CNB: requiescat in pace?

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Despite its failure to predict variation in secondary chemistry for many metabolites and taxa, the Carbon/Nutrient Balance (CNB) hypothesis continues to be invoked with regularity. According to Lerdau and Coley (2002), the value of the hypothesis is that it explains phytochemical phenotypic plasticity outside of an evolutionary context. We contend that the CNB hypothesis impedes a more comprehensive understanding of plant biology and that an approach divorcing plant chemistry from function is inherently limited in its ability to predict responses of plants to the environmental conditions in which they have evolved. Although it is possible for a mechanistically flawed hypothesis to predict phenomena, such hypotheses almost always suffer a lack of generality. Lerdau and Coley argue that the limitations of the CNB model are its strengths, not its weaknesses, and set forth refinements that, in essence, further limit its applicability. We address these refinements and explore alternative adaptive explanations for adjustments of secondary metabolism in relation to resource availability and environmental variation. In view of its flawed nature and the existence of alternative approaches, CNB no longer warrants consideration as a viable hypothesis.

Background

The Carbon/Nutrient Balance hypothesis (CNB or CNBH), as first described in this journal by Bryant et al. (1983), consisted of a collection of ideas revolving around the notion that resources influence the evolution of defense allocation. Among the ideas put forth in that paper was one that would dominate research on chemical defense for the next 20 years. That idea was that the relative availability of carbon and nutrient resources could govern relative investment by plants in carbon- and nutrient-based secondary compounds. By altering light and nutrient availability, two resources known to vary naturally, one could in theory determine which and in what relative amounts secondary metabolites would be produced. Failures of the hypothesis to account for outcomes in certain species or for certain secondary compounds began to accumulate shortly after the hypothesis was proposed (reviewed in Herms and Mattson 1992, Karban and Baldwin 1997, Ko-

richeva et al. 1998, Hamilton et al. 2001). These shortcomings were addressed by a series of modifications that limited applicability of the hypothesis to the point where Hamilton et al. (2001) recommended its use be discontinued. In response to that assessment, Lerdau and Coley, in this issue, maintain that the hypothesis in a highly reduced form remains a useful tool for the study of plastic responses. In this reply, we will address the fundamental difficulties associated with the hypothesis. We will then specifically address the refinements identified by Lerdau and Coley. Finally, we will argue that CNB impedes understanding of resource-based variations in secondary compounds, as it has forestalled research on alternative hypotheses, a few of which we outline.

CNB has become an evolution-free hypothesis

When Bryant et al. (1983) introduced the CNB hypothesis, they used resource availability to explain inter- and intra-specific patterns of secondary metabolites. At the interspecific level, variations in concentrations of secondary metabolites were attributed to evolutionary forces. They stated that, "Low-resource-adapted woody plants...have slow growth rates which preclude the possibility of escape from the high-risk browsing zone by rapid vertical growth. Such trees and shrubs have responded evolutionarily to browsing by increased production of thorny leaves or twigs or repellent secondary metabolites." Intraspecific phenological patterns of growth and chemical defense were also thought to be adaptive by Bryant and his co-authors: "In spring there are often strong and competing selective pressures for use of carbon by deciduous species, on the one hand for rapid leaf production so as to maximize photosynthesis throughout the growing season, and on the other hand to effectively protect leaves against removal by herbi-

vores. The resulting pattern of allocation of carbon depends upon the balance of these selective forces." In contrast, phenotypic responses in secondary metabolites to carbon and nutrient resource limitation in the environment were not explicitly described in adaptive terms.

