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Peter van den Hurk

Harold M.I. Kerkkamp

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Peter van den Hurk [a*], Harald M.I. Kerkkamp [b]

[A] Department of Biological Sciences, Clemson University, Clemson, SC 20624, USA
[B] Institute of Biology, University of Leiden, Leiden 2300 RA, The Netherlands.

*Corresponding author:
Dr. Peter van den Hurk
Department of Biological Sciences, Clemson University
132 Long Hall, Clemson, SC 29634, USA
Phone: +1.864-656-3594
pvdhurk@clemson.edu

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Abstract

While it has been known for a while that some snake species are extremely sensitive to acetaminophen, the underlying mechanism for this toxicity has not been reported. To investigate if essential detoxification enzymes are missing in snake species that are responsible for biotransformation of acetaminophen in other vertebrate species, livers were collected from a variety of snake species, together with samples from alligator, snapping turtle, cat, rat, and cattle. Subcellular fractions were analyzed for enzymatic activities of phenol-type sulfotransferase and UDP-glucuronosyltransferase, total glutathione S-transferase, and N-acetyltransferase. The results showed that none of the snake species, together with the cat samples, had any phenol-type glucuronidation activity, and that this activity was much lower in alligator and turtle samples than in the mammalian species. Combined with the lack of N-acetyltransferase activity in snakes and cats, this would explain the accumulation of the aminophenol metabolite, which induces methemoglobinemia and subsequent suffocation of snakes and cats after acetaminophen exposure. While previous investigations have concluded that in cats the gene for the phenol-type glucuronosyltransferase isoform has turned into a pseudogene because of several point mutations, evaluation of genomic information for snake species revealed that they have only 2 genes that may code for glucuronosyltransferase isoforms. Similarity of these genes with mammalian genes is less than 50%, and suggests that the expressed enzymes may act on other types of substrates than aromatic amines. This indicates that the extreme sensitivity for acetaminophen in snakes is based on a different phylogenetic origin than the sensitivity observed in cats.

Key words: Acetaminophen, toxicity, biotransformation, UDP-glucuronosyltransferase, N-acetyltransferase, phylogeny, snake, Reptilia, Mammalia.

1. Introduction

After the accidental introduction of the brown treesnake (*Boiga irregularis*) on the island of Guam in the 1950's, the population of this species rapidly expanded because of the lack of natural predators and the presence of a bountiful array of prey species (Savidge, 1987). After several decades of expansion, a number of indigenous bird species are now considered extinct on the island, and the brown treesnake is considered a nuisance species for human activities (Rodda et al. 1999; Burnett et al., 2012). This triggered investigations into possible methods to contain and eradicate this invasive species. A variety of general wildlife pesticides was tested for their toxicity to the brown treesnake, together with several human therapeutic drugs that were known to be toxic to some vertebrate species. Surprisingly, the brown treesnake proved to be very sensitive to low doses of acetaminophen, and to a lesser extent to aspirin, but not

ibuprofen (Savarie et al., 2000). A dose of only 80 mg acetaminophen per animal did kill 100 % of the tested snakes within 12-24 h. This knowledge has since been used to control the brown tree snake population on Guam by lacing dead mice with acetaminophen, and distributing this bait in habitats where the snakes reside. Brown treesnakes do eat dead carrion, and therefore this has proven to be an effective and relatively safe management strategy (Johnston et al., 2002).

From a comparative toxicology point of view it was unexpected that this species is so sensitive to acetaminophen. The compound is used as a common over-the-counter analgesic, and has relatively low toxicity to humans and most other mammals (Bertolini et al., 2006). After absorption and distribution, a large amount of the compound is processed in the liver, where specific isoforms of two enzymes, sulfotransferase (SULT) and UDP-glucuronosyltransferase (UGT), rapidly conjugate the acetaminophen with a sulfonate group or a sugar group, and thus ready the poorly water soluble substrate for excretion in urine or bile (Bertolini et al., 2006). If these enzymatic pathways become saturated, another liver enzyme (cytochrome P-450-2E1) can turn the acetaminophen into a highly reactive quinone (NAPQI), which can cause liver damage, as seen in people who overdose on the drug (James et al., 2003). The reactive quinone can be neutralized by the anti-oxidant glutathione, with help of the enzyme glutathione S-transferase. But if this pathway becomes saturated after an excessive dose, and accumulation of NAPQI occurs, serious liver damage will ensue (McGill & Jaeschke, 2013).

