Pedobiologia 50 (2007) 563–576

Available online at www.sciencedirect.com
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Pedobiologia
www.elsevier.de/pedobi

Rhododendron thickets alter N cycling and soil extracellular enzyme activities in southern Appalachian hardwood forests

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Received 29 September 2006; accepted 1 October 2006

KEYWORDS
Ericoid mycorrhiza;
Protein–tannin complex;
Polyphenol oxidase;
Protease;
Leaf litter

Summary

Rhododendron maximum L., a spreading understory shrub, inhibits overstory regeneration and alters forest community structure in southern Appalachian hardwood forests. Using paired plots and reciprocal litter transplants in forests with and without *R. maximum* cover, we examined the influence of *R. maximum* on litter mass and quality, N cycling and soil extracellular enzymes. Standing stocks of soil organic matter, soil N, leaf litter mass and fine root biomass were greater in forests with *R. maximum* than those without. Tannin extracts from *R. maximum* foliage, and leaf litter and fine roots collected under *R. maximum* had a relatively high capacity to precipitate protein compared to extracts from trees. Across the growing season, soil inorganic N availability was generally lower under *R. maximum*, mostly due to reduced NO_3^- availability. Our data suggest that *R. maximum* litter alters N cycling through the formation of recalcitrant polyphenol–organic N complexes. Soil extracellular enzymes indicate the potential processing rates of organic substrates. Between forest types, polyphenol oxidase activity was greatest in *R. maximum* O horizons, regardless of litter type, suggesting that the local microbial community can better degrade and access protein–tannin-complexed N. Protease activity did not differ between forest types, but was greater on *R. maximum* leaf litter than hardwood leaf litter. The alteration of the N cycle via the formation of polyphenol–organic N complexes may contribute to hardwood seedling suppression, while the enzymatic release of these complexes by ericoid mycorrhizal fungi may increase N acquisition for *R. maximum* and contribute to its expansion in southern Appalachian forests.

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doi:10.1016/j.pedobi.2006.10.001

Introduction

Ericaceous plants inhibit forest regeneration in several systems worldwide (Mallik, 2003; Nilssen and Wardle, 2005); however, the precise mechanisms by which they do so are unknown (Hättenschwiler and Vitousek, 2000; Mallik, 2003). Plants in the Ericaceae produce foliage and roots rich in polyphenols (Gallet and Lebreton, 1995; Preston, 1999). Plant polyphenols (e.g., tannins) are structurally and functionally diverse (Waterman and Mole, 1994), and influence litter and soil chemistry, and ecosystem N cycling (Hättenschwiler and Vitousek, 2000; Kraus et al., 2003b). Ericaceous understories often result in lower N mineralization and decomposition rates (DeLuca et al., 2002; Read et al., 2004), and polyphenolic concentrations of plant litter are negatively correlated with soil fertility or productivity (Nicolai, 1988; Northup et al., 1995; Côté, 2000).

The formation of polyphenol–organic N complexes, and their influence on the N cycle, may be one mechanism of tree suppression by ericaceous understories. These complexes decrease protein and plant material decomposition rates (Basaraba and Starkey, 1966; Benoit et al., 1968), resulting in organic matter accumulation (Handley, 1961) and lower N mineralization rates (Bradley et al., 2000; Fierer et al., 2001; Castells et al., 2003; Kraus et al., 2004a). Ericaceous plants may control N cycling in ecosystems with low inorganic N availability by increasing the formation of polyphenol–organic N complexes, excluding other plant species from this portion of the N pool, and hence contributing to their suppression (Northup et al., 1998; Preston, 1999; Mallik, 2003). The ericoid mycorrhizal (ERM) fungi of ericaceous plants can degrade and access complexed organic N to a greater extent than can ectomycorrhizal (ECM) fungi (Bending and Read, 1996a, 1996b, 1997). Therefore, while altering patterns of N availability for other plant species, ericaceous plants may access polyphenol-complexed N through the saprotrophic capacity of ERM fungi. ERM fungi produce extracellular enzymes which can degrade polyphenol–organic N complexes, but soil activities are not well documented in forests containing ericaceous understories.

Rhododendron maximum L., a dominant ericaceous understory shrub of southern Appalachian forests, accounts for as much as 18–34 mg ha^{-1} in aboveground biomass (Baker and Van Lear, 1998). *R. maximum* forms dense thickets that spread by layering and root sprouts (Monk et al., 1985), and thicket cover has increased since fire suppression and the loss of *Castanea dentata* to the chestnut blight (Phillips and Murdy, 1985). The success of *R. maximum* interests forest ecologists because it suppresses conifer and hardwood regeneration (Phillips and Murdy, 1985; Nilsen et al., 1999; Beckage et al., 2000; Nilsen et al., 2001), reduces understory species diversity (Baker and Van Lear, 1998) and changes forest community composition (Lambers and Clark, 2003). *R. maximum* leaf litter has a slow decomposition rate relative to the leaf litter of other forest species (Hoover and Crossley, 1995; Hunter et al., 2003), and *R. maximum* increases soil organic matter (Boettcher and Kalisz, 1990) and decreases inorganic N availability (Nilsen et al., 2001; Boettcher and Kalisz, 1990). Therefore, *R. maximum* may influence soil N processes through its litter quality, possibly through the precipitation of organic N by polyphenols. However, the role of *R. maximum* litter polyphenols on N cycling, and the activities of soil extracellular enzymes that degrade polyphenol–organic N complexes have not been addressed.

We explored the effect of *R. maximum* thickets on N cycling and soil extracellular enzyme activities in a southern Appalachian hardwood forest. First, we evaluated standing soil organic mass and N, litter mass and inorganic N availability. We hypothesized that O horizon mass and N, leaf litter mass and standing root biomass would be greater, and available inorganic N would be lower in forests with *R. maximum* thickets compared to those without. Our second objective was to evaluate the reactivity of polyphenols from foliage, leaf litter and fine roots. We expected that polyphenols from *R. maximum* plant tissues would have a greater reactivity relative to those from hardwood species. Our final objectives were to measure activities of extracellular enzymes that degrade polyphenol–organic N complexes (polyphenol oxidases, PPO) and organic N (proteases) in soils and on reciprocally placed leaf litter. Since ERM fungi have a greater capacity to produce PPO and proteases in pure culture, than do ECM fungi, we expected greater activities of these enzymes in forest soils with *R. maximum* thickets compared to those without. We also expected that the placement of experimental leaf litter treatments under *R. maximum* would result in greater enzyme activities than those placed in forests without *R. maximum* because of local differences in microbial communities.