Since the introduction of the CNB hypothesis, the non-adaptive aspects of the model increasingly became the focus of interest, and, as advocates of the hypothesis, Lerdau and Coley (2002) now consider exclusion of adaptation to be one of the model's most important contributions: "One of the key insights of the CNBH is that it does *not* make claims for optimality, but simply argues that resources in excess of growth demands will be shunted into defenses". They further maintain that defenses are manufactured only with resources in surplus to the needs of growth even when diversion of resources from growth into defense would increase plant fitness: "...physiology may put constraints on the range of plastic responses that are possible, perhaps compromising plant performance under certain circumstances. The CNBH clearly points out this non-adaptive alternative..." (Lerdau and Coley 2002). As a consequence, natural selection cannot alter the resource allocation pattern because plant biochemical pathways are constrained by physiology: "it is certainly possible that plasticity could be under selection and that regulatory enzymes would lead to adaptive responses. But it is equally possible that plastic responses are constrained by physiology and resource availability and are not always optimal" (Lerdau and Coley 2002). Based on the premise that phenotypic plasticity is generally unregulated and non-adaptive, the CNB model hypothesizes that the relative abundance of resources drives phenotypic responses in plants: "...many plastic responses of plants to resource variation may be a consequence of substrate availability and not a result of regulated and adaptive responses" (Lerdau and Coley 2002).

However, this neutral mode of plant metabolism clearly does not operate in many plants (Hamilton et al. 2001). Even when CNB appears to explain allocation patterns in a limited number of circumstances, as suggested by Lerdau and Coley, upon closer examination the CNB hypothesis is mechanistically incorrect. In the section that follows, we address those few pathways that appear to be consistent with CNB.

Regulation and physiological constraint

We address two basic premises of the CNB hypothesis as postulated by Lerdau and Coley: first, that phenotypic plasticity of secondary metabolites is an unregulated consequence of substrate availability, and second, that plasticity is constrained to follow allocation patterns predicted by CNB.

Phenotypic plasticity is not a consequence of substrate availability

The CNB premise that secondary metabolism is unregulated and substrate-driven is, in general, not true. As an example, we cite regulation at the interface of primary and secondary metabolism. Phenylalanine ammonia-lyase (PAL) is an enzyme that links primary and secondary metabolite portions of the shikimic acid pathway, a biochemical pathway which Lerdau and Coley (2002) identify as being exemplary of the CNB hypothesis. Phenylalanine, an amino acid primary metabolite produced from shikimic acid, is converted by PAL into cinnamic acid, a building block for many secondary metabolites, including tannins, lignins, and flavonoids.

Plasticity in the rate of conversion of phenylalanine to cinnamic acid by PAL is highly regulated and is not passively determined by the availability of phenylalanine substrate. A multigene family codes for different forms of PAL enzymes; that PAL activity is regulated is evidenced by predictably differential expression of these genes in different tissues, at different stages of plant development, in response to environmental stimuli, and in response to complex interactions between development and environmental stimuli (Hrazdina 1992, Kervinen et al. 1998, Kubasek et al. 1998). In parsley (*Petroselinum sativum*), an adaptive phenotypic response in flavonoid synthesis to protect against ultraviolet light damage requires coordinated regulation of a complex of enzymes in primary and secondary metabolic pathways, including PAL (Logemann et al. 2000). In tobacco (*Nicotiana tobacum*), PAL activity is also inhibited by its product, cinnamic acid, indicating that negative feedback regulation prevents the passive flow of phenylalanine substrate into secondary metabolism (Blount et al. 2000). PAL may also be regulated through inhibition by flavonoids synthesized further downstream in the pathway (Seigler 1998). The delicate coordination of PAL by environmental and developmental signals operating through multigene control would seem entirely inconsistent with the passive response to resource supply postulated by CNB.

Plants are not constrained to follow CNB allocation patterns

Lerdau and Coley (2002) suggest that "physiology may put constraints on the range of plastic responses that are possible". Although they do not spell out the nature of this physiological constraint, presumably it prevents resource allocation patterns that deviate from that postulated by the CNB hypothesis, which is to allocate resources to defense only if they are not needed for growth. To contradict this premise, we use examples of within-species variation in resource allocation to con-

densed tannins, because Lerdau and Coley cite these compounds as conforming particularly well to the CNB hypothesis (see Hamilton et al. 2001 for more examples).

