

Intestinal capacity of P-glycoprotein is higher in the juniper specialist, *Neotoma stephensi*, than the sympatric generalist, *Neotoma albigula*

Adam K. Green^a, Shannon L. Haley^b, M. Denise Dearing^b, David M. Barnes^c,
William H. Karasov^{a,*}

^aDepartment of Wildlife Ecology, University of Wisconsin-Madison, Madison, 1630 Linden Dr., WI 53706, USA

^bDepartment of Biology, University of Utah, Salt Lake City, UT 84112, USA

^cDepartment of Animal Sciences, University of Wisconsin-Madison, Madison, WI 53706, USA

Received 24 June 2004; received in revised form 23 September 2004; accepted 24 September 2004

Abstract

Permeability-glycoprotein (Pgp) is a membrane-bound, ATP-dependent, transport protein that excludes many cytotoxic compounds including plant metabolites and pollutants from the barrier epithelia of many tissues including the small intestine. We hypothesized that intestinal Pgp capacity would be higher in *Neotoma stephensi*, a specialist on *Juniperus monosperma* known to be high in plant toxins, than the sympatric generalist, *Neotoma albigula*, which consumes juniper in the field, but is unable to tolerate a high juniper diet. We measured Pgp activity as the difference in accumulation of a known Pgp substrate, digoxin, between everted sections of small intestine exposed to ethanol vehicle control and a maximal level of a known competitive inhibitor of Pgp, cyclosporin A. We estimated intestinal capacity by averaging Pgp activity along the intestine and multiplying by total small intestine mass. These first measures of Pgp in wild mammals show a significant difference among species with the juniper specialist, *N. stephensi*, exhibiting a 2.4 fold higher capacity than the generalist, *N. albigula*. This result suggests that Pgp may play a role in the ability of *N. stephensi* to tolerate juniper.

© 2004 Elsevier Inc. All rights reserved.

Keywords: P-glycoprotein; *Neotoma*; Woodrat; *Juniperus monosperma*; Multi-drug resistance

1. Introduction

Biochemical defenses against ingested toxins vary greatly among species and populations within species (Walker, 1980; Mead et al., 1985; Van Straalen, 1994; Fossi et al., 1995). A general hypothesis regarding this variation is that populations or species that are evolutionarily exposed to toxins and have developed a tolerance, should exhibit higher levels of biochemical defenses. Much of the evidence in support of this hypothesis comes from studies of the phase I and II biotransformation enzymes among major dietary groups such as omnivorous and piscivorous birds (Knight et al., 1981; Walker and Knight, 1981; Ronis and Walker,

1985; Fossi et al., 1995). Lower mixed function oxidase activity in piscivorous birds has been attributed to a lower evolutionary exposure to dietary toxins (Walker and Knight, 1981) and increased omnivory has been correlated with increased CYP 2B activity, which is responsible for detoxification of xenobiotics (Knight et al., 1981; Ronis and Walker, 1985; Fossi et al., 1995). None of these broad scale comparisons, however, accounted for phylogeny (Walker, 1980; Walker and Knight, 1981; Ronis and Walker, 1985). Furthermore, there have been no comparisons within other dietary groups (e.g., herbivores). Although we might expect dietary specialists and generalists to differ in defense mechanisms that act on a small range of compounds that differ in their diets, predicting the outcome for a more general defense mechanism that recognizes a broad range of substrates is more difficult. Without data to the contrary we cannot assume that a specialist and generalist differ in

* Corresponding author. Tel.: +1 608 263 9319; fax: +1 608 262 6099.

E-mail address: wkarasov@wisc.edu (W.H. Karasov).

overall toxin ingestion, which precludes formulating a general hypothesis regarding dietary specialization.

As more general biochemical defenses have been discovered and described in different species (Taipalensuu et al., 2001; Scotto, 2003), their pattern of variation among populations or species can be evaluated within the above context. A recently described general biochemical defense mechanism is Permeability-glycoprotein (Pgp), which has affinity for numerous and varied compounds that include toxins of plant origin (Ford and Hait, 1990). Pgp is a membrane bound transporter that actively expels toxins that diffuse into the cell membranes of barrier epithelial cells in the small intestine, blood–brain barrier, blood testis barrier, and in organs that routinely deal with toxins such as liver and kidney (Gottesman and Pastan, 1993; Sarkar, 1995; Hunter and Hirst, 1997; Brady et al., 2002).

Woodrats of the *Neotoma* genus provide an excellent opportunity to investigate a general defense mechanism like Pgp in a wild mammalian herbivore system. Both dietary generalists and specialists that consume toxin laden plants, exist within *Neotoma* (Atsatt and Ingram, 1983; Dial, 1988; Edwards et al., 2001). *Neotoma albigula*, a generalist, and *N. stephensi*, a juniper specialist, are sympatric and differ in their dietary inclusion (Dial, 1988) and tolerance (Dearing et al., 2000) of *Juniperus monosperma* leaves. Although we lack a complete understanding of the chemical composition in either the specialist or generalist diet, there is evidence indicating a difference in overall toxin load. *J. monosperma* is high in numerous terpenoids and phenolic compounds (Adams et al., 1981; Adams, 1994), and the dominant monoterpene, alpha-pinene, is known to cause toxicity in mammals (Sperling et al., 1967; Savolainen and Pfaffli, 1978; Dearing et al., 2000). Although *N. albigula* also consumes *J. monosperma* (24% of diet), 29% of the diet is made up of *Yucca baileyi* (Dial, 1988). There are no data on the toxicity of *Y. baileyi*, so we must rely on data from a congener. *Yucca shidigera* is used as a feeding supplement in domestic mammals (i.e. pigs, cattle, rabbits) and results indicate only positive effects including mass gain and increased digestive efficiency (Hussain and Cheeke, 1995; Hussain et al., 1996; Killeen et al., 1998; Min et al., 2001; Hong et al., 2001). Added to the dietary data are physiological comparisons between *N. stephensi* and *N. albigula* gavaged with alpha-pinene that indicate *N. stephensi* exhibits a lower plasma concentration of alpha-pinene (Sorensen and Dearing, 2003), and an increased excretion of unchanged alpha-pinene in the feces (Sorensen et al., 2004). The authors implicated a difference in intestinal absorption possibly mediated by Pgp. Together these data led us to hypothesize that *N. stephensi* will exhibit higher Pgp activity than *N. albigula*.

