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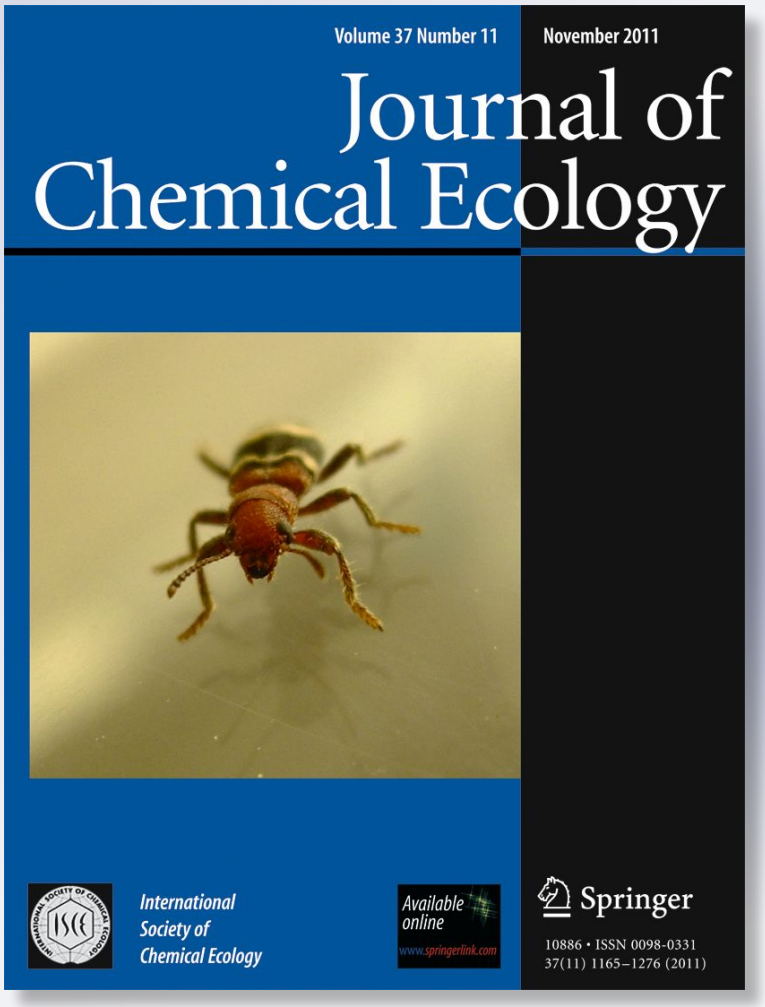

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Inhibition of Snowshoe Hare Succinate Dehydrogenase Activity as a Mechanism of Deterrence for Papyriferic Acid in Birch

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Abstract The plant secondary metabolite papyriferic acid (PA) deters browsing by snowshoe hares (*Lepus americanus*) on the juvenile developmental stage of the Alaska paper birch (*Betula neoalaskana*). However, the physiological mechanism that reduces browsing remains unknown. We used pharmacological assays and molecular modeling to test the hypothesis that inhibition of succinate dehydrogenase (SDH) is a mode of action (MOA) of toxicity of PA in snowshoe hares. We tested this hypothesis by measuring the effect of PA on the activity of SDH in liver mitochondria isolated from wild hares. In addition, we used molecular modeling to determine the specific binding site of PA on SDH. We found that PA inhibits SDH from hares by an uncompetitive mechanism in a dose-dependent manner. Molecular modeling suggests that inhibition of SDH is a result of binding of PA at the ubiquinone binding sites in complex II. Our results provide a MOA for toxicity that may be responsible for the concentration-dependent anti-feedant effects of PA. We propose that snowshoe hares reduce the dose-dependent toxic consequences of PA by relying on efflux transporters and metabolizing enzymes that lower systemic exposure to dietary PA.

Key Words Chemical defense · Enzyme inhibition · Mode of action · Papyriferic acid · Succinate dehydrogenase · Snowshoe hare

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Introduction

The interaction between woody plants and the snowshoe hare (*Lepus americanus*) in winter in boreal North America has received significant attention as a model of chemically mediated diet selection by mammalian herbivores (Bryant and Kuropat, 1980; Bryant, 1981; Reichardt et al., 1984, 1990; Clausen et al., 1986; Sinclair et al., 1988a; Jogia et al., 1989; Williams et al., 1992). Lipophilic plant secondary metabolites (PSMs) such as resins and essential oils appear to control the daily intake of winter-dormant woody browse by snowshoe hares. Specifically, the glandular trichomes on the current-annual-growth (CAG) twigs of juvenile Alaska paper birch (*Betula neoalaskana*, synonym *B. resinifera*; taxonomy of Dugle, 1966) and *B. pendula* produce resin that is rich in the triterpene papyriferic acid (Reichardt, 1981; Lapinjoki et al., 1991; Raatikainen et al., 1992; Taipale et al., 1994; Julkunen-Tiitto et al., 1996). Papyriferic acid (PA) deters feeding by the snowshoe hare (Reichardt et al., 1984) and correlative evidence suggests that PA also deters feeding by other boreal mammals (Jia et al., 1997; Pusenius et al., 2002).

The physiological mechanism underlying the effect of birch resin on intake by the hare remains an open question. Sinclair et al. (1988b) have shown that the resin of the shrub birch *B. glandulosa*, which also contains PA (Williams et al., 1992), reduces the apparent digestibility

of protein by half. However, both theoretical analysis (Moran and Hamilton, 1980) and experimental studies (Kuijper et al., 2004) have concluded that reduced protein digestibility is likely to cause compensatory feeding in small mammals (Meyer et al., 2010), and therefore, an increase in daily intake by herbivores. Compensatory feeding is likely in the case of ceacalids, such as the snowshoe hare, that rapidly pass plant biomass through the gut (Kuijper et al., 2004; Stott, 2008). Therefore, an alternative mechanism is needed to explain the negative effects of birch resin on intake by hares.

Hares may reduce intake to maintain concentrations of toxic PSMs below a deleterious threshold in the blood (Wiggins et al., 2003; Boyle et al., 2005; Sorensen et al., 2005; McLean and Duncan, 2006; Mclean et al., 2007; Torregrossa and Dearing, 2009). For example, in no-choice feeding trials with PA-rich CAG twigs of juvenile *B. neoalaskana*, food intake was reduced sufficiently that the hares would rapidly starve to death if an alternative, PA-free, food was not available (Reichardt et al., 1984).