This well-studied pathology profile of acetaminophen in humans could explain the observed toxicity in the brown treesnake if snakes are missing any of the essential enzymes in the detoxification pathway of the compound. However, from experiments in which brown treesnakes were dosed with acetaminophen, it became clear that they did not die from acute liver failure, but from anemic hypoxia (Clark et al., 2018). This rare phenomenon is also seen in feline species, like cats, when they are exposed to acetaminophen (Court & Greenblatt, 1997). The sensitivity of cats to acetaminophen has been explained by the lack of a functional isoform of the UDP-glucuronosyltransferases which conjugates acetaminophen in other mammalian species. The gene for this isozyme has several point mutations in cats, which has turned the gene into a pseudo-gene (Court & Greenblatt, 2000). Because the lack of a functional phenol-type UGT isoform leads to acute toxicity in cats, the objective of the current study was to investigate if the underlying mechanism for acetaminophen toxicity in cats is the same as in the brown treesnake and other snake species, or if phylogenetic signals leading to differentiated enzyme expressions are responsible for the sensitivity of reptilians to acetaminophen. The approach to answer this question was to collect liver samples from a variety of snake species, and several other vertebrates for comparison, and measure the activities of the enzymes involved in acetaminophen metabolism. In addition, we explored existing data in GenBank of

investigated or closely related species to compare genetic information on the genes involved in these enzymatic pathways.

2. Materials and Methods

2.1 Tissue samples

Eastern diamondback rattlesnake (*Crotalus adamanteus*) samples (n=2) were kindly donated by Darin Rokyta's lab (Florida State University, Tallahassee, FL), ball python (*Python regius*) and corn snake (*Pantherophis guttatus*) samples (both n=1) were obtained from University of Texas Arlington (Todd Castoe lab). Liver samples (n=1 each) of several Colubridae snakes (*Nerodia clarkii compressicauda*, *Phyllorhynchus decurtatus*, *Rhinocheilus lecontei*, *Thamnophis marcianus*) and a cottonmouth (*Agkistrodon piscivorus*) were donated by Chris Parkinson's lab (University of Central Florida, Orlando, FL). Samples of Burmese python (*Python bivittatus,* n=4) were obtained with the help of Frank Mazzotti's lab (University of Florida, Davie, FL). American alligator (*Alligator mississippiensis*) samples (n=5) were supplied by Lou Gillette's lab (Medical University of South Carolina, Charleston, SC). Snapping turtles (*Chelydra serpentine,* n=4) were collected locally in the Reedy River near Greenville, SC, rat (*Rattus norvegicus*) samples (n=4) were obtained from the Godley Snell animal use facility at Clemson University, cat (*Felis catus*) livers (n=4) were dissected from euthanized feral cats at the Oconee Animal Shelter (Seneca, SC) and heifer (*Bos taurus*) livers (n=6) were obtained from the veterinary school at the University of Georgia (Athens, GA). All liver tissue samples were flash frozen in liquid nitrogen and stored at -80°C until use.

Livers were thawed on ice and approximately 2 g of liver tissue was homogenized with a Polytron tissue grinder in 10 ml ice-cold Tris buffer (0.05 M, pH 7.4), containing 0.25 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT) and 0.2 mM phenylmethanesulfonyl fluoride (PMSF). Smaller samples were homogenized with a glass Potter-Elvehjem homogenizer in 2 mL of chilled homogenization buffer. Samples were spun for 20 min at 10,000 g at 4º C to remove cell debris, connective tissue and fat, followed by a 60 min cold spin at 100,000 g. The cytosolic supernatant was collected and separated in aliquots; the microsomal pellet was then resuspended in 1 ml Hepes buffer (0.01 M, pH 7.4), containing 0.25 M sucrose, 0.1 mM EDTA, 0.1 mM DTT, and 5% glycerol. Samples were frozen and stored at -80º C until use.

