Plant-Herbivore Interactions between North American Porcupines (*Erethizon dorsatum*) and Trembling Aspens (*Populus tremuloides*)

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March 2005

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

ABSTRACT

Plant-herbivore interactions play a significant role in the structure and functioning of ecosystems. Co-evolutionary theory suggests that plant defenses evolved due to herbivores and herbivore pressure can shape the genetic composition of their food resources. We used interactions between North American porcupines (*Erethizon dorsatum*) and trembling aspens (*Populus tremuloides*) as a system to investigate this theory's important assumption that herbivores select food sources based on genetically controlled traits. We confirmed that porcupines exhibit intra-specific food selection and that this is linked to the genetic composition of the aspens. We also demonstrated that variation in phenolic glycosides and condensed tannins are strong components of this selection, thereby creating an important link between genetics, plant chemistry, and mammalian herbivory. We investigated potential impacts of porcupine herbivory on aspen using fluctuating asymmetry, however we did not detect any stress on heavily eaten trees, thereby questioning the validity of this tool for this study system.

RÉSUMÉ

Les interactions plantes-herbivores jouent un rôle fondamental dans les écosystèmes. La théorie de co-évolution suggère que les défenses chimiques des plantes ont évolué en fonction de la pression d'herbivorie et que les herbivores ont le potentiel de modifier la composition génétique de leurs ressources alimentaires. Nous avons utilisé les interactions entre le porc-épic d'Amérique (*Erethizon dorsatum*) et le peuplier faux-tremble (*Populus tremuloides*) comme système afin d'examiner la prémisse importante de cette théorie, que les choix alimentaires des herbivores sont basés sur des traits étant sous un contrôle génétique. Nous avons démontré que les porcs-épics effectuent une sélection alimentaire intra-spécifique, qui est liée à la composition génétique des trembles. Nous avons également établi que la variation des glucosides phénoliques est importante dans cette sélection, invoquant ainsi un lien entre la génétique des plantes, leur chimie et l'herbivorie des mammifères. Nous avons examiné l'impact de l'herbivorie sur les trembles avec une analyse d'asymétrie fluctuante, mais les résultats n'ont détecté aucun stress sur les arbres consommés, remettant en question la validité de cet outil pour ce système d'étude.

PREFACE

This MSc. consists of three chapters. The first is a general introduction to the thesis and a literature review on subject matter pertinent to the thesis but outside the scope of a journal article. This material is in accordance with the requirements of thesis submission and should be used to help the reader familiarize him/herself with background theory and information related to the topic.

Contribution of Authors

Although chapters two and three have co-authors, the entire thesis is to be considered as having been written by the student. The contribution of Dominique Berteaux in both chapters two and three is directionial ??? in nature and limited to the normal supervisory roles of assistance with project design, fieldwork and statistical analysis, as well as feedback on earlier versions of the manuscripts. The role of Jim Fyles in chapter two is also supervisory in nature and includes help with analyses, as well as preparation of, and feedback on, the manuscript. The contribution of Rick Lindroth is related to his expertise in biochemical analyses and interpretation of results.

ACKNOWLEDGEMENTS

Throughout my degree, I often heard myself say “I’m the luckiest girl in the world”. This was not in reference to my overworked and underpaid student status, or the mass hours spent battling biting insects to photograph 577 aspen ramets (6 times each) in the field, or the thousands of test tubes I pipetted, re-pipetted, and then pipetted again. Although I learned from each of those experiences (some more character building than others), it is to my supervisors that I owe my deepest gratitude. And so, the oft-repeated sentence from above is in relation to the fact that I lucked out by having the two best supervisors ever.

Both in their own ways, and both with their own styles, Dr. Dominique Berteaux and Dr. Jim Fyles offered me inspiration, motivation, direction, creativity, reasoning, and so much support. Advice flowed freely, whether it was scientific, philosophical, spiritual, intellectual or emotional. Their continued understanding, encouragement, enthusiasm and financial support made this whole process possible and I can’t thank either of them enough for believing in me.

My gratitude to, and respect for, Dominique are unquantifiable. His laid-back supervisory style allowed me to develop and trust my own scientific instincts and his commitment to sound science and discovery taught me how to be rigorous, thorough and precise in all aspects of this project. His kindness and generosity extended well beyond his role as supervisor and I thank him also for enriching many other aspects my life.

There is also a suite of others who contributed to the success of this project. I'd like to thank Dr. Rick Lindroth and all the members of the Lindroth lab in Madison, Wisconsin, for all the painstaking hours spent teaching me how to perform biochemical assays and interpreting the results. In addition, I have endless gratitude to Dr. Christopher Cole for his genetic analyses of several aspen ramets which strengthened this project and removed several uncomfortable question marks from my data. A huge portion of this work was made possible due to their kindness and hospitality throughout my stay in Madison.

The staff at Parc National du Bic also deserves a big thank you for their welcoming nature throughout my field work in the park. And of course, the many field assistants who devoted their summers, their minds and their bodies to making this project possible. In particular, thanks to Jackie Hass, Caroline Trudeau, Isabelle Turcotte, and Patrick Bergeron whose drive and dedication in the field were unparralled. Also, a big thank you to Ilya Klvana for discussing ideas, trying new things, and all the help and direction he gave with my field work. Alain Caron's help and advice with statistical analyses contributed a great deal to this project as well.

And finally, although perhaps not directly implicated in the findings of this research, there are a few people out there whose unconditional, love, support, and patience can not go unnoticed. My unbelievable family- Mom, Bill, Dad and Rob, never in a million years could I have made it this far without you. Jenn, your unfaltering love and loyalty has gotten me over many hurdles, thanks for always believing in me.

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CHAPTER I: INTRODUCTION AND LITERATURE REVIEW

1.0. Introduction

Interactions between plants and animals have been under investigation for several decades. The ability of herbivores to shape the ecosystems in which they live, and the way plants attempt to defend themselves from herbivore attack are paramount to the study of plant-herbivore interactions. The fact that different plants produce distinct defensive chemicals that may affect different herbivores in different ways is widely acepted (Rhoades 1979, Tuomi 1992, Freeland and Janzen 1974, Bryant et al. 1991). However, the evolutionary significance of the existence and diversity of these chemicals, as well as an herbivore's ability to impose selection on defensive traits is still under debate.

Many authors have attempted to provide evidence for the idea that host plants and their herbivores have evolved together in a co-evolutionary race (Elrich and Raven 1964, Rhoades 1979 and 2005, Mauricio 2001), while others point to resource constraints as the limiting factor in plant investment to defense (Loomis 1953, Bryant et al. 1983, Herms and Mattson 1992). Both schools of thought have provided ample evidence for their theories although much of it is site and/or species specific. This has resulted in the lack of a unifying theory in understanding chemical defenses of plants and their interactions with herbivores.

The recent increase in our knowledge and understanding of plant genetics and the improvement in molecular techniques to identify plants at

the genome level have spurred a new wave of plant-animal studies. Still, little research has explicitly examined plant chemical defense, plant genetics, and the impact of herbivores within one study system, information necessary to provide evidence for co-evolutionary theory. Furthermore, much of this research pertains to insect herbivores and therefore does not shed light on the more complex role of mammals in plant-herbivore interactions.

There is thus an important gap in the literature given that mammalian herbivores are primary consumers, important links in the food chain, and thus key components of almost all terrestrial ecosystems. The study described in this thesis attempts to bridge this gap by examining the variation in defensive chemistry of trembling aspens (*Populus tremuloides*) in relation to plant genetics, and how this variation may affect food choices of the North American porcupine (*Erethizon dorsatum*), an important mammalian herbivore in the temperate and boreal forest.

1.1. Plant Defensive Chemistry

Stahl's 1888 paper was perhaps the first to suggest that certain chemical properties of plants evolved to help decrease pressure by herbivores (Rhoades 1979). However, it was Fraenkel's (1959) paper on the "raison d'être" for the existence of secondary plant substances, that inspired numerous researchers to examine secondary metabolites and plant defensive chemistry in terms of how and why they've evolved, as

well as how they may effect their invertebrate and vertebrate consumers (Herms and Mattson 1992).

All plants, across all taxa, contain primary substances (often referred to as the nutritive plant parts), such as vitamins, minerals, and amino acids (Fraenkel 1959). These primary substances are used to meet the daily requirements of life such as respiration, digestion, excretion, and photosynthesis, and are found in practically all cells, tissues and organs of plants (Berenbaum 1995).

In addition to primary substances, plants contain a vast array of what are known as “secondary” substances (Fraenkel 1959). Unlike primary substances, the distribution of secondary metabolites is limited, both within and among different plants. Although derived from metabolites involved in primary physiological processes (Berenbaum 1995), the large variation of secondary substances across plant parts, individuals, species, families and genera suggests that they do not play a role in the metabolic functions of plants (Fraenkel 1959). Instead, they are responsible for the biosynthesis, accumulation, transport and storage of the metabolic products of several pathways (Herms and Mattson 1992). However, there is excessive variation in the nature, quantity and distribution of secondary metabolites (both within and between plants), making it difficult to pinpoint the exact function of these substances (Berenbaum 1995).

Despite this, it is widely accepted that specific secondary metabolites do in fact have defensive functions (Fraenkel 1959, Erlich and Raven 1967, Freeland and Jansen 1974, Rhoades 1979, Tuomi 1992).

These compounds can be broken down into two distinct categories; toxic secondary substances such as cyanide, phytoecdysone ecdysterone, and ouabain, that actively poison herbivores if ingested; and inhibitory secondary compounds including tannins and other phenolics that hinder digestive processes (Rhoades 1979). These inhibitory compounds tend to be the most widespread defensive plant secondary metabolites (Rhoades 1979).

"Phenolics" encompass a broad range of compounds containing a hydroxyl group attached to an aromatic ring. They are present in almost all plants and accumulate in all plant parts, however the function of many phenolic compounds is still unknown (Levin 1967, Raven et al. 1999). Simple phenolics exhibit considerable biological activity, including antifungal and antibacterial properties, others can act as estrogens when ingested by mammals, and still others have been shown to decrease the palatability of plants to herbivores (Markham 1971, Palo 1984).

One very important and well studied group of phenolics is the tannins. Tannins are found in high concentrations in all classes of vascular plants, and show a wide structural divergence and a diverse distribution among and within individual species (Swain 1979, Raven et al. 1999). Tannins are said to inhibit attack on lignified tissues by fungi and bacteria and to serve as a defense against herbivores by reducing the nutritional availability of soluble plant proteins and polysaccharides. Tannins also reduce the activity of the digestive enzymes and symbiotic microorganisms within the herbivore's own gut (Swain 1979).

Although the defensive functions of tannins are widely accepted, it has been suggested that these compounds evolved not as a defense to herbivores, but to protect plants against fungal and bacterial attack (Swain 1979). Actually, the defensive functions of secondary compounds as an explanation for the evolution of plant secondary metabolism, is still under debate (Tuomi 1992), and has spurred research efforts in two main directions. The first stresses the notion that defensive functions contribute to the evolution of secondary metabolites, whereas the second concerns itself with how other factors, such as resource availability, can constrain secondary metabolism and in turn, defensive responses (Tuomi 1992).

The remainder of this literature review concentrates on theories that have emerged from the fist group however, the latter category includes theories such as the growth-differentiation balance hypothesis (Loomis1953), the carbon/nutrient balance hypothesis (Bryant et al. 1983), and the environmental constraint hypothesis (described in Herms and Mattson 1992), as well as a host of others (see Berenbaum 1995 for a list of chemical defense theories and Herms and Mattson 1992 for a review).

Contrary to the resource availability theories mentioned, the optimal defense theory sees herbivory as the primary force shaping quantitative patterns of secondary metabolism (Herms and Mattson1992). This theory suggests that a plant can only allocate a certain proportion of its resources to defense, while the rest are used to meet the needs of other biological processes, such as growth. Optimal defense theory assumes 1) that organisms evolve and allocate defenses in such a way as to maximize

individual inclusive fitness and 2) that the production of defenses incurs a cost to the fitness of the organism (Rhoades 1979). Because defense is costly, it is only expected to arise when the payoffs exceed the costs. Therefore, plants evolving under similar resource conditions can evolve different growth rates, as a function of herbivory pressure (Herms and Mattson 1992).

Extensive research on interactions between antagonist herbivores and their host plants have given rise to the aforementioned theory. In turn, optimal defense theory (and its major assumptions) has been tested across a variety of plant taxa, herbivores, and environmental conditions (see Rhoades 1979 for an extensive review). Although some of the critical assumptions of the model are difficult to test, optimal defense theory has offered a framework within which to examine notions of the co-evolutionary theory that plant chemical defense has arisen in response to herbivore pressure (Rhoades 1979).

The co-evolutionary theory was first proposed by Elrich and Raven in 1964, as a way to explain the diversity of secondary compounds in plants. They proposed that this diversity arose to counter the variation in both herbivore species and herbivore performance. For this theory to hold, two assumptions must be met. First, any selection imposed by herbivores must act on traits that confer resistance (ie: chemical defense) and must cause divergence in these traits. Second, selection imposed on herbivores by these plant traits must cause herbivores to diverge in traits that counteract the resistance traits of plants (Mauricio 2001). A working

problem with this theory lies in the fact that most authors who attempt to test it, do so without strongly adhering to both assumptions (Mauricio 2001).