Rigorous testing of the toxicity hypothesis requires knowledge of the presumed toxin's mode of action (MOA) and the concentration of PSM that elicits toxicity and ultimately reduces intake. The link between concentration (pharmacokinetics) and the MOA that elicits a response (pharmacodynamics) is at the heart of the field of pharmacology, and it is used to predict variable response of individuals and populations of humans to drugs (McLean and Duncan, 2006; Sorensen et al., 2006). Likewise, ecologists are typically interested in predicting the variable response of populations of herbivores to plants. In the case of birch and snowshoe hares, there is correlative evidence that populations of birch and hares are involved in a biochemical interaction that has resulted in a mosaic of co-evolution (Bryant et al., 1994, 2009; Swihart and Bryant, 2001). Yet, as is the case in the majority of studies on interactions between plants and vertebrate herbivores (Provenza et al., 1994; Provenza, 1995; Lawler et al., 1998; Forbey and Foley, 2009; McLean et al., 2009), the MOA for chemicals in birch has not been investigated in hares. We propose that pharmacological approaches that reveal the MOA of PSMs (Forbey and Foley, 2009; Sotka et al., 2009) will help ecologists scale up variation observed in plant chemistry and tolerance to PSMs by herbivores from individuals to populations.

Our objective was to use the birch-snowshoe hare system to identify the MOA of the resinous dammarane triterpene, papyriferic acid. This study builds on recent evidence that the MOA of PA in domestic rabbits, laboratory rats, and oxen involves uncompetitive inhibition of succinate dehydrogenase (SDH, McLean et al., 2009). Succinate dehydrogenase is part of both the citric acid cycle and respiratory electron transfer chain in mitochondria (Rutter et al., 2010). Inhibition of SDH interferes with cellular energy production and may be detrimental to health of animals (Wallace and Starkov, 2000). We tested our hypothesis that PA inhibits snowshoe hare SDH in a dose-dependent manner by measuring the effect of PA on the oxidation of succinate in mitochondria isolated from wild hares. In addition, we used molecular modeling to examine the hypothesis that PA inhibits SDH by binding to the ubiquinone sites on complex II.

Methods and Materials

Chemicals Papyriferic acid was prepared from the ether extract of the resin of CAG twigs of winter-dormant *B. neoalaskana* saplings (Reichardt, 1981). Authenticity of PA was confirmed by using ^{1}H NMR with a standard Triple Resonance Inverse probe (Bruker Biospin, Billerica, MA, USA) and Quadrupole Time of Flight mass spectrometry (Q-TOF, Bruker Daltonics, Billerica, MA, USA). The molecular mass (604.367 Dalton) and the ^{1}H spectral data were in agreement with data previously reported for PA (Reichardt, 1981). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburg, PA, USA) and were of ACS grade.

Laboratory Animals Six-week old male Wistar rats and C57Bl/6 mice were purchased from Simonsen Laboratories (Gilroy, CA, USA). Animals were housed in polycarbonate cages and provided with an NIH-07 diet and water *ad libitum* for 7 d prior to tissue collection. Rats and mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Upon dissection of each euthanized animal, the liver was perfused with 10 ml ice cold 0.9% NaCl. Samples were snap-frozen in liquid nitrogen and stored at −80°C until use. The experiments were approved by the Boise State University Institutional Animal Use and Care Committee (006-AC10-002).

Snowshoe Hares Snowshoe hares were trapped near the Bonanza Creek Experimental Forest, approximately 20 km SW of Fairbanks, AK, USA (64° 41′ 37″ N, 148° 17′ 30″ W) using #3 Havahart live-traps baited with 300 g of fresh carrots and alfalfa cubes. The hares were sacrificed near the site of capture using cervical dislocation, and liver samples were collected immediately from each animal and snap-frozen in liquid nitrogen. Samples then were transported in a liquid nitrogen dry shipper and stored at −80°C until use. The hare experiments were approved by the Boise State University and University of Alaska-Fairbanks Institutional Animal Use and Care Committees (006-AC10-004 and 130970–5, respectively).

Mitochondria Preparation Mitochondria were prepared by thawing liver samples on ice and rinsing the tissue with cold 0.9% NaCl solution to remove blood. Tissues were cut into small pieces, homogenized in 5 volumes of isolation buffer containing 0.32 M sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4, and centrifuged for 10 min (1000×g, 4°C). Supernatants were transferred to clean vials and centrifuged for 15 min (12000xg, 4°C), followed by washing the pellets with isolation buffer and recentrifuging for 15 min (12000xg, 4°C). The final pellets from the same animal were resuspended in isolation buffer, pooled, and stored at −80°C until use.

Succinate Dehydrogenase (SDH) Activity Assay SDH activity was determined as described previously (Rosen et al., 1987; McLean et al., 2009) with adaptations for a 96-well format. Different concentrations of succinate (0–10 mM) were incubated with mitochondria to examine the enzyme kinetics of SDH with and without the addition of various concentrations of PA (10, 40, 80, and 160 μM). A single concentration of malonic acid (MA, 80 μM), a competitive inhibitor of SDH (Wojtovich and Brookes, 2008), was used as a control. In a 96 well plate, 5 μl of mitochondrial preparation were mixed with 5 μl of buffer containing the desired concentration of PA or MA and 220 μl of reaction media containing 2, 6-dichlorophenol indophenol (DCPIP) and sodium azide. The mixture was incubated for 5 min at 37°C before initiating the reactions by the addition of 20 μl of buffer that contained the desired concentration of succinate. The total volume of the reaction mixture was 250 μl, and the final concentrations of DCPIP and sodium azide were 100 μM and 4 mM, respectively. Immediately after the addition of succinate, the plate was placed in a BioTek Synergy MX plate reader (BioTek, Winooski, VT, USA) pre-heated to 37°C, and reaction rates were measured for 3 min at 600 nm. Protein content of the mitochondrial preparations was measured with a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. The reaction rates for each experiment were normalized by protein concentrations and expressed as ΔAU/min/mg protein.

Molecular Modeling We used molecular docking to predict the predominant binding mode(s) of PA with SDA. Successful docking methods search high-dimensional spaces of proteins and use a scoring function that ranks candidate dockings of compounds (i.e., ligands) to the protein. Docking can be used to virtually screen a large number of compounds, rank the results, and propose structural hypotheses that describe how the compounds interact with the target protein. A typical docking experiment involves computationally preparing the protein and the ligand, running the docking software, and evaluating the results. Advanced descriptions and specific methods used in our experiment are described in Supplementary Material 1.

