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THE INFLUENCE OF PLANT SECONDARY METABOLITES ON THE NUTRITIONAL ECOLOGY OF HERBIVOROUS TERRESTRIAL VERTEBRATES

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■ **Abstract** Plant secondary metabolites (PSMs) significantly impact the nutritional ecology of terrestrial vertebrate herbivores. Herbivores have a wide range of mechanisms (herbivore offenses) to mitigate the negative effects of PSMs. We discuss several behavioral and physiological offenses used by terrestrial vertebrates. Several newly recognized herbivore offenses such as regulated absorption and regulation of toxin intake are presented. We give a detailed description of the biotransformation system with respect to PSMs. We also summarize recent findings of plant–animal interactions for lizards, birds, and mammals. Finally, we discuss some new tools that can be applied to long-standing questions of plant–vertebrate interactions.

INTRODUCTION

Herbivores are faced with a food resource of poor quality not only because plants are low in nutrients but also because they produce plant secondary metabolites (PSMs) that have wide-ranging physiological effects from direct toxicity to digestion impairment. We review the significant impact of PSMs on the nutritional ecology of terrestrial vertebrate herbivores by beginning with a very brief introduction to PSMs. We follow with an extensive review of herbivore offenses (Karban & Agrawal 2002) including numerous behavioral and physiological mechanisms herbivores use to deal with PSMs. We finish with a short summary of the current state of research for three classes of vertebrate herbivores and novel tools for future studies. A goal of this review is to point out new avenues for research in the area of plant–animal interactions.

Plant Secondary Metabolites

Understanding the effects of PSMs on herbivore foraging may seem overwhelming given the bewildering variety of compounds. This diversity has led to different classification schemes based on chemical characteristics (e.g., terpenoid, phenolic, alkaloid), modes of action (toxins versus digestibility reducers), or plant apparency (abundance). Although these classifications have been useful in formulating some broad theories of plant defense, the more we learn, the less useful such broad classifications become (Foley et al. 1999, Foley & McArthur 1994). Most progress in the past decade in understanding how PSMs influence vertebrate herbivores has come from systems where the plant chemistry is well characterized through partnerships between chemists and ecologists (e.g., Clausen et al. 1986, Jakubas & Gullion 1990, Lawler et al. 2000, Moore et al. 2004). We believe that understanding the precise nature of the chemistry mediating plant–vertebrate interactions is extremely valuable. However, detailed chemical characterization may remain beyond many ecological studies, which will continue to consider PSMs in broad chemical groups. Readers desiring more detail on PSM chemistry are referred to Rosenthal & Berenbaum (1991).

MECHANISMS

In this section, we present three general mechanisms (avoidance, regulation, biotransformation) used by vertebrate herbivores to mitigate the effects of PSMs. Of the three sections, ecologists may be least familiar with biotransformation. We provide details of the molecular biology of biotransformation as a primer to aid ecologists attempting to access the pharmacological literature.

Avoidance

DECOMPOSITION Herbivores that cache food before consumption may behaviorally circumvent or reduce the effects of PSMs if the compounds degrade during storage. Behavioral reduction of PSMs prior to ingestion has a number of advantages. The detoxification system is energetically demanding (Sorensen et al. 2005a). If animals can reduce the dose of toxins consumed through food storage, they may save significant quantities of energy that would be lost during detoxification (e.g., endogenous conjugates). Second, ingestion of lower doses may reduce the likelihood of the formation of toxic intermediate metabolites or free radicals. Lastly, behavioral manipulation of PSMs may enhance diet breadth by allowing herbivores to consume plants containing toxins or concentrations of toxins that they would otherwise be unable to process.

A few examples of behavioral manipulation are documented. Meadow voles (*Microtus pennsylvanicus*) snip branches from conifer trees and delay consumption for a few days, during which time the PSMs decrease (Roy & Bergeron 1990). Beavers (*Castor canadensis*) soak twigs in water for days prior to consumption.

The phenolics in the twigs are reduced through the leaching process. Beavers prefer leached twigs to fresh twigs (Muller-Schwarze et al. 2001). Pikas (*Ochotona princeps*) also manipulate the PSMs in their diet prior to consumption (Dearing 1997). In the summer, pikas preferentially collect leaves high in phenolics for consumption during the winter. During storage, the phenolic concentrations of leaves decay to acceptable levels. An added benefit of collecting leaves high in phenolics for storage is that these leaves preserve better, that is, there is less decomposition of the cache than leaves lacking phenolics.

TANNIN-BINDING SALIVARY PROTEINS (TBSP) Many herbivores produce salivary proteins that may assuage the effects of tannins. Some tannins (condensed or hydrolyzable) react with proline-rich proteins secreted in saliva, binding to form a complex that is usually insoluble and unabsorbed (Lu & Bennick 1998). Proline has a secondary amine group that gives it a rigid conformation and disrupts the alpha-helical structure of proteins. This makes carbonyl groups in the protein available for hydrogen bonding with the phenolic group of tannins (Santos-Buelga & Scalbert 2000). The interactions are stronger with high-molecular weight condensed tannins that possess many phenolic groups.