Several authors have contributed evidence to the theory of plant-herbivore co-evolution, and Rhoades (1979) reviewed them extensively. Recently, the role of herbivores as selective agents has gained renewed interest with an increased understanding of the relationship between plant chemistry and genetics (see Snyder 2002, Bailey et al. 2004, Oreilly-Wapstra 2004). However, Mauricio (2001) contends that the majority of studies exemplifying that herbivores exhibit selective pressure on plant traits only demonstrate the potential of herbivores to do so, rather than demonstrating the actual occurrence of selective pressure. Rhoades (1979) also expressed that many of the aforementioned theories are based on limited evidence and should not be regarded as truth. He stressed that many of these theories are based on site and/or species specific attributes and questioned the extent to which any of them can be seen as general phenomena.

Characterizing the evolution of plant chemical defense is further complicated by the notion that plant chemistry is not static and that herbivory can stimulate induced responses in plants. In a meta-analysis examining damage-induced changes in woody plants, Nykanen and Koricheva (2004) reported that concentrations of phenolics, and the protein-precipitation capacity of tannins, tend to increase as a response to herbivory. These induced responses can also be affected by plant

characteristics such as fast versus slow growing plants, deciduous versus conifer species, and early versus late season foliage. This idea is supported by several other plant-insect studies including Haukioja (1991), Karban and Baldwin (1997), Wold and Marquis (1997), Kaitaniemi et al (1998), Boege (2004).

Although as of yet, there is no unifying theory to explain the existence of the diversity of secondary compounds in plants, it seems clear that future research should orient itself to testing specific theories, while ensuring that all assumptions of the models are met. Tuomi (1992) suggested that GDBH could provide such a framework, whereas Berenbaum (1995) advocates that future studies on co-evolution must focus on the genetics of chemical defense. In conjunction with Berenbaum, Mauricio (2001) proposed an ecological genetics approach to the study of plant-herbivore co-evolution. This approach to studying plant-herbivore interactions has received widespread attention in recent years, particularly with insects (see Lindroth et al. 1986, Mauricio and Rausher 1997, Mauricio 2001, Fornoni 2004). Similar analyses with vertebrate herbivores are few and yet, are required for a greater understanding of plant-herbivore interactions, and in order to better formulate a unified theory on the evolution of plant defense.

1.2. Mammalian Herbivory

Herbivore pressure can affect forest succession, tree growth, and distribution of energy to different plant parts. Furthermore, selective

herbivory can alter plant community composition, stand age structure, and the spatial distribution of habitat resources (Bailey et al. 2004).

Mammalian herbivores in particular, have been shown to shape forest ecosystems by browsing, trampling, and redistributing nutrients (Kielland and Bryant 1998, O'Reilley-Wapstra et al. 2004, Bailey et al. unpublished manuscript). Despite the important role of mammalian herbivores, most plant-herbivore research has concentrated on plant-insect communities. The result is a multitude of literature discussing the impacts of insect herbivores on their hosts, defense strategies of hosts to reduce these impacts, the evolution of new traits in insects to combat these new defenses, and several new hypotheses on the evolution of plant defenses (see review by Mattson et al. 1988).

Research on plant-mammal interactions has not gained the same wide-spread attention. This may be due to the more complex nutritional requirements of mammals, the long-term implications of multi-generational mammal studies, the difficulty of re-creating similar field conditions for mammals in experimental settings, and the difficulty in establishing patterns and causes of mammalian food selection. Nonetheless, although few, some studies do exist and most of them reiterate similar findings; mammalian herbivores have the ability to impose changes on the ecosystems in which they reside.

Kielland and Bryant (1998) show that moose, browsing preferentially on *Salix* sp., favors the growth of *Alnus* sp., thereby altering

the composition of the forest, and accelerating successional change. It has also been suggested that microtine rodents play an important role in the mortality of plants in boreal areas, especially in peak population years, and that sex selection increases male mortality of *Salix* species (Elmqvist et al. 1988). And beavers (*Castor Canadensis*) have long been referred to as "ecosystem engineers" (Bailey et al. 2004). Several species of hares have also been investigated and food selection related to secondary compounds is evident (Bryant et al. 1983, Clausen et al. 1986, Lindroth and Hwang 1996b, Lindroth 2000).

Although the aforementioned studies exemplify that many mammals are selective foragers, and that selection is related to the chemical defense compounds of plants, they do not necessarily imply that this behavior imposes selection pressure on the host plants. To do so, research must show first that the mammalian herbivores exhibit selective foraging, second, that this selection is a function of, and therefore acts on, defensive traits of plants, third that these defensive traits are under genetic control, and finally, that herbivory pressure reduces the fitness of the host plant (Mauricio 2001).

Research with mammals meeting these criteria is minimal, however several recent studies have attempted to examine the potential of mammalian herbivores as selective agents, in conjunction with the co-evolutionary hypothesis. Snyder (1992), Bailey et al. (2004), O'Reilley-Wapstra et al. (2004) and many others have provided building blocks for

evidence of this theory. In any plant-herbivore system, this requires an extensive understanding of the herbivore's food selection process and its ability to impose changes on its environmental surroundings, as well as measures on the variation in, and genetic basis of, the plant's secondary chemistry.

The remainder of this review focuses on these aforementioned characteristics of a plant-herbivore system by examining foraging decisions and impacts of North American porcupines, and the chemical and genetic bases of plant defense in trembling aspens.

1.3. Porcupine Feeding Patterns

The North American Porcupine, an arboreal, folivorous mammal, consumes great quantities of plant material throughout the year and exhibits substantial seasonal changes in its diet. Leaves, buds, and fruits of deciduous trees and forbs form the majority of its summer food intake, while the winter diet consists mainly of inner bark of trees and conifer foliage (Roze 1989). Winter is a period of critical weight loss as porcupines subside on a nutritionally poor diet from November to April inclusive. By the end of this season (early May), the average mass of adult porcupines in our study site is 6.8 kg, 27% less than the average of 9.4 kg found in mid October (Berteaux et al. in press).

Porcupines are known to be selective feeders. During the period when body weight and energy levels are low, they select specific tree

components over others based on levels of phenols and overall nutritional content (Roze 1989). Throughout the year, porcupines exhibit intra-specific selection according to biochemical differences which may be related to the genetic make-up of the tree (Snyder and Linhart 1997). These animals also display inter-species food selection based on nitrogen content and levels of crude protein (Roze 1989).

During spring, one of their preferred food sources in Parc National du Bic is the trembling aspen. Porcupines climb into the aspen canopy where they can remain for several hours, feeding on buds and leaves. They tend to sit on large branches, break off smaller ones, and feed on the foliage, discarding the remaining branches and petioles. They can eat the leaves from an entire tree limb in one evening's feeding, potentially altering the growth patterns, architecture and health of the tree (Diner, personal observation). When they climb they scratch the tree trunks, ultimately leaving scars, and therefore, a record of where they've been.

These climbing scars can be used as an index to determine differential herbivory pressure imposed on trembling aspens and to investigate the variation in chemical defense compounds of scarred versus unscarred trees.

1.4. Aspen Biology and Chemical Defense

Trembling aspens are considered "ecologically successful" due to their large geographic range, population density, and ability to thrive in a diversity of habitat types (Lindroth 2001). In addition, they are among the

few North American tree species that form naturally occurring, multistemmed clones (Barnes 1964). A clone consists of an aggregation of stems (ramets) produced asexually from a single sexually produced individual (the genet). In aspen, a clone is formed from the root system of the seedling genet, following an event that destroys the genet (Perela 2003). One clone can contain up to several thousand individual ramets (Kemperman 1977). Therefore it is the clone, and not the individual tree, that is the basic unit of any aspen stand (Kemperman 1977). Members of a clone are practically identical but can be distinguished from those of a neighboring clone by electrophoresis (Cheliak and Patel 1984), chemotaxonomy (Blake 1964), aerial photography (Blake 1964) or a variety of field observations (Barnes 1969).

P. tremuloides exhibits remarkable interclonal genetic variation with respect to growth rate, leaf morphology, timing of leaf flush and senescence, and resistance to insects, diseases, drought and pollution (Lindroth and Hwang 1996a, Osier et al. 2000). Interestingly, levels of secondary metabolites appear to be much more variable among aspen genotypes than are those of primary metabolites or mineral nutrients (Lindroth 2000).

The dominant secondary metabolites of aspen are phenolic compounds, produced via the shikimic acid pathway. These include phenolic glycosides and condensed tannins, which occur in leaf, bark, and root tissues, and coniferyl benzoate, which occurs only in flower buds (Lindroth 2000). Trembling aspen contains four phenolic glycosides;

salicin, salicortin, tremuloidin, and tremulacin. Of these, salicin and tremuloidin generally occur in concentrations <1% dry leaf weight whereas levels of salicortin and tremulacin (compounds containing the cyclohexenone functional group), are much higher, typically 1 to 8% each, and occasionally attain 15% (Lindroth 2000). In addition, they exhibit greater toxicity and reduce herbivore performance in a dose-dependant fashion (Lindroth and Hwang 1996b). Studies have shown that foliar concentrations of phenolic glycosides vary significantly over time and among clones and that temporal changes in phenolic glycoside concentrations are strongly dependant upon clone (Hwang and Lindroth 1998, Osier et al 2000).

The second major class of phenolics produced in aspen is condensed tannins. These compounds can make up nearly 30% of dry leaf weight (Lindroth 2000) and vary from 3% to nearly 30% of dry leaf weight among clones (Lindroth and Hwang 1996a,b, Osier et al. 2000). And finally, concentrations of coniferyl benzoate range from 0% to 7% dry weight in flower buds (Jakubas et al. 1989, Lindroth 2000).

According to Lindroth and Hwang (1996a), levels of tremulacin and salicortin show greater interclonal variation than intra-clonal variation. In contrast, levels of condensed tannins are fairly uniform among clones and exhibit little within-clone variation. In addition, there is a positive correlation between concentrations of tremulacin and salicortin, and levels of salicortin are negatively correlated with levels of condensed tannins (Lindroth and Hwang 1996a).

Although aspens rely on both chemical defense and tolerance, the former is known to be of critical importance in protection from insect herbivores (Lindroth 2000). Across clones, variation in phenolic glycoside concentrations often explains most of the variation in insect herbivore performance in both garden and field studies (Hemming and Lindroth 1995, Lindroth and Hwang 1996b, Hwang and Lindroth 1997, 1998, Osier et al. 2000). Studies indicate that at toxic doses, phenolic glycosides cause the formation of degenerative lesions in the midgets of insects, and that although condensed tannins have little impact against aspen-adapted insects, they may deter feeding by unadapted insects (Lindroth and Hwang 1996b). Furthermore, Jakubas et al. (1989) found that concentrations of coniferyl benzoate above 1.8% deter aspen feeding by ruffed grouse (*Bonasa umbellus*).

Although less is known about the protective role of aspen secondary compounds against mammalian herbivores, studies have shown that phenolic glycosides are more efficacious than tannins as defenses (Erwin et al 2001). For example, Reichart et al. (1990) found that balsam poplar (*Populus balsamifera*), a close relative of trembling aspen, is best protected from browsing by hares by a derivative of salicylic acid, but is not affected by tannins. Furthermore, opposums (*Trichosurus vulpecula*) avoid *Populus* sp. containing high concentrations of salicin and related compounds (Markham 1971, Edwards 1978), and it has been suggested that adventitious shoots of aspen are unpalatable to snowshoe hares (*Lepus americanus*) because they contain higher levels of phenolic

and terpene resins than do twigs of mature trees (Lindroth and Hwang 1996b).

Few mammalian herbivores respond strongly to non phenolic glycoside secondary compounds in aspen. However, beavers (*Castor Canadensis*) show preference for aspens with low levels of an unknown phenolic compound. The avoidance of this compound, which is found in high concentrations in juvenile tissues, can result in beavers selecting larger rather than smaller trees, a behavior contradictory to what is normally observed in this species (Basey et al. 1990). In addition, recent work has demonstrated that condensed tannins likely play an important role in cottonwood (*Populus* spp.) selection by beavers (Bailey et al. 2004). And finally, it has been shown that both tannin and nontannin phenolics affect diet selection of ruminant browsers (Lindroth and Hwang 1996b, Erwin et al. 2001).

If aspen secondary metabolites are effective deterrents to feeding by particular herbivores, clonal variation in herbivore preference is likely to be more strongly determined by secondary than primary chemical composition (Lindroth 2000). Understanding the quantitative variation of these secondary compounds is essential given how strongly it affects the interactions between aspen and its associated herbivores (Lindroth and Hwang 1996b, Osier et al. 2000).

1.5 Structure of Thesis

The underlying theme throughout this project is the relationship between vegetation and mammalian herbivores, an important force in the structure and functioning of most terrestrial ecosystems. Despite their importance, mammals have not been extensively studied in plant-herbivore interactions and many of the theories that exist to explain the evolution of plant defenses are based on plant-insect systems.

