HIGHLIGHTED STUDENT RESEARCH

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Inducible phenotypic plasticity in plants regulates aquatic ecosystem functioning

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Abstract

Differences among individuals within species affect community and ecosystem processes in many systems, and may rival the importance of differences between species. Intraspecific variation consists of both plastic and genetic components that are regulated by different processes and operate on different time scales. Therefore, probing which mechanisms can affect traits sufficiently strongly to affect ecosystem processes is fundamental to understanding the consequences of individual variation. We find that a dominant deciduous tree of Pacific Northwest riparian ecosystems, red alder, exhibits strong and synergistic responses to nutrient resources and herbivory stress. These induced responses, which include shifting nutrient and plant secondary metabolite composition, have cascading effects on aquatic ecosystem function. Defense responses suppress leaf litter decomposition in small streams, thus altering the rate of energy capture for one of the most abundant terrestrial carbon sources entering aquatic systems. We find that alder responses to herbivory stress largely depend on availability of soil nutrients, with modification of the highly cytotoxic diarylheptanoid group of secondary metabolites being favored in nutrient-poor environments and modification of the typically dose-dependent ellagitannins being favored in nutrient-rich environments. Importantly, these findings identify traits for herbivore resistance in alder trees and demonstrate that plastic responses occurring within a species and over short time scales substantially alter a key function of an adjacent ecosystem. Furthermore, demonstrating plasticity among alder secondary metabolites lends insight into this system, in which decomposer communities are known to adjust to the secondary chemistry of local alder trees to facilitate rapid decomposition of locally derived leaf litter.

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We document that plasticity in secondary metabolites occurring within plants over short times scales affects the rate of leaf decomposition in adjacent river ecosystems. This work changes our understanding of the temporal stability and regulation of this ecosystem function.

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Introduction

Populations survive spatial and temporal fluctuations in their environment by shifting genetic composition or individual phenotype. Phenotypic variation within species across both time and space has wide ranging implications for communities and ecosystem functions, such as energy flux among trophic levels. Recent evidence suggests variation within species can rival that of shifts in species composition in their effects (Madritch and Hunter 2002; Crutsinger et al. 2008; Jackrel and Wootton 2014; Jackrel et al. 2016; Martin and Blossey 2013; Schweitzer et al. 2005). However, in nearly all of these studies that evaluate the ecosystem-wide implications of intraspecific diversity, diversity has been assumed to be due either to fixed genetic differences, a combination of fixed genetic and phenotypically plastic differences, or demographic population structure (Crutsinger et al. 2006; Barbour et al. 2009; Hersch-Green et al. 2011; LeRoy et al.

2007; Rudolf and Rasmussen 2013a, b). While phenotypic plasticity is an important response to fluctuating environments (Agrawal 1998; Kovach-Orr and Fussmann 2012; Agrawal 1999; Hendry 2015; Miner et al. 2005), few studies have evaluated the extent to which inducible phenotypic responses extend beyond population-level dynamics to affect entire communities and ecosystems.

We aimed to test the importance of plastic responses on ecosystem functioning both within and across ecosystem boundaries using forest trees belonging to a natural population of genetically variable individuals. As major generators of primary production, the energy from tree leaves is a key driver of ecosystem functions both in forests, but also, in adjacent aquatic ecosystems that receive influxes of leaf litter (Anderson and Sedell 1979; Tank et al. 2010). Indeed, there is substantial evidence that genetically controlled leaf traits of riparian trees strongly regulate multiple ecosystem functions in terrestrial and aquatic environments (LeRoy et al. 2006, 2007; Schweitzer et al. 2004, 2008). Further, as sessile, long-lived organisms, trees experience substantial temporal fluctuations in their environment and often respond via plasticity. A major component of plasticity in plants consists of inducible defense responses aimed towards minimizing damage from herbivores (Strauss and Agrawal 1999; Agrawal 2007). Defense methods often include chemical deterrents and toxins such as tannins, cardenolides, and glucosinolates (Mithöfer and Boland 2012). Plants are also known to employ other methods, such as the reallocation of valuable nutrients from leaves to less accessible storage organs such as the roots and stem (Gómez et al. 2010). Such inducible defenses are also often context dependent. For example, a plant's defensive response may be contingent on abiotic factors, such as availability of the nutrients, water, and sunlight needed to engage the defensive response (Ballaré 2014; Stamp 2003).

We evaluate plasticity in a riparian tree, red alder (*Alnus rubra*). Red alder and other plants that are highly apparent to herbivores due to numerical dominance and/or longevity are generally predicted to produce dose-dependent quantitative defenses, such as tannins (Feeny 1976). In this system, we have previously found that trees vary in their secondary metabolites at exceptionally fine spatial scales, and this variation is a key driver of locally accelerated ecosystem functions (Jackrel and Wootton 2014; Jackrel et al. 2016). Such geographic patterns may develop over decadal (or longer) timespans due to genetic differences among red alder trees. However, as these trees induce plastic responses to environmental stresses over annual or even seasonal time-scales, aquatic communities may further respond to such plasticity. We previously demonstrated that experimentally applied mock herbivore stress and fertilizer treatments ultimately leads to suppressed aquatic ecosystem function via declines in leaf decomposition rate (Jackrel and Wootton 2015a). We

had found prior evidence that leaf carbon and nitrogen content in part explains this shift in decomposition rate. Now after collecting additional data on 62 leaf secondary metabolites and further analyzing the original experiment, here we demonstrate that the mechanism underlying reduced aquatic ecosystem function is caused by both rapidly induced shifts in red alder leaf secondary metabolites and leaf nutrients. Plastic responses can, therefore, create a diverse landscape of plant traits and by evaluating the cascading implications of such inducible traits, we may improve our understanding of a wide range of ecosystem functions, such as leaf litter breakdown and nutrient cycling. Further, identifying changes in leaf chemistry in response to these manipulations, particularly of understudied classes of secondary metabolites, provides insight into the many important traits involved in plant resistance to herbivory. Our results highlight the role of ellagitannins in Pacific Northwest ecosystems, providing further evidence in support of the importance of this understudied class of secondary metabolites in plant defense ecology (Salminen and Karonen 2011).

