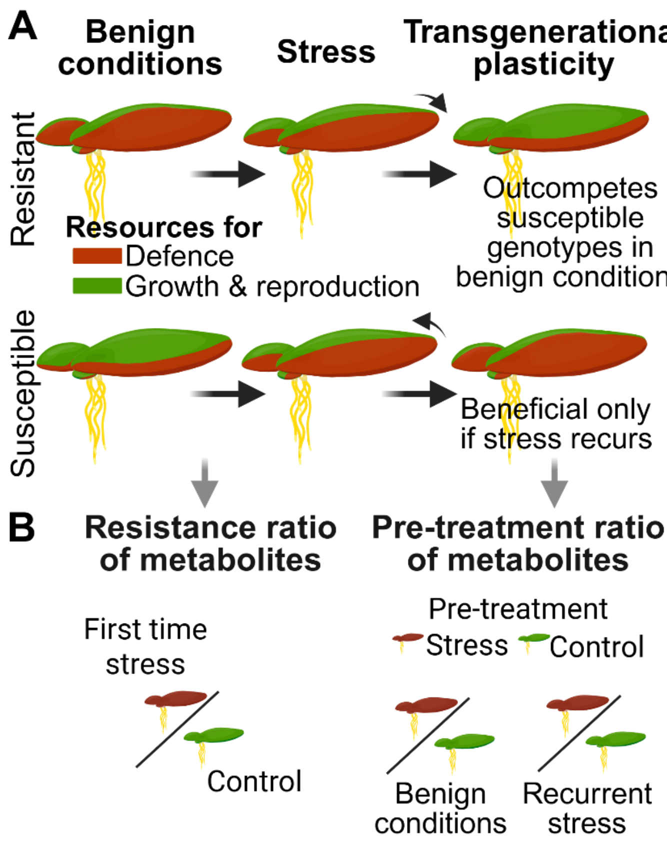

FIGURE 3. TRANSGENERATIONAL RESOURCE ALLOCATION IS DEFINED BY THE RESISTANCE OF A GENOTYPE.

A Stress resistant genotypes are less affected by stress and re-allocate reproduction resources to populate water bodies fast, while susceptible genotypes allocate resources into defence, outperforming under recurrent stress. **B** Metabolite comparison between first time stressed and control plants (resistance ratio), and ancestrally stressed plants and control plants (pre-treatment ratio) show the physiological changes underlying the trade-offs of transgenerational plasticity. Control refers to plants grown under benign conditions. Diagonal lines between stressed and control plants refer to the comparison or ratios estimated for statistical analysis.

ii.c. The link between the patterns and mechanism of transgenerational effects

The patterns of transgenerational plasticity in plant physiology, transgenerational retention and transgenerational priming of metabolites, offer insights into the mechanisms underlying transgenerational plasticity. Physiological changes could result from vertical transmission of metabolites or epigenetic regulation of the biosynthetic machinery (Rasmann et al., 2012). During clonal reproduction, parental fronds of *S. polyrhiza* remain physically connected to the offspring through the stipe (Landolt, 1986), which increases the likelihood of transmitting metabolites vertically (Latzel et al., 2016). To mitigate the transmission, we severed the stipe after two to four days when moving the new generation to a new tube as part of the single descendant propagation, limiting the time for vertical transmission of metabolites. Despite this separation, the likelihood of transmitting these transgenerationally plastic metabolites remains. Although we did not attempt to identify the epigenetic mechanisms that drive transgenerational plasticity in *S. polyrhiza*, we analysed the transgenerational retention and priming patterns, as well as the expression of biosynthetic genes to recognize epigenetic priming events (Rasmann et al., 2012). Under aphid herbivory, we observed both transgenerational retention and transgenerational priming of the amine tyramine (Manuscript III). Surprisingly, the expression levels of two of the three annotated tyrosine decarboxylases (*TYDCs*), genes that encode the enzymes to synthesize tyramine, revealed that, while *TYDC1* retained the enhanced expression after ancestral stress, *TYDC2* was transgenerationally

primed under recurrent aphid herbivory, aligning with the observed physiological changes. The transgenerational regulation of biosynthetic machinery could result from metabolite feedback loops (Erb & Kliebenstein, 2020; Li et al., 2024c). However, these signalling loops require sustained or threshold concentrations of the metabolite, similar to the induction phase (Bheda, 2020; Li et al., 2024a), disappearing during the recovery phase in transgenerational experiments. Therefore, the observed transgenerational priming can only be explained due to the regulation of epigenetic marks (Grossniklaus et al., 2013). Although further studies should explore the specific epigenetic marks ruling transgenerational plasticity in *S. polyrhiza*, these results suggest that epigenetic regulation plays a key role in transgenerational plasticity of clonal plants.