Materials and methods

Site description

Our study sites are mature southern Appalachian northern hardwood forests located in the Coweeta Hydrologic Laboratory (a Long-Term Ecological

Research (LTER) site) and Nantahala National Forest in North Carolina, USA (35° 03′N, 83° 25′W, Swank and Crossley, 1988). We established five blocks of paired 5 m × 5 m plots with N–NW aspects along a 2.5 km span of a high elevation ridge (1430–1460 m). The hardwood forest overstory composition is continuous within plot pairs; one plot of each pair contains a dense understory *R. maximum* thicket, while the other does not (hereafter referred to as Hdwd+Rmax and Hdwd plots, respectively). The overstory consists of *Quercus rubra* L., *Betula lenta* L. and *Betula alleghaniensis* Britt., *Acer rubrum* L. and *Fraxinus americana* L. The Hdwd plot understory is dominated by *Acer pennsylvanicum* L., *Amelanchier arborea* (Michaux f.) Fernald and *Castanea dentata* (Marshall) Borkh. Annual herbs and ferns are present, but the Hdwd plots lack ERM host plants. In contrast, Hdwd+Rmax plots contain a dense *R. maximum* thicket and are devoid of other understory species and herbs, with the exception of occasional achlorophylous plants, *Monotropa uniflora* L. and *Conopholis americana* L. Wallroth. Soils are inceptisols, formed in residuum of igneous and metamorphic rock, including the Burton, Plott, Craggey series (Humic and Humic Lithic Dystrudepts), and soil series are consistent within each block. Depth to lithic contact ranges from 20 to 80 cm. Two blocks are adjacent to the LTER watershed 5–27 which has a mean annual temperature of 9.4 °C, and evenly distributed annual precipitation of over 250 cm (L. Swift, unpubl. data).

Organic horizon stores and soil nutrients

In each plot, three randomly located samples of organic horizons and fine woody debris (FWD) were extracted using a square template (0.0613 m^2) and a serrated knife. Samples were separated into three categories; FWD (bark, twigs and stems <10 cm diameter), Oi horizon, and a pooled Oe and Oa horizon (Oe/a), and dried at 60 °C. Soil samples (five cores per plot, homogenized by horizon) were dried, ball-mill ground and analyzed for total C and N (Micro Dumas combustion analysis). We measured pH on a subset of unground Oe/a and A soils (H_2O; 1:10 O horizon, 1:5 A horizon). The A horizon soils were further measured through atomic absorption spectrophotometry for exchangeable cations (K, Mg and Ca; NH_4OAc extraction) and available P (double acid extraction) (Robertson et al., 1999).

Inorganic N availability

We assayed N availability using a resin bag approach (Binkley and Matson, 1983). Resin bags contained 2.5 g wet mass of both cation and anion exchange resin beads (AG 50W-X8 H^+ and AG 1-X8 Cl^-, Bio-Rad, Hercules, CA) sewn into square nylon bags (2.5 cm × 2.5 cm), re-charged with 1 N HCl and rinsed with DI water. For each monthly measurement, four resin bags were randomly placed in each plot of four blocks, ~5 cm below surface into a 45°-angled slice, targeting a high-density root zone while minimizing the severing of roots above the bag. Bags were removed after 28 days, rinsed with DI water, and individually extracted with 1 M KCl. Extracts were analyzed for NH_4^+-N and NO_3^--N, and corrected with blank bags, using a continuous flow colorimetric assay (Technicon AutoAnalyzer). Resin-bag extractable N (NH_4^+ and NO_3^-) was measured once in 2004 (September) and monthly during the 2005 growing season (April–August).

Leaf litter inputs and root biomass

We collected current year leaf litter in October 2004, when leaf fall was complete, from three randomly located areas in each plot using a 0.158 m^2 template. Although evergreen, leaf loss from *R. maximum* peaks in the fall (Monk et al., 1985), and we collected only fresh leaf litter (green or yellow) to avoid overestimating *R. maximum* litter inputs. Leaf litter was sorted by species, and dried at 60 °C.

We sampled total standing root biomass and peak season root standing biomass (≤1 mm), a proxy for fine root litter inputs (Joslin and Henderson, 1987; Gill and Jackson, 2000) in July 2004. Four randomly collected soil cores (4 cm diameter, 11 cm deep, from each plot), were placed on ice and frozen until sorted. We sorted roots into three size classes (coarse >1 mm, fine 0.5–1 mm and very fine <0.5 mm). Coarse and fine roots were washed, dried at 60 °C and weighed. Very fine root length was determined using a line-intersect technique, from which mass was determined using specific root length (SRL, cm g^{-1}) (Hendrick and Pregitzer, 1993).

Foliar and fine root total C and N

We sampled green foliage of all commonly occurring tree species by pooling samples from 3 to 5 individuals of each species at each block in July 2004. Fine and very fine roots were collected from soil cores (above), sorted from organic material, and pooled by plot. Green foliage and fine roots were washed, kept on ice, flash-frozen in liquid N and lyophilized. All samples were ball-mill ground into a fine powder for total C and N analysis (Micro

Dumas combustion) and for tannin extraction (see below).

Tannin protein precipitation

We used the radial diffusion assay to measure tannin reactivity in green foliage, fine roots and leaf litter (Hagerman, 1987). This method provides a relative measure of tannin protein precipitation on a dry mass basis. Foliar and root sampling are as described above. Leaf litter was collected after leaf fall (October 2004) and pooled within each plot, air dried and ball-mill ground into a fine powder. Agarose-protein plates were created as described in Hagerman (1987). We used two protein types bovine serum albumin (BSA, fraction V, lyophylized powder, Sigma A4503) and gelatin (Knox brand), added to the agarose solution at concentrations of 1 mg (gelatin) or 0.5 mg (BSA) ml^{-1} buffer, respectively. The volume of precipitate from applied 50% aqueous methanol extracts of plant tissues was measured with digital calipers.