The consequences of binding tannins with salivary proteins are twofold. Tannins are inactivated as toxins, enabling the animal to eat tannin-rich browse. The evidence that herbivores have developed specific TBSPs to bind the particular tannins found in their diet is conflicting (Hagerman & Robbins 1993, McArthur et al. 1995). However, the increased secretion of TBSPs results in protein loss in feces, which can affect nitrogen digestibility (Skopec et al. 2004) and body weight (Santos-Buelga & Scalbert 2000). Furthermore in some species with TBSPs, the rate of secretion of protein is so low as to question their role in defense against dietary tannins (McArthur et al. 1995).

PERMEABILITY GLYCOPROTEIN AND CYTOCHROME P4503A Regulated absorption of PSMs by gut cells is a unique and only recently acknowledged herbivore offense (Sorensen & Dearing 2003, Sorensen et al. 2004). Absorption of PSMs across intestinal cells can be regulated by two molecular mechanisms, a glycoprotein transporter (permeability glycoprotein, or P-gp) and a biotransformation enzyme (cytochrome P450 3A, or CYP3A) that function either in tandem or independently to reduce the amount of PSMs absorbed. P-gp is one of a group of transporters that remove foreign substances from cells (Hoffmann & Kroemer 2004, Lin & Yamazaki 2003). It was originally discovered in tumor cells, in which it is responsible for resistance to several anticancer drugs (e.g., *Vinca* alkaloids, taxanes). P-gp is one of a large superfamily of ATP-binding cassette transporters that are highly conserved and found in all species from bacteria to mammals. P-gp is highly expressed on the apical surface of cells in the lower intestine with increasing levels from the duodenum to colon. P-gp pumps PSMs (e.g., digoxin, morphine) back into the lumen of the gut (Hoffmann & Kroemer 2004, Lin & Yamazaki 2003). P-gp acts on hydrophobic substances, usually also containing a

hydrophilic region, with a tertiary nitrogen and aromatic ring. P-gp is also present in liver and renal tubular cells, where it pumps substances into the bile and urine, respectively, and is in the luminal side of the capillary endothelium that forms the blood-brain barrier (Lee & Bendayan 2004). Overall, the role of P-gp seems to be a defense against xenobiotics such as PSMs, by opposing their absorption, hastening their excretion, and protecting the brain. For example, mdr1a knockout mice that lack P-gp are more sensitive to neurotoxicity from oral ivermectin than are wild-type mice (Lin & Yamazaki 2003).

CYP3A is a biotransformation enzyme that metabolizes about 50% of all drugs and many PSMs. It is expressed in large amounts in liver cells but also in intestinal cells where it usually acts in concert with P-gp to reduce the absorption and bioavailability of PSMs (Cummins et al. 2002, Johnson et al. 2001). This joint defense is most effective against toxins that are taken in low doses and which are slowly absorbed, such as the fungal metabolite, cyclosporine (Lin & Yamazaki 2003). The K_m values of P-gp and CYP3A are <100 μM, so PSMs present in high concentrations that rapidly enter intestinal cells will saturate both transporter and enzyme, resulting in dose-dependent absorption. For very lipophilic PSMs, diffusion into intestinal cells is likely to be faster than P-gp can return them to the lumen; however, the effect of the pump will be to increase the time that the PSM will spend in the cell, where it is exposed to metabolism by CYP3A. The result is more extensive metabolism of the PSM rather than it being excreted unchanged in the feces.

Only recently have P-gps been examined within the context of plant–herbivore interactions. Several studies comparing performance of woodrats that specialize on juniper (*Neotoma stephensi*) to that of a generalist woodrat (*N. albigula*) suggest P-gps may play a role in dietary specialization. First, the specialist had lower blood levels after oral dosing with α-pinene (a juniper PSM) than the generalist, although the rates of biotransformation were not different (Sorensen & Dearing 2003). The specialist also excreted more α-pinene unchanged in the feces compared to the generalist (Sorensen et al. 2004). Lastly, in *in vitro* tests, the intestinal P-gp capacity of the specialist was significantly greater than the generalist (Green et al. 2004). Taken together, these studies suggest that the ability of *Neotoma stephensi* to specialize on juniper may be in part due to P-gp and/or CYP3A that reduce absorption of juniper toxins.

Many PSMs can act as activators, inducers, or inhibitors of P-gp and CYP3A4 (Zhou et al. 2004). Exposure to enzyme inhibitors can also trigger induction. Both P-gp and CYP3A can be induced by PSMs that bind the nuclear pregnane X receptor (PXR) of liver and intestinal cells (Dresser et al. 2003, Kullak-Ublick & Becker 2003, Moore et al. 2000). St. John's wort (a common nutraceutical used for depression in humans) decreases the bioavailability of cyclosporine and digoxin, probably through induction of P-gp and CYP3A by the flavonoids, hyperforin and hypericin (Mueller et al. 2004, Zhou et al. 2004). The potential outcome of these P-gp and CYP3A interactions may be complex in herbivores that consume plants with myriad PSMs.