This thesis adds valuable insides into the factors that shape transgenerational plasticity. Using immense experimental setups as well as exhaustive statistical analyses, I was able to characterize and predict transgenerational plasticity, but also to suggest that epigenetic mechanisms shaped the transgenerational effects. While epigenetic marks in the absence of genetic change cause transgenerational plasticity (Cubas et al., 1999; Dias & Ressler, 2014; Gallego-Bartolomé et al., 2018; Manning et al., 2006; Ong-Abdullah et al., 2015; Rechavi et al., 2014), screening of epigenetic marks underlying transgenerational plasticity has rarely shown causality (Quiobe et al., 2025). These findings indicate that focus on a single epigenetic mechanism rarely shows the causal mark of transgenerational plasticity. Instead, a more comprehensive approach that examines multiple epigenetic mechanisms and their interactions with candidate genes presents a promising direction for further research. By addressing this gap, we can begin to piece together the intricate molecular network underlying transgenerational plasticity.

iii. Ecological implications of transgenerational plasticity

Transgenerational plasticity affects not only the evolution of organisms but also their ecological dynamics. By influencing the fitness of future generations of specific genotypes, transgenerational plasticity can alter genotype frequencies within populations. For instance, if transgenerational plasticity benefits resistant genotypes in the absence of recurrent stress, their offspring will dominate in relative stable environments, while susceptible genotypes will thrive in fluctuating environments, driving changes in population structure (**Figure 4**). However, changes in population dynamics influence the whole community of organisms, interacting directly, or indirectly. For example, Knight et al. (2005) found that fishes eating dragon fly larvae close to terrestrial flowering plants increased pollination in comparison to plants near water bodies without fishes, as dragon flies eat insect pollinators. In duckweeds, snails benefitted plants instead of reducing their fitness when consuming the *S. polyrhiza* competitor, the green algae (Böttner et al., 2024). Similarly, herbivory of the native waterlily aphid herbivore reduced

plant populations, increasing phytoplankton and boosting daphnia (Schäfer et al., 2024). These data suggests that transgenerational effects on plant fitness and morphology have intra- and inter-organism repercussions.

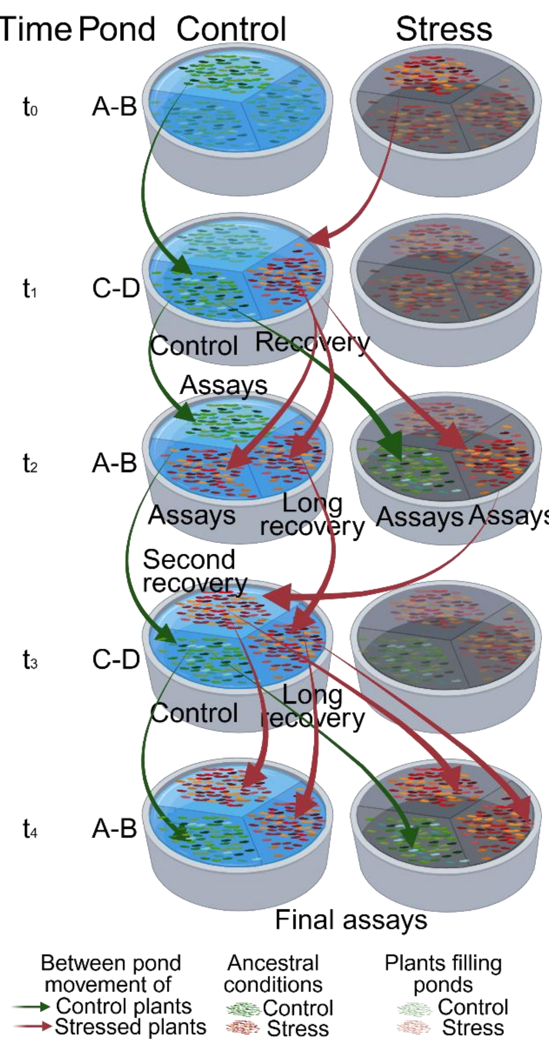

FIGURE 4. THE FREQUENCY OF RECURRENT STRESS INDUCING TRANSGENERATIONAL PLASTICITY MODELS POPULATION DYNAMICS.