Soil extracellular enzyme activities

PPO activities were assayed with 3,4 dihydroxy-L-phenylalanine (L-DOPA) (Sigma D928), which serves as a general substrate for the oxygen oxidoreductase enzymes laccase (EC 1.10.3.2), catechol oxidase (EC 1.10.3.1) and tyrosinase (EC 1.14.18.1). All enzymes share some degree of overlap in substrate affinity, and oxidize polyphenolic compounds coupled by the four electron reduction of O_2 to H_2O (Thurston, 1994). Protease (EC 3.4.) activity was measured with azocoll (azo dye-impregnated collagen, Sigma A4341), a general protease substrate (Colpaert and Van Leare, 1996), that tests for the activity of both exo- and endopeptidases.

From June–August, five soil samples (2 cm diameter) were randomly sampled and homogenized by horizon from each plot. Oi horizon samples were cut into ~1 cm^2 pieces, and Oe/a and A horizons were sieved (4 and 2 mm sieve, respectively). Soils were placed on ice, transported to the laboratory and analyzed within 6 h. Extracellular enzymes were extracted with 50 mM NaOAc buffer, pH 5.0 following methods of Decker et al. (1999). A 2 ml aliquot of extract was combined with 2 ml substrate in buffer (10 mM L-DOPA or 0.01 g azocoll), for each sample with four analytical replicates, a substrate blank and a soil-free blank. PPO samples were incubated at room temperature in the dark for 1 h, centrifuged and measured at 460 nm. Protease samples were incubated at 37 °C for 2 h at 200 rpm, placed in ice water, centrifuged and measured at 520 nm. An extinction coefficient for PPO was generated with laccase (Sigma 40442), and protease activity was calibrated against a standard curve using protease (Sigma P6110). Soil subsamples were dried and weighed to correct for water content.

Reciprocal litter extracellular enzyme activity

We conducted a reciprocal leaf litter study to test for the effect of plot (Hdwd+Rmax vs. Hdwd) and leaf litter composition on extracellular enzyme activities. Leaf litter was collected after leaf fall (October 2003), and mixed at four blocks into four leaf litter treatments: (1) *R. maximum* leaf litter only, (2) *R. maximum* and hardwood species leaf litter, (3) hardwood species leaf litter from Hdwd+Rmax plots and (4) hardwood species leaf litter from Hdwd plots, designated as R, R+H, H (Hdwd+Rmax) and H (Hdwd), respectively. At each plot, leaf litter from each of the four treatments were placed into one 15 cm × 10 cm litter bag with 2 mm screen (allowing for in-growth of roots and fungal hyphae and movement of micro- and mesofauna) and placed randomly at the Oa horizon surface in each plot. We placed wire mesh cages over the bags to prevent incident leaf litter coming in contact with the bag. Extracellular enzymes were extracted from leaf litter after 10 months (August 2004) and 21 months (July 2005), and assayed for PPO and protease activities. Enzyme extraction and analysis were the same as above, except data were expressed on a specific leaf area (SLA, g cm^{-2}) basis to account for the effect of differential decomposition rates on surface area to leaf mass relationships among litter treatments. We used Scion Image software (Scion Corporation, National Institute of Health) to create relationships between mass and surface area.

Statistical analyses

We tested all data for normality. Percent C and N were arcsine-root transformed, and resin-bag N data were log transformed. All data were analyzed with SAS software (SAS Institute Inc., Cary NC).

First, using a single-factor (fixed effect) ANOVA with a blocking factor (random effect) design, we tested the effect of plot type (Hdwd+Rmax vs. Hdwd) ($n = 2$) on the following: A horizon nutrients, total leaf litter mass, hardwood leaf litter, FWD, very fine root length, root biomass by root class, root litter, and protein precipitation by leaf

litter and fine root extracts. Using the same design, we tested for the effect of plant species ($n = 8$) on protein precipitation by foliar extracts. Tukey's HSD was used as a post-hoc test for significant differences ($P < 0.05$) among species.

Second, we used a split-plot (fixed effect) ANOVA with a blocking factor (random effect) design to test for plot and within-plot effects. In this design, the whole-plot factor was plot type (Hdwd+Rmax, Hdwd) and the split-plot factor was either soil horizon ($n = 2$–3), species ($n = 9$), or leaf litter treatment ($n = 4$). We examined percent C, percent N, C:N, pH, O horizon mass, and enzyme activities by soil horizon, leaf litter mass by plant species, and extracellular enzyme activities on treatments of reciprocally placed leaf litter. If within-plot effects were significant, we used a post-hoc least-square means separation technique.

Finally, we used a two-factor split-plot (fixed effects) ANOVA with a blocking factor (random effect) design to test for the effect of plot type, sampling date and N type (NH_4^+ or NO_3^-) (and the interaction of these factors) on N availability. In this design, the whole-plot factor was plot type ($n = 2$), and sampling date ($n = 5$) and N type ($n = 2$) were the split-plot factors. We used all data from April–August 2005 and due to non-continuous sampling we excluded data from September 2004. If interactions were present, data from each sampling date were analyzed separately to test for the effect of plot and N type.

Results

Organic horizon stores and soil nutrients

The storage and chemistry of O horizons was altered in the presence of *R. maximum* thickets. The mass of the O horizons was significantly greater in the Hdwd+Rmax plots ($P < 0.0001$) (Table 1). FWD in Hdwd+Rmax plots (283.5 (75.9) $g\,m^{-2}$, mean (SE)) was not different from that in Hdwd plots (226.9 (44.8) $g\,m^{-2}$) ($P = 0.51$). Both percent C and N had a significant horizon by plot interaction (both $P = 0.01$), and C:N ratios were significantly different by horizon ($P < 0.0001$) and presence of *R. maximum* ($P = 0.01$), with greater C:N in the O and A horizons in Hdwd+Rmax plots (Table 1). Soil pH was not different between plots in the Oe/a horizons, but A horizon pH was lower in Hdwd+Rmax plots compared to the Hdwd plots ($P = 0.014$). A horizon available P (1.01 (0.11) and 0.75 (0.07) $mg\,kg^{-1}$), exchangeable Ca (41.4 (15.2) and 61.5 (25.4) $mg\,kg^{-1}$), exchangeable Mg (23.6 (5.4) and 21.1 (2.3) $mg\,kg^{-1}$) and exchangeable K (62.1 (7.4) and 54.9 (5.8) $mg\,kg^{-1}$) in Hdwd+Rmax and Hdwd plots, respectively, were not significantly different.