In quaking aspen (*Populus tremuloides*), responses in growth rate and condensed tannin concentration to CO_2 and nutrient treatments vary among aspen clones (Lindroth et al. 2001). These significant genotype × CO_2 and genotype × nutrient interactions indicate that phenotypic plasticity in resource allocation to growth and defense is variable within a species and is not subject to a physiological constraint as postulated by Lerdau and Coley at least within the range of conditions examined. Moreover, this variation in phenotypic response differs among genotypes, indicating that condensed tannin levels at the whole-plant level are regulated and not driven solely by resource pools as predicted by CNB. Genetic variation in phenotypic plasticity of secondary chemistry is potentially subject to natural selection, a prerequisite for the evolution of adaptive patterns in secondary metabolite production (Agrawal 2001).

Plants are also not physiologically constrained to allocate to defense only those resources in excess of growth needs. Negative correlations between growth and condensed tannin concentration have been observed in plants, suggesting that there is a tradeoff between growth and tannin production (Coley 1986, Sagers and Coley 1995). Plants in these studies were grown in common environments so that variation in resource allocation was due to genotypic and phenotypic variation among individuals and not plasticity in response to resource availability; however, these examples suggest that condensed tannins are produced with resources at the expense of growth demands in these species, not with resources in excess of growth demands, as CNB explicitly assumes.

Lerdau and Coley argue that tradeoffs between growth and defense help to explain phenotypic patterns based on the CNB hypothesis (e.g. "condensed tannins and lignins are produced from phenylalanine and therefore compete directly with protein synthesis for nitrogen"), but it is important to keep in mind that the concept of physiological tradeoffs is not exclusive to CNB; rather, it is a key component of explicitly evolutionary hypotheses. Physiological tradeoffs are integral to evolutionary explanations of phenotypic responses, including plasticity in resource allocation to growth, storage, dispersal, and reproduction, as well as defense (Herms and Mattson 1992, Bazzaz and Grace 1997, Zera and Harshman 2001). We therefore agree with Lerdau and Coley that "a more complete understanding of the biosynthetic pathways would clarify where one would expect to find competition for resources and hence tradeoffs between investment in growth or defense, or between different classes of defenses," but contend that identifying tradeoffs neither

excludes evolutionary hypotheses nor confirms CNB.

CNB – refine or decline?

Lerdau and Coley (2002) offer three refinements that are intended to strengthen the CNB hypothesis: 1) "CNBH is unlikely to be useful for understanding the regulation of compounds that are present in vanishingly small quantities", 2) "the major resource pools involved in production of a compound may lie in synthesis and/or storage, rather than in its skeleton", and 3) "secondary metabolites produced by pathways other than the shikimate pathway do not always (and may never) follow the predictions of the CNBH." However, these refinements are primarily ad hoc modifications that do not address the fundamental problems with the premises of the CNB hypothesis as we have already discussed. As a result, these attempts to strengthen the model serve only to highlight problems inherent to the CNB hypothesis:

CNB is no longer a general model

Since the CNB hypothesis was introduced, its domain of applicability has progressively shrunk as the model has been repeatedly refined (Hamilton et al. 2001). Refinements 1 and 3 narrow the domain still further. The CNB hypothesis now applies only to shikimate-derived compounds that "make up a substantial fraction of leaf biomass or energy allocation" (Lerdau and Coley 2002). However, not even all compounds from the shikimate pathway can be expected to follow the predictions of CNB. Lerdau and Coley state earlier in their paper that, "...even within the shikimate pathway, condensed tannins and lignins respond to nitrogen fertilization as predicted by the CNBH, but hydrolyzable tannins do not." We suggest that CNB is no longer a general model for resource allocation but simply a description of the behavior of a small fraction of the total secondary metabolite diversity in plants.