To assess how transgenerational plasticity influences genotype frequencies based on the time interval between ancestral and recurrent stress, reciprocal transplant experiments can be conducted every five generations (three to four weeks). After recovery from the pre-treatment (ancestral conditions), plants are either maintained under benign (control) or recurrent stress conditions, representing stable and fluctuating environments, respectively. Following assays, second time stressed plants are returned to recovery, and finally plants under stable and fluctuating environments undergo further assays. These assays include measurements of surface area in the population, and genotype frequency analysis of resistant and susceptible genotypes using pool-seq. The movement of plants between ponds introduces a founder effect, while "plants filling ponds" maintain consistent surface coverage and nutrient usage throughout the experiments. Pond divisions allow movement of nutrients and microbiota, but not plants.

iii.a. Transgenerational effects on plant – herbivore interactions

Transgenerational effects on an organismal fitness can reverberate on the performance of interacting organisms. Evidence of maternal effects altering both the organism of focus and its interacting species is well-documented (Baker et al., 2019; Holeski et al., 2012; Luna et al., 2011; Rasmann et al., 2012; Verhoeven & van Gurp, 2012). For example, *Polygonum persicaria*, when grown under shade conditions, enhanced the competitive ability of its offspring, suppressing neighbouring plants and altering community dynamics (Baker et al., 2019). Similarly, choice experiments on the larvae of *Spodoptera exigua* showed that offspring of unstressed *Taraxacum officinale* parents were preferred over offspring whose parents had been treated with jasmonic acid (Verhoeven & van Gurp, 2012). We found that ancestral abiotic and biotic stress in single-descendant lineages caused transgenerational changes in plant fitness, but also altered the fitness of its herbivore, the waterlily aphid: plants exposed to both ancestral copper excess and aphid herbivory boosted aphid fitness (manuscripts II and III).

These data demonstrate that ancestral exposure to stress can critically impact population conformation and shift ecological dynamics, even several generations after the stress has occurred.

To identified potential molecular changes affecting aphid fitness we screened for amino acids, amines, phenylpropanoids and phytohormones that could be shared under both ancestral copper excess and aphid herbivory. Although not exactly the same metabolite nor the same transgenerational patter, we observed that both stresses transgenerationally enhanced jasmonates in single descendant lineages: while ancestral copper excess transgenerationally retained the jasmonate precursor OPDA (manuscript II), ancestral aphid herbivory transgenerationally primed jasmonic acid and jasmonic acid isoleucine (manuscript III). Jasmonates play a crucial role in signalling antioxidant regulation and osmolyte synthesis under abiotic stress (Aslam et al., 2021; Riemann et al., 2015), but more importantly, they activate several pathways that favour herbivory defence (Gasperini & Howe, 2024; Li et al., 2022; Machado et al., 2016; Yang et al., 2012). To corroborate the unexpected effect of jasmonates in aphid reproduction, we supplemented plants with methyl jasmonate in three different setups, considering short and long jasmonate induction time (manuscript II and III, respectively), and the presence and absence of DMSO to dissolve the methyl jasmonate (manuscript II). All setups showed that enhanced jasmonates increased both per capita and population growth rates (manuscript II and III). Interestingly, genome-wide association studies in *S. polyrhiza* resistance to the herbicide diquat identified that genotypes carrying a 184 bp deletion in the lipoxygenase LOX2.1 gene, which is involved in jasmonate biosynthesis (Chauvin et al., 2016), exhibited higher resistance to diquat than homozygous genotypes lacking the deletion, suggesting that jasmonates may make *S. polyrhiza* more vulnerable to diquat (Höfer et al., 2024). These findings suggest that jasmonates play an uncharacterized role in *S. polyrhiza*, which aphids may manipulate (**Figure 5**), warranting further investigation.