Leaf litter and root biomass

Total leaf litter mass was greater in Hdwd+Rmax than Hdwd plots ($P < 0.0001$, Table 2). The *R. maximum* leaf litter in Hdwd+Rmax plots was responsible for the difference as total hardwood litter inputs were not significantly different between plots ($P = 0.69$). *Q. rubra* and *R. maximum* were the greatest contributors of leaf litter and *Q. rubra* leaf litter mass was not different between Hdwd+Rmax and Hdwd plots (Table 2). Total standing root biomass was greater (but not significantly different) in Hdwd+Rmax plots ($P = 0.26$). There were no differences between plots in coarse ($P = 0.97$) and fine ($P = 0.57$) standing root biomass. However, very fine root (<0.5 mm diameter) standing biomass was greater in Hdwd+Rmax plots ($P = 0.0001$) (Table 3). The difference in very fine root biomass between Hdwd+Rmax and Hdwd plots, as well as the respective difference in SRL (6957.6 (800.8) and 4188.3 (276.4) $cm\,g^{-1}$) resulted in a significant

Table 1. Soil characteristics in hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax)[a]

	Soil horizon and plot type					
	Oi		Oe/a		A	
	Hdwd+Rmax	Hdwd	Hdwd+Rmax	Hdwd	Hdwd+Rmax	Hdwd
Mass ($g\,m^{-2}$)	546.3 (23.9)a	322.5 (48.9)b	2476 (305.0)a	469.7 (79.6)b		
Percent total C	47.9 (0.2)a	43.2 (0.8)b	29.1 (4.1)a	15.3 (1.1)b	8.4 (0.9)a	9.3 (0.6)a
Percent total N	1.9 (0.1)a	2.0 (0.08)a	1.2 (0.1)a	0.8 (0.04)b	0.36 (0.04)b	0.46 (0.02)a
C:N	25.3 (1.1)a	21.8 (0.6)b	24.5 (0.8)a	18.8 (0.8)b	23.5 (0.8)a	20.1 (0.8)b
PH			4.08 (0.1)a	4.46 (0.1)a	4.99 (0.2)b	5.68 (0.2)a

[a]Values are means and (SE) from $n = 4$ sampling blocks; different letters between Hdwd+Rmax and Hdwd plots within a soil horizon indicate a significant difference, $\alpha < 0.05$.

Table 2. Leaf litter mass and species composition in hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax)[a,b,c]

	Leaf litter mass ($g\,m^{-2}$)	
	Plot type	
Species	Hdwd+Rmax	Hdwd
Amelanchier arborea	13.0 (3.6)a	10.0 (3.2)a
Acer pennsylvanicum	0	7.0 (1.5)
Acer rubrum	12.2 (3.6)a	20.2 (10.1)a
Betula alleghaniensis	2.3 (1.1)b	10.8 (3.8)a
Betula lenta	22.6 (10.8)b	31.1 (12.3)a
Castanea dentata	0.11(0.1)b	1.5 (0.5)a
Fraxinus americana	0.34 (0.2)b	3.56 (1.9)a
Quercus rubra	146.6 (13.9)a	124.7 (13.0)a
Rhododendron maximum	112.4 (8.6)	0
Total leaf litter mass[d]	336.8 (12.9)A	235.5 (18.6)B

[a]Values are mean and (SE) of $n = 4$ sampling blocks.
[b]Data possessed a significant plot by species interaction ($p<0.0001$).
[c]Species' leaf litter mass in the same row followed by a different letter are significantly different between forest plots ($\alpha<0.05$) Total leaf litter mass by plot is significantly different ($\alpha<0.05$).
[d]Total leaf litter also contains unidentifiable leaves, and species not included in individual species analysis.

Table 3. Standing root biomass and estimate of fine root litter inputs in hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax)[a]

	Plot type	
	Root biomass ($g\,m^{-2}$)	
Root class[b]	Hdwd+Rmax	Hdwd
Very fine	197.1 (11.1)a	125.2 (10.9)b
Fine	98.4 (12.5)a	90.2 (8.0)a
Coarse	203.4 (43.1)a	201.6 (38.9)a
Total	498.9 (47.7)a	417.0 (47.9)a
Root litter[c]	295.5 (19.8)a	215.4 (14.4)b

[a]Values are means (SE), values in the same row followed by a different letter are significantly different ($\alpha<0.05$); $n = 4$ sampling blocks.
[b]Very fine < 0.5 mm, fine = 0.5–1 mm, coarse > 1 mm, root litter ≤ 1 mm.
[c]Root litter inputs estimated by peak season fine root biomass (Joslin and Henderson, 1987; Gill and Jackson, 2000).

difference in very fine root length density (13,711.2 (775) and 5244.8 (457) $m\,m^{-2}$, $P<0.0001$). Using peak season standing fine root biomass (≤1 mm diameter) we estimated greater root litter inputs in Hdwd+Rmax plots compared to Hdwd plots ($P = 0.0038$) (Table 3).

Foliar and root C:N

Foliar C:N varied among species ($P<0.0001$). *R. maximum* foliage had a significantly greater C:N ratio (45.3 (1.4)) than other species (range 24.7–33.0), which were not significantly different from one another (data not shown). Fine root C:N was not significantly different in Hdwd+Rmax and Hdwd plots, 38.9 (2.6) and 35.0 (2.0), respectively ($P = 0.27$).

Protein precipitation by tannin extracts

Protein precipitation by tannin extracts varied among species with both gelatin and BSA protein (both $P<0.01$) (Fig. 1). With gelatin, *A. rubrum* extracts precipitated the greatest volume, while *A. arborea*, *B. alleghaniensis*, *B. lenta* and *F. americana* precipitated the least (Fig. 1). With BSA, *A. rubrum*, *Castanea dentata*, *Q. rubra* and *R. maximum* extracts precipitated the greatest volume, while *A. arborea* precipitated the least. Hdwd+Rmax plot leaf litter extracts precipitated a greater volume of gelatin and BSA than extracts from Hdwd plot leaf litter ($P = 0.01$, $P = 0.007$ respectively) (Fig. 1). Fine root extracts from Hdwd+Rmax plots precipitated more gelatin and BSA than those from Hdwd plots ($P = 0.02$ and $P = 0.03$, respectively) (Fig. 1).