MICROBIAL DETOXIFICATION Many propose that PSMs are detoxified by microbes in foregut fermenting herbivores (e.g., ruminants, kangaroos, hoatzin). The diverse microbial populations in the foregut can perform many reactions, which can both reduce and increase the toxicity of ingested PSMs (Duncan et al. 2000, Foley et al. 1999). However, there remain too few examples to judge whether foregut microbial detoxification is an important driver of diet diversification in wild herbivores. The best example comes from agricultural systems where Jones & Megarrity (1986) showed that acquisition of a specific microorganism (*Synergistes jonesii*; Allison et al. 1992) enabled goats to consume greater quantities of *Leucaena leucocephala* containing mimosine, a toxic nonprotein amino acid. Nonetheless, agriculturalists are focusing on identifying and manipulating the microbial population of the foregut of sheep and cattle to degrade PSMs because they realize that breeding programs to eliminate PSMs from forage plants can be counter-productive (McSweeney et al. 2002).

Several recent studies (Jones et al. 2000, 2001) conclude that microbial populations from wild browsers had no greater ability to degrade tanniniferous foods than did those from domesticated cattle. This coupled with the reluctance of authorities to allow the release of genetically modified microorganisms to degrade toxic compounds (Gregg et al. 1998) suggests that enhancement of microbial detoxification is more likely to come from manipulating rumen populations rather than discovering new, highly specific organisms. In contrast, recent studies (Krause et al. 2004) have started to use community genome approaches to identify specific changes in the rumen microbiology of sheep eating tannin-rich forages, and ecologists should see rapid progress in understanding the extent of microbial response to PSM-rich diets (at least in agricultural systems) over the next few years.

Regulation of PSM Intake

Browsing mammals encounter a diverse range of PSMs in the majority of the foods they consume. Therefore, complete avoidance of PSMs in the diet is not likely to be a realistic strategy, as animals would need to exclude most available plants from their diet. The vast majority of PSMs that animals ingest are not so acutely toxic that a single bite would be lethal or severely detrimental, but large amounts of even low-potency compounds can be harmful. Consequently, animals must have mechanisms that allow them to detect and regulate their intake of PSMs to ensure that low-potency toxins do not cause damage. Understanding the sorts of mechanisms that animals use to regulate toxin intake is vital if we are to integrate the effects of PSMs with broader theories of feeding. For example, is the regulation of PSM intake part of the same mechanisms that animals use to recognize nutrient deficiencies or are there separate regulatory mechanisms imposed on normal feeding patterns? By understanding the interactions and tradeoffs between the intake of nutrients and PSMs, we can identify general patterns so that each new compound studied does not have to be treated as a special case. Below we review evidence that animals regulate PSM intake and describe potential mechanisms permitting regulation.

EVIDENCE OF REGULATED INTAKE The evidence that the intake of PSMs is regulated comes largely from dose response studies in which purified PSMs are fed across a range of concentrations (5 to 10 times range) to captive animals. For example, both common ringtail (*Pseudocheirus peregrinus*) and brushtail (*Trichosurus vulpecula*) possums adjusted food intake on diets containing different concentrations of jensenone such that there was no difference in the daily intake of jensenone. Ruffed grouse (*Bonasa umbellus*) also regulate their intake of coniferyl benzoate (CB) over a fivefold range of dietary concentrations (Jakubas et al. 1993). However, it is worth noting that regulation does not appear to be as precise when animals feed on natural plant diets containing the same compounds. We present three mechanisms that we think animals may use to regulate their intake of PSMs.

NAUSEOUS FEEDBACK CAUSING CONDITIONED FOOD AVERSIONS Conditioned food aversions (CFAs) have long been known to be powerful influences on the diets of herbivores (Provenza et al. 1998). CFAs arise when an animal makes an association between the taste or smell of a plant and some negative consequence—usually illness. Provenza and coworkers (e.g., Provenza et al. 2003) have been instrumental in demonstrating that CFAs influence diet choices of large domestic animals such as sheep, goats, and cattle and that CFAs are a useful practical method of protecting free-ranging livestock from being poisoned. In Provenza's view, CFAs are balanced by the possibility of animals forming conditioned preferences—they associate the flavor of food with positive consequences such as enhanced energy or protein status (Provenza et al. 1998).

In spite of the large volume of laboratory data that support a central role for CFAs in herbivore foraging, there are several caveats to consider. First, there have been few direct demonstrations that the attenuation of nauseous sensations allows herbivores to increase consumption. Lawler et al. (1998) showed that administration of the drug ondansetron [a specific serotonin ($5HT_3$) receptor antagonist that reduces nausea and vomiting in humans] led to greater intakes of the PSM jensenone in two herbivorous marsupials and attributed the effect to reduction in nauseous sensations. Similarly sheep consumed more endophyte-infected fescue when dosed with a dopamine receptor antagonist that reduces nausea and vomiting in humans (Aldrich et al. 1993).