In general, we prepared the mitochondrial respiratory membrane protein complex II (C-II) for molecular docking experiments by obtaining the crystal structure of the protein and using standard approaches to optimize the protein for docking. C-II contains four proteins, the flavoprotein containing the SDH enzyme domain (Chain A, SDHA), the iron-sulfur protein (Chain B, SDHB), the large (Chain C, SDHC; cytochrome b large, CybL) and small (Chain D, SDHD, CybS) trans-membrane proteins. Chains B, C, and D are relevant to binding of ubiquinone, a natural substrate for C-II.

The compounds 2-thenoyltrifluoroacetone (TTFA), ubiquinone-5 (UbQ), α-tocopheryl succinate (TOS), and PA were prepared for docking by using steps similar to those used to prepare the protein. The co-crystal structure coordinates of TTFA and UbQ (Sun et al., 2005) were chosen to validate docking experiments (Supplementary Material 1). In addition, the predicted UbQ binding free energy provides a natural substrate reference to compare to the binding strength of PA. The binding energy for TOS, a known inhibitor of the UbQ binding sites in C-II (Dong et al., 2008), was used in the docking experiment for comparison. Grid maps were created using AutoGrid version 4.2 (Morris et al., 2009) to describe interaction energies for each compound with SDH, one for each atom type in the compound. An affinity grid was created that centered on the protein ubiquinone binding site on C-II, specifically the proximal (Qp) and distal (Qd) sites.

AutoDock version 4.2 (Morris et al., 2009) with the Lamarckian genetic algorithm was used to simulate compound-SDH docking. TTFA was used as a positive control to optimize docking parameters to the Qp and Qd sites of C-II (PDB code 1ZP0). Conformational clustering was used to determine how often a given binding mode was predicted across all the dockings that were run. Docking results were obtained from the lowest binding energy conformations of the most populated cluster. The final docked structures were visualized by using visual molecular dynamics (VMD, Humphrey et al., 1996).

Statistical Analysis Reaction velocities and enzyme kinetics were determined using Microsoft Excel. V_{max} and K_m of the enzyme reaction were derived from Lineweaver-Burk plots. The inhibition constants (K_{iEIS}) were derived from the intercepts on axis of the secondary plots of $1/V_{max}$ vs. inhibitor concentration. The initial experiments indicated that the reaction rate reached maximum velocity with 5 mM succinate in the incubation mixture. Thus, 10 mM succinate was used to ensure enough substrate in the reaction to examine inhibition by PA at various concentrations (10–

160 μM). Percent inhibition by PA or MA was calculated for each inhibitor treatment. V_{max} (without inhibitor) or apparent $V_{max\ app}$ (with inhibitor), K_m (without inhibitor) or apparent $K_{m\ app}$ (with inhibitor), K_{cat}/K_m (catalytic efficiency), K_{iEIS}, and percent inhibition were compared among animal species within inhibitor treatments and within animal species among inhibitor treatments using a one-way analysis of variance (ANOVA). Tukey post-hoc tests were used for comparisons among multiple groups, and Dunnett post-hoc tests were used for comparison between treatments and control. The level of significance was $P<0.05$. Data for V_{max}, K_m and K_{iEIS} are presented as mean±standard error of the mean (SEM).

Results

Kinetics of SDH from Liver Tissues Excellent linearity was observed for progress curves during the 3 min period monitored. There was negligible reaction when succinate was omitted from the incubation mixture. The reaction rate increased with increases in succinate concentration and reached a maximum velocity at 5 mM succinate. Significant differences in V_{max} and K_m were observed among hares, mice, and rats (Table 1). The catalytic efficiencies (k_{cat}/K_m) for the enzyme from snowshoe hares and mice (18.6±1.5 and 15.9±0.9 min^{-1} mM^{-1}, respectively) were higher than the value for rats (13.5±0.6 min^{-1} mM^{-1}). Significant differences in $V_{max\ app}$ were observed among snowshoe hares, mice, and rats for all inhibitor treatments (Table 1). The $K_{m\ app}$ was significantly lower for enzyme from rats compared to enzyme from mice or snowshoe hares at low concentrations of PA, but the $K_{m\ app}$ did not differ when PA was 40 μM or higher or when MA was the inhibitor (Table 2). No difference was found between $K_{m\ app}$ for enzyme from snowshoe hares and mice for any inhibitor treatment.

Inhibition of SDH by PA All concentrations of PA (10–160 μM) inhibited SDH from all three animal species (Supplementary Material 2). PA appeared to act as an uncompetitive SDH inhibitor as indicated by the similar slopes in the Lineweaver-Burk plots (Supplementary Material 3). MA acted as a competitive inhibitor (Supplementary Material 3). Incubation with even very low concentrations of PA (10 μM) decreased $V_{max\ app}$ in a concentration-dependent manner in all animal species (Table 1). Similar patterns also were found for $K_{m\ app}$ (Table 2); K_m decreased with increasing concentration of PA, but $K_{m\ app}$ increased in the presence of MA.

The secondary plot of $1/V_{max}$ vs PA concentrations was linear for the enzyme from all animal species (Supplementary Material 4), confirming that PA is an uncompetitive inhibitor of SDH. The intercept on the X-axis of this plot indicates that K_{iEIS} for snowshoe hares (54.2±6.3 μM), mice (74.8±2.2 μM), and rats (106.7±1.4 μM) differ significantly ($P<0.05$; One-way ANOVA with Tukeys HSD test). SDH from snowshoe hares was more susceptible to PA inhibition (i.e., lower K_{iEIS}) compared to mice and rats (Supplementary Material 4).

The percent inhibition of SDH activity by PA was dose-dependent in all species (Fig. 1), with the enzyme from snowshoe hare most susceptible to inhibition. The inhibition of SDH by 80 μM MA was comparable to inhibition by 40 μM PA, but was significantly lower than inhibition by 80 μM PA, indicating that PA is a stronger SDH inhibitor than MA (Fig. 1).