Inorganic N availability peaked in August in Hdwd plots, and peaked in spring in the Hdwd+Rmax plots. In general, NH_4^+-N increased between April and July, and decreased in August. There were significant time by plot ($P = 0.046$) and time by N type ($P = 0.0008$) interactions with resin-bag-captured N (April–August) (Fig. 2). Because of the interaction with time, we analyzed each month independently for the effect of plot on NH_4^+-N, NO_3^--N and total inorganic N. In the Hdwd plots, NO_3^--N was greater than Hdwd+Rmax plots in May, June and July ($P<0.05$), while there were only trends of greater NH_4^+-N availability in September ($P = 0.13$), April ($P = 0.06$), July ($P = 0.13$), August ($P = 0.09$). Total inorganic N was significantly greater in Hdwd plots in April ($P = 0.04$) and August ($P = 0.01$) and higher, but not significant in both May ($P = 0.08$) and July ($P = 0.07$) (Fig. 2).

Soil extracellular enzyme activity

In both plots, PPO activity decreased with soil depth. There was a significant horizon by plot interaction with soil PPO activity ($P = 0.0005$) with greater PPO activity in Hdwd+Rmax plots in the Oi and Oe/a horizons. There was no difference in

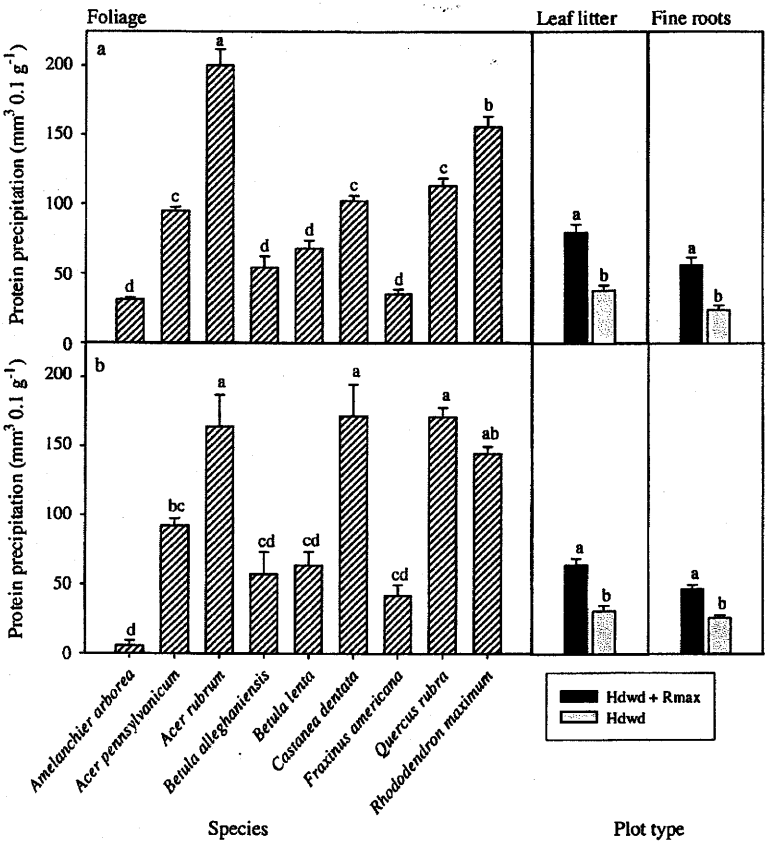

Figure 1. Protein precipitation of foliage (by species), and root and leaf litter (by plot) extracts from hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax) for (a) gelatin and (b) BSA protein types. Bars indicated by a different letter (within tissue group) are significantly different ($P<0.05$) with $n = 5$ sampling blocks.

PPO activity between plots in the A horizon (Fig. 3). Soil protease activity decreased with depth ($P = 0.0042$) but was not different between plots (Fig. 3).

Extracellular enzyme activity of reciprocally placed leaf litter

PPO activity was greater when litter treatments were placed in Hdwd+Rmax plots compared to Hdwd plots after 10 months (non-significant, $P = 0.13$) with a block effect ($P<0.0001$) (Fig. 4), and this pattern strengthened after 21 months (marginally significant, $P = 0.06$). Although the effect of litter was not statistically significant ($P = 0.09$), there was a trend of greater PPO activity on the R and R+H litter types compared to the H only litter, especially in the Hdwd+Rmax plots. Protease activity had a strong response to the litter types ($P = 0.005$ and $P<0.0001$, 10 and 21 months, respectively). After 21 months, R litter had the greatest protease activity, followed by R+H and the H litter groups (Fig. 4).

Discussion

Rhododendron maximum inhibits overstory regeneration

R. maximum clearly inhibits overstory regeneration in southern Appalachian forests, although the contributing factors are not fully understood (Nilsen et al., 1999; Beckage et al., 2000; Nilsen et al., 2001). While reduced photosynthetically active radiation under *R. maximum* contributes to seedling mortality, low light and seedling shade intolerance do not entirely explain the lack of regeneration (Clinton and Vose, 1996; Lambers and Clark, 2003). Additional mechanisms contributing to suppression are not immediately obvious. Inhibition of seed rain and germination (allelopathy) does not occur (Nilsen et al., 1999; Lei et al., 2002), and

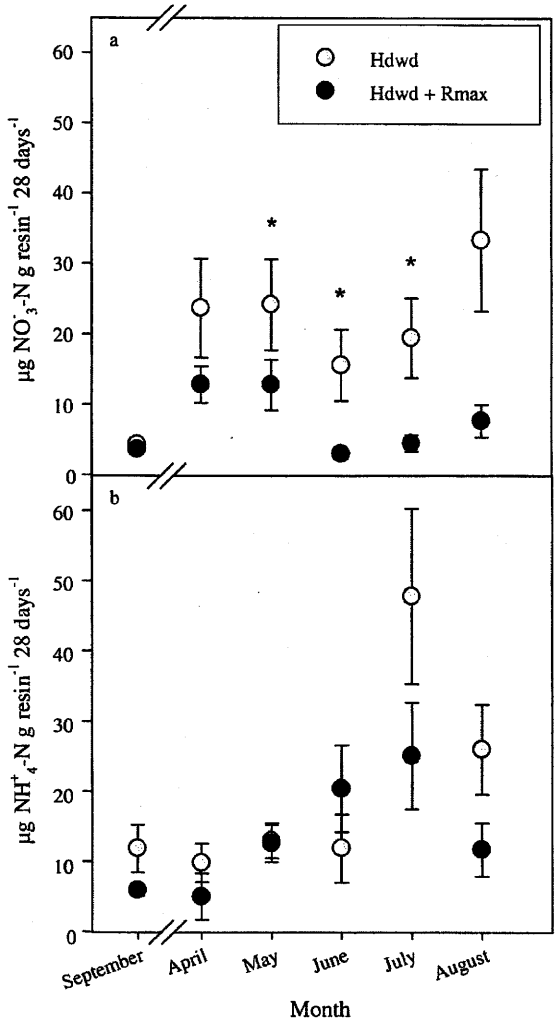

Figure 2. Monthly resin bag extractable N in hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax) for (a) NO_3^--N and (b) NH_4^+-N. Asterisks denote a significant difference between plot type at each sampling date ($P<0.05$); $n = 4$ sampling blocks.