The goal of this thesis is therefore to examine the chemical defenses of trembling aspens in relation to herbivory pressure by North American porcupines. More specifically, chapter two first investigates the idea that porcupines select certain aspen ramets over others, and then attempts to determine the reason(s) for this intra-specific feeding behavior by quantifying the chemical variation found in aspen trees. Chapter two also explores the potential that the genetic make-up of aspen clones determines their chemical variation, which in turn, influences feeding choices of porcupines. The overall objective of this chapter is therefore to establish a link between aspen chemistry and genetics, and porcupine herbivory, while the chapter's secondary goal is to investigate the idea that porcupines may act as selective agents on aspen stands.

In order to follow up on this idea, chapter three, although only a small part of a larger study, attempts to quantify the impacts of porcupine feeding behavior on aspen trees. This chapter is based on the idea that if porcupines are to be seen as selective agents, they must have a negative impact on the fitness of the aspen stand. As such, we attempt to measure

the effects of intensive porcupine feeding using an analysis of fluctuating asymmetry of trembling aspen leaves.

We hope that the extensive knowledge gained in this project, in relation to porcupine food selection and their responses to the chemical defenses of trembling aspens, will shed light on the role of mammalian herbivores in the forest ecosystem, as well as their potential to act as selective agents on specific plant traits.

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MANUSCRIPT TO BE SUBMITTED TO *ECOLOGY*

Chapter II:

From Genetics to Chemistry to Herbivory: Interactions between North American Porcupines (*Erethizon dorsatum*) and Trembling Aspens (*Populus tremuloides*)

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Running Head: Genes, Chemistry and Porcupine Herbivory

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2.0. Abstract

Mammalian herbivores play a key role in forest ecosystems which are partly shaped by their browsing, trampling, and redistribution of nutrients. Despite this, previous research has not been done linked plant genotype, plant chemistry, and feeding preferences of mammalian herbivores. We examine this relationship through the North American porcupine, an arboreal, folivorous mammal, and the trembling aspen, a preferred food source of porcupines and one of the few clonal species of North American trees. Although porcupines consume the buds and leaves of these trees, not all aspens are used equally. Preference for certain ramets was determined through visual examination of porcupine scars left on tree bark, as well as through controlled feeding experiments. We analyzed the causes of this selection via the clonal composition of these aspens, as well as biochemical analyses. The results show that two phenolic glycosides (tremulacin and salicortin), which are both under genetic control, are the chemical variables that most influence feeding choices by porcupines. This study demonstrates that the clonal structure of the aspen stand affects the chemical defense system of aspen trees, which in turn, influences the amount of herbivory pressure faced by any given aspen ramet. This raises the notion that porcupines may act as selective agents on the genetic composition of the aspen stand.

Keywords: North American porcupine, trembling aspen, clone, phenolic glycoside, mammalian herbivory, selective herbivory, plant-animal interactions, defensive chemistry

2.1. Introduction

In order to protect themselves from the negative impacts of herbivory, plants have developed a suite of physical and chemical defenses (Fraenkel 1959, Freeland and Janzen 1974, Rosenthal and Berenbaum, 1991, Mauricio 2001). Chemical defenses (phenolics in particular) can also show induced responses to herbivory and therefore, variation in concentration of secondary compounds can change throughout the life of a plant, as a function of herbivory pressure (Haukioja 1991, Karban and Baldwin 1997, Wold and Marquis 1997, Kaitaniemi et al 1998, Boege 2004, Nykanen and Koricheva 2004).

Plant chemical defenses can be under genetic or environmental control (Snyder 1992). For traits under genetic control, a herbivore can act as a selective agent (Snyder 1992, Mauricio and Rausher 1997, O'Reilley-Wapstra et al. 2004), thereby changing the genetic structure of a stand. Although this idea has been investigated extensively with plant-insect systems (Lindroth et al. 1986, Mauricio and Rausher 1997, Fornoni 2004) there is an absence of research that links plant genotype, plant chemistry, and feeding preferences of mammalian herbivores (see Snyder 1992; Bailey et al. 2004; Laitinen et al. 2004; O'Reilley-Wapstra et al. 2004).

Here we examine this relationship via the North American porcupine (*Erethizon dorsatum*), an arboreal, folivorous mammal, and the trembling aspen (*Populus tremuloides),* one of its preferred food sources. This plant-herbivore system is an excellent study model for five reasons.

First, porcupines are generalist herbivores that demonstrate food selection at the species (Roze 1989), individual (Snyder and Linhart 1997), and plant part (Roze 1989) levels. This is important because an organism can only impose selective pressure if it consistently shows a preference for one (or several) trait(s) over others. Second, the asexual reproduction of trembling aspen makes it one of the few North American tree species that form naturally occurring multi-stemmed clones containing up to several thousands ramets (Kemperman 1977). This offers the opportunity to examine a natural stand that contains multiple individuals (ramets) of the same genetic stock, as well as multiple units (clones) of differing genetic composition (Barnes 1966; Wall 1971). Third, *P. tremuloides* has a strong chemical defense system whose secondary metabolites (mainly phenolic glycosides and condensed tannins) are well known for deterring aspen feeding insects (Lindroth 2000) and some mammals (Basey et al. 1990, Bailey et al. 2004, Bailey et al. unpublished manuscript). In addition, variability in phenolic glycosides is known to be potentially high across clones but low within clones (Hemming and Lindroth 1995, Lindroth and Hwang 1996, Hwang and Lindroth 1997, Osier and Lindroth 2001). This implies that variation in this defensive trait is at least partly under genetic control, a fundamental component of determining if an animal has the potential to act as a selective agent (Mauricio 2001). Fourth, porcupines are medium-sized mammals that can be temporarily kept in enclosures to perform food choice experiments. And finally, when porcupines climb trees to access leaves, they scratch the tree trunks with their claws, leaving

scars which offer a natural record of herbivory pressure on individual ramets. This gives a unique archive from which to collect information on porcupine food selection.

We used this study system to meet three objectives through a combination of field observations, feeding trial experiments, and laboratory analyses of plant chemistry and genetics. First, do porcupines exhibit intra-species food selection on trembling aspens? Second, is this intra-species selection explained by plant chemical variables? Third, is herbivory-relevant plant chemistry under genetic control?

2.2. Materials and Methods

2.2.1 Study Site

We worked from 6 June - 25 August, 2002 and from 5 May - 5 July, 2003, in Parc National du Bic (48°21.529'N, 68°45.60'W), Quebec, Canada, at the southern limit of the boreal forest. Details on the topography, vegetation, and climate of the study area, and on the natural history of the porcupine population, are available in Berteaux et al. (in press).

We performed field work in a 2.2 hectare patch of forest that was dominated by trembling aspens, of relatively uniform topography, and heavily used for porcupine feeding. We tagged all aspen stems with a minimum circumference of 20cm present in our study site (n = 577). We excluded smaller trees because they are never climbed by porcupines.

We measured the circumference of each tree, and determined its spatial position (± 1 m) using a Theodolith.

2.2.2. Identification of Clones

We characterized the clonal structure of the aspen stand in two steps. We first used the phenological and morphological characteristics of ramets to delineate clones and then performed electrophoretic identification on a sub-sample of trees to validate field procedures.

We delineated clones using field techniques described in Barnes (1969) and Kemperman (1977). In May 2003, we examined all trees daily, for bud break, flowering and leaf flush. We recorded the phenology of these events and determined the sex of flowers. We also examined tree bark for differences in color, texture, and susceptibility to frost cracks and disease, and characterized stem and branch form (straight, undulated or twisted), branching habit (upwards, downwards or horizontal), and stem fork (presence or absence; if present, height of lowest fork). Using these qualitative characteristics of aspen trees, two observers independently clumped ramets into clones according to the observed spatial variation in ramet characteristics. Eighty-one per cent (n = 467) of ramets were classified into the same set of 16 clones. We excluded from subsequent analyses the remaining 110 ramets that were not classified similarly by the two independent observers. Electrophoretic identification of 24 ramets (see appendix 1 for laboratory techniques) confirmed the field delineation.

2.2.3. Determination of Food Selection

We first evaluated climbing scars left by porcupines on aspen bark to quantify the intensity of use of individual aspen ramets by porcupines. We then used feeding trials on captive porcupines to verify if the variability in use of ramets by porcupines corresponded to differential food selection. Scratches observed on aspen bark were attributed to porcupine climbing based on two criteria: they are oriented diagonally on the tree trunk (due to the position of the forepaws when climbing) and they are clumped in groups (multiple scars are left simultaneously when several nails of a given paw dig into the bark). Only scars fitting these patterns were included in the analysis (see appendix 2, fig. 1 for a fresh climbing scar).

We measured the density of climbing scars on a given ramet with two 64 cm^2 quadrats located on areas of the trunk considered visually to contain the highest number of porcupine scars (appendix 2, Fig. 3). We selected this stratified sampling strategy (rather than a random approach) because, according to preliminary observations, it was the most efficient at capturing the among-ramet variability in scar density. We used the average number of scars per quadrat as an index of tree use because climbing scars remain on tree trunks for many years (D. Berteaux, personal communication). We performed a regression analysis to remove any effect caused by tree size. We considered residual values as an index of tree use independent of tree age (Appendix 3). Bark texture was inappropriate to register claw marks on 67 ramets, therefore use was quantified on 400 ramets (70% of ramets contained in the study site).

We captured five porcupines from 25 May - 10 June, 2003 (four adult males and one juvenile female) in the area surrounding the study site, and housed them separately in 1.5 m^3 cages to perform feeding trials. Each cage contained a rubber pipe, which provided shelter to the porcupine. Cages were placed in a forest stand close to the capture locations and porcupines were released at their site of capture immediately after finishing the experiment (on average, 19 days after their initial capture).

We conducted the experiment over a period of 12 consecutive nights from 16 June - 28 June, 2003. Each night porcupines were offered the choice between leaves coming from highly scarred aspens and leaves coming from aspens containing few scars. The experiment was replicated at the clonal (or genotype) level (n = 3) and at the ramet (or phenotype) level (n = 4). Therefore, 24 ramets (two “treatments” x three genotypes x four phenotypes) were used. Electrophoretic identification was performed on these 24 ramets (see appendix 1 for methods).

Each evening, we selected one bundle from two different ramets belonging to two different clones. We chose bundles according to the categorical index of climbing scars described in appendix 3. We considered trees in categories 1-3 as low scarred and those in categories 4-6 as highly scarred. We tied branches from individual ramets into equal size bundles containing a similar quantity of leaves. We placed bundles in cages so that they were equally accessible to porcupines. Once bundles were placed in cages, porcupines were continuously observed for one hour, after which the percentage of leaves eaten in each bundle was

estimated. Additional estimates were also performed one and a half, four, and 24 hours after bundles were placed in cages. In addition to the aspen leaves offered for the experiment, porcupines were fed daily with dandelions, apples, grass, clover, and aspen leaves from trees not used in this study, in order to assure that their nutritional requirements were met.

2.3. Plant Chemistry

In July 2003, we analyzed the chemical composition of leaves from 252 aspen ramets representing the diversity of genotypes and phenotypes present on our study site. We measured the content of nutrients (nitrogen and carbohydrates- starch and sugar) and secondary compounds (condensed tannins and phenolic glycosides- tremulacin and salicortin) in order to assess the quality of leaves to porcupines. Tremulacin and salicortin were *a priori* identified as potentially important secondary compounds because they are abundant in trembling aspens and known to deter aspen-feeding insects (Lindroth 2000). The leaf sampling methodology and laboratory procedures used for chemical analysis are detailed in appendix 4.

2.4. Statistical Analyses

2.4.1. Differential use of aspen ramets by porcupines

If aspen genotypes differ in their level of secondary compounds, the clonal structure of an aspen stand potentially imposes a strong spatial structure in terms of food quality to porcupines. We therefore tested the

null hypothesis that scarred ramets were distributed randomly with respect to unscarred ramets. We reasoned that rejecting this null hypothesis would demonstrate a spatial patterning to ramet use by porcupines, and would justify the investigation of the effect of clone on porcupine food selection.

We used a marked point pattern approach, an extension of Diggle's Randomization Procedure (Diggle 1983), to examine the distribution of scarred vs. non scarred trees. First, we organized data into categories of scarred vs. unscarred and then we generated a marked point pattern of all trees, using UTM coordinates. The Diggle's randomization procedure calculates the observed category to category distances and generates a cumulative frequency graph. It then simulates 99 independent permutations of the marked point process under the null hypothesis that the point pattern observed for aspen trees visited by porcupines is a completely random point process from the point pattern observed for aspen trees not visited by porcupines. The observed frequency distributions of the category to category distances are then compared to those from the permutations that define the lower and upper limits of a completely random distribution.

In a second step, we investigated the influence of clonal structure on tree use by porcupines by comparing the observed to expected proportion of scarred versus non-scarred trees in each clone, using a Chi square test.

2.4.2. Feeding Experiment

We calculated the mean value of the four estimates of percentage of leaves eaten by porcupines obtained at each feeding trial as an index of consumption for each porcupine/leaf bundle. We used a nested ANOVA to test for any significant effect of "treatment" (e.g. level of use in the field) on preferences between aspen bundles.