Methods

Study sites

We chose red alder (*A. rubra*) as our focal tree species because it is the most abundant deciduous tree in riparian zones and early successional forests of the Pacific Northwest, including our study sites along the South Fork Pysht River of the Olympic Peninsula, Washington, USA. This abundant pioneer species enriches soils for further plant succession by fixing atmospheric nitrogen via symbiotic root associations with the bacterium, *Frankia alni* (Luken and Fonda 1983). Our study sites along the Pysht River were dominated by red alder, as well as a small number of bigleaf maple (*Acer macrophyllum*) and western hemlock (*Tsuga heterophylla*). Understory vegetation was predominately vine maple (*Acer cinereus*), salmonberry (*Rubus spectabilis*), salal (*Gaultheria shallon*) and sword fern (*Polystichum munitum*). The naturally recruited population of red alder trees used in this experiment grew alongside logging roads and walking trails of the Merrill & Ring Tree Farm (48.09°N, 124.12°W) and were the same trees described in Jackrel and Wootton (2015a). Insect damage on these alder trees was common and included defoliation, skeletonization, rolled and folded leaves, leaf miner scars, and galls. In earlier work (Jackrel and Wootton 2015a), we had measured only leaf C:N:P content and decomposition rates. These results motivated us to carry out subsequent work, in which we characterized the relative abundance of 62 leaf secondary metabolite compounds in each of these red alder trees from surplus leaf material archived from these experiments. With

these additional data, we could further analyze our experiment to identify the responses of leaf chemistry in our environmental treatments, and to probe the relative importance of phenotypically plastic shifts in concentrations of nutrients and leaf metabolites on decomposition in streams and soils.

Experimental design

We employed the following experimental design to test the effects of induced plasticity in a natural field system. As such, we incorporate the standing fixed genetic difference in natural forests. Extensive knowledge has been gained from highly controlled common garden experiments, which are essential to disentangle environment × genotype interactions. However, grounding principles in natural field experiments is necessary to assess whether such effects that may be observable in highly controlled systems do indeed have observable effects in complex, 'noisy' natural systems. We emphasize that our experimental design precludes us from partitioning the exact contributions of fixed genetic differences versus phenotypic differences.

Leaf herbivory damage and soil nutrient availability could both induce plastic changes in leaf traits such as production of secondary metabolites or reallocation of nutrients, and they may interact. Such induced changes in leaf nutritional quality may influence the foraging decisions, energy assimilation abilities, and growth rates of the terrestrial herbivores that contributed to these leaf chemistry changes, as well as soil and freshwater invertebrate and microbial decomposers residing in habitats where leaf litter provides a major component of the base primary productivity. To test the main and interactive effects of herbivory and fertilizer treatments, we implemented a 2 × 2 experimental design in May of 2012. Our experimental units were 84 red alder individuals organized into 21 blocks, with four trees per block. Note, due to sample damage and loss over the course of the experiment, we report results from 78 alder trees. The average size of the trees used in our experiment (15.7 ± 1.4 cm trunk diameter at 1.5 m above ground) correlates with a 15 year-old tree growing on a site of average quality (Worthington et al. 1960). We surveyed each tree for insect damage (four branches per tree, 10 leaves per branch) and found 75% of surveyed leaves showed visible signs of insect damage prior to the experiment. However, trees were of similar size ($F_{3,82} = 0.31$, $P = 0.82$) and had similar levels of insect damage ($F_{3,82} = 1.56$, $P = 0.21$) across treatment group assignments. We chose experimental trees growing a minimum of 100 m apart from any other experimental tree to minimize cross contamination of methyl jasmonate (the chemical we used to induce plant defenses). Methyl jasmonate is a volatile chemical released naturally from damaged plant tissue and may be

recognized by neighboring plants and used as an early warning signal for nearby plants to bolster defense prior to an herbivory event (Bruin et al. 1995). Each of our experimental blocks of trees was, therefore, a minimum of 400 m in length (see Fig. 1 for illustration). We also used a paired sampling design, collecting leaves from each tree immediately before and after treatments, to account for whether leaf composition may change due to environmental fluctuations over the course of the experiment, such as increased temperature, reduced precipitation, and the emergence of terrestrial herbivores. Our control group aids in differentiating between changes occurring due to treatment versus those due to environmental change over time.

Immediately prior to implementing our experimental treatments, we collected three to five fresh green leaves from each tree for pre-treatment measurements of nutrients and secondary metabolites. Leaves were oven dried for 48 h immediately after collection, ground in a mortar, and stored at room temperature until further mass spectrometry analyses. We then implemented our fertilizer treatment which consisted of a single addition of 10 g triple superphosphate fertilizer (P_2O_5) spread beneath two trees per block on 23-May-2012, which was either 10 or 26 days prior to the start of the mock herbivory treatment depending on tree block. Lack of phosphorus often limits nitrogen-fixers such as red alder, and similar phosphorus additions have increased growth in young red alder (Brown et al. 2011) and speckled alder (*Alnus incana*) in northeastern North America (Gökkaya et al. 2006). We then applied our mock herbivory treatment to two trees per block: one tree that had previously received the fertilizer, and one that had not. Our treatment consisted of mechanical wounding and applying the plant hormone, methyl jasmonate, to chemically signal the threat of herbivory. We wounded every third leaf below 2 m in height by punching two 6.35 mm holes using an office hole punch, and we brushed each punched leaf with 50 μL of a 100 mM methyl jasmonate solution in 10% ethanol and
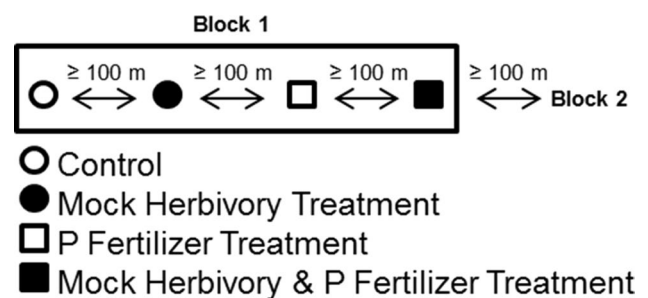

Fig. 1 Illustration of 2 × 2 randomized block design of fertilizer and mock herbivory treatments applied to 84 naturally recruited red alder (*Alnus rubra*) trees growing alongside logging roads and trails in Northwest Washington

0.125% Triton-X. By repeating this procedure three times over a 6-day period in June (with the same leaves treated each time), by the end of the treatment, one-third of leaves below 2 m were given six hole punches and brushed with methyl jasmonate three times. This treatment was intended to mimic a sustained herbivory threat, rather than a one-time pulse of damage, which may fail to induce strong defense responses in plants (Mithöfer et al. 2005). We left the remaining two-thirds of leaves untouched for use in leaf pack experiments.