Second, there have been few demonstrations of CFAs where both the compounds responsible for the distinctive taste of the plant and the nausea-inducing factors are naturally present in the same plant; nearly all studies rely on using the artificial agent LiCl to induce nausea. In contrast, Lawler et al. (1998) demonstrated an ecologically relevant example in *Eucalyptus* where the concentrations of the dominant *Eucalyptus* terpene, 1,8-cineole, covaried with the concentrations of a nausea-inducing agent jensenone. More examples like this would build confidence in the ecological relevance of CFAs.

Finally, the ability of animals to form CFAs to PSMs must be tested under the more complex foraging scenarios commonly experienced by free-ranging animals. For example, Duncan & Young (2002) showed that the ability of animals to

make associations between illness and a particular food were reduced when all test foods were offered during the learning period, as would be the case in free-ranging animals. Caution is prescribed in extrapolating results of simple conditioning experiments carried out with captive animals to free-ranging herbivores (Duncan & Young 2002).

PRE-INGESTIVE EFFECTS: TASTE AND TRIGEMINAL STIMULATION Many studies have demonstrated that foods can be repellent to herbivores without inducing CFAs. Humans experience this when eating foods containing hot peppers. The vanilloid compound in the peppers, capsaicin, irritates trigeminal nerves in the mouth, generating a burning sensation and, if it is too intense, food consumption is reduced. However, the experience rarely leads to aversion to such dishes in the future. Similarly, it is clear that animals can use trigeminal feedback to regulate their intakes of these irritant PSMs. For example, Jakubas et al. (1993) showed that CB was a trigeminal irritant in birds and that ruffed grouse could regulate their intake of CB in aspen.

Compounds that are bitter, but not necessarily irritant, may also be effective deterrents at high concentrations and lead to thresholds in the ingestion of the PSM. For example, the intensely bitter phenolic glycoside, salicin, is an effective antifeedant in willows (*Salix* sp.) for common brushtail possums in the field. However, the effect was due to "taste" rather than postingestive effects because direct infusion of salicin into the stomach had no effect on subsequent salicin intake (Pass & Foley 2000).

Recent studies confirm that molecular differences in the vanilloid receptor between birds and mammals may explain why mammals but not birds are repelled by a hot, peppery taste (Jordt & Julius 2002). In contrast, bitter substances are significantly more repellent to birds. Recent advances in understanding the molecular basis of bitter tastes (e.g., Margolskee 2002) offer exciting opportunities to understand why some animals are more repelled by bitter compounds than others and to learn how the taste of PSMs integrates with other food components. The differences between mammals and birds in their susceptibility to different repellent PSMs is of particular interest in understanding the role of PSMs in fruit dispersal, particularly the possibility of directed dispersal of fruits by either birds or mammals (Cipollini & Levey 1997a, Tewksbury & Nabhan 2001).

FEEDBACK FROM DETOXICATION LIMITATIONS Recent experiments show that limits to the rate of detoxication of ingested PSMs can constrain feeding rates of mammalian herbivores. However, it remains unclear how animals translate physiological effects into changes in feeding behavior. For example, brushtail possums fed diets rich in benzoic acid ate more when provided with supplemental glycine because the rate of formation of the detoxified excretory product benzoyl glycine (hippuric acid) was enhanced (Marsh et al. 2005). Animals recognized whether diets contained supplemental glycine and modified their feeding accordingly. However, feeding rates did not change when brushtails were dosed with ondansetron

(Marsh et al. 2005), suggesting that nauseous feedback was not responsible for the changes in food intake induced by dietary benzoate.

Biotransformation

PSMs can be eliminated from the body by excretion or chemical change (biotransformation), or a combination of these processes. Because of the way the terrestrial kidney works to conserve water, lipophilic substances are poorly excreted. In the mammalian kidney, the plasma filtered at the glomerulus is nearly completely reabsorbed from the renal tubules, and lipophilic substances are extensively reabsorbed across the renal tubular epithelium leaving polar molecules that cannot permeate the epithelial cells to be excreted in the urine. The most lipophilic PSMs (e.g., monoterpenes) are not excreted without transformation (Boyle et al. 1999, 2000a), whereas more polar PSMs (e.g., quercetin, gallic acid) are excreted unchanged to varying extents (Schwedhelm et al. 2003, Wiggins et al. 2003).

Terrestrial animals have evolved a powerful suite of biotransformation enzymes to convert lipophilic PSMs into more polar metabolites readily excreted in urine or bile. These biotransformation enzymes are broadly categorized into two groups: functionalization (also called Phase 1), in which functional groups are introduced into metabolites, and conjugation (Phase 2), in which adducts are formed with endogenous compounds to further increase polarity. Functionalization and conjugation enzymes can work alone or in tandem, depending on the substrate. The most important are the mixed-function oxidases of the cytochrome P450 family (P450s) (Gonzalez & Nebert 1990, Guengerich 2004). These enzymes are considered to have evolved in part as a response to dietary plant toxins (Gonzalez & Gelboin 1994, Gonzalez & Nebert 1990). Although most drugs lose their activity after biotransformation, there are examples of PSMs whose toxicity is mediated by metabolites.