Table 1 Maximal velocities of succinate dehydrogenase from liver mitochondria of snowshoe hares, C57BL/6 mice, or Wistar rats. Velocities (AU/mg protein/min) were determined in the presence ($V_{max\ app}$) or absence (V_{max}) of the inhibitors papyriferic acid (PA) or malonic acid (MA)

Inhibitor	Snowshoe hares	Mice	Rats
None	1.58±0.08[a]	1.85±0.01[b]	1.19±0.04[c]
PA, 10 μM	1.33±0.03*,[a]	1.58±0.02*,[b]	1.02±0.02*,[c]
PA, 40 μM	0.84±0.01*,[a]	1.07±0.04*,[b]	0.82±0.03*,[c]
PA, 80 μM	0.56±0.01*,[a]	0.84±0.02*,[b]	0.62±0.03*,[c]
PA, 160 μM	0.38±0.02*,[a]	0.56±0.01*,[b]	0.46±0.01*,[c]
MA, 80 μM	1.30±0.13*,[a]	1.75±0.20*,[b]	0.92±0.04*,[c]

Values represent mean±SEM for enzyme from four animals with and without PA and three animals for MA. An asterisk (*) represents a significant differences in means from no inhibitor within an individual species ($P<0.05$; *One-way ANOVA* with *Dunnett test*). Different letters indicate significant differences in means between species for an individual treatment ($P<0.05$; *Tukeys HSD test*).

Table 2 The Michaelis constant for succinate dehydrogenase from liver mitochondria of snowshoe hares, C57BL/6 mice, or Wistar rats. The K_m (mM) was determined in the presence ($K_{m\ app}$) or absence (K_m) of the inhibitors papyriferic acid (PA) or malonic acid (MA)

Inhibitor	Snowshoe hares	Mice	Rats
None	0.28±0.01[a]	0.29±0.01[a]	0.20±0.01[b]
PA, 10 μM	0.26±0.02[a]	0.25±0.01[a]	0.19±0.02[b]
PA, 40 μM	0.18±0.02* [a]	0.20±0.01* [a]	0.18±0.02[a]
PA, 80 μM	0.14±0.02* [a]	0.15±0.01* [a]	0.14±0.01* [a]
PA, 160 μM	0.07±0.01* [a]	0.09±0.01* [a]	0.08±0.01* [a]
MA, 80 μM	1.42±0.29* [a]	1.99±0.42* [a]	1.18±0.21* [a]

Values represent mean±SEM for enzyme from four animals with and without PA and three animals for MA. An asterisk (*) represents a significant differences in means from no inhibitior within an individual species ($P<0.05$; *One-way ANOVA* with *Dunnett test*). Different letters indicate significant differences in means between species for an individual treatment ($P<0.05$; *One-way ANOVA* with

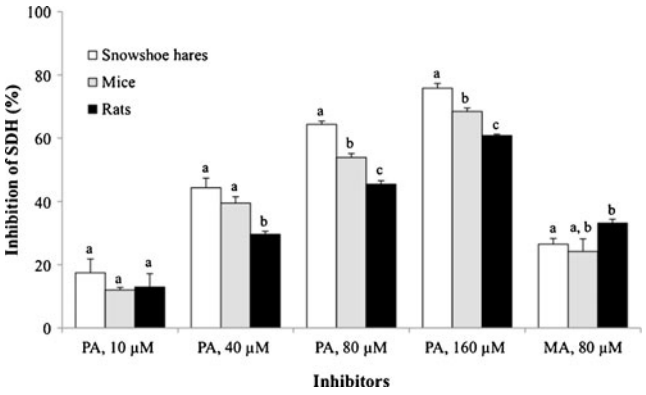

Fig. 1 Inhibition of SDH from snowshoe hares, C57BL/6 mice, and Wistar rats by papyriferic acid (PA) or malonic acid (MA). Inhibition is calculated as a percent of the activity of control samples from the same species. Values represent mean±SEM of 4 animals for PA and 3 for MA. Different letters indicate significant differences in means between species for an individual treatment ($P<0.05$; *Tukeys HSD test*)

Molecular Modeling When evaluating the results of docking, the main criterion to consider is how well the binding mode predicted by the docking matchs structural data, where available. TTFA was used as a positive control to show that AutoDock 4.2 is able to emulate experimental binding with an acceptable degree of accuracy and precision, and to validate further docking experiments with other inhibitors. Specifically, TTFA was re-docked to the proximal UbQ-binding site (Qp) and the distal UbQ-binding site (Qd) of C-II crystal structure (Fig. 2). The overall root mean square deviation of the predicted binding pose compared to the crystal structure pose is 2.67 Å for the Qp site and 2.81 Å for the Qd site. The differences between docked and crystal poses lie primarily in the placement and orientation of the thenoyl group, which is thought to interact with both binding sites through mediating water molecules. Additionally, UbQ was re-docked back to the Qp site. The docked quinone ring coordinates are in good agreement with those in the crystal structure (PDB code 1ZOY, Fig. 2).

Molecular modeling reveals that PA binds to both Qp and Qd sites in a fashion similar to TOS (Fig. 3). In the Qp site, the carbocyclic rings of PA reside in the same binding groove as the phenyl ring of TOS. The carboxylate groups of PA and TOS reside in the central binding pocket where the UbQ ring and the TTFA thenoyl ring are bound, making hydrogen bonds and electrostatic contacts with SER42 and ARG46, and interacting with TYR91 through bridging water. Similar to the isoprenoid tail of UbQ, the remaining hydrophobic side chain of TOS loops around the hydrophobic binding grooves adjacent to the central binding pocket. In contrast, PA adopts a horizontal configuration due to the favorable polar interactions between the carbocyclic ring ester and hydroxyl groups and ARG93, TRP173, and ASN174 residues.

In the Qd site, the carbocyclic rings of PA and the phenyl ring of TOS bind in the same location where the thenoyl ring of TTFA binds, at the center of the binding site. The succinate ester moieties of PA and TOS extend towards the bottom of the binding site. The end carboxylate groups of PA and TOS nearly overlap, indicating key electrostatic interactions and hydrogen bonding with positively charged LYS135 and LYS128 residues. Due to its large size, the carbocyclic rings of PA readily interact with TYR61 and TRP134 side chains without water mediation.

The predicted binding free energies indicate that PA has a higher affinity for both the Qp and Qd sites than TTFA, UbQ, or TOS (Table 3). Furthermore, UbQ, TOS, and PA have higher affinity for the Qd site than the Qp site, whereas TTFA seems to preferentially bind to the Qp site. These findings are corroborated with the crystal structure electron density data (Sun et al., 2005) and a molecular docking study using AutoDock 3 (Dong et al., 2008).