2.2 Protein assay

All protein concentrations were measured with a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL), using bovine serum albumin (BSA) to prepare the standard curve.

2.3 UDP-glucuronosyltransferase activity

UGT activity was measured using 1-naphthol as a substrate (Mackenzie & Hanninen, 1980), which is a good substrate for the UGT isoform that is also responsible for the conjugation of acetaminophen. The benefit of using 1-naphthol is that this substrate and its metabolite are fluorescent, which lowers the detection limit in spectrophotometric analysis (Soikkeli et al., 2011). After method development experiments with varying concentrations of substrate, cofactor, microsomes, and Brij58, and different incubation times, the final assays were performed in a 250 µl reaction mixture, containing 0.1 M sodium phosphate buffer pH 7.4, 5 mM magnesium chloride, 25 µg of microsomal protein, treated with 0.3 mg/mg Brij 58, 0.08 mM 1-naphthol (20 µl from a 1mM stock solution in 5% DMSO), and 0.1 mM uridine-diphosphoglucuronic acid (UDPGA). Negative controls consisted of the complete reaction mixture, but with a subsample of mixed microsomes that was boiled for 5 minutes to denature all proteins and thus inhibit any enzyme activity. The reaction was started by adding the UDPGA to the reaction mix. The reaction was performed at room temperature in an all-black 96-well microplate, with 3 replicate wells per sample. The production of the glucuronidated conjugate of 1-naphthol was measured at 293/335 nm (excitation/emission) over 30 min at 2 min intervals, using the kinetic option in SoftMax Pro software with a SpectraMax Gemini plate reader from Molecular Devices. The results are presented as V_{max} values (mUnits/min).

2.4 Sulfotransferase activity

Phenol-type sulfotransferase activity was measured based on the method published by Arand et al. (1987). After initial experiments to optimize substrate, cytosol, and cofactor concentrations, and incubation times, the assay mixture for the reported results consisted of 80 µl of a 1M potassium phosphate buffer, pH 7.4, 200 µl of cytosol, adjusted to 1 mg/ml protein, and 100 µl of 3'-phosphoadenosine-5'-phosphosulfate (PAPS) of 0.1 mM, which was purified by retaining it on a Sep Pak Accell Plus QMA column (Waters Corporation, Milford, MA), and eluted with 150 mM sodium chloride. Negative controls consisted of the complete reaction mixture, but a subsample of mixed cytosol was boiled for 5 minutes to denature all proteins and thus inhibit any enzyme activity. The reaction was started by adding 20 µl of 1mM β-naphthol in 5% dimethyl sulfoxide. The reaction mixture was incubated for 10 min. at 37°C, after which the reaction was stopped by adding 600 µl of 0.4 M glycine solution, acidified with 10% trichloroacetic acid to pH 2.2. The reaction products were separated by adding 5 ml of chloroform, vortexing for 30 sec. and centrifuging at 2000 g for 5 min. to separate the phases. One hundred µl of the upper phase was mixed with 140 µl of 1 N sodium hydroxide in all-black 96-microwell plates, and fluorescence was measured at 285/335 nm excitation/emission wavelengths in a Biotek plate reader. Reaction rates were calculated using a 0.625 – 10 mM standard curve of 2-naphthyl sulfate potassium salt.