FUNCTIONALIZATION The most common functionalization reactions are oxidations by P450s, although other reactions (reduction, hydrolysis) can be important, depending on the chemical structure of the PSM. P450s are a large family of enzymes with different but overlapping substrate specificities and a common mechanism of action (Guengerich 2004). They are located in the smooth endoplasmic reticulum where the membrane phospholipid provides an environment suitable for their activity. The liver is the major organ for P450 activity but extrahepatic sites, especially the gastrointestinal tract, are also important (Ding & Kaminsky 2003). P450s have a general requirement for molecular oxygen, NADPH, and cytochrome P450 reductase, and the reaction results in the introduction of an oxygen atom in the form R-H to ROH. The oxidized metabolites may be similar to the parent molecule, e.g., alcohols and acids formed from hydrocarbons such as monoterpenes (Boyle 2000b, Boyle et al. 1999) or may become unrecognizable through cleavage of the carbon skeleton (e.g., phenols and hydroxy acids formed from complex phenolics such as tannins and flavonoids) (Schwedhelm et al. 2003,

Zhou et al. 2003). Analysis of the literature on human P450s with substrates and inhibitors reveals that there are five primary P450s for drug metabolism (CYP: 2D6, 3A4, 2C, 1A2, 2B6) and four for other substances such as PSMs (CYP: 1A1, 1B1, 2E1, 2A6; Rendic 2002). Comparison of P450s from humans and the puffer fish (*Takifugu rubripes*) indicates that the overall pattern of P450 genes has been well conserved for 420 million years (Nelson 2003).

Despite the general conservation of P450 genes, the activities of P450 enzymes can vary widely between and within species due to differences in allelic forms and expression (Gonzalez & Kimura 2003, Guengerich 2002, Wilkinson 2004). For example, specialist marsupial folivores, whose diets are high in monoterpenes, are able to more extensively oxidize monoterpenes than generalist folivores (Boyle 2000a; Boyle et al. 1999, 2000b, 2001). In vitro, the P450s from marsupial folivores have greater activity in oxidizing terpenes compared to rat or human P450s (Pass et al. 2001, 2002).

Metabolism does not always detoxify PSMs as many adverse reactions are due to the formation of reactive and toxic metabolites (Gonzalez & Gelboin 1994). For example, the pyrrolizidine alkaloids (Fu et al. 2004) and aflatoxin B_1 form toxic intermediates after biotransformation.

CONJUGATION Conjugation reactions involve the addition of an endogenous molecule directly to a PSM or a metabolite formed from a functionalization reaction. The most common conjugates are glucuronides and sulfates but glutathione may also be an important conjugate. These conjugates bind to PSMs forming highly polar products that are readily excreted in urine or bile (Shipkova et al. 2003), although they require active transport to leave the cell (Konig et al. 1999). Conjugation is more energetically costly compared with functionalization in that an endogenous compound, the conjugate, is typically excreted from the body as part of the process.

Conjugation with glucuronides occurs through any of ~50 enzymes in the mammalian superfamily of UDP-glucuronosyltransferases (UGTs). These enzymes catalyze reactions between activated UDP-glucuronic acid and the -COOH, -OH and $-NH_2$ groups of a vast array of endogenous and exogenous substrates such as steroid hormones and the phenolic metabolites of flavonoids and tannins (Miners et al. 2004, Radominska-Pandya et al. 1999). UGTs are located in the endoplasmic reticulum of liver, gut, and kidney, and substrates must be lipophilic to reach the membrane-bound enzyme. This is demonstrated by the differential glucuronidation of terpene metabolites in marsupial folivores, in which the less polar hydroxyterpenes are extensively conjugated, whereas the more polar hydroxyacid metabolites are excreted in the free form (Boyle et al. 1999, 2000).

The different forms of UGTs are proposed to have evolved in part in response to the challenge of dietary PSMs (Bock 2003). There are marked species differences in the pattern and rate of glucuronidation (Walton et al. 2001). Interestingly, the cat and related carnivores are deficient in UGT activity and are highly sensitive to toxicity of certain nondietary UGT substrates such as acetaminophen and morphine

(Court & Greenblatt 2000). In the koala (*Phascolarctos cinereus*), which specializes on *Eucalyptus*, there is negligible conjugation of terpene metabolites, which instead are extensively oxidized to enable their excretion (Boyle et al. 2000a, 2001). However, the koala does glucuronidate phenolic metabolites (McLean et al. 2003), possibly because they are not readily oxidized by P450 enzymes.