CNB is no longer a testable hypothesis

According to refinement 2, synthesis and storage costs must be accounted for before the CNB hypothesis can be adequately tested. However, it is unclear how the costs of producing storage tissues are to be quantified and incorporated into the CNB hypothesis. For example, monoterpenes require specialized storage tissues (Lerdau and Gershenzon 1997), whereas specialized cells are required for the synthesis and deposition of condensed tannins (Gruber et al. 1999). How are the carbon/nutrient costs of these two different tissues to

be compared, and what common currency of carbon/ nutrient costs is to be used to predict which secondary metabolites will increase or decrease in response to carbon/nutrient availability? In other words, what compounds are to be considered "carbon-based" and "nutrient-based" compounds under the refined CNB? Refinement 2 implies that researchers must first estimate costs associated with specialized tissues involved in secondary metabolite production, transport, and storage before they can formulate testable hypotheses.

In addition, as Lerdau and Coley (2002) admit, "accounting for all resource pools" may be "particularly complicated for compounds such as alkaloids which include both nitrogen and carbon and are products of numerous interconnected biosynthetic pathways." In reality, many compounds, including tannins, are products of more than one biosynthetic pathway (Seigler 1998). Condensed tannins are comprised of flavonoid subunits, which in turn are derived from shimikic acid and malonyl-CoA; malonyl-CoA is also a precursor for fatty acids, waxes, and polyketides. According to the refinements of the CNB hypothesis, researchers will presumably need to devise carbon/nutrient formulas for each compound to be studied, incorporating the relative contributions of each pathway. This is an operationally daunting task.

CNB is no longer internally consistent

In refinement 3, Lerdau and Coley suggest that the shikimate pathway follows the CNB hypothesis because "important differences exist in either the reliance on different resources or the regulation of different biosynthetic pathways." By incorporating regulation as a mechanism to explain why CNB should work for some secondary metabolites and not others, the CNB hypothesis becomes logically inconsistent with its basic premise that plasticity in secondary metabolism production is often unregulated ("...many plastic responses of plants to resource variation may be a consequence of substrate availability and not a result of regulated and adaptive responses", Lerdau and Coley 2002).

CNB is no longer viable

The CNB hypothesis describes the phenotypic responses of only a limited number of secondary metabolites and thus is of little utility as a practical tool to formulate predictions. It promotes the classification of secondary metabolites as carbon- and nutrient-based, a concept especially difficult to define under the new refinements. The hypothesis is logically inconsistent. In short, the CNB hypothesis is no longer a viable framework for studying the effects of resource availability on secondary metabolite production.

Secondary metabolites are adaptations to variable environments

Lerdau and Coley (2002) argue that plasticity in secondary metabolite production is generally not adaptive because phenotypic responses to environmental variation do not correspond to patterns observed between species. To test the hypothesis that plasticity is adaptive, they compare phenotypic changes in tannin concentration against interspecific patterns predicted by the Resource Availability Hypothesis, concluding that "many examples exist where plastic responses do not mirror the optimal patterns seen across species". We suggest that a more successful approach to studying changes in secondary metabolites is to consider how these changes affect plant performance relative to specific environmental stresses.

CNB interprets plasticity in secondary metabolites as passive consequences of resource availability, but plasticity can be adaptive with respect to stresses associated or correlated with changes in light and nutrients. We provide examples of adaptive hypotheses to explain secondary chemical responses to light and nutrients, again focusing on tannins and shikimate-derived compounds because of their putative concordance with CNB. Let us emphasize that by offering these hypotheses, we are not arguing that resource limitation is unimportant in determining secondary metabolite levels in plants because resource limitation most likely affects all physiological processes (Bazzaz and Grace 1997).