2.4.3. Biochemical Content and Preferences

After log transforming all data that did not fit a normal distribution, we used MANOVAs to examine the relationship between chemical content of aspen leaves and porcupine feeding preferences. We lumped salicortin and tremulacin (subsequently called "phenolic glycosides") together because they were strongly correlated ($R^2 = 0.724$). We compared the mean differences in the chemical variables between preferred and non-preferred trees using a MANOVA. We then examined these same differences in relation to porcupine scarring with a second MANOVA. In the latter, continuous scar values from the 252 trees were transformed into binary categorical data as scarred and unscarred.

In addition, we analyzed what percentage of the variation in scars could be explained via biochemical content using multiple regression with scar data and chemical variables.

We used ANOVAS to investigate the relationship between chemical variables and clone. First, we tested for an effect of clone on all chemical variables, using all 252 trees. We then repeated these analyses using only trees from the feeding experiment.

The interpretation of our results may be constrained by the clonal nature of the aspen stand under study. Replicate observations of clones, because they come from similar areas and from the same parental stock, are not strictly independent and must be considered as pseudoreplicates. This reduces our ability to attribute chemical differences between clones to genetic structure. Nevertheless, manipulative experimental research on aspen clonal chemistry consistently shows a strong genetic affect on chemistry, and thus supports our interpretation of the genetic effect of clone on chemistry. This problem of pseudo replication is an inherent feature of any "natural" experiment with clonal species.

2.5. Results

2.5.1. Distribution of Scarred Trees and Clonal Structure of Aspen Stand

Of the 510 ramets in our study site with readable bark, 299 (59%) showed signs of porcupine climbing. Amongst these, the number of scars ranges from 1-55 per quadrat (average: 8.70 ± 0.48 SE). The distribution of scarred trees was not spatially random with respect to unscarred trees, as shown by the fact that the cumulative frequency distribution of the observed marked point pattern was not contained by the lower and upper envelopes (Fig. 1b).

We identified 16 clones among the 400 ramets with readable bark that were recognizable at the clonal level. Of these, clone size varied from five to 81 ramets, with a mean of 25.1 ± 5.3 ramets per clone. Number of

scars per clone ranged from 0-79, while on average, 48.9% ± 7.2 of the ramets within each clone contained climbing scars. The observed frequencies of scarred trees in each clone were significantly different from the expected frequencies. We therefore conclude that some aspen clones were more intensively used by porcupines than others (X^2 = 138.03, *df* = 15, $P < 0.001$, Fig. 1c).

2.5.2. Feeding Experiment

In the feeding experiment, leaves from highly scarred trees were preferred over leaves from trees with few scars. In 10 of 12 trials, the porcupines consumed a greater percentage of leaves from highly scarred trees. The mean consumption of these trees was 38% ± 0.1 of total leaf matter, whereas that of trees with a low number of scars was 18.1% ± 0.1.

For four of the twelve trials (those between clones m and f) however, preference was either minimal or reversed (Fig. 2). Despite this, it is clear that selection existed and an ANOVA indicates a significant overall preference for scarred trees $F_{(1,96)} = 23.4$ ($P < 0.001$), which shows that porcupines exhibit significant preferences and that scar data are a good index of herbivory

2.5.3. Chemical Variation and Feeding Preference

The presence or absence of porcupine scars on aspen tree trunks was partly related to the levels of phenolic glycosides and condensed

tannins in the tree. A MANOVA showed no significant difference in the levels of nitrogen ($F_{1,250}$ = 0.32, P = 0.57), sugar ($F_{1,250}$ = 0.84, P = 0.36), and starch ($F_{1,250}$ = 1.44, P = 0.23) between scarred and unscarred trees. However, significant differences were found in condensed tannins ($F_{1,250}$ = 19.14, P < 0.001) and total phenolics ($F_{1,250}$ = 44.93, P < 0.001). Wilk's Lambda test shows an overall effect of scarred vs. non scarred trees in terms of overall variation of chemicals measured ($F_{5,246}$ = 10.70, P < 0.001) (Fig. 3a).

The number of scars found on a tree can be partially attributed to condensed tannins and phenolic glycosides. A multiple regression analysis, using only these two variables, shows a positive correlation between the number of scars and the levels of these two chemical compounds (R^2 = 0.19 and P < 0.001). This relationship can be represented by the equation: $y = 1.04x_1 + (-7.68x_2) + 1.0385$ (with x_1= tannins and x_2 = phenolics), which shows that it is the concentration of phenolics that have a greater effect on porcupine food selection.

The data from the feeding experiment does not show a relationship between preference and biochemistry. The results of a MANOVA, comparing chemistry between preferred and non-preferred trees, show no significant differences in any of the chemical variables measured (condensed tannins: $F_{3,20}$ = 1.16, P = 0.35; total phenolics: $F_{3,20}$ = 1.68, P = 0.20). Wilk's Lambda test shows no overall effect of preference ($F_{5,16}$ = 1.51, P = 0.24) (Fig. 3b).

Although the significant effect of phenolic glycosides on preference between scarred and unscarred trees is not seen with the feeding experiment data, the trend that preferred trees are low in phenolics, relative to non-preferred trees, is consistent with the pattern seen with scar data (Figure 3b). In addition, variation in phenolic glycosides across clones is greater in the sample of 510 ramets (3.92 times) than in the sub-sample of 24 ramets used in the feeding experiment (2.08 times), suggesting that perhaps a large variation in phenolic glycosides is required for porcupines to be able to make food choices.

2.5.4. Clones and Chemistry

There was a significant relationship between clonal structure and chemistry. Examination of all 252 trees from the sub-sample shows significant differences in chemical variables between clones. The largest variations were found in condensed tannins and total phenolics. The means of these two chemical variables varied across clones 2.70 and 3.92 times respectively. By comparison, the other compounds differed only from one to two-fold between clones (nitrogen 1.26, sugar 1.60 and starch 1.94). ANOVA results and variation in chemical variables across clones are contained in appendix 5, table 1.

The same test, using only the trees from the feeding experiment showed similar results. However, the variation in chemicals between clones is slightly different. Here, levels of starch vary by the most between clones (2.2 times), and the variation in phenolics is second highest (2.09

times). Condensed tannins and sugar vary almost to the same degree, at 1.55 and 1.53 times respectively, while the variation in nitrogen is only 1.29 times (Appendix 5, table 1).

2.6. Discussion

This work clearly demonstrates that porcupines exhibit intra-species food selection. We demonstrated that porcupines select certain aspens over others through the spatial distribution of porcupine climbing scars and the distribution of scarred trees among clones. The feeding experiment confirmed that porcupines feed selectively on certain aspen ramets and also showed that porcupine climbing scars can be used as an index of herbivory pressure in future studies. This unequal consumption of aspens offers an indication that summer feeding by porcupines may have the ability to impose changes on the aspen stand from which they feed.

In order for porcupines to act as selective agents they must be selecting for a genetically-determined trait. Our results from scar data demonstrate that porcupines select leaves with lower levels of phenolic glycosides and higher levels of condensed tannins (Fig. 3a). Phenolic glycosides are more likely than tannins to be the driving force behind porcupine selection. Although high amounts of condensed tannins have been shown to deter several species of mammals including black-tailed tree rats (*Thallomys nigricauda)*, brushtail possums (*Trichosorus vulpecula*), and beavers (*Castor canadensis*) (Downs et. al. 2003, Marsh et. al. 2003, Bailey et al. 2004), other research has indicated positive

associations between aspen-feeding insects and foliar concentration of condensed tannins. These studies have suggested that this positive association may be due to the covariance between phenolic glycosides and condensed tannins, rather than a preference for high levels of condensed tannins (Hemming and Lindroth 1995). In addition, other studies with trembling aspens show an increase in condensed tannin concentrations after defoliation (Peters and Constabel 2002, Osier and Lindroth 2001, 2004), suggesting that the positive correlation between porcupine herbivory and condensed tannins may be a result, rather than a cause, of herbivory. These ideas bring into question the role of condensed tannins in porcupine food selection and, coupled with our multiple regression data (which show a stronger relationship between phenolic glycosides and herbivory than between condensed tannins and herbivory), allow us to isolate phenolic glycosides as being the main deterrent to porcupine herbivory, amongst the chemical variables that were measured.

This result is not surprising when compared with other studies. Tremulacin and salicortin in aspens have been shown to reduce the performance of gypsy moth (*Lymantria dispar* L.) and forest tent caterpillar (*Malacosoma disstria* Hbn) larvae (Hemming and Lindroth 1985, Osier and Lindroth 2004). Research examining the relationship between mammalian herbivory and phenolics is uncommon, however it has been shown that low concentrations of tremulacin were associated with a 7.5-fold increase in the probability of aspens being browsed by elk (Bailey et. al unpublished manuscript). Studies with snowshoe hares (*Lepus americanus*), mountain

hares (*L. timidus*), and European hares (*L. europaeus*), also show a negative correlation between phenolics and voluntary food intake (Sinclair and Smith 1984, Iason and Palo 1991). The common ringtail possum (*Pseudocheirus peregrinus*), the koala (*Phascolarctos cinereus*), and the silver-gray brushtail possum (*Trichosurus vulpecula*), all arboreal folivores also avoid foliage with high phenolic content (Edwards 1978, Lawler et al. 1998, Pass and Foley 2000, O'Reilley-Wapstra et al.2004).

Furthermore, phenolics are known to show induced responses to insect herbivores (Haukioja 1991, Karban and Baldwin 1997, Wold and Marquis 1997, Kaitaniemi et al 1998, Boege 2004, Nykanen and Koricheva 2004), and it is therefore possible that the variation observed in these chemicals is a result of herbivory pressure by aspen-feeding insects. Nonetheless, because porcupines tend to avoid trees with lower levels of phenolics, the induced response of increasing phenolic concentrations would only serve to attract porcupine herbivory, rather than deter it.

For porcupine herbivory to shape the genetic composition of an aspen stand, two other issues must be resolved. First, phenolic glycosides must be under some degree of genetic control and second, this trait must be correlated with herbivore damage and plant fitness (O'Reilly-Wapstra et. al. 2004).

If the variation in foliar concentration of phenolic glycosides in trembling aspens is determined, at least in part, by the genetic make-up of the ramet, we would expect to find a larger variation in these concentrations across clones, and little or no variation within clones. Using

the 24 ramets in the feeding experiment, our results are consistent with this hypothesis, with a 2.1-fold variation in phenolic glycosides across clones and only a 1.9-fold (coefficient of variation = 0.23) mean variation within clones. However, the same analysis with all 252 ramets yielded different results. Here the across clone variation is 3.9-fold whereas the mean within clone variation is 4.8-fold. It is important to note, however, that of the 16 clones in this study, only four clones showed a variation higher than this mean, whereas the other twelve showed variation well below this mean. In addition, the c.v. is relatively low at 0.38. It is possible then that the high variation found in certain clones is responsible for this high mean value and that this 4.8-fold difference is not a good reflection of within clone variation of phenolic glycosides.

Other studies show that levels of phenolic glycosides are said to differ greatly among genotypes, but are much less responsive to resource availability (Erwin et. al. 2001). Lindroth and Hwang (1996) reported marked variation in foliar concentrations of tremulacin (5.9- fold) and salicortin (10.3- fold) across 31 clones in Michigan, U.S.A. The authors also noted a significantly reduced variation of these chemical variables within clones (*c.v.* of 0.31 for tremulacin and 0.27 for salicortin). Furthermore, a study designed to evaluate the effects of plant genotype, nutrient availability, and defoliation on the foliar chemistry of *P. tremuloides,* reported that aspen genotype accounted for 93% of the variation in phenolic glycoside concentrations (Osier and Lindroth 2001). And finally, Bailey et al. (unpublished manuscript) found that through

asexual reproduction (the predominant method of reproduction in aspen), 100% of the variation in tremulacin is genetically based.

We have shown that the chemical composition of trembling aspens influences the amount of porcupine herbivory experienced by any given ramet. More specifically, we have shown that the foliar variation of tremulacin and salicortin plays the most important role in porcupine-aspen food selection. We have also demonstrated that these two secondary metabolites are under a high degree of genetic control. If porcupines select for trees with low levels of phenolic glycosides, which are under genetic control, are porcupines acting as selective agents?

To answer this question, we need to show that the plant defensive trait is correlated with herbivore damage and plant fitness (O'Reilley-Wapstra et al. 2004). Therefore we need to show that porcupine herbivory has a negative impact on the growth and fitness of aspens. The asexual reproduction and resultant clonal unit of *P. tremuloides* makes it difficult to measure the effects of herbivory on the fitness of an individual. Studies that have done so have either used long-term exclosures (Baker et al. 1997, Bailey et al. unpublished manuscript) or grown clones in a garden environment (Osier and Lindroth 2004). Jelinski and Cheliak (1992) suggest that the growth pattern of clonal units buffers negative impacts, spreads the risk of death, and retards selection, thereby making it more difficult to quantify the long-term effects of selective herbivory on the fitness of the aspen genotype. However, visual observation of highly selected trees shows decreased foliage and a largely reduced canopy

(Appendix 4, Fig. 2). This is important since artificial defoliation of aspen trees has been shown to suppress plant growth (Osier and Lindroth 2004).