Conjugation reactions with sulfate are similarly catalyzed by a large family of cytosolic sulfotransferase enzymes (SULTs) that act on a vast array of endogenous and exogenous substrates, including phenols, benzylic alcohols, and hydroxylamines (Blanchard et al. 2004, Glatt & Meinl 2004, Kauffman 2004). The sulfonate group (SO_3^-) is transferred from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) and forms a sulfate ester (SO_4) only if the acceptor is an OH group. Phenols (e.g., gallic acid) can form both sulfate and glucuronide conjugates (Yasuda et al. 2000). The proportions vary depending on the relative activities of the transferases and their cofactors (Glatt & Meinl 2004) as well as on species and dose. For example, the rabbit excretes paracetamol mainly as glucuronides, whereas the rat mainly excretes sulfates at low doses and glucuronides at high doses (Walton et al. 2001).

Glutathione (GSH) forms conjugates with electrophilic toxins, which otherwise could form adducts with nucleophilic sites on proteins and DNA leading to toxicity (Armstrong 1997, Eaton & Bammler 1999). The resulting glutathione conjugates are either excreted in bile or undergo further metabolism to mercapturic acids, which are excreted in urine. Some PSMs (e.g., formylphloroglucinols; McLean et al. 2004) bind GSH rapidly, but others (e.g., isothiocyanates from glucosinolates; Mithen et al. 2000) need catalysis by glutathione *S*-transferases (GSTs). There are several GST forms that occur both in the cytoplasm for soluble electrophiles and membranes for more lipophilic substrates (Armstrong 1997, Eaton & Bammler 1999).

Because of the serious consequences of genotoxicity, where a single molecular hit can potentially result in mutation or cancer, it is perhaps significant that there is a large overcapacity of GSH conjugating activity, both in GSH and GST concentrations (Rinaldi et al. 2002). This system protects cells from endogenous electrophiles (e.g., the fatty acid oxidation product, 4-hydroxy-2-nonenal) and PSMs (e.g., aflatoxin). PSMs can alter GST activity by inhibition (e.g., curcumin) and induction (e.g., isothiocyanates, goitrin; Eaton & Bammler 1999). The wide species differences in susceptibility to aflatoxin B_1, a secondary metabolite produced by some strains of *Aspergillus*, is due to variations in the relative activities of metabolic activation to the electrophilic aflatoxin B_1-8,9-epoxide and its detoxification by GST (Klein et al. 2000).

Although conjugation is usually considered to be a detoxifying reaction, with some substrates the glucuronide or sulfate can generate a reactive electrophile that forms adducts with proteins and DNA resulting in toxicity to the cell or genome. Examples of reactive conjugates of PSMs are the acyl glucuronide of salicylic acid (Spahn-Langguth & Benet 1992) and the sulfate ester of safrole (Kauffman 2004).

INDUCTION AND INHIBITION OF METABOLISM The activity of enzymes and transporters can be modified by PSMs that inhibit their function or stimulate the synthesis of more active protein (a process called "induction"), or both effects in succession (Hollenberg 2002). Inhibition can be due to competition between substrates for limited enzyme or an irreversible "mechanism-based" reaction where the inhibitor is oxidized by the P450 to a reactive metabolite that binds covalently to the active site of the enzyme. One example of a mechanism-based inhibitor is 6′, 7′-dihydroxybergamottin, a PSM in grapefruit juice that destroys CYP3A in enterocytes, requiring three days for recovery of enzyme activity (Greenblatt et al. 2003).

Induction of P450 enzymes occurs via several nuclear receptors. The nuclear receptors activate target genes in a similar manner to the steroid hormone receptors (Wang & LeCluyse 2003). Polycyclic aromatic hydrocarbons and dietary constituents were the first substances found to induce metabolism, and now many drugs and natural products are recognized as inducing agents (Harris et al. 2003, Hollenberg 2002).

Conjugation enzymes are inducible as well as subject to inhibition. PSMs such as hyperforin, β-napthoflavone and indole-3-carbinol induce UGTs as well as CYPs via the nuclear receptors AhR, PXR, and CAR (Bock & Kohle 2004). Induction of sulfonation is not as marked (Coughtrie & Johnston 2001) but glutathione transferases are upregulated by their electrophilic substrates (Rinaldi et al. 2002). Sulfonation is potently inhibited by the polyphenols quercetin, epigallocatechin and epicatechin (Antonio et al. 2003). Glucuronidation and glutathione conjugation do not seem to be as subject to inhibition by PSMs (Eaton & Bammler 1999, Radominska-Pandya et al. 1999), although glutathione conjugates with hydrophobic substituents (e.g., isothiocyanates) bind and inhibit the active site of GSTs (Armstrong 1997).

Because of the multiplicity of enzyme forms, PSMs do not uniformly induce or inhibit all functionalization and conjugation enzymes (Manson et al. 1997). PSMs can have complex effects on P450 regulation, depending on the substances and amounts consumed, target organ, and species (Zhou et al. 2003). There is also large individual variability in the inhibition and induction of P450 enzymes (Lin & Lu 2001) and polymorphism in transporters (Ieiri et al. 2004). The consequences for herbivores eating large amounts of PSMs that can act as inhibitors of metabolism (during the feeding session) and inducers (on the following days) will be complex interactions.