soil factors such as moisture, temperature, exchangeable cations and pH are only subtly different or not consistently different between thicket and non-thicket areas, nor among studies (Boettcher and Kalisz, 1990; Clinton and Vose, 1996; Nilsen et al., 2001; Clinton, 2003; Beier et al., 2005). Our results suggest that *R. maximum* influences the N cycle through increases in leaf and root litter, and litter tannins forming complexes with organic N. Furthermore, these changes to N dynamics may not be as detrimental to *R. maximum* if the saprotrophic capacity of ERM fungi is superior to that of the ECM and arbuscular mycorrhizal fungi of hardwood forest trees.

Soil C and N, litter inputs

Soils under *R. maximum* thickets have greater organic mass and N in the O horizon, and lower percent N in the A horizon, compared to forests without thickets. Hardwood forest O horizon mass in our study is similar to a previous report from a Coweeta northern hardwood stand ($615.3\,g\,m^{-2}$; Knoepp et al., 2000). *R. maximum* O horizon mass is 280% greater than hardwood forests; similarly, O horizon total N is 286% due to both great O horizon mass and Oe/a horizon percent N. In comparison, in Kentucky, *R. maximum* increased O horizon mass by 9% and 76%, and O horizon N by 9% and 83% in *Tsuga canadensis* and *Liriodendron tulipifera* cove forests, respectively (Boettcher and Kalisz, 1990). *R. maximum* thickets vary in density (Baker and Van Lear, 1998), and our selection of dense thickets along with differences in forest community structure may explain the greater impact of *R. maximum* on O horizon mass and N content in our study. In contrast to the O horizons, A horizon percent N is lower in forest soils with *R. maximum* compared to those without.

Organic matter accumulates under *R. maximum* due to greater leaf and root litter inputs and slower decomposition relative to other species. *R. maximum* typically retains leaves for 6 years, losing only 9% of standing crop of leaves annually (Monk et al., 1985), but it increases total leaf litter mass by 43%, and estimated root litter mass by 39% in our study sites. The accumulation of organic matter under *R. maximum* is also due to the slow rate of litter decomposition (Hoover and Crossley, 1995; Hunter et al., 2003). *R. maximum* foliage is sclerophyllous, and in our study, C:N of *R. maximum* foliage is greater than all other measured species. In previous studies at Coweeta, the DOC:DON ratio of fresh *R. maximum* litter leachate was almost twice that of the dominant tree species (Qualls et al., 1991), and freshly fallen *R. maximum* leaf litter C:N was greater than *Liriodendron tulipifera* and *Quercus prinus* (Hunter et al., 2003). The high C:N in *R. maximum* leaf litter is likely related to low foliar N concentrations and high N remobilization during leaf senescence (Monk et al., 1985), and high concentrations of polyphenols (Hunter et al., 2003). Similarly, we found tannin extracts from *R. maximum* leaf litter and roots have a greater protein precipitation capacity than those from hardwood tissues (see below).

Protein precipitation by tannin extracts

Regardless of species tannin extracts from green foliage precipitated the greatest volume of protein,

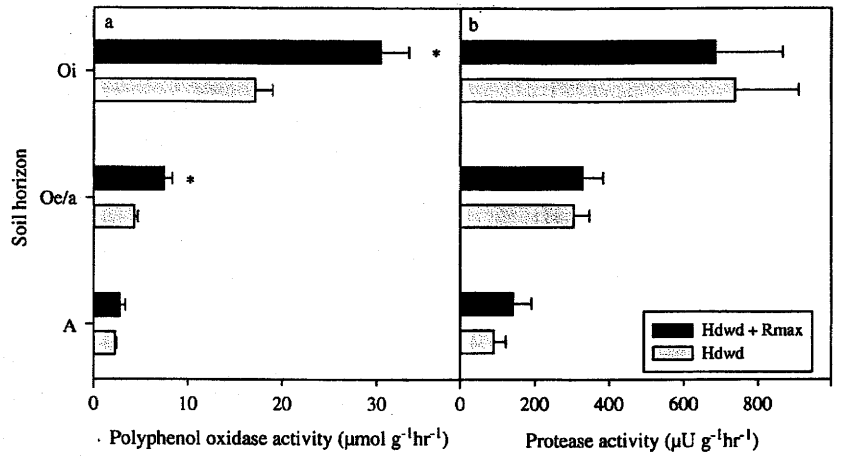

Figure 3. Soil extracellular enzyme activities in hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax) of (a) polyphenol oxidase (PPO) and (b) protease. Asterisks denote a significant difference in enzyme activity between plot types at each soil horizon ($P<0.05$); $n = 4$ sampling blocks.

followed by extracts from fresh roots and leaf litter. From the few existing examples in the literature, tannin concentrations and tannin precipitation capacity are generally greater in foliage than roots of woody plants (Preston, 1999; Kraus et al., 2004b; but see Gallet and Lebreton, 1995) and are even lower in litter and humus (Baldwin and Schulze, 1984; Preston, 1999), possibly due to leaching losses (Baldwin and Schulze, 1984; Nicolai, 1988). *R. maximum* and *Q. rubra* contributed the most leaf litter mass in our study, and both had a relatively high foliar precipitation capacities compared to other species. However, *A. rubrum* foliar extracts had the greatest (gelatin), or were among the greatest (BSA) in protein precipitation capacities of species tested. *Acer* species foliar extracts have high astringency, possibly due to the unique chemistry of their hydrolysable tannins (Bate-Smith, 1977), and *Acer* species foliar tannins have greater reactivity or concentration relative to the northern hardwood species *Q. rubra*, *F. americana* and *B. alleghaniensis* (Baldwin and Schulze, 1984; Côté, 2000; Lovett et al., 2004).