Discussion

We showed that inhibition of mitochondrial SDH is one mode of action of a secondary metabolite in birch that could deter feeding by snowshoe hares. Papyriferic acid, the major PSM of the juveniles of *B. neoalaskana* and *B. pendula*, inhibits SDH by an uncompetitive mechanism in a dose-dependent manner (Supplementary Material 2, 3, and 4). In addition, molecular modeling strongly supports the notion that inhibition of SDH activity is a result of PA targeting the ubiquinone binding sites rather than the catalytic succinate binding site in C-II (Fig. 3). PA and TOS bind to both ubiquinone sites in a similar fashion, which is distinct from TTFA and UbQ binding due to structural differences. However, the core binding regions are consistently occupied by all four ligands, indicating that TTFA, TOS and PA all disrupt ubiquinone binding. The predicted binding free energies generated by AutoDock show that PA is a significantly more potent ligand than TTFA and TOS at both sites (Table 3). Several factors may contribute to the higher potency of PA. First, PA has more polar ester, hydroxyl, and carboxylate groups than TTFA and TOS to enhance the hydrogen bond and electrostatic interactions with binding site polar and positively charged residues. Second, the large ring scaffold of PA increases potential π-π interactions and minimizes the need for water mediation. The *in vitro* and computer modeling results provide a mode of action for toxicity that may be responsible for the concentration-dependent anti-feedant effects of PA observed in snowshoe hares (Reichardt et al.,

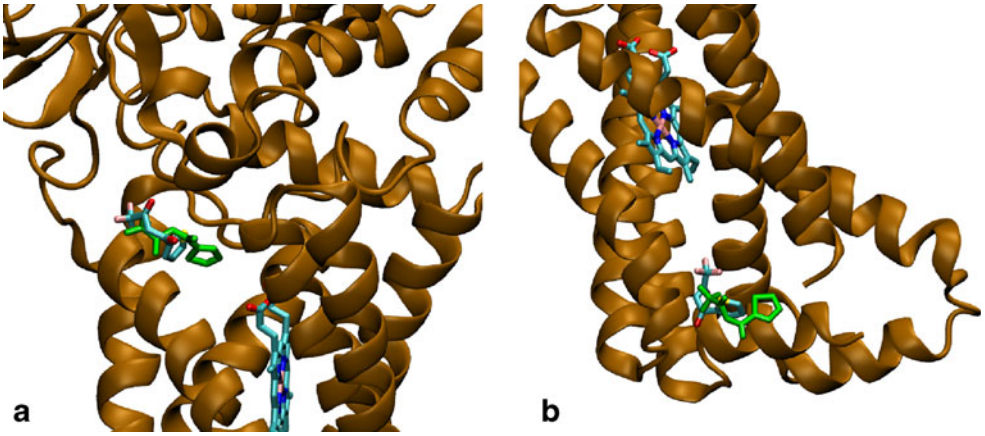

Fig. 2 Re-docking of 2-Thenoyltrifluoroacetone (TTFA) to the crystal structure of Complex II (C-II, PDB code 1ZP0). The predicted TTFA binding pose is colored by element and the crystal structure TTFA binding pose in green. The heme group is shown in cyan. (**a**) Qp site and (**b**) Qd site

1984). We predict that mechanisms that lower systemic concentrations of PA would reduce the toxicity of PA and explain the variable tolerance to PA observed between and within species that consume birch.

Inhibition of SDH may be a general mechanism of action of PA. PA inhibited SDH in a species that has had an ecological history with birch (snowshoe hares, this study) and those that are considered naïve to the chemicals in birch (ox, rabbits, rats, and mice, this study and in McLean et al. 2009). In every species tested thus far, inhibition of SDH by PA was dose dependent, uncompetitive, and more potent than a well-known competitive inhibitor of PA, malonic acid (Pardee and Potter, 1949; Greene and Greenamyre, 1995). From an evolutionary perspective, our *in vitro* results may at first appear somewhat puzzling. One might expect that animals with a 6000 year ecological history of consuming PA (Bryant et al., 2009), would have lower sensitivity to its MOA than naïve species. Lower sensitivity would provide a plausible explanation for the capacity of some hare populations to eat a PA-rich diet