Compensatory defense in response to increased attractiveness to herbivores

Plants respond to light availability by configuring their leaves to maximize photosynthetic potential (Lambers et al. 1998). Shade leaves are thinner and partition more nitrogen to metabolites involved in harvesting light, whereas sun leaves are thicker and invest more nitrogen in the dark reactions of photosynthesis (e.g. ribulose-bis-phosphate carboxylase). Most importantly, however, sun leaves contain on average nearly twice as much organic nitrogen per unit area as shade leaves (Evans and Poorter 2001). As nitrogen (really, protein) is often a limiting resource for insects (Scriber 1984), sun leaves should be more nutritionally rewarding per unit area than shaded leaves. Indeed, feeding and fitness of *Malacosoma disstria* Hbn. larvae were higher on sugar maple crown leaves than on shade leaves from the same trees (Fortin and Mauffette 2002). Not only is the protein content of sun leaves high, starch content is also elevated (Koricheva et al. 1998).

To offset such changes in the nutritional value of leaves caused by light availability, plants may adjust concentrations of anti-herbivore chemicals. For example, variation in tannin concentration, which seems for

the most part to conform to CNB, may be adaptive in this regard. By virtue of their protein-binding capacity (which includes binding of ribulose-bis-phosphate carboxylase; Min et al. 2000, and references therein), tannins are effective when their abundance is commensurate with the amount of protein to be rendered indigestible. In this light (forgiving the pun), the plastic tannin responses of individual plants to shading might more appropriately be investigated as an adaptive adjustment in defense.

Protection against oxidative stress

Increases in secondary metabolites under high light conditions may in part serve to protect plants from reactive oxygen species (Frankel and Berenbaum 1999). Photosynthesis generates vast amounts of oxygen, one mole for each mole of CO_2 fixed, and oxygen poses a serious threat to cells, which typically function overall in a reduced state. The photosynthetic generation of oxygen increases as a function of light availability. It is now well known that secondary metabolites can act as potent antioxidants (Grace and Logan 2000). Antioxidant activity is highly correlated with total phenolic content both among species (Saleem et al. 2001, Kaur and Kapoor 2002) and among genotypes within a species (Connor et al. 2002), and even tannins and lignins exhibit antioxidant properties under certain conditions (Noferi et al. 1997, Hagerman et al. 1998, Kamoun et al. 1999).

Protection against ultraviolet light

Most of the secondary metabolites that vary according to CNB also are effective at absorbing ultraviolet light (UV). Because UV radiation, particularly UV-B (280–320 nm), generates a variety of destructive free radicals, reducing penetration of these wavelengths to the interior of cells is beneficial. UV intensity can be attenuated by foliage; thus, it is not surprising that UV-absorbing secondary metabolites often increase in full sunlight (Meijkamp et al. 1999). In addition to the benefit of intercepting UV, these secondary metabolites may scavenge UV-generated radicals (Kostina et al. 2001).

Enhancement of nutrient uptake

Plants encountering low resource availability respond by actively increasing functions to enhance resource acquisition (Boggs 1997). Condensed tannin production in the pine tree *Pinus muricata* appears to be one such adaptation in response to low soil nutrient availability (Northrup et al. 1995). Tannins in the leaf litter of *P. muricata* complex with litter proteins, preventing miner-

alization of organic nitrogen to ammonia and nitrate. By maintaining litter nitrogen in a dissolved organic state, tannins prevent nitrogen from being used by competing soil organisms while keeping it accessible to beneficial mycorrhizae associated with pine. Organic nitrogen also does not leach below the root zone as does nitrogen in the form of nitrate. Northrup et al. (1995) also found that the leaf concentration of condensed tannins in *P. muricata* trees was negatively correlated with soil fertility along a natural gradient of habitats in California. *P. muricata* trees growing in acidic, nutrient-poor soils produced higher concentrations of condensed tannins (and as a consequence, have higher tannin concentrations in their litter) than individuals growing in more fertile soils. Although a common garden or transplantation study would be needed to determine whether the variation in tannin production is due to plastic responses or differences in constitutive tannin levels, this study suggests that plasticity in tannin production may be an adaptation to nutrient limitation.