To test this hypothesis, we measured Pgp activity in the small intestine, which is the first Pgp expressing organ that could serve as a barrier to absorption of ingested toxins. To strengthen our comparison within an evolutionary/phylogenetic context, we included the more distantly related

congener (Edwards et al., 2001; Edwards and Bradley, 2002), *N. cinerea*, that also consumes a generalist diet (Johnson and Hansen, 1979; Haufler and Nagy, 1984; Frase and Sera, 1993). Among the three species we compared intestinal Pgp capacity, which we define as the activity of Pgp over the entire small intestine.

2. Materials and methods

2.1. Animals

Specialist (*Neotoma stephensi*) (2 male, 3 female) and generalist (*N. albigula*) (4 male, 3 female) woodrats were trapped outside the south border of Wupatki National Park, 45 km NE of Flagstaff, AZ (35°30' N 111°27' W) from the same area with equal access to *J. monosperma*. The diets of these species were described in an extensive study by Dial (1988). *Neotoma cinerea* (2 male, 3 female) were trapped at a private residence near Heber City, Summit, UT. *N. cinerea* is a dietary generalist (Johnson and Hansen, 1979; Haufler and Nagy, 1984; Frase and Sera, 1993). The caches of the *N. cinerea* suggested they were foraging on *Opuntia* clades, sagebrush (*Artemisia tridentata*) and various non-native garden plants. Animals were transported to the University of Utah Animal Facility and were housed individually in shoe box cages (48×27×20 cm) with bedding and cotton batting at 20 °C on a 12L:12D photoperiod. All animals were fed Teklad ground rabbit chow (formula 2120) and water ad libitum. Animals were in captivity for 6–12 months prior to experiments. All experiments on woodrats conformed to University of Utah IACUC protocol #04-02012.

2.2. Chemicals

[³H]-digoxin and [¹⁴C]-polyethylene glycol were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). [¹⁴C]-D-glucose and [³H]-L-glucose were purchased from Moravak (Brea, CA, USA). Phloridzin was purchased from Sigma Aldrich (St. Louis, MO, USA) and cyclosporin A (CsA) was purchased from Qbiogene (Carlsbad, CA, USA).

2.3. Western blot analysis for Pgp

Pgp has previously been found in numerous tissues including the small intestine of Sprague–Dawley rat (i.e. Ambudkar et al., 1999). We conducted Western blot analysis to verify that a Pgp homolog was present in the small intestine of the three species of woodrat in this study.

Brush border membrane vesicles (BBMV) for analysis of membrane-bound Pgp were prepared from frozen tissues as previously described (Barnes, 2001). Frozen tissues were homogenized with a SDT 1810 Tekmar Tissumizer (Cincinnati, OH, USA) in ice-cold homogenization buffer (300 mM mannitol, 12 mM Tris/HCl, 5 mM EGTA, pH 7.1–7.2

sterile filtered, with, 2 $\mu\text{g}/\text{mL}$ aprotinin, 2 $\mu\text{g}/\text{mL}$ leupeptin, 1mM PMSF). The homogenate was centrifuged at $1000\times g$ for 5 min at 4 °C. The supernatant was made 10 mM with MgCl_2 , placed on ice for 15 min, then centrifuged at $2300\times g$ for 15 min at 4 °C. Membrane vesicles were isolated by centrifugation at $23,000\times g$ for 30 min at 4 °C, re-suspended in storage buffer (300mM mannitol, 20 mM HEPES, 10 mM Tris/HCl, 4 mM MgCl_2 , pH 7.4, sterile filtered), aliquoted, and stored at -70 °C until analyzed.

For the immunodetection of Pgp, 10–30 μg of solubilized BBMV protein were electrophoresed on vertical 7% polyacrylamide mini-gels and Western blotting of the separated proteins was performed using standard protocols. We used rabbit polyclonal primary antibodies raised against human Pgp (C-219, Signet Pathology Systems, Dedham, MA, USA) followed by anti-rabbit IgG conjugated with horse-radish peroxidase (HRP). Pgp is highly conserved among a diversity of species and the C-219 antibody has been used to detect Pgp homologs in mammals (Georges et al., 1990; Kamath and Morris, 1998; Veau et al., 2002), birds (Barnes, 2001), fish (Doi et al., 2001), and even a mollusk (Keppler and Ringwood, 2001). Immunoreactive proteins were visualized using photographic film. Membrane vesicles prepared from the rat hepatoma cell line, H-4-II-E, were run with the samples from *Neotoma* species to verify the molecular weight of Pgp. We have used this method previously to detect Pgp homologues in a variety of species including Japanese quail (*Coturnix japonica*), chicken, and American robin (*Turdus migratorius*) (unpublished data). We used the gels only to verify presence of a Pgp homolog, not to quantify protein expression.

2.4. Measures of Pgp activity using tissue accumulation of digoxin

For tissue preparation and mounting, we followed closely the procedures described in (Karasov and Diamond, 1983; Karasov et al., 1985). Briefly, animals were euthanized and the intestine from the stomach to the proximal end of the caecal attachment was quickly removed (4–6 min) and flushed with ice-cold Ringer solution. Solution composition (in mM) was 50 mannitol, 100 NaCl, 4.7 KCl, 2.5 CaCl_2 , 1.2 KH_2PO_4 , 1.2 MgSO_4 , and 20 NaHCO_3 gassed with 95% O_2 and 5% CO_2 . Osmolality (mosM) was 290. After measuring intestine length, the intestine was everted, and short sleeves (1.5 cm long) were cut and then mounted on stainless steel rods by tying the tissue down over grooves exactly 1-cm apart. During this preparatory stage, mounted and un-mounted tissues were kept in cold Ringer gassed with 95% O_2 and 5% CO_2 .

Just prior to a flux measurement, a rod with a mounted tissue was pre-incubated in 37 °C Ringer solution bubbled with 95% $\text{O}_2/5\%$ CO_2 for 2 min (Pgp assays) or 6 min (D-glucose uptake). Pre-incubation solutions also contained either test compounds or ethanol vehicle control to maximize tissue exposure to our treatments. The tissue was then