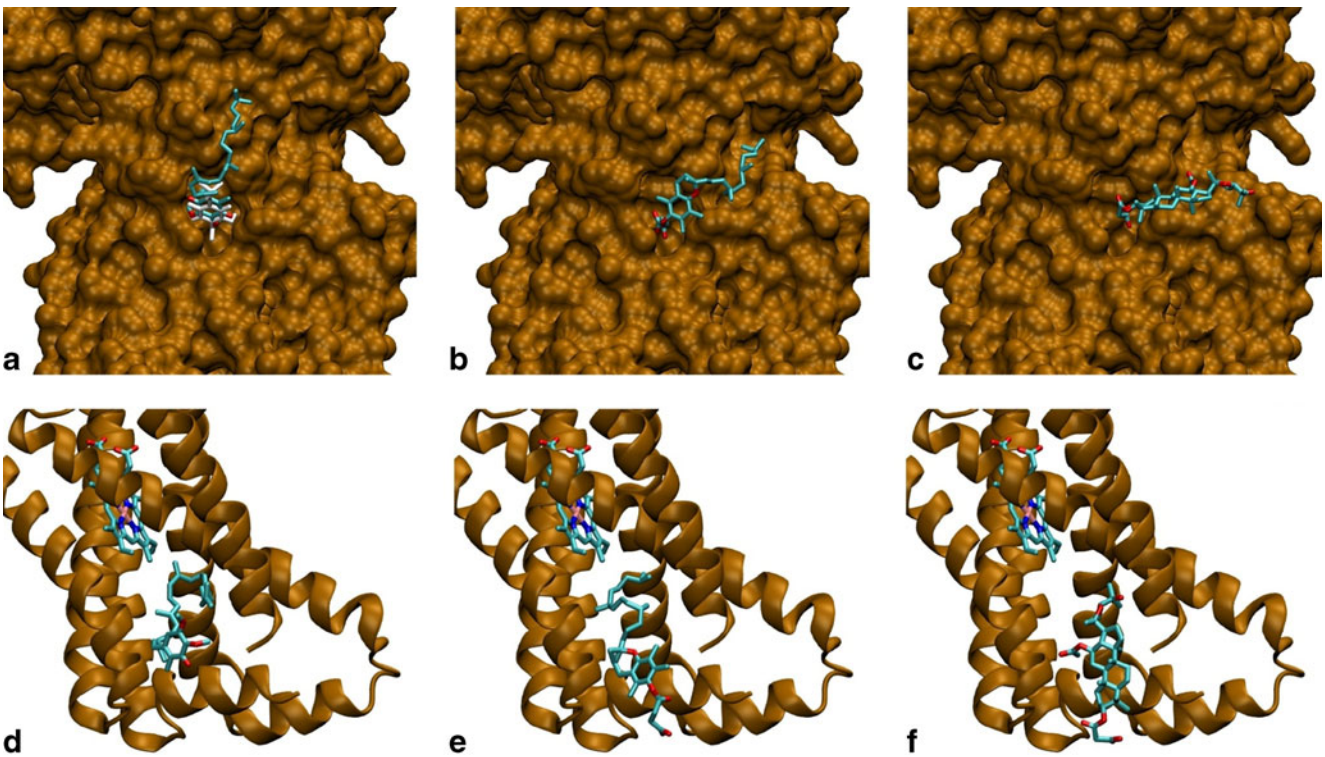

Fig. 3 Predicted binding poses of (**a**) Ubiquinone-5 (UbQ, cyan) and crystal structure UbQ (white), (**b**) α-tocopheryl succinate (TOS), and (**c**) papyriferic acid (PA) for the Qp site rendered in molecular surface representation, and (**d**), (**e**), (**f**) following the same order for the Qd site rendered in cartoon representation. The heme group is shown in cyan situated above the docked ligands

Table 3 Interaction energies (Kcal/mol) for the best-ranked docking conformations for 2-Thenoyltrifluoroacetone (TTFA), Ubiquinone-5 (UbQ), α-Tocopheryl succinate (TOS), and papyriferic acid (PA) in the Qp- and Qd-binding sites of succinate dehydrogenase. More negative interaction energies are indicative of higher binding affinity

Ligand	Qp site (Kcal/mol)	Qd site (Kcal/mol)
TTFA	−4.13	−3.99
UQ5	−5.15	−8.30
TOS	−6.55	−10.58
PA	−8.98	−12.55

(Bryant et al., 1994). In contrast, however, we have shown that snowshoe hare mitochondria are more sensitive *in vitro* than the naïve species tested.

In vitro sensitivity to PA inhibition may not translate to *in vivo* sensitivity for several reasons. First, the gut mitochondria of hares may be less sensitive to inhibition by PA than liver mitochondria. If absorption of PA by hares is low, as is the case in rats (McLean et al., 2009), then PA concentrations could be very low in the liver, and the gut would be a more plausible site of action than the liver. Moreover, the population of snowshoe hares we studied may not represent the species as a whole. Species do differ in their sensitivity to SDH inhibition (this study and Mogi et al., 2009), and we predict that the Alaska population of hares used here may have had higher sensitivity to SDH inhibition due to their "intermediate" tolerance to birch than would have been found in more tolerant populations (Swihart and Bryant, 2001; Bryant et al., 2009). Future studies could be performed to determine whether a geographical mosaic exists for sensitivity to SDH inhibition by PA.

An alternative explanation for our results is that the *in vitro* sensitivity to PA by hares does not account for *in vivo* mechanisms that can influence the systemic dose of PA that interacts with SDH in naturally feeding animals. The dose-dependent inhibition found in a variety of species suggest that variation in tolerance of PA by species or populations could be associated with physiological mechanisms that reduce systemic concentrations of PA rather than mechanisms that influence the sensitivity to inhibition of SDH by PA. Hares can behaviorally reduce the systemic dose of PA by avoiding the highest concentration of PA found in plant parts (Reichardt et al., 1984). In addition, enzymes that regulate absorption and metabolism of chemicals can dictate the concentration of ingested chemicals at the site of action and ultimately influence susceptibility to those chemicals (Danhof et al., 2007; Megarbane et al., 2008; Ploeger et al., 2009; Dahl et al., 2010). Efflux transporters and metabolizing enzymes could regulate the cellular concentrations of ingested PSMs in herbivores (Dearing et al., 2005; McLean and Duncan, 2006; Sorensen and Dearing, 2006; Sorensen et al., 2006; Forbey and Foley, 2009). Additional studies are needed to test whether efflux transporters and metabolizing enzymes can explain the disconnect between sensitivity to PA by snowshoe hares observed in these *in vitro* assays compared to the *in vivo* tolerance to PA by some populations of hares (Swihart and Bryant, 2001; Bryant et al., 2009).

This study is the first to our knowledge to confirm a mode of action of PA in an herbivore. Identifying the mode of action provides the necessary mechanistic data to help explain Thompson's (2005) geographic mosaic of selection in the birch-snowshoe hare system (Bryant et al., 1994, 2009; Swihart and Bryant, 2001). We have established a relatively simple protocol for testing SDH inhibition as a general mechanism of resistance in birch against other herbivores. SDH inhibition also may be a general mechanism of toxicity of other PSMs in other plant-herbivore systems. In addition, we have showcased the value of molecular modeling to further elucidate the mechanism of toxicity of PSMs and other ecologically relevant molecules. This approach has been fruitful in predicting binding of PSMs to metabolizing enzymes in insects (Baudry et al., 2003; Rupasinghe et al., 2003; Jones et al., 2010; Mao et al., 2007) and could prove equally valuable in screening the binding between PSMs and efflux transporters and metabolizing enzymes as well as the mode of action of PSMs in vertebrates.

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References

BAUDRY, J., LI, W. M., PAN, L. P., BERENBAUM, M. R., and SCHULER, M. A. 2003. Molecular docking of substrates and inhibitors in the catalytic site of CYP6B1, an insect cytochrome P450 monooxygenase. *Protein Eng.* 16:577–587.

BOYLE, R. R., MCLEAN, S., BRANDON, S., and WIGGINS, N. 2005. Rapid absorption of dietary 1,8-cineole results in critical blood concentration of cineole and immediate cessation of eating in the common brushtail possum (*Trichosurus vulpecula*). *J. Chem. Ecol.* 31:2775–2790.

BRYANT, J. P. 1981. Phytochemical deterrence of snowshoe hare browsing by adventitious shoots of 4 Alaskan trees. *Science* 213:889–890.

Bryant, J. P. and Kuropat, P. J. 1980. Selection of winter forage by sub-arctic browsing vertebrates—the role of plant chemistry. *Annu. Rev. Ecol. Evol. Syst.* 11:261–285.

Bryant, J. P., Swihart, R. K., Reichardt, P. B., and Newton, L. 1994. Biogeography of woody plant-chemical defense against snowshoe hare browsing—comparison of Alaska and eastern North America. *Oikos* 70:385–395.

Bryant, J. P., Clausen, T. P., Swihart, R. K., Landhausser, S. M., Stevens, M. T., Hawkins, C. D. B., Carriere, S., Kirilenko, A. P., Veitch, A. M., Popko, R. A., Cleland, D. T., Williams, J. H., Jakubas, W. J., Carlson, M. R., Bodony, K. L., Cebrian, M., Paragi, T. F., Picone, P. M., Moore, J. E., Packee, E. C., and Malone, T. 2009. Fire drives transcontinental variation in tree birch defense against browsing by snowshoe hares. *Am. Nat.* 174:13–23.