Immediately after the completion of these experimental treatments, we collected additional green leaves for both our post-treatment nutrient and secondary metabolite measurements, as well as our leaf pack experiments described below. Green leaves, compared to dried senescent leaves, decompose more rapidly, support greater invertebrate diversity, and regularly fall into streams in large quantities during the summer growing season (Stout et al. 1985). In the Olympic Peninsula of Washington, alder leaves that are still green in color often comprise over half of the leaf litter entering small streams (Jackrel and Wootton 2014). By manually detaching green leaves rather than collecting naturally detached freshly fallen green leaves (i.e., "greenfall"), we ensured that leaves were derived from the experimentally treated branches from the correct trees. Further, this approach ensured that we could collect a large number of leaves that had been attached to their parent tree for the same length of time. This approach standardized the length of the experimental treatment by controlling for the amount of time leaves remained on the tree after starting treatments. Lastly, the potential complication of leaf chemistry changes that occur during autumn leaf senescence is inherently less of a concern for greenfall that has not undergone the process of senescence. When collecting alder leaves, we chose leaves with little or no natural herbivore damage and sealed them in plastic bags during transport to the laboratory. All leaves collected from trees in the herbivory treated group were adjacent to leaves given the herbivory treatment (i.e., we did not use leaves directly receiving the hole punches and methyl jasmonate). As was done prior to the experimental treatment, an additional three to five leaves that were collected immediately after the experimental treatment were oven dried for 48 h, ground in a mortar, and stored at room temperature until further mass spectrometry analyses.

We assembled the remaining leaves collected after the experimental treatment into leaf packs, each containing 12 leaves from a single tree. One pack per tree was designated for streams, and a second pack per tree was designated for soils. We recorded initial weights of leaf packs prior to deployment in either streams or soil. Leaf packs were deployed for 17–21 days at five locations in the Pysht River. All packs per experimental block were deployed at the same location in the Pysht River and locations with slightly slower decomposition rates were incubated for up to four extra days. Any mass gain or loss due to sedimentation or other abiotic processes was controlled by our randomized intermixing of leaf packs within blocks that minimized any systematic differences in biotic and abiotic conditions affecting each group. After leaf packs were removed from the stream, leaves were gently suspended in water to dislodge any accumulated sediment and invertebrates from the samples, and then blotted dry with paper towels and weighed to the nearest 0.01 g. Leaf packs deployed in the soil were incubated for either 56 or 67 days at 21 deployment sites that were watered periodically to accelerate decomposition. Each of these soil deployment sites was located beneath the canopy of one of the experimental trees, selected at random, per experimental block of four trees. Therefore, due to this experimental design, the soil decomposition experiment had an extra 'home' versus 'away' treatment of leaf packs deployed beneath their parent tree versus a different tree. After incubation in soil, we oven dried and weighed the leaves to the nearest 0.01 g.

CNP measurements

Using the fresh green leaves we collected from each tree immediately before and after the experimental fertilizer and herbivory treatments, we measured percent nitrogen, percent carbon, and $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios using a Costech 4010 Elemental Analyzer combustion system coupled to a Thermo DeltaV Plus IRMS via a Thermo Conflo IV interface at the University of Chicago Stable Isotope Ratio facility (Jackrel and Wootton 2015a). We also measured phosphorus using a modified ashing, acid-hydrolysis, and phosphomolybdate-blue spectrophotometric protocol of Monaghan and Ruttenberg (1999) [see Jackrel and Wootton (2015a) for details of modifications].

Secondary metabolite analyses

Using the fresh green leaves collected from each tree immediately before and after our experimental fertilizer and herbivory treatments, we measured secondary metabolites via mass spectrometry. We extracted 100 mg of leaf powder in 10 mL 70% methanol and auto injected samples through a Zorbax SB-C18 2.1 × 150 mm, 3.5 µm column on an Agilent Q-TOF LC–MS with dual ESI (Agilent 6520) with the following parameters: 325 °C gas temperature, 9 mL min^{-1} drying gas, 5 eV fixed collision energy, 40 psig nebulizer, 120 V skimmer voltage, 750 V OCT 1 RF Vpp, 170 V fragmentor, and 4500 V capillary voltage. Mass accuracy was within 2–5 ppm. Samples were eluted with 0.1% formic acid in water (A) and 100% methanol (B) using the following separation gradient: 5 min of 100% A at a rate of 0.2 mL min^{-1}, followed by a gradient from 100 to 60% A (0 to 40% B) over

20 min at a rate of 0.1 mL min^{-1}. Flow was then held for 5 min at 60% A (40% B) at a rate of 0.3 mL min^{-1}. Then a gradient from 60 to 0% A (40 to 100% B) was carried out over 15 min at 0.3 mL min^{-1}, and then held for 10 min at 0% A (100% B) at 0.3 mL min^{-1}. Compounds were characterized using retention times and fragmentation patterns of chromatograms with automatic agile integration in Agilent Mass Hunter Software (Qualitative Analysis B6 2012). We also used additional software for heuristic filtering of the obtained molecular formulas (Kind and Fiehn 2007). Commercial standards including oregonin (Sigma-Aldrich, CAS # 55303-93-0) and curcumin (Sigma-Aldrich, CAS # 458-37-7) were used to aid in characterization. We quantified compounds by recording base peak chromatogram (BPC) abundance for each peak, standardized abundance as BPC per milligram of leaf material, and report relative abundance of each compound as the log transformed value of BPC per milligram of leaf material.

As we describe further in our results, certain compounds were quite abundant compared to others (see mean BPC% area reported in Table A1). Abundant compounds that change substantially in peak area are not necessarily those compounds showing the greatest percent changes. For example, rare flavonoids may not have as much biological relevance at very low concentrations, and yet occasionally receive high factor loads (see compounds # 60 and # 61, Appendix B1) in our discriminant models, which we describe in the next section. Alternatively, organisms could be more sensitive to changes in relative abundance of rare compounds because of limited exposure to these compounds. Therefore, while we have not excluded any rare compounds from our analyses, when interpreting our results, we have more strongly considered compounds with both larger factor loadings and of sizable relative abundance that we have defined here as a minimum mean 0.20 BPC% Area (see Table A1).