2.5 Glutathione S-transferase activity

GST activity was measured as the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) by cytosolic protein (Habig et al., 1974). Assay method was optimized using varying substrate, cytosol and CDNB concentrations over different incubation times, and final reaction mixtures consisted of 250 µl contained 0.1 M HEPES buffer (pH 7.6), 1 mM glutathione (GSH), and 25 µg of cytosolic protein. Negative controls consisted of the complete reaction mixture, with a subsample of mixed cytosol that was boiled for 5 minutes to denature all proteins and thus inhibit any enzyme activity. The reaction was started by adding CDNB (1 mM final concentration). Formation of the CDNB conjugate was measured at room temperature by taking absorption readings on a SpectraMax 190 plate reader (Molecular Devices Corporation, CA) using the V_{max} kinetics option at 9 s intervals for 2 min at 344 nm, and was quantified by using the molar absorptivity of 9.6 mM^{-1} for the enzymatic product.

2.6 N-acetyltransferase activity

N-acetyltransferase activity was measured according to Andres et al. (1985). After method optimization experiments with varying substrate, cytosol and cofactor concentrations, and incubation times, the final reaction assay mixture included 60 µl of cytosol of 2 mg/ml protein, and 20 µl of working solution, consisting of 0.25 M Tris buffer (pH 7.5), 4 mM 1,4-dithiothreitol, 4 mM ethylenediaminetetraacetic acid, 22.5 mM acetyl phosphate, 5 units of phosphotransacetylase/ml of working solution, and 500 µM of p-aminobenzoic acid. Negative controls consisted of the complete reaction mixture, but with a subsample of mixed cytosol that was boiled for 5 minutes to denature all proteins and thus inhibit any enzyme activity. After the reaction mixture was allowed to come to room temp, the reaction was started by adding 20 µl of 1 mM acetyl-coenzyme A, and transferring the reaction tube to a 37°C heating block. Reaction tubes were incubated for 30 min. The reaction in each tube was stopped by adding 50 µl of 20% trichloroacetic acid. Tubes were centrifuged at 20,000 g for 3 min to pellet the denatured proteins, after which 500 µl of 5% dimethylaminobenzaldehyde in acetonitrile was added. Samples were vortexed and spun again at 14,000 rpm for 1 min, after which 2 replicates of 250 µl were transferred to a clear 96-well microplate and absorption was measured at 450 nm.

2.7 Data analysis

Because only a limited amount of liver samples could be obtained for the snake species, with only one specimen for several species, data for the snake species were grouped into the following clusters, based on taxonomic and ecophysiological relatedness: the venomous snakes (*Crotalus adamanteus, Agkistrodon piscivorus*, n=3 for the group), the python samples (*Python bivittatus, Python regius,* n=5), the water snakes (WS) (*Nerodia clarkii compressicauda*,

Thamnophis marcianus, n=2), and other snakes (*Phyllorhynchus decurtatus*, *Rhinocheilus lecontei*, *Pantherophis guttatus,* n=4) . All data were analyzed using the GraphPad Prism 4 software package. After sample mean and standard error were calculated data were log transformed to approach homogeneity of variance before statistical analysis. One-way ANOVA was applied on transformed data, followed by Tukey's Multiple Comparison Test to identify significant differences between species ($p<0.05$). To analyze the genomic information, relevant glucuronosyltransferase isoforms sequences were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). The program CLC Main workbench version 7.6.4 was used for BLASTing and alignment of the found glucuronosyltransferase sequences.