Leaf litter tannin extracts from Hdwd+Rmax plots precipitated more protein than those from Hdwd plots. Relative to Hdwd+Rmax plots, Hdwd plots receive greater leaf litter mass from species whose foliar samples have relatively low protein precipitation (e.g., *Betula* species and *F. americana*), and phenolics from deciduous leaves are rapidly lost during decay relative to evergreen leaves (Kuiters and Sarink, 1987). In Hdwd+Rmax plots, *R. maximum* accounts for over 30% of leaf litter mass and the sclerophyllous nature of *R. maximum* leaf litter allows for greater retention of tannins relative to hardwood leaf litter. In fact, after one year of decomposition, *R. maximum* leaf litter contains greater concentrations of total phenolics, condensed tannins and hydrolysable tannins than *L. tulipifera* and *Q. prinus* leaf litter (Hunter et al., 2003).

Root litter tannins are a significant source of tannins in forest soils (Hättenschwiler and Vitousek, 2000; Kraus et al., 2003b; Kraus et al., 2004a). Even if tannin concentrations in roots are lower than those in leaves, rapid root turnover could make fine roots a greater contributor of soil tannin (Kraus et al., 2003b). In our study, root extracts from Hdwd+Rmax plots precipitate more protein than those from Hdwd plots; therefore, *R. maximum* roots may contribute to the greater protein precipitation capacity.

Inorganic N availability

We predicted lower availability of inorganic N in forests with *R. maximum* than without because litter polyphenols can decrease the availability of organic N for microbial acquisition and subsequent mineralization. During the growing season, resin bag extractable NO_3^--N was lower in Hdwd+Rmax plots compared to Hdwd plots during three months of the growing season, while NH_4^+-N and total inorganic N were generally lower overall. Nitrification rates are inhibited by polyphenols (Baldwin et al., 1983) and are negatively correlated with forest stand age, ericaceous cover and soil polyphenol concentrations in coniferous Swedish forests (DeLuca et al., 2002). In the southern Appalachians, the presence of *R. maximum* lowered soil NO_3^- concentrations (Nilsen et al., 2001)

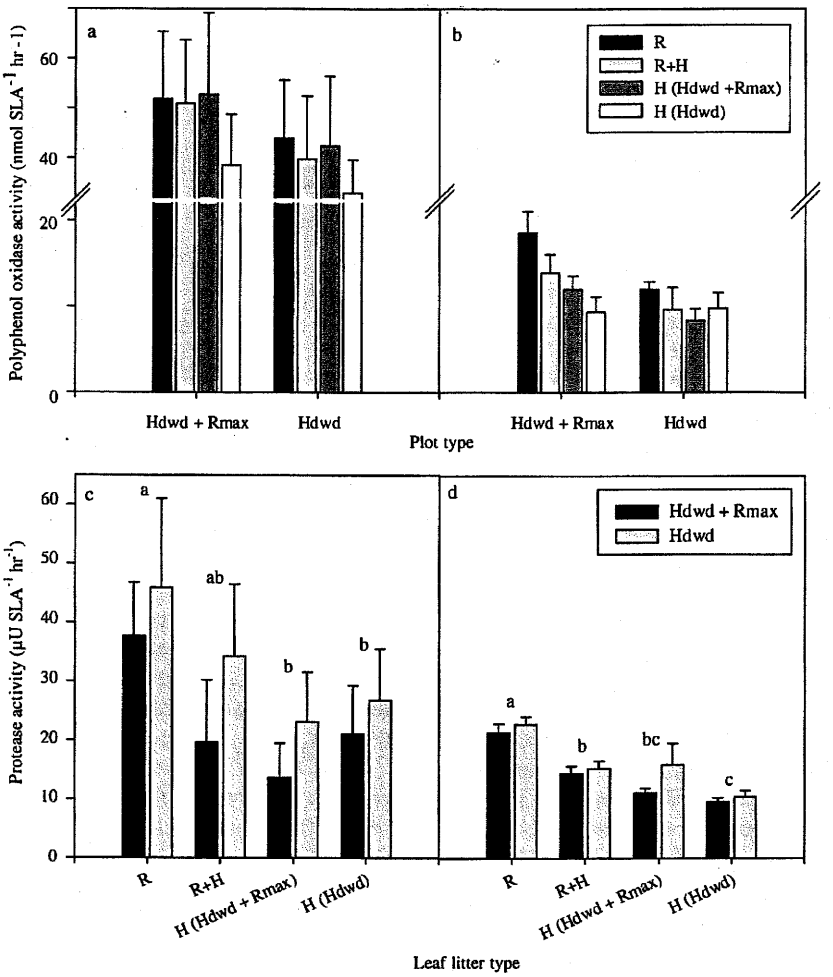

Figure 4. Extracellular enzyme activities on reciprocally placed leaf litter in hardwood forest plots (Hdwd) with and without *Rhododendron maximum* (Rmax): (a) polyphenol oxidase activity, 10 months, (b) polyphenol oxidase activity, 21 months, (c) protease activitiy, 10 months and (d) protease activity, 21 months. Significant differences ($P<0.05$) in protease activity among litter types denoted by different letters; $n = 4$ sampling blocks. Leaf litter treatments are as follows: R = *R. maximum* leaf litter only, R+H = *R. maximum* and hardwood species' leaf litter, H (Hdwd+Rmax) = "hardwood species" leaf litter from Hdwd+Rmax plots and H (Hdwd) = "hardwood species" leaf litter from Hdwd plots.

and reduced N mineralization rates by 60–100% in cove forests (Boettcher and Kalisz, 1990; but see Nilsen et al., 2001).

Across the Coweeta basin, N mineralization is influenced more by forest type than by climate; forests with *R. maximum* as a dominant species have one of the lowest rates of N mineralization relative to total soil N of those reported (Knoepp and Swank, 1998; Knoepp et al., 2000). Our results support the premise that forest composition can influence inorganic N availability. In forests with *R. maximum*, we observed lower inorganic N availability and shifts in soil N distribution across horizons compared to forests without *R. maximum*. Greater N storage in the O horizons under *R. maximum* was related to both greater organic mass and greater percent N in the Oe/a horizons. Since *R. maximum* litter extracts have a greater capacity to complex protein, organic N may be complexed with tannins, increasing stored organic N in the Oe/a horizons and decreasing N mineralization rates. O horizon storage of N under *R. maximum* could also be enhanced by biological immobilization.