HERBIVORES AND PSMs

Below we give an overview of recent research on how PSMs influence diet selection in the major groups of terrestrial herbivores, e.g., the lizards, birds, and mammals. Some groups such as the lizards have received relatively little attention in this area compared to others such as mammals. The disparity in research among groups

precludes reasonable speculation regarding the relative importance of PSMs to one group versus another. Clearly more research is necessary with respect to the comparative physiology of biotransformation mechanisms used by each group prior to the establishment of generalizations.

Lizards

Herbivory is relatively rare in lizards; only 3% of all species are herbivorous (120 species; Cooper & Vitt 2002). The diets of several herbivorous lizards are documented and several are known to eat or avoid plants with notable PSMs. For example, the chuckwalla (*Sauromalus obesus*) preferentially feeds on the flowers of creosote bush (*Larrera tridentata*) while avoiding the resinous leaves that contain high levels of toxins (Nagy 1973). Despite extensive documentation on diet selection by lizards, there are very few studies that examine the role that PSMs play in food choice.

Of the herbivorous lizards, those that have received the most attention with respect to the role that PSMs play in diet selection are the herbivorous whiptail lizards of the southern Caribbean, *Cnemidophorus murinus* and *C. arubensis*. Studies on both species revealed that whiptails avoided some PSMs such as tannins and alkaloids, but were less deterred by other PSMs such as cyanide (Dearing & Schall 1992, Schall & Ressel 1991). Furthermore cyanide was ingested at extremely high doses with no apparent effect (Schall & Ressel 1991). Caribbean whiptails are capable of discriminating plant odors from those of nonfood items (Cooper et al. 2002). The ability to detect plant chemical cues in whiptails as well as other herbivorous lizards appears to be a trait that evolved with herbivory (Cooper 2003). Herbivorous whiptails can detect differences in concentrations of PSMs, e.g., quinine. The acceptable concentration of quinine in experimental food varied seasonally and may be a function of the availability of other less toxic foods (Schall 1990). These studies indicate that PSMs influence diet selection and that herbivorous whiptails have physiological capabilities for detecting PSMs. Given that these two species of *Cnemidophorus* are not considered to be as specialized for herbivory as some of the iguanans, it is likely that the more specialized lizards also possess such traits and may have other novel traits for detecting and dealing with PSMs.

Little is known about the detoxification mechanisms that herbivorous lizards employ in processing toxins. Obviously, much more research is needed on how PSMs influence diet selection in herbivorous lizards.

Birds

Herbivory is also rare among birds, presumably due to mass tradeoffs associated with flight. However, there are a number of herbivorous birds such as geese, grouse, some parrots, and the hoatzin (*Opisthocomus hoazin*) that feed on terrestrial plants. PSMs play a significant role in diet selection of herbivorous bird species. The hoatzin, for example, is a folivore that inhabits riparian areas of South America.

Although selective in its diet, many of the plants consumed by the hoatzin contain PSMs (Dominguez-Bello et al. 1994). The hoatzin's ability to detoxify certain PSMs may be a consequence of foregut fermentation rather than biotransformation by its own enzymes. The hoatzin has an atypically enlarged crop to house symbiotic bacteria that ferment fiber. It has been proposed that these bacteria in the crop biotransform PSMs before they reach the absorptive tissue in the small intestine (Grajal et al. 1989); however, this has not yet been directly tested. Furthermore, the spectrum of compounds that these fermentative bacteria can process may be limited to alkaloids rather than tannins (Jones & Palmer 2000). Diet selection of the hoatzin is consistent with this hypothesis in that it tends to avoid tannin-rich forages and selects young leaves, which typically are defended by PSMs like alkaloids.

The ruffed grouse has likely received the most attention with respect to detoxification of PSMs. Ruffed grouse consume aspen flowers (*Populus tremuloides*) as well as leaves from a variety of evergreen shrubs and ferns. CB, a phenylpropanoid ester present in aspen, is a primary determinant of feeding (Jakubas et al. 1989). Grouse can detect CB concentration differences and feed selectively on flowers low in CB. Detoxification of CB by grouse occurs through some functionalization reactions but also extensively via conjugation with both glucuronic acid and ornithine (Jakubas et al. 1993). The energetic cost of detoxification of aspen flowers with low levels of CB, typical of that consumed by grouse in the field, was enormous. Grouse lost 10% of metabolizable energy (e.g., energy not lost in urine or feces) in simply the detoxification conjugates (glucuronic acid and ornithine) excreted. This cost is an underestimate of the total energy used in detoxification as it does not account for energy used in enzymatic reactions that attach the conjugate to the PSM or functionalization reactions. Furthermore, losses of the amino acid, ornithine, as a conjugate increased the minimum nitrogen requirement. Another study on grouse consuming leaves from several different species of shrubs and at different doses revealed that usage of conjugation pathways varies among plant species but also within species at different doses (Hewitt & Kirkpatrick 1997). Surprisingly, the shift in pathway usage within a particular food type does not appear to be related to a limitation of conjugates or saturation of the detoxification pathway (Hewitt & Kirkpatrick 1997).