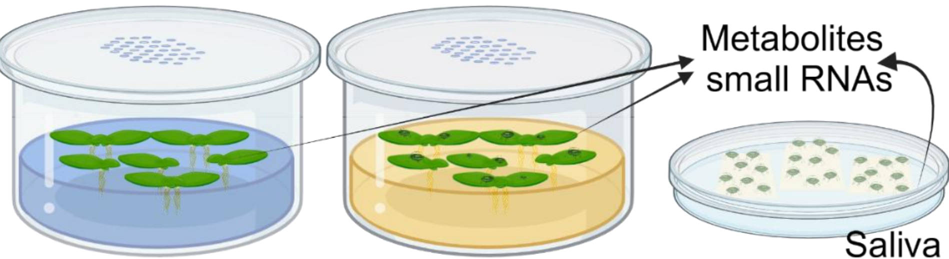

FIGURE 5. IDENTIFYING CANDIDATE EFFECTORS INTRODUCED BY APHIDS TO MODULATE PLANT GENE EXPRESSION.
To determine whether aphids manipulate plant responses, both aphid saliva and plant material are collected for metabolite screening and small RNA sequencing or RT-PCR. Saliva can be collected from aphids feeding on a sucrose solution or directly from the plant if using Electrical Penetration Graphs (EPG) while the plant is immobilized in agar.

Aphid herbivory-induced transgenerational plasticity may not be completely maladaptive. In addition to the transgenerationally induced jasmonates, ancestral aphid herbivory also transgenerationally primed and retained elevated tyramine concentrations, an amine that is also enhanced in *S. polyrhiza* by snail herbivory (Malacrinò et al., 2024). The external application of tyramine to plants tended to increased plant fitness under aphid herbivory, suggesting an adaptive role for this compound (manuscript III). Moreover, aphids feeding on these plants, as well as those fed a sucrose and tyramine solution, demonstrated that tyramine negatively affects the waterlily aphid, further supporting the amine potential as a defensive mechanism (manuscript III). Since aphids may lack the ability to convert tyramine into dopamine or revert it to L-tyrosine (Fuchs et al., 2014; Rabatel et al., 2013), either tyramine itself or its derivative, octopamine, could intoxicate aphids (Orchard, 1982; Rosikon et al., 2023). Further investigation into the metabolites involved in the L-tyrosine pathways in aphids, along with behavioural changes following tyramine and octopamine feeding, will help clarify how these metabolites influence aphid fitness. Overall, the findings suggest that, despite the negative effects of jasmonates, transgenerationally enhanced tyramine may provide an adaptive response against herbivory.

iii.b. Transgenerational plasticity under natural environments

Controlled experiments under standardized laboratory conditions are essential to identify pure changes, but these changes may differ from those observed in natural conditions. For instance, determining whether transgenerational plasticity occurs and affects the organismal phenotype and fitness is only possible under single descendant lineages. Single descendant lineages minimize confounding effects arising from within-lineage variation, thereby preventing selection of beneficial variants (Baugh & Day, 2020). Additionally, to exclude the vertical transmission of microbes, we surface-sterilized plants (manuscript I) and cleaned aphids over multiple generations (manuscript III). However, under natural conditions, where both selection and drift occur, the effects may differ considerably. Single-descendant lineages of genotype Sp004 exposed to ancestral copper excess under controlled laboratory and natural conditions showed opposite fitness effects under recurrent copper excess (manuscript II). Under controlled conditions, Sp004 consistently exhibited negative fitness effects from ancestral copper excess under recurrent stress (manuscripts I and II), while under outdoor conditions, ancestral stressed enhanced fitness under recurrent copper excess (manuscript II). Microbes likely upregulate the production of defence and growth metabolites in *S. polyrhiza* (Zhao et al., 2017), decreasing the damage of oxidative stresses such as cadmium (Mu et al., 2024). Therefore, vertical transmission of beneficial microbes can protect plants under recurrent stress. These data suggest that the interaction between microbiota and plants through generations plays a crucial role in shaping transgenerational plasticity under natural environments.

Plants overcome the maladaptive effects of aphid-induced transgenerational plasticity under natural conditions. To evaluate whether the maladaptive effects of ancestral aphid herbivory persisted under natural conditions, we grew *S. polyrhiza* genotype Sp102 in large monoclonal populations, where both, vertical transmission of microbes and selection could occur (manuscript III). Interestingly, concentrations of the transgenerationally plastic tyramine indoors were also enhanced outdoors, picking along with aphid density and returning to basal levels after winter. Furthermore, the elevated concentrations were retained in plants for approximately five generations in the absence of herbivory. In contrast, jasmonate concentrations, which favoured aphid fitness, remained similar under both control and aphid herbivory conditions, but with 10-fold higher levels. As a result, ancestral herbivory seemed to reduce aphid fitness instead of benefitting the insect (manuscript III). These observations suggest that in natural conditions, plants may overcome the maladaptive effects in two non-exclusive manners: through vertical transmission of microbiota, likely boosting jasmonates in comparison to indoor conditions, or by selection of intra-genotype transgenerational variants or microbe-induced variants. These data suggests that outdoor conditions facilitate the induction of additional traits that help plants overcome the negative impacts of transgenerational plasticity.