Clausen, T. P., Reichardt, P. B., and Bryant, J. P. 1986. Pinosylvin and pinosylvin methyl-ether as feeding deterrents in green alder. *J. Chem. Ecol.* 12:2117–2131.

Dahl, S. G., Aarons, L., Gundert-Remy, U., Karlsson, M. O., Schneider, Y. J., Steimer, J. L., and Troconiz, I. F. 2010. Incorporating physiological and biochemical mechanisms into pharmacokinetic-pharmacodynamic models: A conceptual framework. *Basic Clin. Pharmacol. Toxicol.* 106:2–12.

Danhof, M., de Jjongh, J., de Lange, E. C. M., Della Pasqua, O., Ploeger, B. A., and Voskuyl, R. A. 2007. Mechanism-based pharmacokinetic-pharmacodynamic modeling: Biophase distribution, receptor theory, and dynamical systems analysis. *Annu. Rev. Pharmacol. Toxicol.* 47:357–400.

Dearing, M. D., Foley, W. J., and McLean, S. 2005. The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. *Annu. Rev. Ecol. Evol. Syst.* 36:169–189.

Dong, L. F., Low, P., Dyason, J. C., Wang, X. F., Prochazka, L., Witting, P. K., Freeman, R., Swettenham, E., Valis, K., Liu, J., Zobalova, R., Turanek, J., Spitz, D. R., Domann, F. E., Scheffler, I. E., Ralph, S. J., and Neuzil, J. 2008. [alpha]-tocopheryl succinate induces apoptosis by targeting ubiquinone-binding sites in mitochondrial respiratory complex II. *Oncogene*:4324–4335.

Dugle, J. R. 1966. A taxonomic study of western Canadian species in the genus *Betula*. *Can. J. Bot.* 44:929–1007.

Forbey, J. S. and Foley, W. J. 2009. Pharmecology: A pharmacological approach to understanding plant-herbivore interactions: An introduction to the symposium. *Integr. Comp. Biol.* 49:267–273.

Greene, J. G. and Greenamyre, J. T. 1995. Characterization of the excitotoxic potential of the reversible succinate-dehydrogenase inhibitor malonate. *J. Neurochem.* 64:430–436.

Humphrey, W., Dalke, A., and Schulten, K. 1996. Vmd: Visual molecular dynamics. *J. Mol. Graph.* 14:33–38.

Jia, J. B., Niemela, P., Rousi, M., and Harkonen, S. 1997. Selective browsing of moose (*Alces alces*) on birch (*Betula pendula*) clones. *Scand. J. Forest Res.* 12:33–40.

Jogia, M. K., Sinclair, A. R. E., and Andersen, R. J. 1989. An antifeedant in balsam poplar inhibits browsing by snowshoe hares. *Oecologia* 79:189–192.

Jones, R. T., Bakker, S. E., Stone, D., Shuttleworth, S. N., Boundy, S., McCart, C., Daborn, P. J., Ffrench-Constant, R. H., and van den Elsen, J. M. H. 2010. Homology modelling of drosophila cytochrome P450 enzymes associated with insecticide resistance. *Pest Manag. Sci.* 66:1106–1115.

Julkunen-Tiitto, R., Rousi, M., Bryant, J., Sorsa, S., Keinanen, M., and Sikanen, H. 1996. Chemical diversity of several *Betulaceae* species: Comparison of phenolics and terpenoids in northern birch stems. *Trees-Struct. Funct.* 11:16–22.

Kuijper, D. P. J., van Wieren, S. E., and Bakker, J. P. 2004. Digestive strategies in two sympatrically occurring lagomorphs. *J. Zool.* 264:171–178.

Lapinjoki, S. P., Elo, H. A., and Taipale, H. T. 1991. Development and structure of resin glands on tissues of *Betula pendula* Roth. during growth. *New Phytol.* 117:219–223.

Lawler, I. R., Foley, W. J., Pass, G. J., and Eschler, B. M. 1998. Administration of a $5HT_3$ receptor antagonist increases the intake of diets containing *Eucalyptus* secondary metabolites by marsupials. *J. Comp. Physiol. B. Biochem. Syst. Environ. Physiol.* 168:611–618.

Mao, W., Schuler, M. A., and Berenbaum, M. R. 2007. Cytochrome P450s in *Papilio multicaudatus* and the transition from oligophagy to polyphagy in the *Papilionidae*. *Insect Mol. Biol.* 16:481–490.

McLean, S. and Duncan, A. J. 2006. Pharmacological perspectives on the detoxification of plant secondary metabolites: Implications for ingestive behavior of herbivores. *J. Chem. Ecol.* 32:1213–1228.

McLean, S., Boyle, R. R., Brandon, S., Davies, N. W., and Sorensen, J. S. 2007. Pharmacokinetics of 1,8-cineole, a dietary toxin, in the brushtail possum (*Trichosurus vulpecula*): Significance for feeding. *Xenobiotica* 37:903–922.

McLean, S., Richards, S. M., Cover, S. L., Brandon, S., Davies, N. W., Bryant, J. P., and Clausen, T. P. 2009. Papyriferic acid, an antifeedant triterpene from birch trees, inhibits succinate dehydrogenase from liver mitochondria. *J. Chem. Ecol.* 35:1252–1261.

Megarbane, B., Aslani, A. A., Deye, N., and Baud, F. J. 2008. Pharmacokinetic/pharmacodynamic modeling of cardiac toxicity in human acute overdoses: Utility and limitations. *Expert Opin. Drug Metab. Toxicol.* 4:569–579.

Meyer, K., Hummel, J., and Clauss, M. 2010. The relationship between forage cell wall content and voluntary food intake in mammalian herbivores. *Mammal Rev.* 40:221–245.

Mogi, T., Kawakami, T., Arai, H., Igarashi, Y., Matsushita, K., Mori, M., Shiomi, K., Omura, S., Harada, S., and Kita, K. 2009. Siccanin rediscovered as a species-selective succinate dehydrogenase inhibitor. *J. Biochem.* 146:383–387.

Moran, N. and Hamilton, W. D. 1980. Low nutritive quality as defense against herbivores. *J. Theor. Biol.* 86:247–254.

Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., and Olson, A. J. 2009. Autodock4 and Autodocktools4: Automated docking with selective receptor flexibility. *J. Comput. Chem.* 30:2785–2791.

Pardee, A. B. and Potter, V. R. 1949. Malonate inhibition of oxidations in the Krebs tricarboxylic acid cycle. *J. Biol. Chem.* 178:241–250.

Ploeger, B. A., van derGraaf, P. H., and Danhof, M. 2009. Incorporating receptor theory in mechanism-based pharmacokinetic-pharmacodynamic (PK-PD) modeling. *Drug Metab. Pharmacokinet.* 24:3–15.

Provenza, F. D. 1995. Postingestive feedback as an elementary determinant of food preference and intake in ruminants. *J. Range Manag.* 48:2–17.

Provenza, F. D., Ortegareyes, L., Scott, C. B., Lynch, J. J., and Burritt, E. A. 1994. Antiemetic drugs attenuate food aversions in sheep. *J. Anim. Sci.* 72:1989–1994.

Pusenius, J., Prittinen, K., Heimonen, J., Koivunoro, K., Rousi, M., and Roininen, H. 2002. Choice of voles among genotypes of birch seedlings: Its relationship with seedling quality and preference of insects. *Oecologia* 130:426–432.

Raatikainen, O. J., Taipale, H. T., Peltari, A., and Lapinjoki, S. P. 1992. An electron-microscope study of resin production and secretion by the glands of seedlings of *Betula pendula* Roth. *New Phytol.* 122:537–543.

Reichardt, P. B. 1981. Papyriferic acid—a triterpenoid from Alaskan paper birch. *J. Org. Chem.* 46:4576–4578.

Reichardt, P. B., Bryant, J. P., Clausen, T. P., and Wieland, G. D. 1984. Defense of winter-dormant Alaska paper birch against snowshoe hares. *Oecologia* 65:58–69.

REICHARDT, P. B., BRYANT, J. P., MATTES, B. R., CLAUSEN, T. P., CHAPIN, F. S., and MEYER, M. 1990. Winter chemical defense of Alaskan balsam poplar against snowshoe hares. *J. Chem. Ecol.* 16:1941–1959.

ROSEN, H., RAKITA, R. M., WALTERSDORPH, A. M., and KLEBANOFF, S. J. 1987. Myeloperoxidase-mediated damage to the succinate oxidase system of *Escherichia coli*. Evidence for selective inactivation of the dehydrogenase component. *J. Biol. Chem.* 262:15004–15010.

RUPASINGHE, S., BAUDRY, J., and SCHULER, M. A. 2003. Common active site architecture and binding strategy of four phenylpropanoid P450s from *Arabidopsis thaliana* as revealed by molecular modeling. *Protein Eng.* 16:721–731.

RUTTER, J., WINGE, D. R., and SCHIFFMAN, J. D. 2010. Succinate dehydrogenase—assembly, regulation and role in human disease. *Mitochondrion* 10:393–401.

SINCLAIR, A. R. E., JOGIA, M. K., and ANDERSEN, R. J. 1988a. Camphor from juvenile white spruce as an antifeedant for snowshoe hares. *J. Chem. Ecol.* 14:1505–1514.

SINCLAIR, A. R. E., KREBS, C. J., SMITH, J. N. M., and BOUTIN, S. 1988b. Population biology of snowshoe hares. 3. Nutrition, plant secondary compounds and food limitation. *J. Anim. Ecol.* 57:787–806.

SORENSEN, J. S. and DEARING, M. D. 2006. Efflux transporters as a novel herbivore countermechanism to plant chemical defenses. *J. Chem. Ecol.* 32:1181–1196.

SORENSEN, J. S., HEWARD, E., and DEARING, M. D. 2005. Plant secondary metabolites alter the feeding patterns of a mammalian herbivore (*Neotoma lepida*). *Oecologia* 146:415–422.

SORENSEN, J. S., SKOPEC, M. M., and DEARING, M. D. 2006. Application of pharmacological approaches to plant-mammal interactions. *J. Chem. Ecol.* 32:1229–1246.

SOTKA, E. E., FORBEY, J., HORN, M., POORE, A. G. B., RAUBENHEIMER, D., and WHALEN, K. E. 2009. The emerging role of pharmacology in understanding consumer-prey interactions in marine and freshwater systems. *Integr. Comp. Biol.* 49:291–313.

STOTT, P. 2008. Comparisons of digestive function between the European hare (*Lepus europaeus*) and the European rabbit (*Oryctolagus cuniculus*): Mastication, gut passage, and digestibility. *Mamm. Biol.* 73:276–286.

SUN, F., HUO, X., ZHAI, Y., WANG, A., XU, J., SU, D., BARTLAM, M., and RAO, Z. 2005. Crystal structure of mitochondrial respiratory membrane protein complex II. *Cell* 121:1043–1057.

SWIHART, R. K. and BRYANT, J. P. 2001. Importance of biogeography and ontogeny of woody plants in winter herbivory by mammals. *J. Mammal.* 82:1–21.

TAIPALE, H. T., HARMALA, L., ROUSI, M., and LAPINJOKI, S. P. 1994. Histological and chemical comparison of triterpene and phenolic deterrent contents of juvenile shoots of *Betula* species. *Trees-Struct. Funct.* 8:232–236.

THOMPSON, J. N. 2005. Coevolution: The geographic mosaic of coevolutionary arms races. *Curr. Biol.* 15:R992–R994.

TORREGROSSA, A. M. and DEARING, M. D. 2009. Nutritional toxicology of mammals: Regulated intake of plant secondary compounds. *Funct. Ecol.* 23:48–56.

WALLACE, K. B. and STARKOV, A. A. 2000. Mitochondrial targets of drug toxicity. *Annu. Rev. Pharmacol. Toxicol.* 40:353–388.

WIGGINS, N. L., MCARTHUR, C., MCLEAN, S., and BOYLE, R. 2003. Effects of two plant secondary metabolites, cineole and gallic acid, on nightly feeding patterns of the common brushtail possum. *J. Chem. Ecol.* 29:1447–1464.

WILLIAMS, D. E., SINCLAIR, A. R. E., and ANDERSEN, R. J. 1992. Triterpene constituents of the dwarf birch, *Betula glandulosa*. *Phytochemistry* 31:2321–2324.

WOJTOVICH, A. P. and BROOKES, P. S. 2008. The endogenous mitochondrial complex II inhibitor malonate regulates mitochondrial ATP-sensitive potassium channels: Implications for ischemic preconditioning. *Biochim. Biophys. Acta* 1777:882–889.