Statistical analyses

We determine whether models including secondary metabolite data alone could predict tree treatment group with a discriminant function analysis (DFA; using the lda {MASS} function in R and SPSS IBM Corp. 2013, Armonk, New York, USA). We chose discriminant function analyses because we aimed to answer which variables best separated our predefined treatment groups. This supervised method aims to maximize between-group separation, whereas unsupervised ordination methods instead aim to describe maximal variance in a dataset (Johnson and Wichern 1992). Using DFAs, we identify which chemical compounds differ most among treatment groups. In addition to metabolite data, we also included experimental block as a predictor variable to account for geographic variation. All predictor variables were standardized in scale ($\mu = 0$, $\sigma = 1$). We first consider a single DFA model using the relative abundance of all 62 red alder chemical compounds and curcumin (included as a control for machine drift over time) among all trees immediately prior and after experimental treatment. To more clearly illustrate experimentally driven shifts in secondary metabolites (versus temporal shifts), we also ran a separate model using only the post-treatment data points. Next, we aimed to compare the importance of different classes of metabolites in the herbivore defense response both under the fertilized and unfertilized scenario. We, therefore, ran an additional eight DFA models on subsets of the data to detect shifts due to the herbivory treatment among: (1) chlorogenic acids among fertilized trees, (2) flavonoids among fertilized trees, (3) ellagitannins among fertilized trees, (4) diarylheptanoids among fertilized trees, (5) chlorogenic acids among unfertilized trees, (6) flavonoids among unfertilized trees, (7) ellagitannins among unfertilized trees, and (8) diarylheptanoids among unfertilized trees. Significance of each discriminant function was determined by calculating the Wilk's lambda statistic with a Chi square distribution, in which Wilk's lambda values range between 0 and 1, with smaller values indicating a lower proportion of the total variance in the discriminant scores that is not explained by differences among groups (SPSS IBM Corp. 2013, Armonk, New York, USA). For each significant discriminant function, we calculated which groups differ from each other using post hoc Tukey's HSD tests. Groups that differ significantly are depicted using 50% confidence ellipses of the discriminant scores.

Lastly, we tested which traits best predicted decomposition rates in rivers and soil using linear-mixed effects models using the lme {nlme} function in R. Traits tested included leaf %C, %N, %P, C:P, C:N, N^{15}/N^{14}, C^{13}/C^{12}, and summarized secondary metabolite variation (i.e., using discriminant scores from the model including all classes of metabolites in Appendix B1, as well as discriminant scores from an ellagitannin-only model in Appendix B5). We chose to include discriminant scores from an ellagitannin-only model in addition to a model including all classes of secondary metabolites because our results suggested ellagitannin content was strongly affected by treatment. To calculate leaf mass lost from leaf packs, we converted our final leaf weights to fresh weight using regression equations generated from a laboratory experiment (Jackrel and Wootton 2014). For aquatic leaf packs, we used the equation: fresh mass = 0.941(blotted-dry mass) − 0.00337 ($R^2 = 0.98$) and for soil leaf packs we used the equation: fresh mass = 2.53(oven dried-mass) + 0.275 ($R^2 = 0.79$) (Jackrel and Wootton 2014). For our aquatic model, our random effect was our 21 experimental blocks nested within our five deployment locations. For our soil model, our random effect was only the 21 experimental blocks because we had one deployment location per block (21 deployment locations total) and using both variables
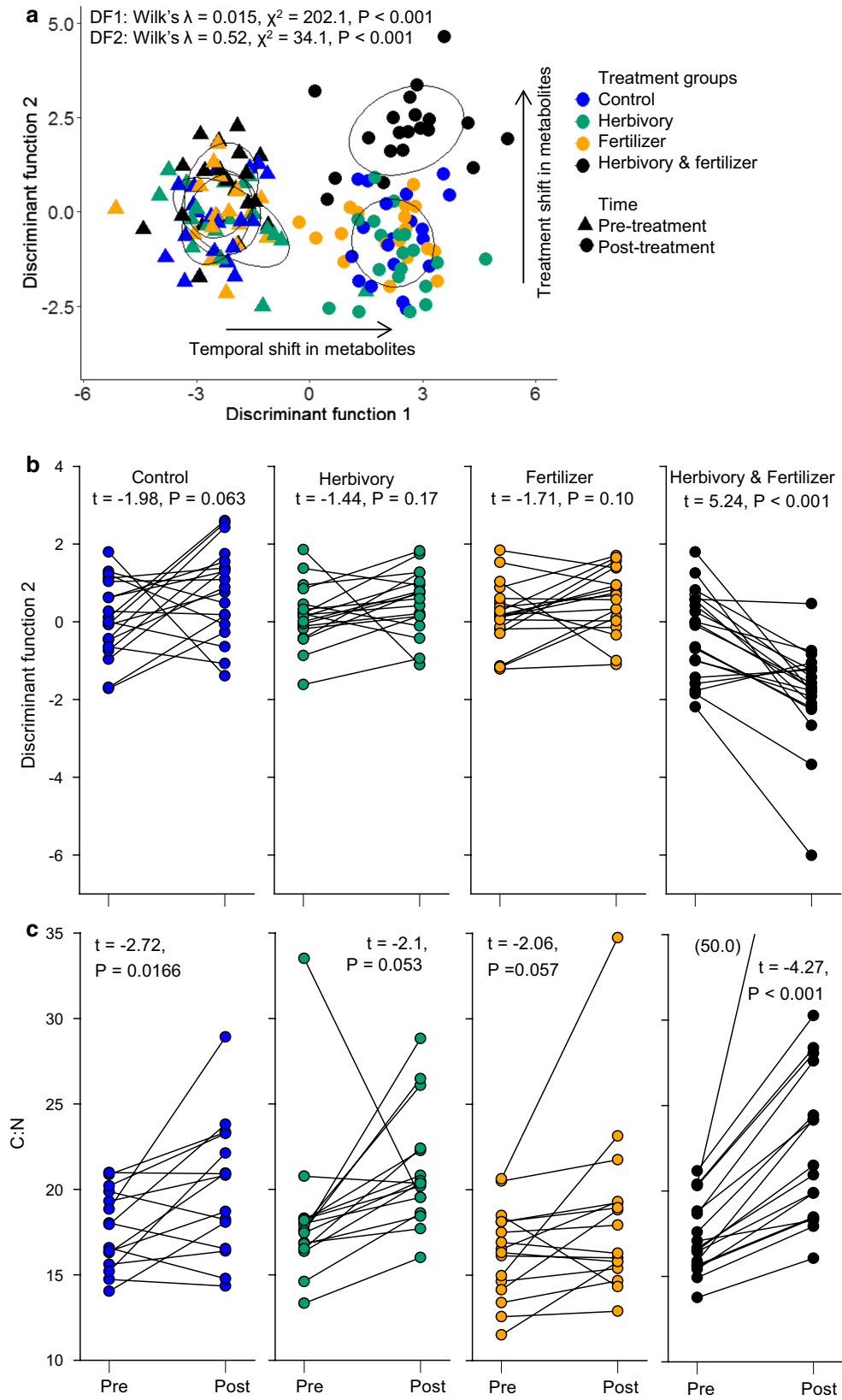
◄**Fig. 2 a** Shifts among leaf secondary metabolites from 78 red alder trees immediately before and after treatments with phosphorus fertilizer and a jasmonate-based mock herbivory stress via discriminant function analysis. Secondary metabolite composition shifts significantly over time and by treatment, as indicated by a model containing relative abundance data of 62 secondary metabolites. Secondary metabolites most strongly weighting discriminant functions are listed in Appendix Table B1. Significant differences between groups were determined with Tukey post hoc tests on discriminant scores and are illustrated with 50% confidence ellipses. **b** The herbivory and fertilizer treatment group, but no other group, shifts significantly in secondary metabolites as illustrated via paired t tests. **c** Similarly, the herbivory and fertilizer treatment group shifts more strongly in leaf C:N than the other treatment groups, as illustrated via paired t tests. A color version of this figure is available online