Finding a direct link between single-species herbivory and plant fitness is confounded by the interacting effects of multiple herbivores and other variables in the ecosystem. For example, there are a multitude of aspen-feeding insects in Parc du Bic and the effects of these populations need to be isolated to obtain an accurate measure of the impacts of porcupine herbivory on trembling aspens

Mammalian food choices are often based on a host of interacting variables and although we have isolated phenolic glycosides as being the main deterrent to porcupine herbivory, it is possible that other factors come into play in this choice. For example, herbivores often have multiple strategies related to selective herbivory and it is likely that they select to maximize energy intake when plants are not well defended whereas they may select to minimize toxic compounds when plant defense systems are strong (Basey et al. 1988, 1990, Bailey et al 2004, and unpublished manuscript).

All these confounding variables make it difficult to assess the potential of porcupines as selective agents in the forest ecosystem. However, if this is the case, herbivory pressure imposed by summer porcupine feeding is likely to induce selection at the genome level within the species, thereby effecting the genetic composition of the trembling aspen stand, rather than the biodiversity of the forest ecosystem. A recent paper concludes that the concentration of tremulacin influenced the

foraging behavior of elk (*Cervus canadensis*), and played a large role in the genetic and chemical future of aspen forests in northern Arizona (Bailey et al. unpublished manuscript).

Although our work can not accurately determine the role of porcupines as selective agents in aspen stands, we have been able to link plant defensive chemistry, plant genotype, and porcupine herbivory by showing that food choices are determined by plant chemistry, that plant chemistry is determined by genetics and that porcupine herbivory may have the potential to negatively impact the growth and fitness of the aspen stand. This work therefore represents an important finding in the continued discussion of the role of mammalian herbivory in ecosystem dynamics and the authors stress that future studies quantifying the impacts of porcupine herbivory on trembling aspens be undertaken in order to complete the link between aspen chemistry and genetics, and porcupine herbivory.

2.7. Figure Legends:

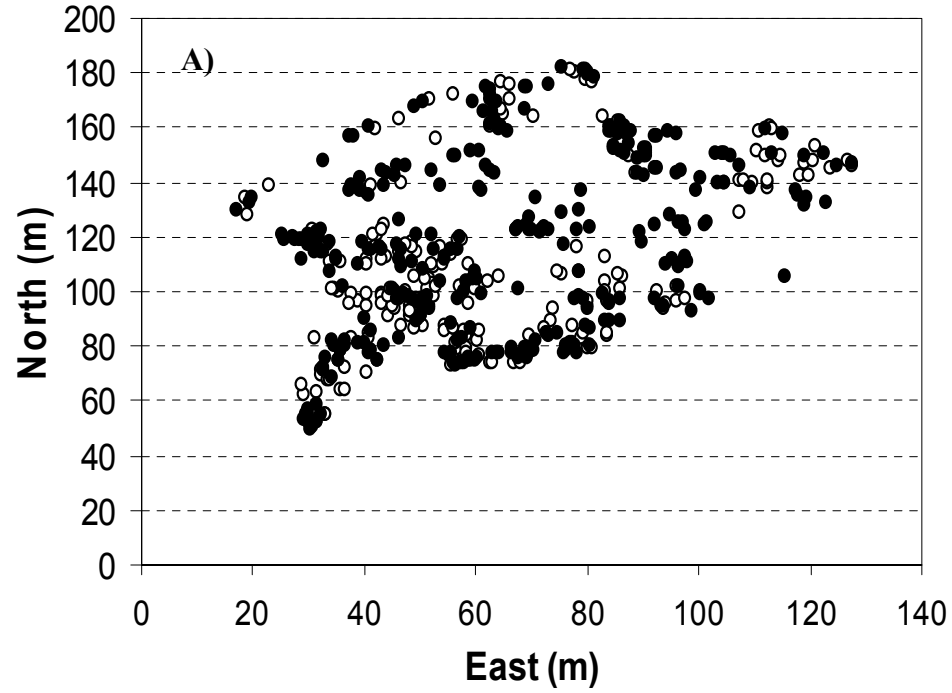
Fig. 1: Spatial distribution of scarred vs. unscarred ramets and relation of scarring to clonal structure in a 2.2 ha trembling aspen stand studied at Bic, Quebec, Canada. A) Point pattern of the 510 trembling aspen ramets whose bark was smooth enough to recognize porcupine climbing scars. Closed circles represent the 299 scarred trees while while open circles represent the 211 trees that contain no visual signs of porcupine feeding. B) Diggle's randomization procedure for all 510 trees, showing the observed number of scarred trees (black triangles) at various distances from unscarred trees. The lower (black circles) and upper (white circles) envelopes represent the expected minimum and maximum numbers of scarred trees at various distances from unscarred trees, as defined by the 99 independent realizations of this procedure. C) Observed (black bars) and expected (white bars) number of scarred ramets in the 16 trembling aspen clones. Observed and expected numbers were derived from the 401 ramets with readable bark that we identified to the clonal level using field techniques.

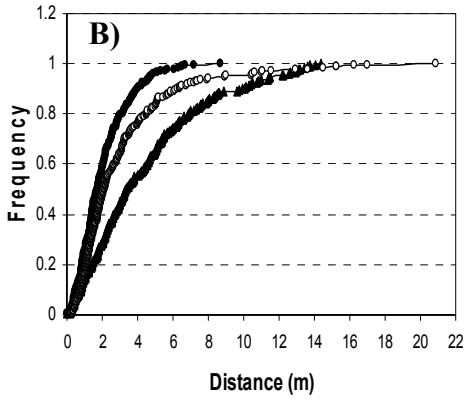
Fig. 2: Average (± SE) consumption of highly scarred (black bars) and low scarred (white bars) trembling aspens by five captive porcupines during the 12 night feeding experiment in June 2003. All 24 ramets originated from our 2.2 ha study site in Parc du Bic, Quebec, Canada and reflect both extremes of tree use by porcupines, as determined during scar data analysis. Numbers 1-12 represent the average consumption of leaf matter

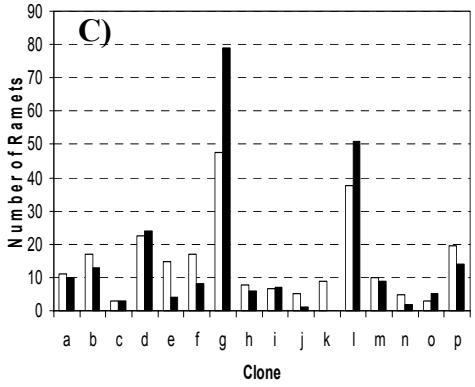
in each feeding trial, categorized by clone, whereas the last two bars represent the overall average consumption. The twelve nights were broken down into three periods of four trials. We used multiple ramets of two clones in each period in order to ensure repeatability of measurements, and also used different clones across each four trial period in order to ensure proper comparison amongst different genetic groups. Error bars show the standard error associated with each mean.

Fig. 3: Average (± SE) content of five chemical variables (Nitrogen, N; Condensed Tannins, CT; Total Phenolics, TP; Sugar, Su; and Starch, St) measured in trembling aspen leaves from our 2.2 ha study site in Parc du Bic, Quebec, Canada. A) Chemical content of 111 aspen ramets bearing porcupine scars (black bars) vs. 141 ramets with no scars (white bars). These 252 trees form a sub-sample from all 16 clones and all degrees of porcupine scarring. Stars represent significant differences ($P < 0.01$). B) Comparison of chemical content in 12 preferred (black bars) vs. 12 non-preferred aspen ramets (white bars) as defined by the results of a feeding experiment in June 2003.

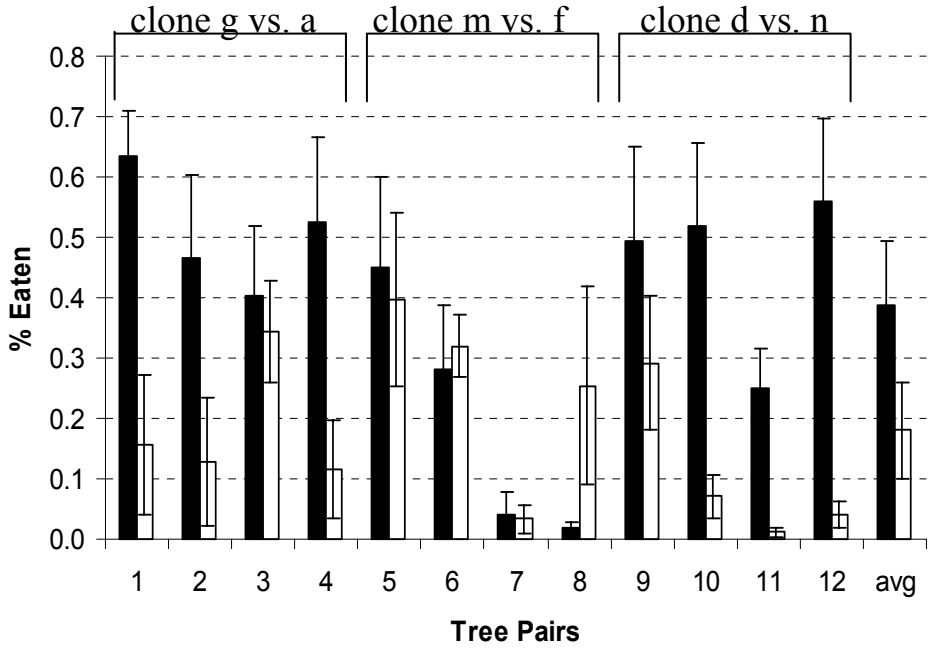
2.8. Figures



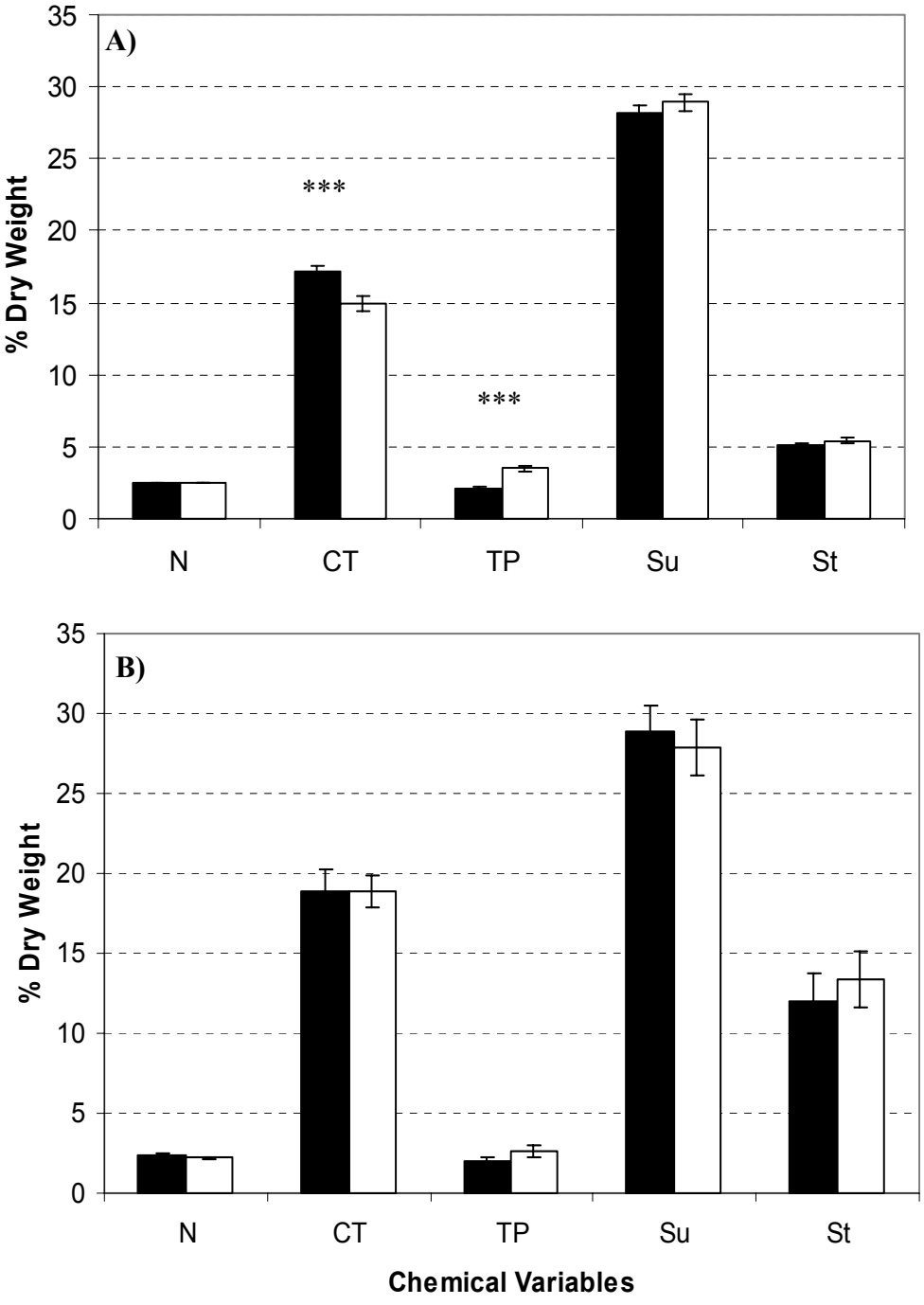




Diner et al. Fig. 1



Diner et al. Fig. 2



Diner et al. Fig. 3

2.9. APPENDIX 1: Genetic analyses

Plant Collections & DNA Extraction

We harvested and stored leaves in the same way as reported in Appendix 4. We performed genetic analyses on 50mg of ground leaf material from each ramet. Homogenization took place in 2 mL microcentrifuge tubes, with the tissue placed between a ceramic sphere and a ceramic cylinder (Bio 101 Systems), and homogenized in a FastPrep FP 120 (Bio 101/Savant) at a speed setting of 4.0 for 90 seconds. Homogenization took place in 500 µL of buffer AP1 from the Qiagen DNeasy 96-well kit, heated to 65 °C. The remainder of the extraction followed the manufacturer's protocol. DNA was quantified using a DynaQuant 2 fluorometer (Hoefer), and diluted to12.5 ng/µL for amplifications. Average yield from the extractions was 100 µL at 130 ng/µL.