3. Results

3.1 UDP-glucuronosyltransferase activity

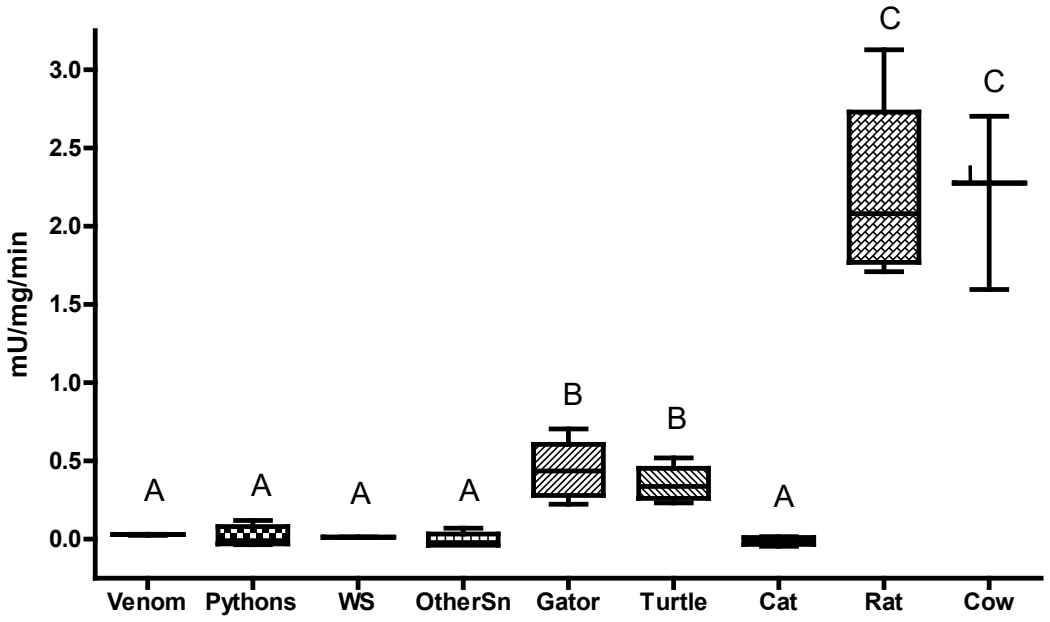

Figure 1. UDP-glucuronosyltransferase activity in microsomes from liver homogenates of snakes and other vertebrates. Species and numbers (n) in each group are listed under *Data Analysis*. Box and whiskers indicate the median, 25-75th percentile and range of data. Groups not connected by the same letter are significantly different from each other ($p < 0.05$).

The results of the glucuronidation assay on 1-naphthol demonstrate that none of the snake samples had any statistically significant glucuronidation activity towards the substrate (Figure 1). This lack of glucuronidation activity was also observed in the cat samples. The alligator and turtle samples had significant glucuronidation activity compared to the snake and cat samples (0.36-0.44 mU/mg/min), but this activity was much lower than in the rat and bovine samples (2.2-2.3 mU/mg/min).

3.2 Sulfotransferase activity

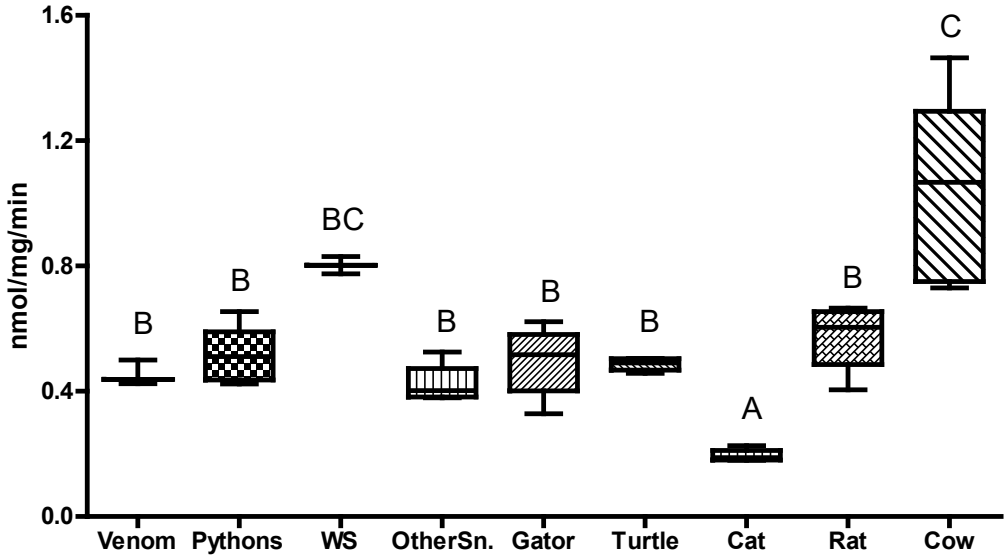

Figure 2. Phenol-type sulfotransferase activity in cytosol from liver homogenates of snakes and other vertebrates. Species and numbers (n) in each group are listed under *Data Analysis*. Box and whiskers indicate the median, 25-75th percentile and range of data. Groups not connected by the same letter are significantly different from each other ($p < 0.05$).