transferred to a flat-bottomed water-jacketed test tube containing 8 mL gassed Ringer with radiolabeled probe and marker solutes at 37 °C. The tube contained a stir bar spinning rapidly (approx. 1000 rpm) to control the unstirred layer thickness (Karasov and Diamond, 1983). Probe molecules used were a specific substrate of Pgp, [^3H]-digoxin (9.1 kBq/mL), for measuring Pgp activity (Sababi et al., 2001; Stephens et al., 2001), or [^{14}C]-D-glucose (1.1 kBq/mL) for measuring D-glucose uptake activity. To correct for non-absorbed probe in adherent mucosal fluid, we used tracer concentrations of a membrane-impermeable marker [^{14}C]-polyethylene glycol (PEG; MW=4000; 1.1 kBq/mL), or in the case of [^{14}C]-D-glucose uptake we used [^3H]-L-glucose (31.4 kBq/mL) to correct for both non-absorbed probe in adherent mucosal fluid and diffusive flux (Karasov and Diamond, 1983). After 12 min (Pgp assay) or 4 min (D-glucose uptake) the incubation was terminated. We used longer incubation times with [^3H]-digoxin (12 min) than are generally used for measuring sugar and amino acid uptake (generally 1–4 min) because digoxin is passively absorbed relatively slowly, and because the target of our measurements was not the accumulation in tissue per se, but a difference in digoxin accumulation in the presence and absence of a competitor, which would reflect digoxin excluded by Pgp (Green et al., in press). After incubation, tissues were then either simply blotted (Pgp assay) or rinsed in stirred cold Ringer and then blotted (D-glucose uptake). The mounted tissue was cut from between the two grooves with a razor blade, weighed, incubated in 1 mL of tissue solubilizer (Solucene-350, Packard, Meriden, CT, USA), and counted in 10 mL of scintillation cocktail (Ecolume, Packard) with 0.5% by volume acetic acid on a Beckman LS 5801 (Beckman Coulter) with counting channels set to minimize spill (counts of the alternate isotope appearing in the same counting channel) and programmed with a standard curve to correct for background quench. Calculation of digoxin accumulation followed (Karasov and Diamond, 1983). For simplicity of expression, we assumed a digoxin concentration of 1 fmol per μL incubation solution (it was actually approximately 0.75 fmol/ μL), yielding units of fmol digoxin accumulated per mg tissue. Total intestine mass was estimated as the product of the summed mass of the 16, 1-cm tissue samples and the ratio of total intestine length: 16 cm.

All experiments were conducted between April 11, 2003 and May 7, 2003 within which we blocked for time and randomized species within each time block. One experiment was conducted each day between 12:00 noon and 16:00 h. Two types of trials, CsA dose response and tissue viability, were used to optimize the method in woodrats. They are described in detail below.

2.5. CsA dose response

First, we measured [^3H]-digoxin uptake in the absence and presence of increasing concentration of CsA (1, 5, 10

μM , and saturated [$\sim 15 \mu\text{M}$] CsA), a known competitive inhibitor of Pgp (Lan et al., 1996). This was done because digoxin accumulation in the tissue is a balance between passive diffusion into and Pgp-mediated export out of the cells. The desired measure of Pgp activity index was the difference in digoxin accumulation per mg tissue between a tissue with Pgp maximally inhibited by the competitor (mimicking a tissue without Pgp) and the accumulation in an adjacent tissue without the competitor (ethanol vehicle control). This estimates the amount of digoxin that would have diffused into the tissue if Pgp were not present. In these trials, we used adjacent sleeves of intestine from the mid-intestinal region. The preincubation was 2 min in control (0.15% ethanol) or CsA-containing Ringer solution and the incubation was 12 min in the same solutions with [^3H]-digoxin.

2.6. Determination of tissue viability and damage

Second, as checks on the structural and functional integrity of the intestinal sleeves, we histologically inspected adjacent tissues in two individuals per species for signs of villus damage (Starck et al., 2000). Also, we measured mediated D-glucose uptake over the same combined pre-incubation and incubation time, using adjacent tissues exposed to CsA (15 μM), vehicle control (0.15% ethanol), and phloridzin (1 mM), a known inhibitor of the intestinal glucose transporter SGLT-1. Histology preparations indicated that woodrat intestinal tissue was not damaged during the experimental procedure for any of the three species. Villi in experimental sleeves were shorter than in sleeves that were not exposed to the experimental procedure, but remained intact (data not shown). Glucose uptake data are presented in Results.

2.7. Total Pgp capacity

Along with a measure of Pgp activity in the mid-gut, we included measures in the proximal and distal regions. To estimate the intestine's total capacity, we computed a weighted average of Pgp activity per mg intestine ($0.25 \times \text{proximal} + 0.5 \times \text{mid} + 0.25 \times \text{distal}$) and multiplied that by the small intestine mass. We tested differences among species by first comparing intestinal Pgp capacity using body mass as a covariate after examining the interaction between body mass and Pgp capacity. Then, because many physiological processes are scaled to body mass, we also scaled Pgp capacity to body mass using $(1 \text{ kg})^{0.54}$, because this is how food (and hence toxin) intake scale with mass in woodrat species (Smith, 1995), and $(2 \text{ kg})^{0.75}$, a generally accepted metabolic mass scalar.

2.8. Statistical analyses

When possible, we analyzed treatment effect with repeated measures, two-factor ANOVA with treatment and

species as factors. When all treatments or concentrations were not represented in all animals we determined treatment effect with a one-way, two-factor ANOVA with treatment and species as the two factors. We also tested differences in intestinal Pgp capacity using ANCOVA with log [intestinal Pgp capacity] with log [body mass] as the covariate after examining the interaction, and using one-way ANOVA on intestinal Pgp capacity scaled to body mass. We used Fisher's LSD for pair-wise comparisons when global tests resulted in $P < 0.05$. Plots of residuals were visually inspected to verify a random distribution. A $P < 0.05$ was considered significant and $0.05 < P < 0.10$ a trend. All data are reported as mean \pm S.E., n = sample size.

3. Results

Western blot analysis confirmed the presence of a Pgp homolog in the small intestine of all three woodrat species (Fig. 1) as expected based on our previous results with other species (unpublished data). The main difference seen among the species was that Pgp from *N. stephensi* migrated further down the blot than either *N. albigula* or *N. cinerea* and we will discuss this subsequently. We removed one *N. cinerea* from the study due to consistently low values for mediated D-glucose uptake indicating a loss of functional integrity in intestinal sleeves.