Gained transgenerational benefits in outdoor conditions are likely mediated by vertical transmission of microbes. Transgenerational plasticity may arise through at least two pathways: one that produces deterministic changes and thus homogenizes the transgenerational effects on individuals of the same genetic background, and another that generates stochastic changes, producing intra-genotype variation and thus allowing selection to act (Huber & Chávez, 2025). Although study in the variance of transgenerational plasticity is scarce, but see Shahmohamadloo et al. (2025), organisms are expected to overcome the maladaptive effects of transgenerational plasticity if ancestral conditions increase phenotypic variation (Herman et al., 2014; O'Dea et al., 2016), a phenomenon described as diversified bet-hedging (Seger & Brockman, 1987). However, variance analysis of 56 different genotypes exposed to ancestral copper excess demonstrated that ancestral stress homogenizes the responses of individual replicates within each genotype, reducing the samples that ceased reproduction under copper excess (manuscript I). These results suggest that transgenerational plasticity likely leads to more deterministic rather than stochastic changes. With this discussed, the beneficial effects of ancestral aphid herbivory outdoors opposed to their negative effects observed indoors were likely due to the plant's interaction with co-existing organisms, such as microbiota. To test the hypothesis that vertically transmitted microbes benefit plants, is necessary to first characterize the microbiota profile in outdoor conditions and then perform single descendant experiments with sterilized plants under control and stress conditions, with and without microbe inoculation during the pre-treatment phase (**Figure 6**). The current

research, showing the aphid fitness and plant physiology differences in Sp102 indoors and outdoors, together with the fitness differences of genotype Sp004 under controlled and natural conditions, strongly suggest that vertical transmission of microbiota promotes in transgenerational plasticity an adaptive role under natural conditions.

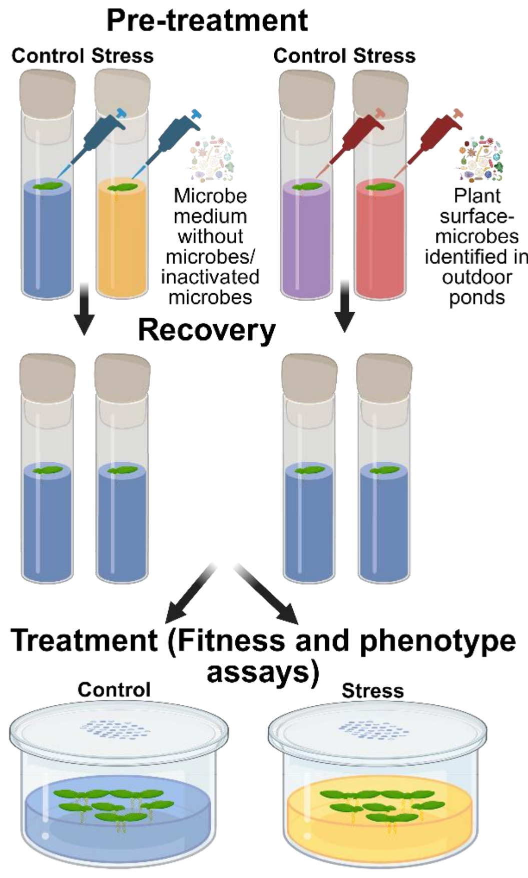

FIGURE 6. THE ROLE OF MICROBE VERTICAL TRANSMISSION IN TRANSGENERATIONAL PLASTICITY.
To assess the transgenerational adaptive role of microbes in plant fitness, it is essential to sterilize a genotype where transgenerational plasticity is associated with costs. The following three key components should be identified: First, the microbiota that directly interact with plants under outdoor conditions, specifically microbes on plant surface. Second, the transgenerationally transmitted microbiota that benefits ancestrally stressed plants under recurrent stress when compared to ancestrally stressed surface-sterile plants. This allows for the identification of microbes with transgenerational benefits. Third, the distinction between the direct effects of the microbiota and their exudates. To achieve this, microbes are inactivated (e.g., by autoclaving), and their medium is applied in an additional control pre-treatment, allowing for separation of microbial effects from the influence of microbial exudates.