PCR Amplifications

PCR amplifications took place in 10 µL reactions containing 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1 % Triton X-100, 2.5 mM $MgCl_2$, 200 µM dNTPs, 100 ng of each primer, and 0.5 units of *Taq* polymerase (Promega, in buffer A). We used a "touchdown" PCR protocol, in which reactions were heated to 92 °C for 5 min; cycled for 9 cycles at 92 °C for 15 sec, 59 °C for 15 sec (dropping by 1 °C each cycle to 50 °C), 72 °C for 30 sec; and

then 20 cycles of 92 °C for 15 sec, 50 °C for 15 sec, 72 °C for 30 sec; with a final extension at 72 °C for 3 min.

We chose four primers (P-575, O-206, W-14, and W-20) to represent independent linkage groups and different repeat unit sizes, from 2 bp to 6 bp, and were labeled with 5Hex or 6-Fam (Table A3.1). These were adequate to identify several of the genets as being unique, and all of the samples for a genet as having the same genotype. Some of the samples could not be distinguished using these four loci, so they were amplified with an additional six loci (O-127, O-149, W-15, O-59, O-26, and O-21).

Electrophoresis & Analysis

Reaction products were diluted in water or TE (at a ratio of 1/10 to 1/80 depending on which primer was used); from these dilutions, 1 µL of a Fam-labeled product and 1 µL of a Hex-labeled product were mixed with 8 µL of formamide and 0.1 µL of Rox-labeled 400HD standard (Applied Biosystems). These standards, included with each sample analyzed, provide a set of 21 ssDNA markers well sized for determining sizes of the PCR products analyzed. Samples were then heated to 95 °C for 5 min and chilled on ice for 2 min. During the initial screening tests for each primer, products were analyzed with an ABI 3100 capillary electrophoresis instrument, while an ABI 3700 was used for all of the population screening tests. Output from the ABI 3700 was analyzed using either Genographer 1.6 (Benham, 2001) or GeneScan 3.7 (Applied Biosystems) software.

Resolution was considerably better than one base, and genotypes were scored by eye.

Table A3.1: Primers used in PCR amplification analysis for the 24 aspen ramets used in the porcupine feeding experiment at Bic, Quebec, Canada, in June 2004. LG = Arabic numeral for linkage group. Motif = repeat unit and number of repeats (if listed) in original study. Left primers were labeled with Hex (bold) or Fam (non-bold). Right primers were unlabeled

Name	*LG*	*Motif*	*Left Primer*	*Right Primer*
ORPM_021	**9**	**[AG]4***	**GGCTGCAGCACCAGAATAAT**	**TGCATCCAAAATTTTCCTCTTT**
ORPM_026	6	[CA]8	GCTGCAGTCAAATTCCAAAA	CGAGCGTCTTCTTCATGGAT
ORPM_059	**14**	**[AT]6**	**TGCTAGTAACTGCGCATTGG**	**GATGTTTTTCGCACGCATTA**
ORPM_127	4	[TG]8	TCAATGAGGGGTGCCATAAT	CTTTCCACTTTTGGCCCTTT
ORPM_149		**[AT]4[CT]4**	**GTCTCTGCCACATGATCCAA**	**CCCGAAATGGATCAAACAAG**
ORPM_206	**19**	**[GCT]7**	**CCGTGGCCATTGACTCTTTA**	**GAACCCATTTGGTGCAAGAT**
PMGC_0575	1	GA	TAAATTCATGTAGATTGACG	CTTACTATTTCATGGTTGTC
WPMS_14		**CGT**	**CAGCCGCAGCCACTGAGAAATC**	**GCCTGCTGAGAAGACTGCCTTGAC**
WPMS_15	5	CCT	CAACAAACCATCAATGAAGAAGAC	AGAGGGTGTTGGGGGTGACTA
WPMS_20		TTCTGG	GTGCGCACATCTATGACTATCG	ATCTTGTAATTCTCCGGGCATCT

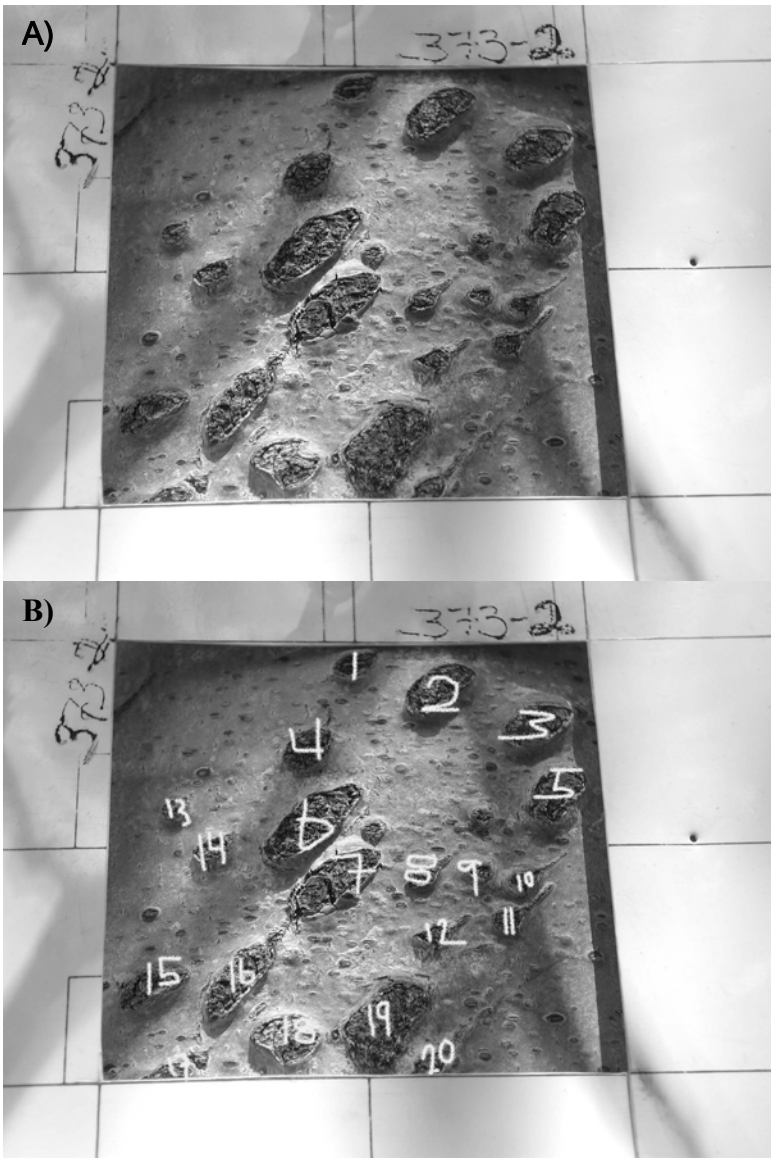
2.10. APPENDIX 2: Porcupine Herbivory on Trembling Aspens

Figure Legends:

Fig.1: Close-up photograph of a fresh porcupine climbing scar on the trunk of a trembling aspen ramet in Parc National du Bic, Quebec, Canada. Scars are oriented diagonally (due to the position of the forepaws when climbing), and are clumped in groups (multiple scars are left simultaneously when several nails of a given paw dig into the bark).

Fig. 2: Photographs demonstrating the field technique used to estimate porcupine herbivory on trembling aspens. A) Photograph of the isolated 64 cm^2 quadrat bearing the highest density of porcupine climbing scars on the aspen ramet. B) The same photograph with each scar attributed to porcupine climbing numbered from 1- 20.

Diner et al. Fig. A1.1

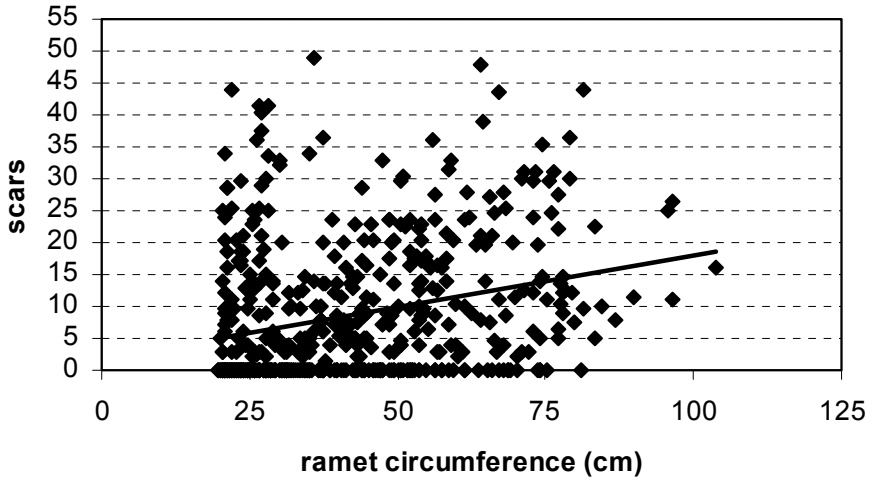


Diner et al. Fig. A1.2

2.11. APPENDIX 3: Categorization of Differential Herbivory

We performed a regression analysis to remove any effect caused by tree size (fig A1). We then used residual values (ranging from -14.99 to +41.28) as the scar index and categorized trees from 1-6 according to the quantity of climbing scars present. We included trees with residual climbing scar values of -14.99 to -5.616 in category 1 (least amount of scars) and divided each subsequent category into increments of 9.37. Therefore, the residual values for trees with the highest number of climbing scars (category 6) fell between +31.90 and +41.28. Sixty-seven trees were excluded from further analysis due to unreadable bark.

Figure A3.1: Positive relationship between number of porcupine climbing scars and circumference of 510 aspen ramets studied at Parc National du Bic, Quebec, Canada. The regression analysis was performed with all tagged aspen ramets whose bark was smooth enough to recognize porcupine climbing scars. $R^2 = 0.0724$.



2.12. APPENDIX 4: Biochemical Analyses

Leaf Harvesting

In July 2003, we selected a sub-sample of 252 trees for biochemical analysis. The selection represented members from all sixteen clones as well as all levels of herbivory, as classified via scar data. We included all ramets from clones containing less than 20 individuals, and randomly selected 50% of the stems from clones containing more than 20 members.

We sampled all leaves during the same period (June 2003) to avoid potentially compounding effects of phenological changes in leaf chemistry. For each sampled ramet, 75 healthy leaves from five different areas of the canopy. We snipped each leaf at the petiole, put them into pre-labeled envelopes, and placed them on dry ice in a cooler. We stored the envelopes at -20° C at the end of each day's sampling, and when all ramets had been sampled, we shipped leaves to the Department of Entomology at the University of Wisconsin, where they were freeze-dried, ground (No. 40 mesh) (Lindroth and Kinney 1998), and stored at -20° C.

Laboratory Analyses

We performed chemical analyses from 5 November - 10 December, 2003. We measured phenolic glycosides using high performance thin-layer chromatography (HPTLC). We extracted leaf samples (25mg of tissue) in methanol and sonicated them for 15 minutes. We then put them through a centrifuge (to remove plant material), and developed duplicate aliquots (2 µl) on HPTLC plates (silica gel 60, 10 X 20cm). We scanned

the plates at 274nm using a Camag Scanner II (Camag Scientific Inc., Wrightsville Beach, North Carolina), and analyzed the chromatograms using Camag TLC software (CATS 3.11). For standards, we used salicortin and tremulacin purified by sequential flash chromatography and thin-layer chromatography, according to Lindroth et al. (1987).

We measured nitrogen levels from 50mg of tissue, with a LECO FP528 nitrogen analyzer, using glycine as the reference standard.

We determined starch and sugar (sucrose + hexose) concentrations using enzymatic hydrolysis and the dinitrosalicylic acid method as in Lindroth et al. (2002). All phytochemical variables are reported in the text and figures as percent dry weight. We divided carbohydrates into total starch vs. total sugars. Starch was first separated from soluble sugars and then enzymatically hydrolyzed to glucose using amyloglucosidase. We quantified glucose concentrations using a modification of the dinitrosalicylic acid method (Lindroth et al 1987 *in* Kopper and Lindroth 2003).