CNB cannot deal with the multifunctionality of secondary metabolites

The adaptive roles of secondary metabolites in compensatory defense, UV protection, or nutrient uptake are not mutually exclusive because secondary metabolites can have multiple functions (Herms and Mattson 1992). Recent studies are starting to elucidate how pleiotropic traits such as secondary metabolites are regulated. DNA microarray analyses have demonstrated that very few genes (including genes involved in secondary metabolite pathways) are regulated by only a single stress factor (Reymond et al. 2000). In contrast, multiple functions are excluded from the CNB hypothesis by its dichotomous categorization of growth versus defense. The artificiality of this dichotomy generates contradictory CNB predictions when compounds function in both growth and defense physiology. For example, lignins are argued by Lerdau and Coley (2002) to be defensive compounds that respond in accordance to the CNB model ("condensed tannins and lignins respond to nitrogen fertilization as predicted by the CNBH"). The categorization of lignins as defensive metabolites is justified because these compounds make plant tissues more difficult to exploit for all heterotrophs except a limited number of microbe species (Hagerman and Butler 1991). However, lignins are also an essential and integral part of growth physiology, especially in woody plants. Without lignins to give mechanical strength to cell walls, a plant could not grow successfully (Hagerman and Butler 1991). How should lignins be classified? If they are classified as growth components, their concentration would be predicted by CNB to increase with

increasing nitrogen fertilization; but, if they are categorized as defense compounds, their concentration would be predicted to decrease under the same conditions. Plant compounds with both primary and secondary metabolite functions are widespread (Herms and Mattson 1992, Berenbaum 1995), but none fall within the domain of the CNB hypothesis because of the logical contradictions they create within the model.

Benefits of the evolutionary approach

The CNB hypothesis is superficially simple to investigate. According to CNB, secondary metabolites are made with surplus resources, and they incur no metabolic cost. Moreover, because CNB assumes no adaptive function for plasticity in secondary metabolite production, there is no necessity to quantify benefits. Thus, plant fitness, the ultimate measure of cost and benefit, need not be quantified. Because measuring plant fitness is especially difficult to quantify in long-lived or iteroparous plants, not having to quantify it is a definite advantage. However, as elegantly as the CNB model may simplify the study of secondary metabolites, it incorrectly describes plant phenotypic responses, conceptually and mechanistically.

We are, of course, not suggesting that resource limitation does not affect resource allocation to different physiological functions, including defense. Bryant et al. (1983) justifiably argued that "the evolutionary response of plants to herbivory is also strongly influenced by other selective pressures in the plant's environment, such as nutrient availability, and can be understood only in this broader context." In this paper, we, too, advocate a broader context – in particular, one that does not focus on resource availability to the exclusion of other selection pressures, as the CNB hypothesis clearly does in its current incarnation. Given the number of plausible alternative explanations for resource-based variations in secondary metabolites (we present just a few examples here), we cannot advocate, as Lerdau and Coley do, that CNB be used as a heuristic tool and invoked as the "first, but not final, step in studying the effects of resource availability". Instead, we advocate an understanding of phenotypic plasticity of secondary metabolites based on hypotheses rooted in the single most powerful theory in biology – evolution – and suggest investigating CNB only as a last resort.

Focusing on the ultimate causes of allocation patterns of secondary metabolites results in the generation of general hypotheses that can be related to plant physiology and evolutionary biology (see Agrawal 2001 for examples). Eventually, plasticity of secondary metabolite production will be fully integrated into evolutionary biology. In the meantime, we suggest that another of Darwin's principles be used as a model: "I have steadily endeavoured to keep my mind free so as to give up any hypothesis, however much beloved (and I cannot resist forming one on every subject), as soon as facts are shown to be opposed to it" (Darwin 1887).

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