The effects of transgenerational plasticity in the evolution and ecology of the clonal duckweed extends to other organisms. The study of transgenerational plasticity has focused on sexually reproducing species (Bell & Hellmann, 2019; Groot et al., 2016; Lin et al., 2024; Sánchez-Tójar et al., 2020), finding limited effects likely due to epigenetic erasure during meiosis (Crevillén et al., 2014; Ono & Kinoshita, 2021; Tao et al., 2017). However, clonal reproduction is not restricted to unicellular organisms, but is also common in plants, and animals (Avise, 2015; Herben & Klimešová, 2020; Hiebert et al., 2021; Jackson, 1985; Klimeš et al., 1997; Tiffney & Niklas, 1985), showing that the transgenerational effects on *S. polyrhiza* are expected in other clonal organisms. Furthermore, many invasive weeds and crops reproduce asexually (McKey et al., 2010; Pyšek, 1997), suggesting that transgenerational plasticity has a huge impact in agriculture. For example, initial stress exposure in herbariums, considering the resistance profile of the genetic variants, can transgenerationally prime defence metabolites like tyramine or jasmonates to reduce plages in fields without the use of pesticides. Additionally, we identified a genetic base in transgenerational plasticity, opening the door to identify the genes controlling reproduction and defence in other asexual, but also

sexually reproducing organisms. Altogether, the data presented in this thesis shows the relevant role of transgenerational plasticity in clonal organisms and facilitates research in sexually reproducing species.

The evidence presented in this section highlights the significant role of transgenerational plasticity in shaping not only individual organism fitness but also ecological interactions and community dynamics. The transgenerational effects observed in clonal *S. polyrhiza* revealed how exposure to both abiotic and biotic stressors can alter the performance of subsequent generations, likely influencing population structure, species interactions, and ecosystem processes. Furthermore, the effects might be influenced by the microbial communities, suggesting that vertical transmission of microbes may serve as an adaptive mechanism under natural conditions. Moreover, the potential applications of these findings extend beyond ecological research, offering insights into agricultural practices, particularly in enhancing crop resilience through transgenerational priming of defence traits. As we deepen our understanding of the molecular mechanisms involved, particularly in jasmonates and other metabolites, we open new avenues for leveraging transgenerational plasticity in both natural and managed ecosystems.

iv. Future perspectives

Although this thesis shows strong evidence of the evolutionary and ecological role of transgenerational plasticity, this phenomenon can be explored in further venues. For example, by identifying and manipulating the epigenetic machinery, by manipulating the genes underlying transgenerational responses or by modulating the selection force on transgenerational plasticity of mix population. In this section I discuss some future approaches to understand more about transgenerational plasticity and its role in organismal evolution.

Identifying the evolutionary and ecological effects of transgenerational plasticity opens the path to characterize the underlying epigenetic mechanisms. Early research focused on the use of inhibitors to examine, for example, DNA methylation or histone modifications. Inhibitors such as 5-azacytidine or zebularine, along with histone deacetylase inhibitors, can alter transgenerational plasticity (Boyko et al., 2010; Herman & Sultan, 2016; Jia et al., 2015; Mladenov et al., 2021; Quan et al., 2022; Verhoeven & van Gurp, 2012). However, inhibitors may lead to inconsistent findings and cause plant toxicity (Agius et al., 2023; Puy et al., 2018). Researchers now rely on advanced techniques such as epiGBS (Van Antro et al., 2023), bisulphite sequencing (Feiner et al., 2022; Sammarco et al., 2023), Oxford nanopore sequencing (Guo et al., 2024) and small RNA sequencing (Baugh & Day, 2020; Morgado et al., 2017) to assess the role of epigenetic marks on transgenerational plasticity. Additional approaches, like chromatin immunoprecipitation sequencing (ChIP-Seq), chromosome conformation capture (Hi-C technologies) and single cell sequencing will help researchers to

study multiple epigenetic mechanisms within the same experiment and characterize the cross-talk of these epigenetic marks (Jia et al., 2015). The rapid development of these technologies makes them increasingly accessible, offering new opportunities to better understand the complex epigenetic regulation of transgenerational plasticity.