We measured condensed tannin concentrations using a modification of the butanol-HCl method of Porter et al (1986), using purified aspen condensed tannins as the reference standard. We performed the extraction on 25 mg of dried plant material with acetone and absorbic acid. We sonicated the samples for 30 minutes and centrifuged them for 10 minutes. We isolated the supernatant into a 2.0 ml microcentrifuge tube and then performed the hydrolyses with a 2% iron reagent (w/v) solution of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2 M HCl. We added the

reagent (0.2ml), along with 1ml of MeOH solution containing the proanthocyanidin, and 6ml of a solution of n-BuOH-conc. HCl (95:5 v/v) to each tube. We then thoroughly mixed the solutions and suspended them in a constant-level water bath run at 95°C and heated for 40 minutes. The solutions were cooled and the visible spectrum recorded between λ = 520 and 580 nm in a 10mm path-length glass cell.

2.13. APPENDIX 5: Chemical Content and Clones

Table A5.1: Comparison of chemical variables across 16 aspen clones studied in the context of herbivory by North American porcupines. The number of trees per clone for which we have chemical data is given as N. The chemical content per variable is expressed as the mean value per clone ± SE. Mean chemical content of the 24 trees used in the feeding experiment are represented in bold. The final five rows give F and P values for ANOVAs comparing clones (bold values are for clones used in the feeding experiment).

Clone	N	Nitrogen	Condensed Tannins	Phenolic Glycosides	Sugar	Starch
A	17	2.34 ± 0.03	20.96 ± 0.71	1.84 ± 0.07	28.88 ± 1.31	5.68 ± 0.38
A	*4*	*2.31 ± 0.07*	*20.17 ± 1.36*	*1.83 ± 0.15*	*22.70 ± 0.84*	*4.20 ± 0.73*
B	15	2.31 ± 0.05	21.95 ± 0.79	1.71 ± 0.09	33.90 ± 1.68	5.83 ± 0.33
C	5	2.67 ± 0.04	12.48 ± 1.50	3.43 ± 0.33	29.57 ± 2.82	5.74 ± 0.29
D	20	2.64 ± 0.04	15.03 ± 0.64	2.23 ± 0.17	24.51 ± 0.97	4.63 ± 0.26
D	*4*	*2.45 ± 0.04*	*15.62 ± 0.53*	*1.90 ± 0.26*	*23.91 ± 1.67*	*3.70 ± 0.90*
E	12	2.61 ± 0.04	8.59 ± 0.88	6.12 ± 0.76	24.75 ± 1.71	3.89 ± 0.52
F	14	2.25 ± 0.06	23.12 ± 0.93	2.08 ± 0.30	33.88 ± 1.07	6.94 ± 0.48
F	*4*	*2.21 ± 0.11*	*24.34 ± 1.69*	*1.85 ± 0.22*	*34.68 ± 0.51*	*8.16 ± 0.72*
G	40	2.62 ± 0.05	18.79 ± 0.61	1.56 ± 0.11	26.78 ± 1.05	5.25 ± 0.24
G	*4*	*2.74 ± 0.12*	*18.77 ± 1.87*	*1.84 ± 0.22*	*30.51 ± 2.83*	*6.51 ± 0.76*
H	13	2.65 ± 0.05	16.65 ± 0.73	1.99 ± 0.16	21.32 ± 1.24	5.18 ± 0.36
I	11	2.54 ± 0.03	13.66 ± 0.78	2.58 ± 0.25	25.10 ± 1.24	6.90 ± 0.65
J	9	2.40 ± 0.06	11.43 ± 0.63	4.36 ± 0.27	29.07 ± 1.70	4.37 ± 0.60
K	15	2.63 ± 0.03	16.02 ± 0.59	2.02 ± 0.11	29.17 ± 1.59	4.66 ± 0.35
L	32	2.35 ± 0.04	14.99 ± 0.65	2.85 ± 0.46	31.02 ± 0.84	4.36 ± 0.35
M	17	2.19 ± 0.04	13.79 ± 1.20	5.85 ± 0.61	30.41 ± 0.88	3.91 ± 0.37
M	*4*	*2.12 ± 0.03*	*18.70 ± 1.00*	*3.84 ± 0.74*	*30.81 ± 2.41*	*5.35 ± 1.17*
N	10	2.15 ± 0.05	14.70 ± 0.95	2.53 ± 0.19	27.65 ± 1.81	7.57 ± 0.72
N	*4*	*2.15 ± 0.12*	*15.77 ± 1.82*	*2.71 ± 0.22*	*27.68 ± 3.61*	*7.36 ± 1.05*
O	5	2.62 ± 0.12	12.98 ± 0.89	3.27 ± 0.57	34.01 ± 0.90	4.31 ± 0.98
P	17	2.39 ± 0.05	12.97 ± 0.77	3.24± 0.62	29.14 ± 1.08	6.24 ± 0.43
ANOVA						
$F_{15,236}$		12.10	18.03	10.95	6.42	5.76
P		<0.001	<0.001	<0.001	<0.001	<0.001
$F_{5,18}$		**6.27**	**4.25**	**4.40**	**3.39**	**3.67**
P		**0.002**	**0.010**	**0.009**	**0.025**	**0.018**

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FROM CAUSES TO CONSEQUENCES

The work presented in the second chapter of this thesis is part of an ongoing study using fluctuating asymmetry (FA) as a tool to investigate the impact of porcupine herbivory on several tree species found in Parc du Bic. This chapter describes the FA study on trembling aspens and discusses the validity of using FA as a tool to monitor herbivory based stress. All studies will then be submitted for publication as one manuscript.

CHAPTER 2: FROM CAUSES TO CONSEQUENCES: FLUCTUATING ASYMMETRY AND PORCUPINE HERBIVORY

3.1. Introduction

Many research tools are used to explore the consequences of mammalian herbivory on plant life history, including cafeteria trials, chemical analyses of plants, or field manipulation of herbivory pressure. Here we test the general hypothesis that plant fluctuating asymmetry (FA) can be a productive tool to study the relations between plants and mammalian herbivores. FA refers to random differences in the right and left sides of traits or organisms that are meant to be symmetrical, and can offer a measure of developmental precision (see Merila and Bjorklund 1995 and Palmer 1996 for extensive reviews of FA definitions and measurements). FA could be useful to study plant-mammal relationships if: 1- plant FA was a significant predictor of food choice by mammalian herbivores (an integrative measure of plant quality), or 2- plant FA indicated the level of stress imposed on plants by herbivores (a warning signal regarding the impact of herbivores on ecosystems).

Several lines of evidence already suggest that plant FA could predict food choice by invertebrate herbivores (Møller 1995, Lempa et al. 2000) and might reflect the level of stress suffered by plants subjected to invertebrate herbivory (Zvereva et al. 1997a, b, Martel et al. 1999). The only study to investigate the relation between plant FA and mammalian herbivory has shown that reindeer grazing increases FA of the wooly

willow (*Salix lanata*) (Olofsson and Strengbom 2000). The first step in testing the hypothesis that FA could be useful to study plant-mammal relations is to look for correlations between plant FA and mammalian herbivory. If plant FA consistently differs according to herbivory pressure, FA could either be a predictor of herbivore plant choice, or could indicate the severity of the stress suffered by plants, depending on the timing of FA measures relative to the herbivory event.

We examined this relationship using porcupines (*Erethizon dorsatum*) and one of their preferred food sources, the trembling aspen (*Populus tremuloides*). Here, we detail the methods and results of the aspen FA analysis, followed by a discussion on the use of FA as a tool. Porcupine-aspen interactions, and the advantages of this study system, are described in detail in chapter one.

3.2. Methods

3.2.1. Study Design

We randomly chose 25 pairs of trees from the 2.2 ha study site described in chapter one. Each pair of trees consisted of a test (many scars) and a control (no scars). Trees of a given pair were of similar size, located in a similar habitat (light availability, slope and soil composition) and were $\leq$ 20 m apart. This ensured that porcupine herbivory was the main variable differing between test and control trees. All subsequent methods were performed in 2002 and 2003 in order to test the repeatability of FA analyses.

We collected 15 leaves from all parts of each tree, using a leaf cutter attached to a collapsible 13 m pole. We harvested leaves in July 2002 and June 2003. When necessary, we used a ladder to access the top of the tree canopy. We dried the leaves between two planks of wood and several layers of newspaper, changing them daily for five days, to guarantee even drying. Once dry, each leaf was numbered and the identity of the source tree was hidden to avoid bias when measuring the specimens.

3.2.2. Leaf Measurements

We measured the distance from the midvein to the outer rim of the leaf on both the left (L) and the right (R) sides. We placed the leaf on graph paper, determined the widest point on each side, and took measurements to the nearest 0.01mm, using a digital caliper.

To quantify measurement error we performed each measurement twice, several days apart. The same researcher took all measurements. We performed all calculations using the average value of these two measures.

3.2.3. Determination of FA

To determine that the size variation in the left and right sides of each leaf represented FA, rather than directional asymmetry or antisymmetry (both of which reflect genetic rather than environmental

determinism), we examined the frequency distribution of R-L for all 1500 leaves measured in 2001 and 2002. FA is determined by a normal distribution around a parametric mean of zero (Palmer and Strobeck 1992).

3.2.4. Statistical Analysis

We performed a regression analysis to determine if FA was affected by leaf size. We plotted the values of R-L against total leaf size R+L, as suggested by Palmer (1994). FA was found to be independent of leaf size ($R^2 = 0.002$) therefore no correction was needed (Fig. 1).

We used a two-way, mixed-model ANOVA with repeated measurements of each side to measure the FA of each tree (Palmer and Strobeck 1992). Sides were the fixed factor and individual leaves were the random factor on each tree. FA of each tree was determined as: $FA = (MS_{sj} - MS_m)/M$, (where MS_{sj} = mean square interactions (side x genotype), MS_m = mean square measurement error, M = number of repeated measurements per side, S = number of sides per individual). We used a one tailed paired t-test with an α value of 0.05 to determine if the leaves belonging to test trees had a higher fluctuating asymmetry than those belonging to control trees. In both years, the measurement error was less than 0.1%.

We performed a regression analysis using porcupine climbing scars as an index of herbivory pressure to determine if FA could be a predictor of porcupine food selection.

A second regression analysis was used to examine the relationship between the FA results from both years, in order to test the repeatability of FA measures.

3.3 Results

3.3.1. Demonstration of FA

We confirmed the presence of FA in aspen leaves. The mean values of L-R were 0.08 ± 0.04 and 0.08 ± 0.05 for 2002 and 2003 respectively. The frequency distribution of L-R for all aspen trees was normally distributed around a parametric mean of zero (Kolmogorov-Smirnov $P < 0.01$) in both years, signifying that the variation in size between the left and right sides represent fluctuating asymmetry rather than directional asymmetry or antisymmetry.

3.3.2 FA and Herbivore Pressure

In both years, degree of FA was independent of herbivory pressure. In 2002, the mean values of FA for scarred trees was less than that of unscarred trees, however this difference is not statistically significant. In 2003 the difference in FA of scarred and unscarred trees was even less pronounced (Table 1). Furthermore, a regression analysis showed no

correlation between porcupine climbing scars and leaf FA of aspen trees ($P < 0.79$, $R^2 = 0.0007$) (Fig. 2).

3.3.3. Repeatability of Measurements

In comparing results from 2002 and 3003 a paired t-test showed no significant difference in the variation of FA from year to year ($P = 0.635$) however, a regression analysis shows no significant relationship between leaf FA from one year to the next ($P = 0.6078$, $R^2 = 0.006$). This signifies that FA values in one year are not very different from those in the subsequent year but that the difference is too great to detect a significant relationship between years.

Furthermore, there is a slight increase in FA of scarred trees between years and a slight decrease between years for control trees, although neither of these differences is significant ($P = 0.70$ and 0.81 for scarred and unscarred trees respectively). Nonetheless, this could signify that plants facing herbivore pressure demonstrate increased FA over time whereas the FA of those trees not exposed to herbivory is less variable.

3.4 Discussion

The results from this study show no correlation between porcupine herbivory and fluctuating asymmetry of trembling aspens. This brings into question the use of FA both as a predictor of herbivore food choices and as a tool to indicate herbivory imposed stress on trees.

3.4.1. FA as a Bioindicator of Stress

If FA is positively correlated with stress, highly stressed individuals should show high levels of FA. We found no difference in leaf FA of aspen trees under porcupine herbivory pressure versus those that were not. This implies that either porcupine feeding does not impose a stress on aspen trees, that this stress is not being detected through the FA analysis we performed, or that there was too little temporal correspondence between when feeding occurred and when symmetry was measured.