would have been redundant. The purpose of these random effect terms in our models are to provide controls for numerous factors we expect might vary by geographic location of the trees, such as the abundance of herbivores and soil moisture, and by the deployment location of the leaf packs, such as accrual of and abrasion by sediments and abundance of aquatic invertebrates. Due to high multicollinearity between nutrient percentages and nutrient ratios, we chose to run two separate models that included either the nutrient percentage data or the nutrient ratio data. This approach minimized multicollinearity in both models to variance inflation factors below ten. For both our starting nutrient percentage model and our starting nutrient ratio model, we selected best fitting models by comparing AIC scores with forward automated model selection using the stepAIC {MASS} function in R. We report a marginal R^2 value for the best fitting mixed-effects model to describe variance explained by the fixed factors (Nakagawa and Schielzeth 2013). We then compared AIC scores of the two best fitting models that included nutrient percentages and nutrient ratios against each other to determine the final best model. For the best fitting model, we report the F-statistic for each main effect, as well as numerator and denominator degrees of freedom using the anova {stats} function with marginal sum of squares in R.

Results

We characterized relative abundance of 62 compounds found in a population of 84 red alder trees. The same compounds were typically found in trees across all four treatments, but at different relative abundances. We characterized 4 chlorogenic acid derivatives, 28 ellagitannins, 7 flavonoids, and 16 diarylheptanoids (Table A1). We also note each compound characterization, molecular mass and formulae of parent molecules, fragmentation patterns, and corresponding peak numbers as illustrated (Fig A1) in Table A1.

Prior to implementing the experimental treatments, secondary metabolite chemistry was similar across treatment

groups (Fig. 2a). Five days after the start of experimental treatments, there were significant temporal (Fig. 2a, b, discriminant function 1) and treatment shifts (Fig. 2a, b, discriminant function 2). The group of trees given both the phosphorus fertilizer and mock herbivory treatment diverged significantly from all other treatment groups (Fig. 2a: discriminant function 2: Wilk's $\lambda = 0.52$, $\chi^2_{152} = 34.1$, $P < 0.001$); paired t test of discriminant scores $t = 5.24$, $P < 0.001$). This model includes a complex set of ellagitannins, diarylheptanoids, and flavonoids weighting these two discriminant functions (see Appendix B1). For example, an ellagitannin (compound #20) was 45% more abundant among trees given the fertilizer and mock herbivory treatment compared to the fertilized only group (94.0 ± 8.3 S.E. BPC compared to 64.6 ± 7.9 S.E. BPC), while the diarylheptanoid Alnuside A-glycoside (compound # 45) was 30% more abundant among trees given the fertilizer and mock herbivory treatment compared to the fertilized only group (111.0 ± 18.1 S.E. BPC compared to 84.6 ± 17.8 S.E. BPC). In addition to these shifts in secondary metabolites, tree leaves across all four treatment groups tended to decline in Nitrogen from immediately prior to the experimental treatments ($\mu = 2.78\%$ N ± 0.45 S.D. or 17.38 C:N ± 3.03) to immediately after completion of the experimental treatments ($\mu = 2.44\%$ N ± 0.54 or 20.97 C:N ± 5.73). The fertilizer and mock herbivory treatment group, which showed the strongest shift in secondary metabolite composition, also declined most sharply in Nitrogen (to an average of 2.24%, or 24.02 C:N, paired t test $t = 4.27$, $P < 0.001$) (Fig. 2c).

The result illustrated in Fig. 2 suggests an interaction between our fertilizer and mock herbivory treatments. Therefore, to determine whether alder trees use different defenses depending on resource availability, we analyzed fertilized and unfertilized trees separately. We found red alder defensive responses resulting from the mock herbivory treatment largely depended on the availability of soil phosphorus. In the fertilized group, the red alder defensive response comprised largely of shifts in ellagitannins (Appendix B3: see standard coefficients for compounds #7, #8, #20, and #32 corresponding to a pair of HHDP-glucose isomers, an uncharacterized ellagitannin, and Tellimagrandin II on DF2). In the absence of excess nutrients, red alder defense instead consisted largely of shifts in diarylheptanoids (Appendix B4: see standard coefficients for compounds #45, #48, #54, #63 corresponding to Alnuside A with glucoside versus xyloside groups, oregonoyl A, and an uncharacterized diarylheptanoid) (Fig. 3). Note however, both classes of compounds still contribute to the fertilized and unfertilized models according to factor loadings, indicating that neither class is insignificant in either scenario.