Accumulation of tracer [^3H]-digoxin, corrected for adherent label, increased sublinearly with increasing concentration of CsA in the solution for intestinal sleeves from all three species (Fig. 2). Cyclosporin A precipitated from solution at around 15 μM , but [^3H]-digoxin accumulation at $\sim 15 \mu\text{M}$ was not significantly higher than at 10 μM . Therefore, in subsequent experiments, we used 10 μM CsA as the maximal inhibitory concentration, and calculated the index of Pgp activity as the difference between accumulation of [^3H]-digoxin in the presence and absence of 10 μM

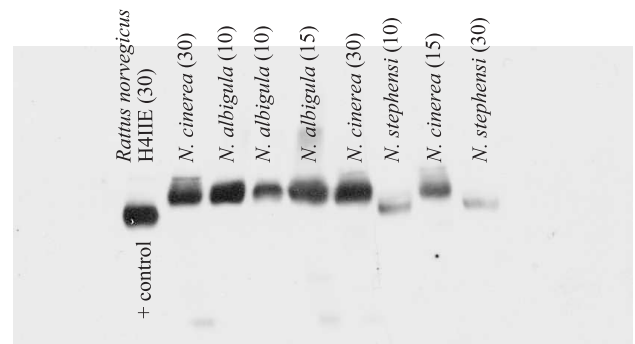


Fig. 1. Western blot analysis of Pgp homologs using protein from brush border membrane vesicles concentrated from mid-intestinal tissue of *N. albigula*, *N. cinerea*, and *N. stephensi*. Membrane vesicles from the rat (*Rattus norvegicus*) hepatoma cell line, H-4-II-E, were used as positive control and to verify molecular weight of the Pgp homolog. Numbers in parentheses are μg of total protein loaded in the lane.

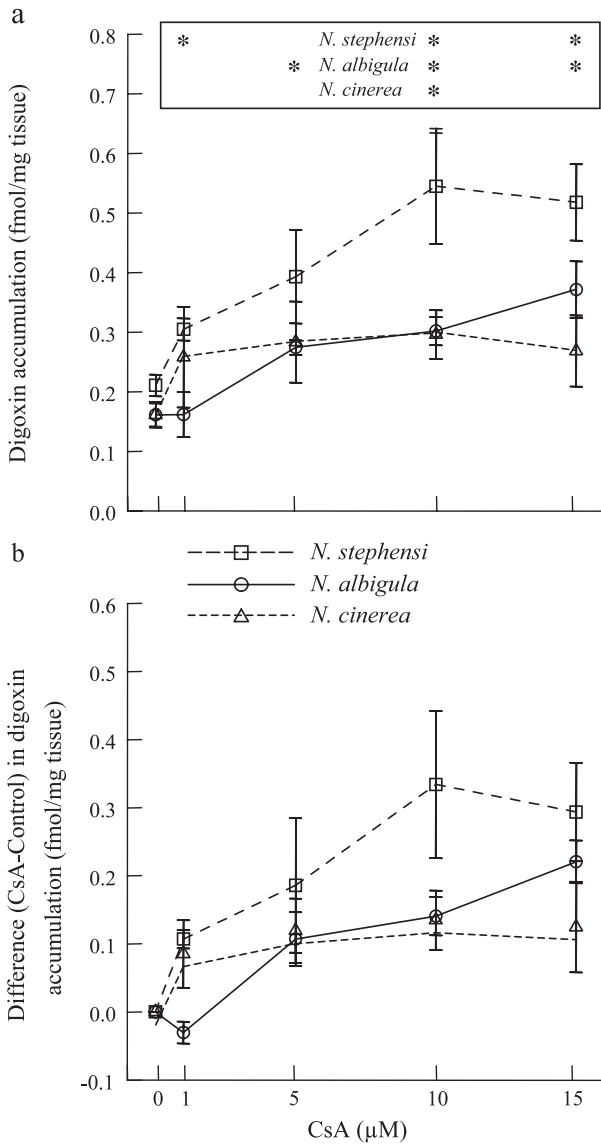


Fig. 2. (a) Digoxin accumulation (fmol/mg tissue) in mid-intestinal sleeves exposed to cyclosporin A (1, 5, 10, ~15 μM) or vehicle control (0 CsA; 0.15% ethanol) and (b) the difference in digoxin accumulation (fmol/mg tissue) between CsA treated and control tissues during 2 min pre-incubations and 12 min incubations in *Neotoma albigula* ($n=4-7$ sleeves/concentration), *N. cinerea* ($n=3-4$ sleeves/concentration), and *N. stephensi* ($n=3-5$ sleeves/concentration). All three species show sublinear increase in digoxin accumulation and in the difference between tissues exposed to CsA and control, and the relationships reach an asymptote by 10 μM (repeated measures ANOVA within species: digoxin accumulation at 10 μM CsA > 0 μM CsA, $P < 0.05$; digoxin accumulation at 10 and 15 μM CsA not significantly different, $P > 0.1$). In the box above the plots, significant differences ($P < 0.05$) from control within species are indicated by (*) based on paired Student's t -tests in (a) or one sample Student's t -tests in (b). Points are mean \pm S.E.

CsA in adjacent intestinal tissues (i.e., digoxin excluded per mg wet tissue mass).

Although we assumed that the enhancement of digoxin accumulation by CsA is due to competitive inhibition of digoxin's exclusion by Pgp, it is also conceivable that CsA acts by depressing ATP generation, which powers the

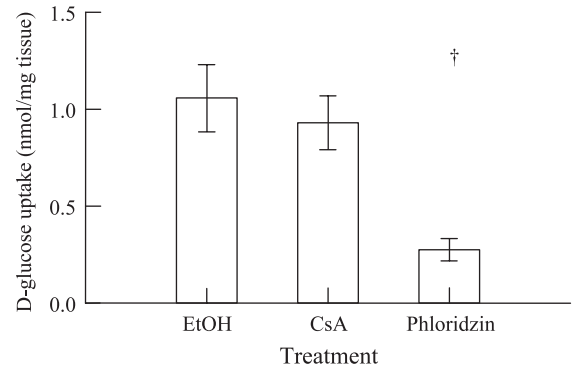


Fig. 3. D-Glucose uptake in mid-intestinal sleeves exposed to vehicle control (0.15% ethanol), CsA (15 μM), and phloridzin (1mM) during 10 min pre-incubation and 4 min incubation in *N. albigula*, *N. cinerea*, and *N. stephensi*. (†) indicates significant differences ($P < 0.01$) from control after significant repeated measures ANOVA ($F_{2,14}=12.217$, $P=0.001$) with no significant interactions.

expulsion of compounds by Pgp (Ambudkar et al., 1999). To test for this nonspecific effect, we compared mediated uptake of 1 mM D-glucose, which is also powered by ATP generation, in the presence and absence of 15 μM CsA (in 0.15% ethanol as vehicle). We also included a measure of D-glucose uptake in the presence of phloridzin (1mM), a potent inhibitor of the D-glucose transporter SGLT-1, to