The mechanisms and genes involved in transgenerational plasticity require experimental validation. Like in any research field, confirming the functional roles of epigenetic marks or genes linked to transgenerational plasticity is essential. Recent advancements in molecular technologies, such as CRISPR-Cas9 in plant engineering (Kumar et al., 2023; Saini et al., 2023), offer new strategies to target specific genomic regions, which reduces fitness and phenotype costs associated with broader genetic modifications (Gallego-Bartolomé et al., 2018). By using tissue-specific or inducible promoters, researchers can control the expression of defence-related genes to, for example, limit the toxicity of defence metabolites or target their accumulation in specific tissues (Koramutla et al., 2016; Lee et al., 2007; Rahamkulov & Bakhsh, 2020). This approach helps reduce plant harm while still enhancing the plant's ability to cope with abiotic or biotic stress. In the case of tyramine, manipulating expression of L-tyrosine decarboxylases in tissues like the phloem–using sucrose-proton symporter promoters (Cao et al., 2013; Sadeghi et al., 2007)–or the cell wall–using Rubisco activase promoters (Rahamkulov & Bakhsh, 2020)–can show where the accumulation of defence molecules affects the waterlily aphid's reproduction. Genetically engineering plants thus provides a powerful tool to explore the role of epigenetic marks and genes in transgenerational plasticity.

Identifying the epigenetic marks and genomic regions that benefits plants the most against future stresses will allow new opportunities for plant breeding programs to create resilient crops (Vázquez-Hernández et al., 2019; Villagómez Aranda et al., 2022). For example, identifying the epigenetic marks that transgenerationally prime plants against biotic and abiotic stress, such as pests or temperature fluctuations, could enable the creation of epimutants. These plants would regulate defence gene expression only under stressful environmental conditions, thus minimizing the costs of defence associated with transgenerational plasticity. Taken together, identifying the underlying mechanisms and pathways of transgenerational plasticity holds the potential to enhance agricultural practices and crop resilience.

Ongoing research into transgenerational plasticity, while not immediately applicable, is crucial for understanding the tools that drive evolution and predicting future evolutionary trajectories. In addition to investigating epigenetic mechanisms, further experiments are needed to confirm the deterministic and stochastic pathways, and the bed-hedging effects of transgenerational plasticity across various clades, to explore the relationship between epimutation rates and the predisposition to evolve via transgenerational plasticity and identify the factors that influence the adaptiveness of this phenomenon under natural conditions. By

advancing research in both molecular and ecological domains, we can uncover strategies to harness the adaptive potential of transgenerational plasticity, thereby deepening our understanding of how organisms adapt to environmental challenges over generations.

Conclusions

The data discussed in the present thesis shows that transgenerational plasticity plays a significant role in shaping the evolutionary trajectory of organisms. Using different genotypes and environments, I show that transgenerational plasticity is heritable, and yet plastic over the years; that it has a genetic base, providing adaptive and maladaptive effects depending on the genotype, and that the adaptiveness in matched and mismatched environments depends on the resistance of the genotype to the stress. The allele frequency dependence allows natural selection to act upon transgenerational plasticity, thus altering the evolution of populations. Furthermore, I show that the transgenerational changes altering an organism fitness and physiology can reverberate not only within populations, but also on co-occurring organisms, altering population and thus, ecosystem dynamics. In the opposite direction, co-existing organisms such as microbiota, may have the potential to modulate transgenerational plasticity, resulting in adaptive effects in natural environments. Therefore, I strongly encourage to continue characterizing transgenerational plasticity in both asexual and sexually reproducing organisms by identifying the epigenetic mechanisms and genes underlying this plasticity, as well as the biotic and abiotic factor that modulate its outputs in ecologically relevant setups.

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Acknowledgements

Alexandra Mireya Chávez Argandoña | CV

Evolutionary biologist analysing fitness, metabolites, and soon epigenomes. Focused on characterizing the pathways and mechanisms through which stress-induced transgenerational plasticity influences the evolution of species.

Johann-Joachim-Becher-Weg 7, 55128 Mainz, Germany

Education

Dr.rer.nat. student **Oct 2020 – Jun 2025**
Johannes Gutenberg-Universität Mainz *Mainz, Germany*
Advisor:
Project: Learning from past generations: Adaptive transgenerational plasticity in clonal plants.

M.Sc. Biology with Minor in Evolution (4 semester degree) **Apr 2018 – Mar 2020**
Universidad del Bío-Bío *Chillán, Chile*
Advisor:
Dissertation title: Were Camelids Carnivores in the Past? Approach from Genes of the Insulin Signalling Pathway and the Sweet/Umami Taste Receptors.
Graduated with distinction.