In addition to models using all available secondary metabolite data (Appendix B3 and B4), we observe a similar pattern when considering only single classes of secondary
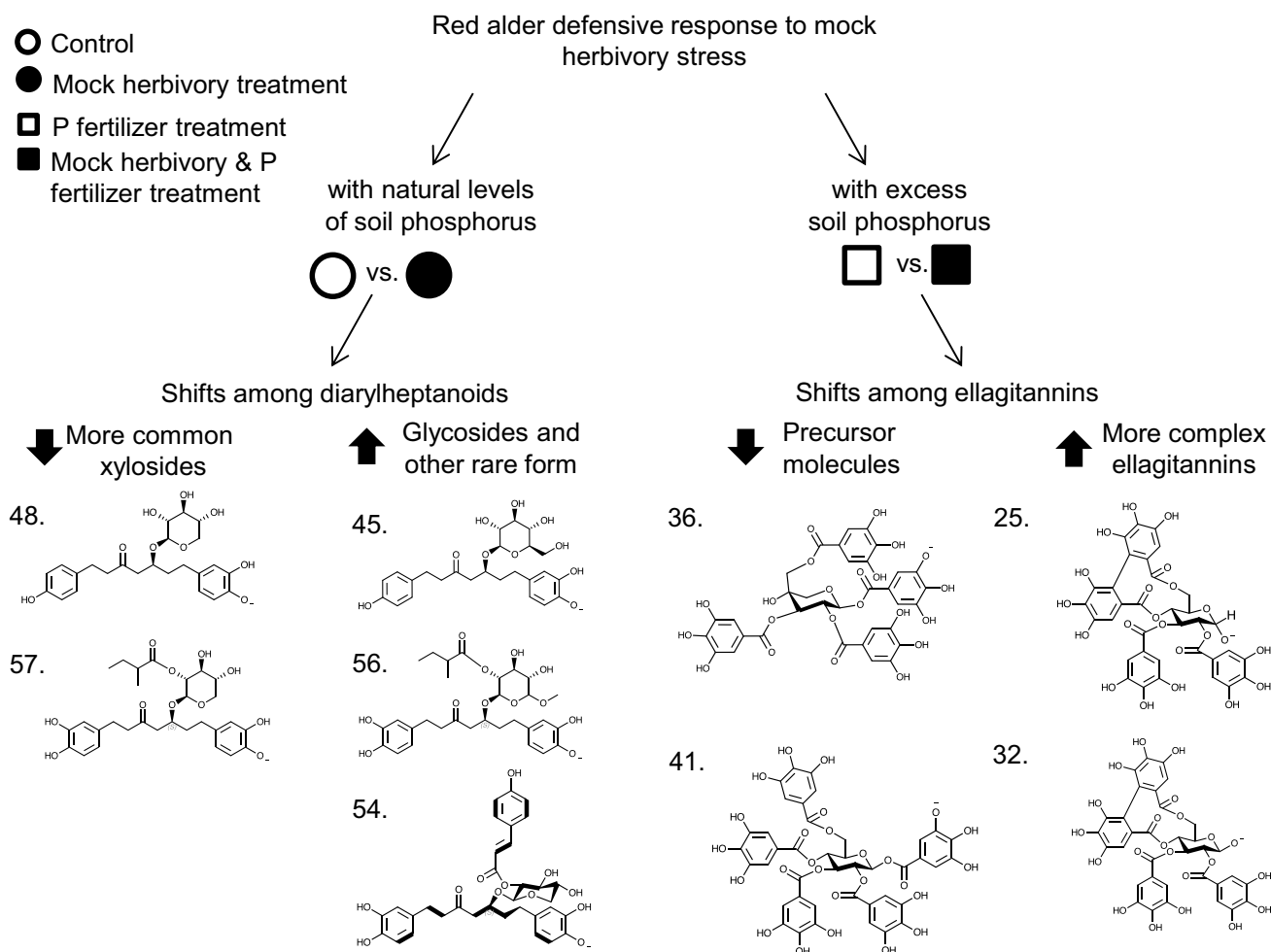
Fig. 3 Illustration of red alder defense response to mock herbivory stress. Alder trees given a jasmonate based herbivore stress in plots with natural levels of soil phosphorus tended to show more shifts among the relative concentrations of the diarylheptanoid group of secondary metabolites (see factor loadings in DF2 of Table B4). In contrast, alder trees undergoing herbivore stress but with excess soil phosphorus fertilizer tended to instead show more shifts among the relative concentrations of ellagitannins (see factor loadings in DF2 of Table B3). Identification numbers listed to the left of each compound can be used find further descriptions of each compound in Table A1

metabolites. When conducting these further analyses on subsets of secondary metabolites, we analyzed the fertilized versus unfertilized groups of trees separately because the effect of the mock herbivory treatment appeared particularly strong when paired with fertilizer. Significant models retaining only ellagitannins distinctly separated the mock herbivory group from the control either among the fertilized (Fig. 4; Appendix B5: discriminant function 2: Wilk's $\lambda = 0.55$, $\chi^2 = 30.2$, $P < 0.001$) or unfertilized group of trees (Appendix B9: discriminant function 2: Wilk's $\lambda = 0.74$, $\chi^2 = 12.6$, $P < 0.001$). Additional significant models retaining only diarylheptanoids also separated the mock herbivory group from the control either among the fertilized (Appendix B6: discriminant function 2: Wilk's $\lambda = 0.88$, $\chi^2 = 5.1$, $P = 0.008$) or unfertilized group of trees (Appendix B10: discriminant function 2: Wilk's $\lambda = 0.84$, $\chi^2 = 6.9$, $P = 0.002$). We did not, however, find any models significantly separating the mock herbivory

versus control groups when using either only flavonoids (see Appendix B7 and B11) or only chlorogenic acid derivatives (see Appendix B8 and B12).

To further illustrate the shift in secondary metabolite composition due to experimental treatment, we ran a separate model using only the post-treatment data points (Fig. 5: DF1 Wilk's $\lambda = 0.04$ and DF2 Wilk's $\lambda = 0.11$, P values < 0.001, Appendix B2). As we reported previously, treatment groups differ in decomposition rates in streams (Jackrel and Wootton 2015a). This ordering in treatment group from the leaves that decomposed most quickly (i.e., fertilizer only) to the leaf group decomposing the least (i.e., fertilizer and mock herbivory) matches shifts in secondary metabolites along discriminant function 1 (Fig. 5). While secondary metabolite chemistry significantly predicts leaf decomposition in streams (Fig. 6a, $F_{1,52} = 9.22$, $R^2 = 0.15$, $P = 0.0037$) metabolite composition does not predict
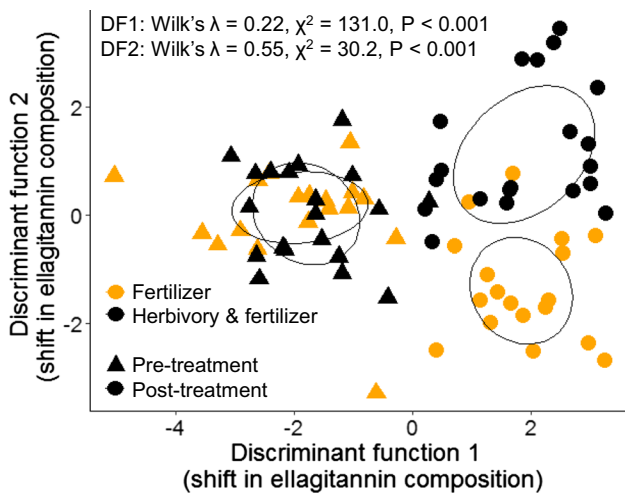
Fig. 4 Among riparian red alder trees receiving a phosphorus fertilizer treatment, those trees receiving versus not receiving a jasmonate-based mock herbivory treatment diverged significantly in the relative concentrations of 27 different ellagitannins. Significant differences between groups were determined with Tukey post hoc tests on discriminant scores and are illustrated with 50% confidence ellipses. Secondary metabolites most strongly influencing this separation are reported in Appendix B5. A color version of this figure is available online
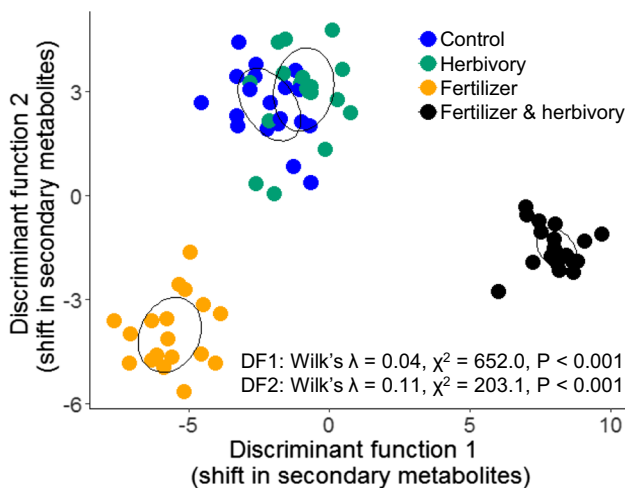