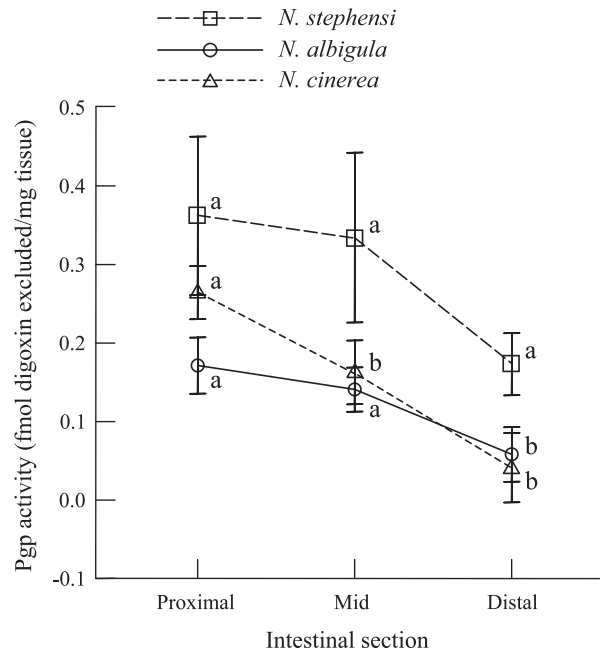


Fig. 4. Variation in Pgp activity (fmol digoxin excluded/mg tissue) along the intestinal tract of *N. albigula* ($n=7$), *N. cinerea* ($n=4$), and *N. stephensi* ($n=5$) as determined by the difference in digoxin accumulation in intestinal sleeves exposed to vehicle control (ethanol) or 10 μM CsA during 2 min pre-incubations and 12 min incubations. *N. albigula* and *N. cinerea*, but not *N. stephensi* exhibit significant differences in Pgp activity from proximal to distal sections of the intestine (repeated measures ANOVA within species, $P=0.005$, $P=0.013$, $P=0.091$, respectively). Different letters (a, b, c) indicate significant differences (pair wise comparisons, $P < 0.05$) among intestinal sections within each species. Points are mean \pm S.E.

Table 1

Body mass, small intestine length and mass from stomach to caeca, and intestinal Pgp capacity of *Neotoma albigula*, *N. cinerea*, and *N. stephensi*

Species	Body mass (g)	Small intestine length (cm)	Small intestine mass (g)	Intestinal Pgp capacity (fmol digoxin excluded)	Intestinal Pgp capacity (fmol digoxin excluded/kg ^{0.54})	Intestinal Pgp capacity (fmol digoxin excluded/kg ^{0.75})
<i>N. albigula</i> (n=7)	192.8±7.3 (a)	38.6±1.6 (a)	3.2±0.3 (a)	414±82 (a)	993±247 (a)	1397±359 (a)
<i>N. cinerea</i> (n=4)	293.9±26.1 (b)	53.5±2.7 (b)	5.2±0.4 (b)	803±49 (a,b)	1563±327 (a,b)	2030±476 (a)
<i>N. stephensi</i> (n=5)	182.0±19.1 (a)	42.2±0.4 (a)	3.4±0.2 (a)	955±186 (b)	2404±293 (b)	3457±425 (b)
Statistics	$F_{2,13}=12.4$ $P=0.001$	$F_{2,13}=19.3$ $P<0.001$	$F_{2,13}=13.6$ $P=0.001$	$F_{2,12}=6.2$ $P=0.014^a$	$F_{2,13}=6.8$ $P=0.010$	$F_{2,13}=6.9$ $P=0.009$

Different letters in parentheses (a or b) indicate significant differences among species ($P<0.05$) within each column from pair wise comparisons following significant one-way ANOVA, unless otherwise noted.

^a ANCOVA of log [intestinal Pgp capacity] with log [body mass] as the covariate.

confirm that our measure was indeed of active D-glucose uptake. D-glucose uptake was significantly depressed by phloridzin ($P=0.004$), but not by CsA ($P=0.148$) (Fig. 3; repeated measures ANOVA, $F_{2,14}=12.217$, $P=0.001$, followed by pair wise comparisons).

N. albigula and *N. cinerea*, but not *N. stephensi* exhibited significant differences in Pgp activity from proximal to distal sections of the intestine (Fig. 4; repeated measures ANOVA within species: $P=0.005$, $P=0.013$, $P=0.091$, respectively). Data on body mass, small intestine length and mass, and intestinal Pgp capacity are summarized in Table 1. We calculated intestinal Pgp capacity by multiplying the weighted average of Pgp activity along the intestine, by intestine mass. Because *N. cinerea* is significantly larger than either *N. stephensi* or *N. albigula* we included log [body mass] as a covariate in an ANCOVA of log [intestinal Pgp capacity]. The scaling (i.e. mass exponent) for the covariate, however, was much higher (1.31) than expected (0.54–0.75). Therefore, we also tested the difference by scaling intestinal Pgp capacity to body mass (kg^{0.54}) based on the scaling of food intake in three *Neotoma* species (Smith, 1995), and to body mass (kg^{0.75}), a generally recognized scaling factor for metabolic processes. By whatever comparative analysis used, *N. stephensi* exhibited a significantly higher intestinal Pgp capacity than *N. albigula* (Table 1).

4. Discussion

In this study, we made the first measures of Pgp activity in wild mammals and the first comparison of intestinal Pgp capacity among mammalian herbivores that differ in dietary specialization. Our data support the hypothesis that the juniper specialist *N. stephensi* has greater Pgp activity along the small intestine (intestinal Pgp capacity) than the sympatric generalist *N. albigula*. First we discuss the validity of the method in *Neotoma*, then the possible reasons for the observed differences in Pgp and potential future research.

These data on Pgp activity are the first in wild mammals, so a concern is the validity of the method and viability of woodrat intestinal tissue throughout the experimental procedure. Evidence that suggests our method was successful in measuring Pgp activity in these woodrats include: (1) A Pgp

homolog is found in woodrat intestinal tissue as indicated by Western blot analysis. (2) We observed the expected sublinear digoxin accumulation with an increasing dose of the known Pgp competitive inhibitor, CsA, that reached an asymptote by 10 μ M in each of the three species. These results match our previous results in chicken and Sprague–Dawley rat (Green et al., in press). (3) Tissues withstood the experimental procedure both structurally, based on histological preparations that show intact villi, and functionally, based on active D-glucose uptake over the experimental time period with or without CsA. An indication of a loss in functional integrity allows us to remove a study animal as was done with one *N. cinerea* in this study.

Pgp expression and activity in rodents can be induced by substrates administered in the diet (Salphati and Benet, 1998; Sandstrom and Lennernas, 1999), and Pgp induction may play a role in how *Neotoma* woodrats deal with dietary toxins. We feel that our data represent basal levels of Pgp activity as all animals were in captivity for 6–12 months prior to the experiment, feeding on the same chow diet. Of interest would be whether there are differences in the ability of each species to induce Pgp when exposed to dietary substrates.