Although protein-precipitation capacity of leaf litter tannins can be a strong predictor of N mineralization rates (Handayanto et al., 1997), precipitation capacity does not reflect the recalcitrance of the protein-tannin complex. These complexes differ widely in recalcitrance, possibly do to

their molecular structure and composition of condensed and hydrolysable tannins (Howard and Howard, 1993; Kraus et al., 2003a). The varying strength of polyphenol–organic N complexes may account for the lack of correlation between foliar chemistry, or tannin protein precipitation, and soil N factors in forest ecosystems. Individual tree species influence N cycling through litter quality (Boerner and Kozlowsky, 1989; Boettcher and Kalisz, 1990; Lovett and Mitchell, 2004), yet standard measures of litter quality (e.g., tannin concentrations) are poor predictors of N cycling characteristics because they do not reflect differences in tannin reactivity (e.g., protein–tannin interactions) (Lovett et al., 2004), nor the strength of the protein-tannin complex.

Extracellular enzyme activity

Soil microorganisms degrade organic matter through the production of diverse extracellular enzymes (Caldwell, 2005). Soil extracellular enzyme activities indicate the potential processing rates of organic substrates, allowing for comparisons between forest communities (Allison and Vitousek, 2004). In forest soils, mycorrhizal fungi comprise a significant amount of microbial biomass (Högberg and Högberg, 2002), and among mycorrhizal fungi, ERM fungi may produce more extracellular enzymes, such as PPO (Read et al., 2004). The production of extracellular PPO is limited to some species of leaf litter and bark decomposing fungi, wood rotting fungi, ERM fungi and some ECM fungi (Dix and Webster, 1995; Read et al., 2004).

We predicted that PPO activity would be greater in *R. maximum* soils because of the presence of ERM fungi and the influence of polyphenols on N availability. In the O horizon, PPO activity was greater in Hdwd+Rmax plots than Hdwd plots. PPO activity is typically greater in litter than soil (Deforest et al., 2004; Gallo et al., 2004), and is positively related to low N mineralization rates and high litter C:N ratios (Gallo et al., 2004). In a field study, soil PPO activities and concentrations of phenolics were greater under the ECM, polyphenol-rich, Mediterranean shrub *Cistus albidus* L., compared to areas between plants (Castells and Peñuelas, 2003). Greater PPO activity under *Cistus albidus* in the above study and in *R. maximum* soils in our study could be attributed to the local microbial community or to greater availability of polyphenolic substrates.

We measured enzyme activities in reciprocally placed leaf litter to test the effects of the local microbial community and leaf litter composition. Although not statistically significant, on average, PPO activity was 21% and 35% greater across the leaf litter treatments after 10 and 21 months, respectively, when they were placed in Hdwd+Rmax plots. Given the high spatial heterogeneity of soil extracellular enzyme activities, it is possible these differences are real and mediated by the local microbial community. The specificity of microbial communities to leaf litter affects on the production of extracellular enzymes (Sugai and Schimel, 1993; Sinsabaugh et al., 2002), decomposition and N cycling (Lovett et al., 2004). In hardwood forests of our study, soil PPO may be an important mechanism for polyphenol degradation and nutrient acquisition. ERM fungi of *R. maximum* may contribute to enzymatic degradation processes, although their precise contribution is unknown.

We hypothesized that soil protease activity would be greatest under *R. maximum*; however, protease activity did not differ between forest types. Unlike PPO, most soil fungi produce proteases (North, 1982; Dix and Webster, 1995), including ERM and ECM fungi (Read et al., 2004). On reciprocally placed leaf litter, protease activities displayed a consistent response to leaf litter type with greater activity in *R. maximum* leaf litter compared to hardwood species leaf litter. Protease activity is positively related to leaf litter protein concentration (Lähdesmäki and Piispanen, 1988) and extracellular proteases are induced by the presence of protein in ERM fungi (Leake and Read, 1991). In our study, protein concentrations may have been greater in R litter compared to H litter after 10 and 21 months of decomposition, inducing the observed patterns in protease activity. These results suggest that protease activity responds to small-scale changes in leaf litter quality, which may have been undetected in the plot level soil assays.

Conclusions

Litter polyphenols may alter the N cycle in *R. maximum* soils by forming recalcitrant complexes with organic N, increasing soil organic N content and lowering inorganic N availability. Ericaceous understories influence N cycling in several ecosystems worldwide; however, most are cold temperate or boreal, and considered N-limited (Nilssen and Wardle, 2005). Our results suggest that an ericaceous understory alters N cycling in a temperate hardwood forest, with relatively high inorganic N availability. The influence of *R. maximum* on N cycling, along with its reduction of

photosynthetically active radiation, may contribute to the suppression of hardwood seedling regeneration in southern Appalachian forests. Furthermore, the slow growth and low N demands of *R. maximum*, and the potential for ERM fungi to acquire of polyphenol-complexed N via extracellular enzymes may explain the shrub's continued success. However, the impacts of polyphenol–organic N complexes on N cycling are not fully understood. Protein–tannin complexes vary in recalcitrance (Howard and Howard, 1993), and we need to understand the turnover and fate of tannin-complexed N derived from ericaceous plants is needed to clarify its importance in N cycling and plant nutrition in forest ecosystems.

Acknowledgments

This research was funded by the Coweeta Long-Term Ecological Research Site (DEB-0218001), the University of Georgia Warnell School of Forestry and Natural Resources and Center for Biodiversity and Ecosystem Processes. We thank Lee Ogden, Dale Porterfield, Chelcy Ford, Brian Higgins, Patrick Bussell, Bruce Caldwell, Barry Clinton, Tamara Kraus, David Coleman, Sarah Covert, Lisa Donovan, Larry Morris, Mark Hunter and Fabio Sartori for their advice and assistance. The authors are especially grateful to Dr. David Coleman for both his assistance and input during Wurzburger's doctoral studies and for his considerable contributions to Hendrick's graduate research program in past years. The authors also acknowledge two anonymous reviewers whose comments and discussions greatly improved the manuscript.

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