Fig. 5 Secondary metabolite composition of 78 riparian red alder trees after receiving a jasmonate based mock herbivory treatment with and without phosphorus fertilizer. Note, only the post-treatment time point is used to best illustrate differences due to treatment. Significant differences between groups were determined with Tukey post hoc tests on discriminant scores and are illustrated with 50% confidence ellipses. Secondary metabolites most strongly influencing this separation are reported in Appendix B2. A color version of this figure is available online

Fig. 6 a Rate of leaf decomposition in streams is predicted by the secondary metabolite composition of red alder tree leaves, which shifted due to experimental treatments of a jasmonate based herbivory stress and/or phosphorus fertilizer. Discriminant function scores on the *x*-axis originate from Fig. 5, and are used here as a summary of secondary metabolite content. Note, we plot the main effects from our aquatic decomposition model using residuals, which factors out our random term of experimental block nested within deployment location. Symbol shape and color depicts the five different rounds of the experiment. **b** Secondary metabolite composition did not predict rate of leaf decomposition in soil. Regression is used to standardize across multiple rounds of experiments by extracting residuals of terrestrial decomposition after factoring out experimental block. A color version of this figure is available online

derived from trees growing in the immediately local area (see a further explanation in Jackrel and Wootton 2015a).

Lastly, using the fertilized trees, which demonstrated the most clear shift in secondary metabolite composition, we found that the best model prediction of leaf decomposition rate in streams included both leaf secondary metabolite composition and C:N (Table 1, secondary metabolites $F_{1,11} = 15.20$, $P = 0.0025$; C:N $F_{1,11} = 17.82$, $P = 0.0014$; marginal $R^2 = 0.34$). This secondary metabolite variable retained in the model included all classes of secondary metabolites (DF2 scores, Fig. 2a, Appendix B1). A second ellagitannin variable (DF2 scores, Fig. 4, Appendix B5) was

decomposition in soils (Fig. 6b). Note, however, that this lack of effect of the herbivory treatment on soils may be due to a confounding effect that consumers tend to prefer leaves
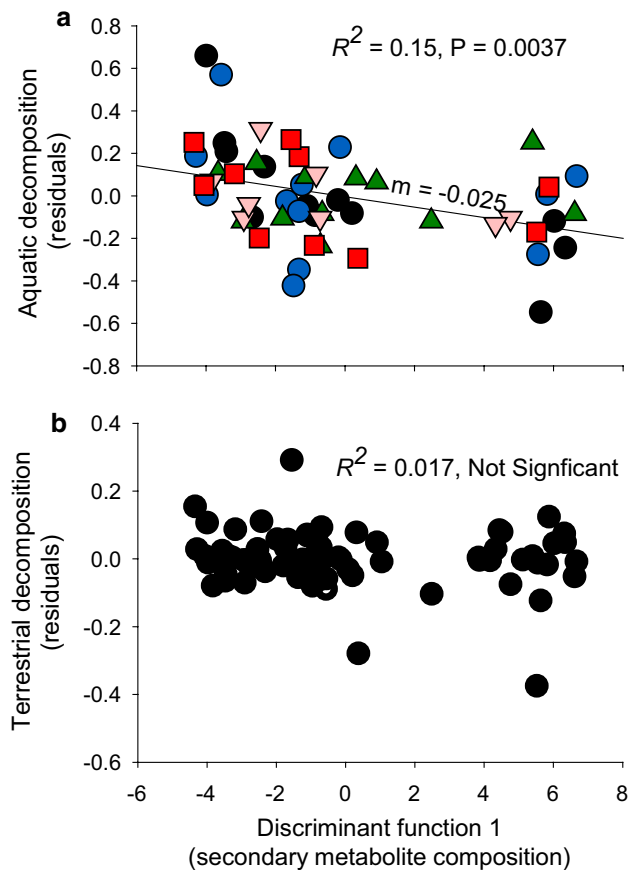
Table 1 Among trees receiving the fertilizer treatment, leaf C:N and secondary metabolite composition strongly predict aquatic leaf decomposition (stepwise mixed-effects linear regression, marginal $R^2 = 0.34$)

Source	F value	P value
C:N	17.82	0.0014
All classes of secondary metabolites	15.20	0.0025

Secondary metabolite composition was entered in the model via discriminant function scores, including discriminant function 2 from a model including all classes of metabolites (Fig. 2, Appendix B1) and discriminant function 2 from an ellagitannin-only model (Fig. 4, Appendix B5). Variables not retained in the model were C:P, $N^{15}N^{14}$, $C^{13}C^{12}$, and discriminant scores from the ellagitannin-only model. The model also controls for random effects with a nested term of experimental block within deployment location

not retained in the model. Our best-fitting model using nutrient percentages rather than nutrient ratios yielded similar results (secondary metabolites $F_{1,11} = 15.33$, $P = 0.0024$; %N $F_{1,11} = 10.8$, $P = 0.007$; marginal $R^2 = 0.32$).