Our finding that the juniper specialist, *N. stephensi*, has significantly higher intestinal Pgp capacity than *N. albigula* adds to the mounting evidence that these two species differ in their treatment of ingested toxins. Dearing et al. (2000) found that *N. albigula* produces a greater volume of more acidic urine compared to *N. stephensi* when on the same diet high in alpha-pinene, the dominant monoterpene in *J. monosperma*. This indicates a difference in the detoxification pathway. Supporting this assertion are preliminary data from S. Haley (unpublished data) suggesting a difference in the activity levels of phase I and II enzymes in liver tissue of these two species. Sorensen and Dearing (2003), using a pharmacokinetic approach, compared the in vivo absorption of alpha-pinene in *N. stephensi* and *N. albigula*. Their results suggested less absorption of alpha-pinene in the specialist than the generalist. Adding to these results is a recent finding that *N. stephensi* excretes more unchanged alpha-pinene in the feces than *N. albigula* (Sorensen et al., 2004). These latter two studies implicated Pgp as a potential mechanism that decreases intestinal absorption of alpha-pinene to a greater extent in the specialist than the generalist.

There are two issues that should be considered when extrapolating our data to the observed difference in juniper tolerance. First, extrapolation of these data presupposes juniper toxins are recognized by Pgp. Establishing a causal link between Pgp and alpha-pinene absorption, for example, requires that we determine whether Pgp recognizes alpha-pinene as a substrate, which is the subject of ongoing research. Second, although intestinal Pgp capacity as we defined it is significantly higher in *N. stephensi*, this does not necessarily translate into decreased toxin absorption at the whole animal level because there may be other transporters that influence the absorption and distribution of toxins. For example, multidrug resistance-associated proteins (MRPs) or MRP-like organic anion transporting polypeptides (OATPs) could be present in intestinal cells (Walters et al., 2000; Cattori et al., 2001) and transport digoxin (Kullak-Ublick and Becker, 2003; Mikkaichi et al., 2004) and thus influence digoxin accumulation. This might explain why the baseline intracellular digoxin levels of the specialist *N. stephensi* were not significantly different from those of the generalist *N. albigula* (see Fig. 2a), rather than being lower as might be expected if Pgp expression were the only factor influencing those levels. Although other factors might influence the baseline digoxin levels in our experiments, to the best of our knowledge the CsA inhibitable component of digoxin uptake is most specific to Pgp, which is why we think that the specialist exhibits higher Pgp activity. But, the diversity of toxin transporting and biotransforming polypeptides underscores how complicated it can be to investigate biochemical defense against ingested toxins. New techniques that can simultaneously measure activities of numerous genes or gene products, such as DNA micro-arrays, may prove to be more powerful in comparative studies than comparisons of only one enzyme or transporter among species.

Identifying the underlying mechanism for the observed difference in Pgp activity requires more study. We found the Pgp homologs among woodrat species migrated differently down the gel (Fig. 1). This could be due to post-translational modification of Pgp such as glycosylation that is not known to affect function (Ambudkar et al., 2003), or differences in isoforms. Future studies should look more closely at specific differences in the Pgp protein in *Neotoma* and how they relate to function. We did not draw any conclusions from relative blot intensity among the species because we had a small sample size limited to mid-intestinal tissue that was not consistently collected from the same region as our activity measures.

An ancillary goal of this research was to continue acquiring data within the *Neotoma* genus to better elucidate the evolutionary trajectory of biochemical defense mechanisms. The intermediate level of intestinal Pgp capacity in the more distantly related generalist *N. cinerea*, however, makes it difficult to determine the ancestral trait in *Neotoma*. It may be that the generalist diets of *N. cinerea* and *N. albigula* are not equivalent in overall toxin load.

Clearly, more data on dietary toxin load and Pgp activity are needed before we can confidently predict Pgp levels based on dietary specialization. Fortunately, there are a number of *Neotoma* species for which we have data on wild diet (i.e. Thompson, 1982; Atsatt and Ingram, 1983; Karasov, 1989; Post, 1992; McMurry et al., 1993), that can be tested to address this relationship.

Variation in the tolerance of plant defensive chemicals among mammals is complicated, probably reflecting the diversity of ingested plant compounds and the diversity of the herbivores' physiological and biochemical adaptations. Studies of the variation among species in phase I and II detoxifying enzymes have thus far dominated the literature (i.e. Walker, 1980; Knight et al., 1981; Ronis and Walker, 1985; Walker and Ronis, 1989; Van Straalen, 1994). Our study underscores that interspecies variation in Pgp activity adds to this complexity. Also, to date there have been 10 ABC-superfamily transport proteins shown to confer cellular resistance to toxins (Taipalensuu et al., 2001; Scotto, 2003). Research on these transporters has been limited to their role in human physiology, so we lack data on non-laboratory animals. The similarities in regulation of the ABC-transporters (Scotto, 2003) and the potential link between Pgp and phase I detoxifying enzymes (Cummins and Benet, 2001; Schuetz and Strom, 2001) suggest a coordinated response that may prove to be very complex. The everted sleeve technique has proven useful to determine the function of Pgp and may be modified to investigate other transporters, providing a tissue-level check of data from in vivo, cell culture, or DNA micro-array techniques.

Acknowledgements

This research was supported by NSF grant IBN-0236402 to M.D.D., and USDA (Hatch) WISO4322 and NSF IBN-9723793 and IBN-0216709 to W.H.K. A.K.G. was supported by an NSF pre-doctoral fellowship. We thank B. Darken, J. Allen, S. Brown, E. Heward, and M. Wong for help with experiments, and S. O'Grady, J. Sorensen, C. Turnbull, and J. McLister for help with animal husbandry. We also thank P. Bandyopadhyay, T. Olivera, D. Bowling, and M. Bastiani for use of equipment and lab space. Three anonymous reviewers improved the manuscript. All research conformed to University of Utah Institutional Animal Care and Use Committee protocols.

References

- Adams, R.P., 1994. Geographic-variation in the volatile terpenoids of *Juniperus monosperma* and *J. osteosperma*. *Biochem. Syst. Ecol.* 22, 65–71.
- Adams, R.P., Zanoni, T.A., Von, R.E., Hogge, L., 1981. The southwestern USA and northern Mexico 1-seeded junipers: their volatile oils and evolution. *Biochem. Syst. Ecol.* 9, 93–96.

- Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramachandra, M., Pastan, I., Gottesman, M.M., 1999. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu. Rev. Pharmacol. Toxicol.* 39, 361–398.
- Ambudkar, S.V., Kimchi-Sarfaty, C., Sauna, Z.E., Gottesman, M.M., 2003. P-glycoprotein: from genomics to mechanism. *Oncogene* 22, 7468–7485.
- Atsatt, P.R., Ingram, T., 1983. Adaptation to oak and other fibrous, phenolic-rich foliage by a small mammal, *Neotoma fuscipes*. *Oecologia* (Berlin) 60, 135–142.
- Barnes, D.M., 2001. E. coli lipopolysaccharide stimulates the expression of P-glycoprotein in the chicken liver, kidney and spleen. *FASEB J.* 15, A647.
- Brady, J.M., Cherrington, N.J., Hartley, D.P., Buist, S.C., Li, N., Klaassen, C.D., 2002. Tissue distribution and chemical induction of multiple drug resistance genes in rats. *Drug Metab. Dispos.* 30, 838–844.
- Cattori, V., van Montfoort, J.E., Stieger, B., Meier, P.J., Hagenbuch, B., 2001. Localization of organic anion transporting polypeptide 4 (Oatp4) in rat liver and comparison of its substrate specificity with Oatp1, Oatp2, and Oatp3. *Eur. J. Physiol.* 443, 188–195.
- Cummins, C.L., Benet, L.Z., 2001. P-glycoprotein (P-gp) plays a significant role in enhancing intestinal metabolism by cytochrome P450 3A4 (CYP 3A4). *FASEB J.* 15, A548.
- Dearing, M.D., Mangione, A.M., Karasov, W.H., 2000. Diet breadth of mammalian herbivores: nutrient versus detoxification constraints. *Oecologia* 123, 397–405.
- Dial, K.P., 1988. Three sympatric species of *Neotoma*: dietary specialization and coexistence. *Oecologia* (Heidelberg) 76, 531–537.
- Doi, A.M., Holmes, E., Kleinow, K.M., 2001. P-glycoprotein in the catfish intestine: inducibility by xenobiotics and functional properties. *Aquat. Toxicol.* 55, 157–170.
- Edwards, C.W., Bradley, R.D., 2002. Molecular systematics of the genus *Neotoma*. *Mol. Phylogenet. Evol.* 25, 489–500.
- Edwards, C.W., Fulhorst, C.F., Bradley, R.D., 2001. Molecular phylogenetics of the *Neotoma albigula* species group: further evidence of a paraphyletic assemblage. *J. Mammal.* 82, 267–279.
- Ford, J.M., Hait, W.N., 1990. Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol. Rev.* 42, 155–199.
- Fossi, M.C., Massi, A., Lari, L., Marsili, L., Focardi, S., Leonzio, C., Renzoni, A., 1995. Interspecies differences in mixed-function oxidase activity in birds-relationship between feeding-habits, detoxication activities and organochlorine accumulation. *Environ. Pollut.* 90, 15–24.
- Frase, B.A., Sera, W.E., 1993. Comparison between plant species in bushy-tailed woodrat middens and in the habitat. *Great Basin Nat.* 53, 373–378.
- Georges, E., Bradley, G., Garipey, J., Ling, V., 1990. Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies. *Proc. Natl. Acad. Sci. U. S. A.* 87, 152–156.
- Gottesman, M.M., Pastan, I., 1993. Biochemistry of multidrug-resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* 62, 385–427.
- Green, A.K., Barnes, D.M., Karasov, W.H., 2005. A new method to measure intestinal activity of P-glycoprotein in avian and mammalian species. *J. Comp. Physiol., B* in press.
- Haufler, J.B., Nagy, J.G., 1984. Summer food-habits of a small mammal community in the pinyon-juniper ecosystem. *Great Basin Nat.* 44, 145–150.
- Hong, J.W., Kim, I.H., Moon, T.H., Kwon, O.S., Lee, S.H., Kim, Y.G., 2001. Effects of yucca extract and (or) far infrared emitted materials supplementation on the growth performance, serum characteristics and ammonia production of growing and finishing pigs. *Asian-Australas. J. Anim. Sci.* 14, 1299–1303.
- Hunter, J., Hirst, B.H., 1997. Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Adv. Drug Deliv. Rev.* 25, 129–157.
- Hussain, I., Cheeke, P.R., 1995. Effect of dietary yucca-schidigera extract on rumen and blood profiles of steers fed concentrate-based or roughage-based diets. *Anim. Feed Sci. Technol.* 51, 231–242.
- Hussain, I., Ismail, A.M., Cheeke, P.R., 1996. Effects of feeding yucca schidigera extract in diets varying in crude protein and urea contents on growth performance and cecum and blood urea and ammonia concentrations of rabbits. *Anim. Feed Sci. Technol.* 62, 121–129.
- Johnson, M.K., Hansen, R.M., 1979. Foods of cottontails and woodrats in south-central Idaho. *J. Mammal.* 60, 213–215.
- Kamath, A.V., Morris, M.E., 1998. Functional expression of P-glycoprotein in the hepatic canalicular membrane of developing rats. *J. Pharm. Sci.* 87, 300–305.
- Karasov, W.H., 1989. Nutritional bottleneck in a herbivore, the desert wood rat (*Neotoma lepida*). *Physiol. Zool.* 62, 1351–1382.
- Karasov, W., Diamond, J.M., 1983. A simple method for measuring intestinal solute uptake in vitro. *J. Comp. Physiol., B* 152, 105–116.
- Karasov, W.H., Solberg, D.H., Diamond, J.M., 1985. What transport adaptations enable mammals to absorb sugars and amino-acids faster than reptiles. *Am. J. Physiol.* 249, G271–G283.
- Keppeler, C., Ringwood, A.H., 2001. Expression of P-glycoprotein in the gills of oysters, *Crassostrea virginica*: seasonal and pollutant related effects. *Aquat. Toxicol.* 54, 195–204.
- Killeen, G.F., Madigan, C.A., Connolly, C.R., Walsh, G.A., Clark, C., Hynes, M.J., Timmins, B.F., James, P., Headon, D.R., Power, R.F., 1998. Antimicrobial saponins of yucca schidigera and the implications of their in vitro properties for their in vivo impact. *J. Agric. Food Chem.* 46, 3178–3186.
- Knight, G.C., Walker, C.H., Cabot, D.C., Harris, M.P., 1981. Activity of two hepatic microsomal enzymes in sea birds. *Comp. Biochem. Physiol., C* 68, 127–132.
- Kullak-Ublick, G.A., Becker, M.B., 2003. Regulation of drug and bile salt transporters in liver and intestine. *Drug Metab. Rev.* 35, 305–317.
- Lan, L.B., Ayes, S., Lyubimov, E., Pashinsky, I., Stein, W.D., 1996. Kinetic parameters for reversal of the multidrug pump as measured for drug accumulation and cell killing. *Cancer Chemother. Pharmacol.* 38, 181–190.
- McMurry, S.T., Lochmiller, R.L., Boggs, J.F., Leslie, D.M.J., Engle, D.M., 1993. Opportunistic foraging of eastern woodrats (*Neotoma floridana*) in manipulated habitats. *Am. Midl. Nat.* 130, 325–337.
- Mead, R.J., Moulden, D.L., Twigg, L.E., 1985. Significance of sulphhydryl compounds in the manifestation of fluoroacetate toxicity to the rat, brush-tailed possum, woylie and western grey-kangaroo. *Aust. J. Biol. Sci.* 38, 139–149.
- Mikkaichi, T., Suzuki, T., Onogawa, T., Tanemoto, M., Mizutamari, H., Okada, M., Chaki, T., Masuda, S., Tokui, T., Eto, N., Abe, M., Satoh, F., Unno, M., Hishinuma, T., Inui, K., Ito, S., Goto, J., Abe, T., 2004. Isolation and characterization of a digoxin transporter and its rat homologue expressed in the kidney. *Proc. Natl. Acad. Sci. U. S. A.* 101, 3569–3574.
- Min, T.S., Kim, J.D., Hyun, Y., Sohn, K.S., Heo, K.N., Han, I.K., 2001. Effects of environmentally friendly agents on growth performance, nutrient digestibility, nutrient excretion and carcass characteristics in growing-finishing pigs. *Asian-Australas. J. Anim. Sci.* 14, 540–547.
- Post, D.M., 1992. Change in nutrient content of foods stored by eastern woodrats (*Neotoma floridana*). *J. Mammal.* 73, 835–839.
- Ronis, M.J.J., Walker, C.H., 1985. Species variations in the metabolism of liposoluble organochlorine compounds by hepatic-microsomal monooxygenase—comparative kinetics in four vertebrate species. *Comp. Biochem. Physiol., C* 82, 445–449.
- Sababi, M., Borgia, O., Hultkvist-Bengtsson, U., 2001. The role of P-glycoprotein in limiting intestinal regional absorption of digoxin in rats. *Eur. J. Pharm. Sci.* 14, 21–27.
- Salphati, L., Benet, L.Z., 1998. Modulation of P-glycoprotein expression by cytochrome P450 3A inducers in male and female rat livers. *Biochem. Pharmacol.* 55, 387–395.