Porcupine feeding habits differ greatly between seasons. In summer, porcupines eat large quantities of leaves, and visual observation of aspen trees bearing a high number of porcupine climbing scars shows decreased foliage and a largely reduced canopy (Fig. 3). To our knowledge, no studies have quantified the impacts of summer porcupine feeding however, artificial defoliation of aspen trees has been shown to suppress plant growth (Osier and Lindroth 2004), suggesting that summer porcupine herbivory is likely to impose a stress on aspens. In contrast, the impacts of winter feeding by porcupines are well known. They debark trees, cutting off the flow of nutrients from roots to canopy, severely stressing and often killing the tree (Curtis 1941, Curtis and Wilson 1953, Storm and Halvorson 1967). Despite this obvious strain, concurrent work by this research group shows no correlation between increased leaf FA and high levels of winter porcupine feeding on paper birch and jack pine (*Pinus banksiana*) trees (unpublished data).

Although the bulk of FA literature shows a positive correlation between stress and FA (Koslov et al. 1996, Zvereva et al. 1997, Koslov and Niemela 1999, Moller 1999), relatively severe environmental stress appears necessary to induce significant FA alterations (Parsons 2000). Furthermore, high levels of leaf FA are characteristic of trees with accelerated leaf growth and do not explicitly indicate that a plant is stressed (Martel et al. 1999, Lempa et al. 2000). It has also been suggested that FA may not be a predictor of lowered fitness as short term environmental perturbations can increase FA (Hochwender and Fritz 1999). It is likely then, that FA is not capable of detecting stress induced by porcupine herbivory.

3.4.2. FA as a predictor of herbivore food choices

It has been suggested that leaves exhibiting high FA may be of higher quality to herbivores than symmetrical leaves (Wilsey et al.1998, Lempa et al. 2000). However, our results do not indicate any significant relationship between porcupine herbivory and leaf FA, indicating that FA is probably not a determinant variable in porcupine food selection.

3.4.3. Repeatability of FA Measures

The comparison of results between years raises some important questions about the use of FA in plant-herbivore studies. Although results from the paired t-test imply between year repeatability of FA measurements, the regression analysis showed no relationship between

the leaf FA of trees from year to year, implying that FA measurements vary over time.

This temporal variation is an important consideration because for FA to be a useful tool in plant-herbivore studies, the variation in FA between years would need to be correlated with a variation in some other biotic factor such as level of herbivory. The measure of herbivory in this study is a static value over both years and therefore we can not examine changes in FA relative to changes in herbivory pressure. However we would expect that stressed trees would exhibit a similar FA from one year to the next. The increase in FA of scarred trees between years may imply that herbivory induced stress increases with time after an herbivory event. However the lack of correlation between FA of scarred trees between years implies otherwise. Therefore, either herbivory is not imposing a stress on aspens, this stress is not being detected in FA measurements, or some other factor is having a stronger effect on the FA of aspen trees.

Previous findings from this research group also question the repeatability of FA measurements. Work with paper birch trees showed a significant relationship of FA between years, with an increase in leaf FA of unscarred trees, and no change in scarred trees (Berteaux et al. unpublished data). A three year experiment with jack pines showed repeatability of FA between the first and third years, but not between the first and second, or first and third years. To our knowledge, these are the only studies that have examined changes in FA over time and the

contradictions within them make it difficult to draw conclusions about the repeatability of FA measures.

The lack of correlation between porcupine herbivory and leaf FA of trembling aspens found in this study questions the validity of using FA as a tool to examine plant-herbivore interactions. This study has also demonstrated the sensitivity of FA analyses and stresses the importance of considering temporal variation when using FA either as a predictive tool or as quantitative measure of stress.

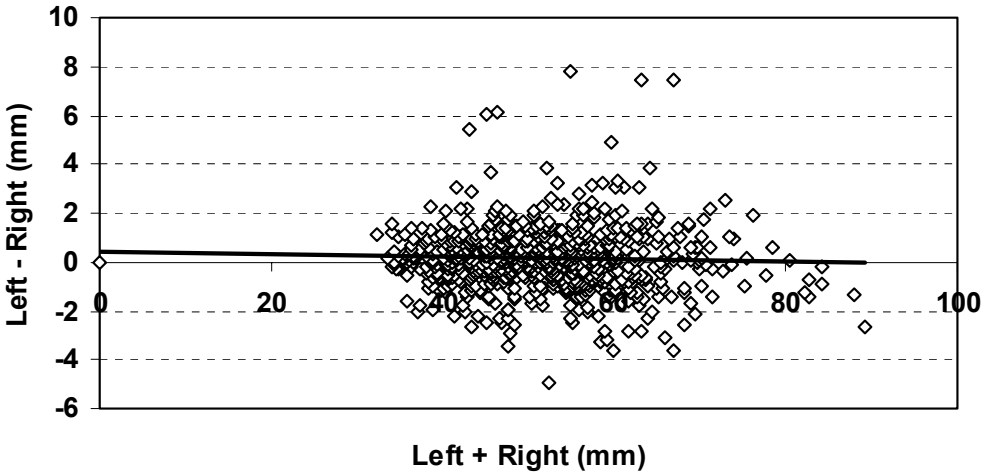
3.5. Figure Legends

Figure 1: Neutral relationship between total leaf size and the difference between left and right sides of each leaf. The regression analysis was performed on all 1500 leaves measured in the FA analysis in 2002 and 2003. $R^2 = 0.002$ exemplifying that an increase in leaf size does not result in an increase of FA.

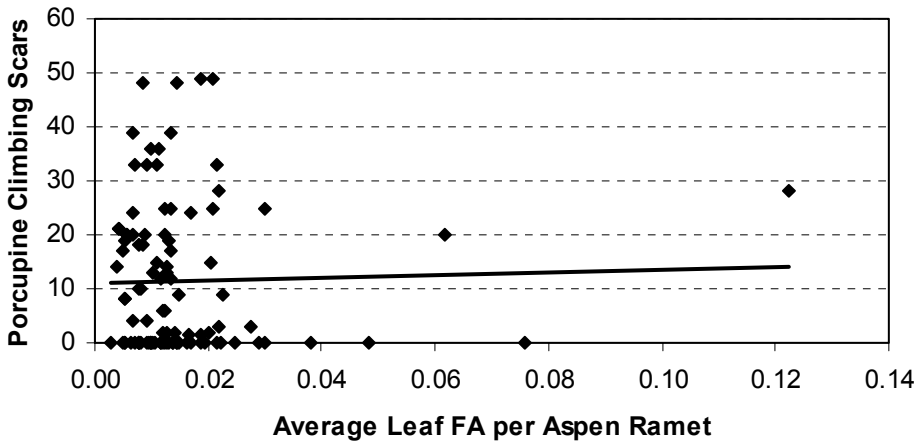
Figure 2: Regression analysis showing no relationship between leaf FA and the porcupine climbing scars used as an index of herbivory. The analysis was performed with the sub sample of 50 aspen ramets from Parc National du Bic, Quebec, Canada, used in the FA analysis. The lack of correlation between these two variables shows no causal relationship between porcupine food selection and aspen leaf FA.

Figure 3: Photographs of the canopies of two trembling aspen ramets in our study site in Parc National du Bic, Quebec, Canada. A) Intact canopy of a ramet bearing no porcupine climbing scars. B) Severely browsed canopy of an aspen ramet bearing a high number of porcupine climbing scars. Large gaps in the canopy represent areas where porcupines gnawed off branches to feed. The tufts of foliage are characteristic of porcupines' tendency to not feed from terminal branches.

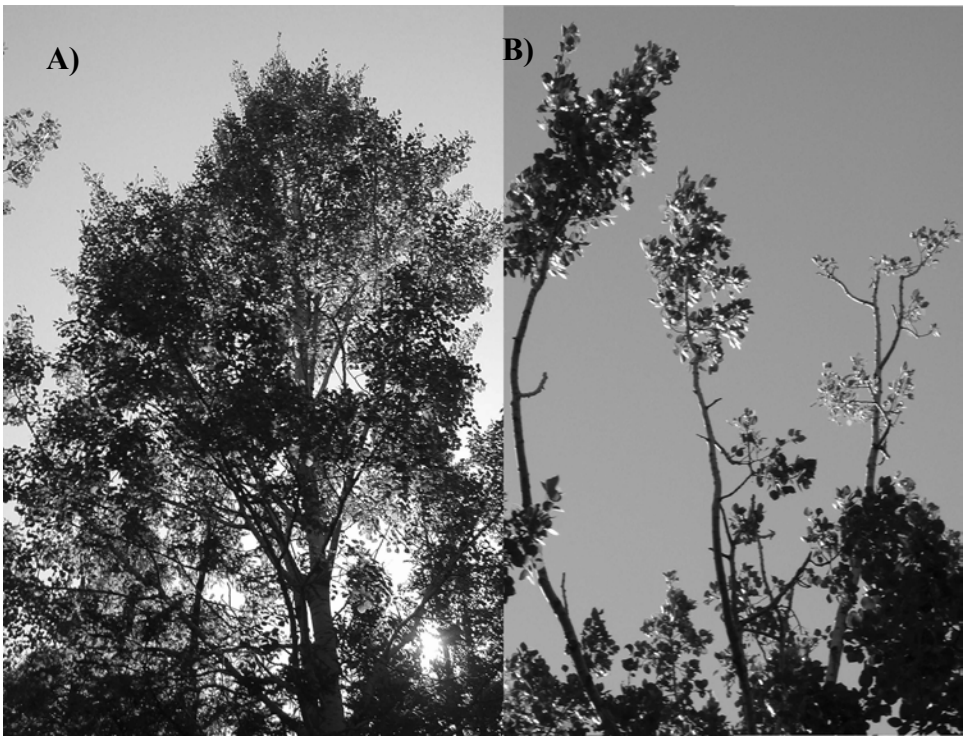
3.6. Figures:



Berteaux et al. Fig 1



Berteaux et al. Fig. 2



Berteaux et al. Fig. 3

Table 1: Results for FA analysis performed on the sub sample of 50 aspen ramets from Parc National du Bic, Quebec, Canada. Mean FA values, standard error, measurement error and P values for test (scarred) and control (unscarred) trees in 2002 and 2003.

	2002		2003	
	Test	Control	Test	Control
Mean	0.014	0.017	0.016	0.016
Standard Error	0.002	0.003	0.005	0.002
P value	0.44		0.99	
ME (% of FA)	0.55%		0.71%	

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GENERAL CONCLUSIONS

The work performed in this thesis addressed two fundamental issues in the study of plant-herbivore interactions. First, our results contribute to a better understanding of how mammals make food choices, and second, we further advanced notions of how mammalian herbivores have the potential to act as selective agents within a forest ecosystem, thereby addressing a fundamental assumption of co-evolutionary theory.

Through the porcupine-aspen study system, we demonstrated that variation in phenolic glycosides is the primary factor responsible for intre-species food selection in a mammalian herbivore. This adds to an understanding of mammalian foraging strategies and is consistent with the findings of several authors (Edwards 1978, Lawler et al. 1998, Pass and Foley 2000, O'Reilley-Wapstra et al 2004, Bailey et al. unpublished manuscript). We have also shown that unlike beavers (Bailey et al. 2004), black-tailed tree rats (Downs 2003), and brushtail possums (Marsh et al. 2003), porcupine food choices do not show a strong relationship to variation in condensed tannins. Given the complex decisions faced by mammals when foraging, understanding how they respond to chemical variation in defensive plant traits is crucial.

To our knowledge, this is one of the first studies to examine porcupine summer feeding strategies (see Roze 1989), and contributes to the existing literature on aspen-mammal interactions (see Edwards 1978, Basey et al. 1988, 1990, Erwin et al. 2001, Bailey et. al. 2004, Bailey et al. unpublished manuscript). This study clearly shows that secondary

chemistry influences the selection of aspen trees by mammalian herbivores. By selecting for trees with low levels of phenolic glycosides, which are in part, influenced by the clonal distribution of aspens, porcupines may be influencing the genetic composition of trembling aspen stands. Therefore, this work creates an important link in the relationship between plant chemistry, genetics, and mammalian herbivory.

We were unable to show any effect on aspen fitness due to herbivore pressure using a fluctuating asymmetry analysis however our work with this tool did raise important implications of using FA in plant herbivore studies.

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APPENDIX A: ANIMAL CARE PERMIT

Université du Québec à Rimouski

CERTIFICAT DE BONS SOINS AUX ANIMAUX

Titulaire du projet :	Dominique Berteaux
Unité de recherche :	Chaire de recherche du Canada en Biologie de la conservation
Adresse :	300 des Ursulines, Rimouski
Titre du projet :	Écologie et comportement des mammifères herbivores
Organisme subventionnaire :	CRSNG et FCAR

Le Comité de protection des animaux de l'Université du Québec à Rimouski (CPA-UQAR) certifie, conjointement avec le titulaire du certificat, que les animaux utilisés pour ce projet seront traités conformément aux principes énoncés par le Conseil canadien de protection des animaux dans son document intitulé «Manuel sur le soin et l'utilisation des animaux d'expérience, volumes I et II», ainsi que selon les directives additionnelles émanant du CPA.

Réservé au CPA

N° de résolution :	CPA12-02-06
Catégorie d'inconfort ou de souffrance :	B
But de l'utilisation des animaux :	1
Période de validité :	Du : 1er avril 2002 au 1er avril 2004

Dominique Berteaux
Titulaire du certificat

Date : 17 janvier 2002

Président du CPA-UQAR

17 fév. 02
Date