PSMs are not thought to play a large role in diet selection by geese (but see Buchsbaum et al. 1984), in contrast to grouse and the hoatzin (Sedinger 1997). A possible cause of this disparity is that geese feed primarily on monocots, which are typically low in PSMs, compared to the dicots high in PSMs fed on by grouse and the hoatzin. Furthermore, monocot feeders are often believed to have lower capacities for detoxification. However, comparisons of detoxification capacities have not been conducted in herbivorous species of birds.

PSMs are thought to be instrumental in fruit selection by frugivorous birds. Because frugivores are not the subject of this review, we direct readers to a few recent works as an introduction into this active and controversial area of research (e.g., Cipollini & Levey 1997a, 1997b; Tewksbury & Nabhan 2001).

Mammals

Herbivory is widespread among mammals, which may in part account for the greater number of studies on mammals compared to lizards and birds. In the past 30 years, numerous studies have demonstrated that PSMs play a significant role in diet selection of mammalian herbivores (e.g., Berger et al. 1977, Foley et al. 1999, Reichardt et al. 1990). Furthermore, detoxification is expensive and comparable to the cost of reproduction in many mammals (Sorensen et al. 2005a). Because there have been far more studies on mammals than lizards or birds, below we synthesize what has been learned in general rather than profiling individual species of mammalian herbivores.

Studies of the role of PSMs on mammalian foraging have been largely influenced by Freeland & Janzen's seminal paper (Freeland & Janzen 1974). In this work, they wedded data from pharmacological studies on laboratory rodents to ecological studies on wild herbivores to produce theory predicting the foraging behavior of herbivores with respect to PSMs. One of their main points explained the paucity of dietary specialization among mammalian herbivores. They suggested that the default state of the mammalian biotransformation system promoted a generalist foraging strategy because this system can process limited quantities of myriad xenobiotics through numerous detoxification pathways. Such a system would force mammals to be generalist feeders to prevent exceeding the capacity of any one of the pathways. A few experimental studies support this hypothesis; herbivores eat more when offered plants with differing PSMs and process the PSMs through different pathways. Unfortunately, all the studies to date in this area have been on a single mammalian herbivore, the brushtail possum (reviewed in Marsh et al. 2005). Studies on other species are needed to evaluate whether limitations of the detoxification system explain the preponderance of dietary generalization in mammalian herbivores.

A recent hypothesis that has emerged from studies of various mammalian herbivores is that specialists use detoxification pathways that are energetically less expensive than generalist feeders. Specifically, specialists appear to rely more on functionalization pathways than on energetically costly conjugation pathways (Boyle et al. 2000a, 2001; Lamb et al. 2004). As functionalization pathways may have greater substrate specificity than conjugation pathways, a potential tradeoff of specialization may be the inability to process novel toxins. Sorensen et al. (2005b) demonstrated such a tradeoff in the juniper specialist, *Neotoma stephensi*, on a novel toxin. Clearly more work on other species and PSMs is necessary to determine the generality of this tradeoff.

TOOLS FOR FUTURE RESEARCH

There is still much to understand about how PSMs mediate diet selection in terrestrial herbivores. In general, we understand little of the mechanisms used by wild herbivores and how these mechanisms provide feedback to influence diet

selection. Rigorously addressing large-scale ecological or evolutionary questions requires more detail on the underlying physiological mechanisms. Below we describe two approaches that will be fruitful for future studies in plant–mammal interactions.

Genomics

The burgeoning field of genomics offers a number of tools to address the role of PSMs in nutritional ecology. The detoxification limitations hypothesis of Freeland & Janzen (Freeland & Janzen 1974) may soon be testable at the level of entire detoxification systems through the use of microarray technology. A single microarray measures gene expression of thousands of genes simultaneously. Commercial arrays profiling the detoxification system have been constructed for toxicology studies in laboratory rats, and microarrays produced for one species have been successfully used for other species (Moody et al. 2002). Thus, it may be possible to use the rat toxicology microarrays on nonmodel herbivore systems such as voles or woodrats. As the technology develops, so will the possibility and affordability of making arrays for herbivores of interest. In addition to microarrays, the rapidly growing numbers of sequencing projects facilitate studies on the evolution of detoxification genes. Questions such as how do functionalization genes change at the molecular level in specialists compared to generalists can be addressed on a large scale using a combination of database mining (e.g., GenBank) and lab work.

Standardized Functional Assays

Studies of the capacity of herbivores to eliminate dietary PSMs would be greatly assisted if we could readily measure the activity of elimination processes such as enzymes, transporters, and renal excretion. The elimination of probe drugs is used in pharmacokinetic studies, but these tests typically require procedures such as intravenous administration, serial blood sampling, and the measurement of the concentrations of drugs and their metabolites (Pelkonen 2002). These methodological complications can be avoided by using a pharmacological response such as time sleeping under anesthesia as a surrogate measure of biotransformation capability. Usually this has been done in comparative studies, such as the effect of capsaicin on hexobarbitone sleeping time (Hamid et al. 1985) and the effect of 7,8-benzoflavone on zoxazolamine paralysis.

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