- Sandstrom, R., Lennernas, H., 1999. Repeated oral rifampicin decreases the jejunal permeability of R/S-verapamil in rats. *Drug Metab. Dispos.* 27, 951–955.
- Sarkar, M.A., 1995. Quercetin not only inhibits P-glycoprotein efflux activity but also inhibits CYP3A isozymes. *Cancer Chemother. Pharmacol.* 36, 448–449.
- Savolainen, H., Pfaffli, P., 1978. Effects of long-term turpentine inhalation on rat brain protein metabolism. *Chem. Biol. Interact.* 21, 271–276.
- Schuetz, E., Strom, S., 2001. Promiscuous regulator of xenobiotic removal. *Nat. Med.* 7, 536–537.
- Scotto, K.W., 2003. Transcriptional regulation of ABC drug transporters. *Oncogene* 22, 7496–7511.
- Smith, F.A., 1995. Scaling of digestive efficiency with body mass in *Neotoma*. *Funct. Ecol.* 9, 299–305.
- Sorensen, J.S., Dearing, M.D., 2003. Elimination of plant toxins by herbivorous woodrats: revisiting an explanation for dietary specialization in mammalian herbivores. *Oecologia* 134, 88–94.
- Sorensen, J.S., Turnbull, C.A., Dearing, M.D., 2004. A specialist herbivore (*Neotoma stephensi*) absorbs fewer plant toxins than does a generalist (*Neotoma albigula*). *Physiol. Biochem. Zool.* 77, 139–148.
- Sperling, F., Marcus, W.L., Collins, C., 1967. Acute effects of turpentine vapor on rats and mice. *Toxicol. Appl. Pharmacol.* 10, 8–20.
- Starck, J.M., Karasov, W.H., Afik, D., 2000. Intestinal nutrient uptake measurements and tissue damage: validating the everted sleeves method. *Physiol. Biochem. Zool.* 73, 454–460.
- Stephens, R.H., O'Neill, C.A., Warhurst, A., Carlson, G.L., Rowland, M., Warhurst, G., 2001. Kinetic profiling of P-glycoprotein-mediated drug efflux in rat and human intestinal epithelia. *J. Pharmacol. Exp. Ther.* 296, 584–591.
- Taipalensuu, J., Tornblom, H., Lindberg, G., Einarsson, C., Sjoqvist, F., Melhus, H., Garberg, P., Sjoström, B., Lundgren, B., Artursson, P., 2001. Correlation of gene expression of ten drug efflux proteins of the ATP-binding cassette transporter family in normal human jejunum and in human intestinal epithelial Caco-2 cell monolayers. *J. Pharmacol. Exp. Ther.* 299, 164–170.
- Thompson, S.D., 1982. Spatial utilization and foraging behavior of the desert woodrat, *Neotoma lepida lepida*. *J. Mammal.* 63, 570–581.
- Van Straalen, N.M., 1994. Biodiversity of ecotoxicological responses in animals. *Neth. J. Zool.* 44, 112–129.
- Veau, C., Faivre, L., Tardivel, S., Soursac, M., Banide, H., Lacour, B., Farinotti, R., 2002. Effect of Interleukin-2 on intestinal P-glycoprotein expression and functionality in mice. *J. Pharmacol. Exp. Ther.* 302, 742–750.
- Walker, C.H., 1980. Species variations in some hepatic microsomal enzymes that metabolize xenobiotics. Bridges, J.W., Chasseaud, L.F. *Progress in Drug Metabolism* vol. 5. John Wiley and Sons, pp. 113–164.
- Walker, C.H., Knight, G.C., 1981. The hepatic microsomal enzymes of sea birds and their interaction with liposoluble pollutants. *Aquat. Toxicol.* 1, 343–354.
- Walker, C.H., Ronis, M.J.J., 1989. The monooxygenases of birds, reptiles and amphibians. *Xenobiotica* 19, 1111–1121.
- Walters, H.C., Craddock, A.L., Fusegawa, H., Willingham, M.C., Dawson, P.A., 2000. Expression transport properties and chromosomal location of organic anion transporter subtype 3. *Am. J. Physiol.* 279, G1188–G1200.