B.Sc. Biology (10 semester degree) **Mar 2012 – Jul 2016**
Universidad Peruana Cayetano Heredia *Lima, Peru*
Thesis dissertation for "Licenciada" degree in Peru **Dec 2017**
Main Advisor:
Secondary Advisors:
Dissertation title: Evaluation of the habitat and the seasonality over the diet of the Andean Bear (*Tremarctos ornatus*) in the district of Corosha, Department of Amazonas. (Translated from Spanish).
Specialisation in Ecology and Molecular Biology

Publications

Chávez A., Schreyer A., Prüsener P., Schäfer M., Xu S., Huber M. Copper-induced transgenerational plasticity in plant defence boosts aphid fitness. https://doi.org/10.1111/pce.15406

Wang Y. Duchen P., **Chávez A.**, Sree K.S., Appenroth K.J., Zhao Hai, Höfer M., Huber M., Xu S. Population genomics and epigenomics of *Spirodela polyrhiza* provide insights into the evolution of facultative asexuality. *Communications Biology* 7, 581 (2024). https://doi.org/10.1038/s42003-024-06266-7

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Marín J., Rivera R., Varas V., Cortés J., Agapito A., Chero A., **Chávez A.**, Johnson W., Orozco-terWengel P. (2018). Genetic variation in coat colour genes MC1R and ASIP provides insights into domestication and management of south american camelids. *Frontiers in Genetics*. 9:487. doi: 10.3389/fgene.2018.00487

Chávez A., Díaz C., Amanzo J.M. (2018). Plants in the diet of the spectacled bear (*Tremarctos ornatus*) in Corosha. *Field guides The Field Museum*. 1(994).

Awards, Funding and Others

Invited speaker - outstanding doctoral project **2020**
Reserach: "Non-genetic inheritance alters stress resistance across generations in the Giant duckweed", International Conference on Duckweed Research and Applications (ICDRA)

Award Painel Sílvio de Almeida Filho: Honorius mention in Animal Genetics **2018**
Research: "Initial Characterization of Genetic Variability and Population Structure of the Andean Bear Tremarctos ornatus) in Peru", XXII International Congress of Genetics (ICG), Brazil

Graduated Studies Scholarship for Proficiency Overseas Students **2018 – 2019**
Universidad del Bío-Bío, Chile

Carlos Ponce Del Prado Grant **2016**
Research grant of $1,500.00 USD, Peru

Universidad Peruana Cayetano Heredia Undergraduate Scholarship **2012 – 2017**
20% discount in university fees for undergraduate students ranked in the top third of the semester, Peru

Presentations in Conferences & Workshops

Transgenerational plasticity of plant defence is relevant for both plant and herbivore fitness **2024**
The international Conference of the German Society for Plant Sciences (Botanik-Tagung) *Halle/Salle, Germany*

Non- genetic Transgenerational Inheritance affects the Resistance of the Giant Duckweed (Spirodela polyrhiza) to Natural Stress. **2022**
International Conference on Duckweed Research and Applications (ICDRA) *Gatersleben, Germany*

Selection and cross resistance in monoclonal *Spirodela polyrhiza* populations under oxidative stress outdoors. **2021**
Epidiverse Conference *Sevilla, Spain*

Were camelids carnivores in the past? An approach from genes of the insulin signalling pathway and sweet/umami taste receptors **2020**
International Conference on Comparative Genomics and Molecular Evolution *Miami, USA*
Note: Cancelled due to corona virus outbreak

Initial Characterization of Genetic Variability and Population Structure of the Andean Bear (*Tremarctos ornatus*) in Peru **2018**
XXII International Congress of Genetics (ICG) *Foz do Iguaçu-PR, Brazil*

Andean Bear (*Tremarctos ornatus*) diet in jalca and montane cloud forest habitats in Corosha, PE **2017**
25th International Conference on Bear Research and Management. IBA *Quito, Ecuador*

Advances in the characterization of genetic variability in *Tremarctos ornatus* (Spanish) **2017**
National Workshop of Capacitation in Stool for Genetic Evaluation and Selection in Elite Alpacas Reproduction. IPEN *Puno, Peru*

Evaluation of spectacled bear (*Tremarctos ornatus*) behaviour *in situ* (Spanish) **2015**
Spectacled Bear Monitoring in the Peruvian Southeast, ACCA *Cusco, Peru*

Evaluation of Amazonic tapir behaviour (*Tapirus terrestris*) in captivity within time ranges **2014**
XXI ALPZA Congress *Lima, Peru*