Discussion

Plant leaves provide essential food resources for multiple communities including terrestrial herbivores, soil decomposers and aquatic decomposers residing in streams and wetlands. Here, we found that terrestrial herbivory stress induces plant phenotypic plasticity via shifts in secondary metabolites and nutrient composition with the cascading implication of reduced leaf litter decomposition in an adjacent ecosystem. Induced plant response to herbivory suppresses energy capture in streams, where aquatic invertebrates and microbes decompose leaf litter at slower rates. The plastic response by red alder trees was quite complex. Red alder trees appear to allocate excess nutrients, via a phosphorus fertilizer, towards shifting secondary metabolite and nutrient composition in their leaves in response to an herbivore stress. The cause of leaf nitrogen decline is not clear, but could be due to (1) the direct costs of secondary metabolite induction, (2) a mechanism to deter herbivory via reallocation to more inaccessible locations, or (3) a deprioritizing of nitrogen fixation under stress. Further, the type of secondary metabolite response was partly resource dependent. With excess phosphorus availability, herbivore defense evidently favors ellagitannin production. Without fertilizer, the defense response is comprised largely of modifications to diarylheptanoids, including shifting away from the typically reported xylose-based diarylheptanoids toward glucose-based diarylheptanoids. Long-lived organisms, such as red alder, typically encounter enemies under varying abiotic conditions through their lifespan. Such synergistic

interactions between biotic and abiotic factors can evidently lead to alternative phenotypes with different consequences on ecosystem functioning: in this case of red alder, increased reliance on the ellagitannin pathway in phosphorus rich soils evidently leads to reduced consumption by aquatic decomposers.

Ellagitannins and diarylheptanoids both appear to be involved in red alder defensive responses against herbivores, but seem to vary in importance depending on soil nutrient conditions. Plants in the control versus treatment group were indistinguishable in ellagitannin and diarylheptanoid content prior to experimental treatments but diverged significantly from each other following the mock herbivory treatments. Plant apparency theory predicts long-lived plants would use dose-dependent chemical deterrents such as ellagitannins to dissuade herbivores. In contrast, the induction of strongly cytotoxic compounds, such as diarylheptanoids, is predicted for short-lived, ephemeral plants (Feeny 1976; Farrand et al. 2014). Our results, however, do support predictions that low metabolic investments (either in unapparent plants, or in our scenario, apparent plants with limited resources), favor more potent toxins (i.e., diarylheptanoids), while greater investment enabled by excess resources favors quantitative defense (i.e., ellagitannins). To determine more precisely the metabolic allocations to these plant defenses, plant physiology experiments and further investigations into the biosynthetic pathways of ellagitannins and diarylheptanoids are required (Niemetz and Gross 2005; Rahman 2012). Diarylheptanoid biosynthesis and ecology remains poorly understood, but interestingly, our mock herbivory treatment induced sharp increases in production of a previously undescribed diarylheptanoid (compound # 45: a modification to Alnuside A that contains glucose rather than the typical xylose sugar is a modification that has not been previously reported). Given the probable role of this compound in herbivore defense paired with the purported anti-cancer properties of other diarylheptanoids in the biomedical literature (Farrand et al. 2014), further investigation into the bioactivity of this compound may be warranted.

Our results reported here on inducible traits align well with our previous finding that natural or constitutive-levels of ellagitannins are a major regulator of aquatic leaf decomposition. We previously found extensive geographic structuring in secondary metabolites, particularly ellagitannins. Further, we had found that this spatial variation in secondary metabolites drives local ecological matching where decomposers on land and in rivers more quickly consumed food resources prepared with locally derived secondary metabolite extracts (Jackrel et al. 2016). These previous results, paired with the result we present here, suggest ellagitannins are an important, and highly inducible, component of red alder defense with extensive consequences for aquatic decomposers and ecosystem functioning. These findings

support the evolving view that ellagitannins, which have traditionally been a neglected class of tannins, may be of greater ecological importance than often appreciated (Salminen and Karonen 2011).

Accumulating evidence that both among and within-species biodiversity can have cascading effects across trophic levels and ecosystem boundaries is an essential step towards our understanding of community ecology (Jackrel and Wootton 2015b). As we unravel the wide ranging implications of individual variation that may rival the effects of species variation, our understanding of the potential temporal and spatial regulations of community and ecosystem processes may shift. To hone in more precisely on the spatial and temporal scales of such regulations, it is essential to determine the contributions of both plastic and genetically driven variation. We had found that despite using a naturally recruited population of trees, which are variable due to fixed genetic differences, we still found that inducible phenotypic plasticity had clear and strong effects on ecosystem function. Future studies should use a common garden approach that controls for plant genotype. Such experimental designs would allow for the partitioning of the exact contribution of fixed genetic versus inducible phenotypic differences towards regulating ecosystem functions. This would be an important direction to pursue because populations of long-lived species that respond to environmental fluctuations via plastic rather than genetic rescue, speed up the potential cascade of indirect interactions and possibly affects the temporal stability of ecosystem functions. Ecosystem functions that are regulated by genetically hardwired, non-plastic variation in individual trees may vary at different scales (i.e., decades) than ecosystem functions regulated by plastic traits. The mechanisms by which a community responds to trait changes may also vary (i.e., rapid responses to plasticity could be the consequence of species sorting among microbial and invertebrate decomposers, whereas more spatially stable, genetically controlled traits may lead to evolutionary changes within species). It is likely that both genetic and plastic components of intraspecific variation in riparian trees are affecting ecosystem functioning. We have previously documented that aquatic decomposer communities are primed to more rapidly decompose leaf litter from immediately local red alder trees, at an extremely fine spatial resolution. This pattern, which we have found is largely driven by spatial variation in secondary metabolites produced to ward off terrestrial herbivores (Jackrel et al. 2016), may develop over decadal timespans due to genetic differences among trees. We now show that induced plastic responses to environmental stresses over annual or even seasonal time-scales have further implications for aquatic communities. Both processes should be studied, and ultimately their effects compared, to determine the relative contributions of genetic and plastic components of individual variation on community and ecosystem processes. Understanding the relative contributions of these components will enable more powerful predictions of stability and resilience of ecosystem functioning.

Author contribution statement SLJ conceived and designed the study, conducted field experiments, generated the nutrient and mass spectra data, performed statistical analyses and wrote the manuscript. TCM design secondary metabolite analyses, generated the mass spectra data, characterized secondary metabolites and assisted with writing the manuscript.

Compliance with ethical standards

Conflict of interest We have no competing interests.

Data availability All leaf decomposition and nutrient data can be found here: http://dx.doi.org/10.5061/dryad.ph812. A subset of mass spectra chromatograms and details about identifying secondary metabolites are available in our supplementary materials. Contact authors for remaining mass spectra.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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