The results of the sulfotransferase assay with β-naphthol showed that the bovine samples had significant higher activity (1.04 nmol/mg/min) than any of the other tested species (Figure 2). On the other hand, cats had significantly lower activity (0.20 nmol/mg/min) than any of the other species. Most snake species had average activity (0.43-0.51 nmol/mg/min), comparable to alligator, turtle and rat samples (0.50, 0.49 and 0.57 nmole/mg/min respectively). The only snakes that had significantly higher activity were the two Natricinae species (*Nerodia* and *Thamnophis,* grouped together as WS) with 0.80 nmol/mg/min.

3.3 Glutathione S-transferase activity

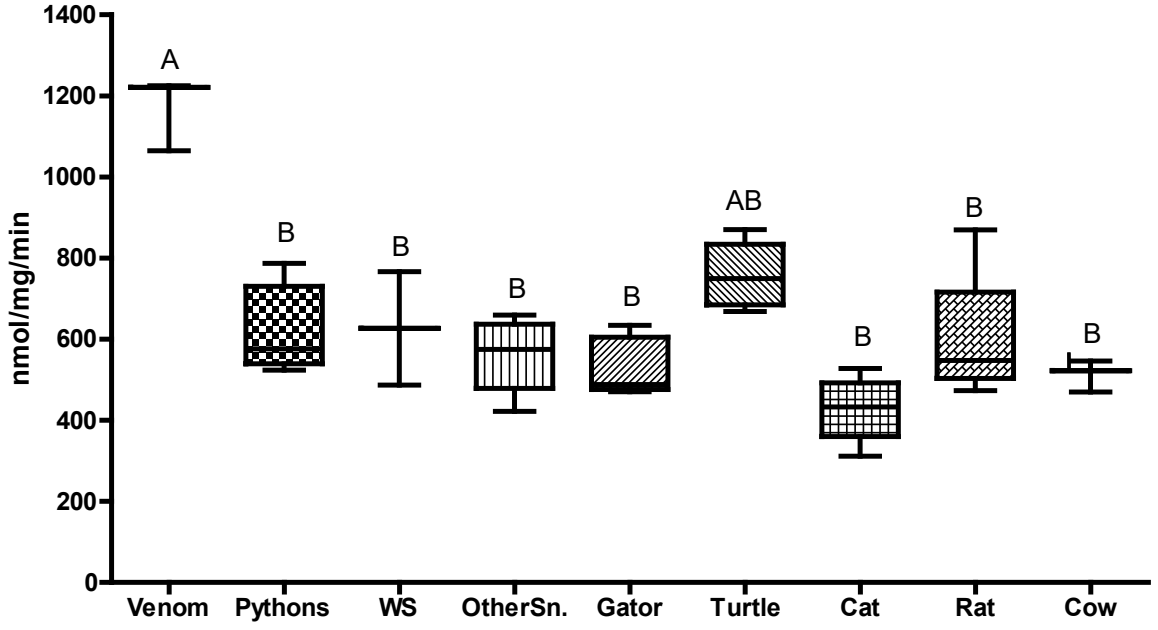

Figure 3. Total glutathione S-transferase activity in cytosol from liver homogenates of snakes and other vertebrates. Species and numbers (n) in each group are listed under *Data Analysis*. Box and whiskers indicate the median, 25-75th percentile and range of data. Groups not connected by the same letter are significantly different from each other ($p < 0.05$).

The glutathione S-transferase assay revealed a fairly constant activity of around 600 nmol/mg/min for most species (Figure 3). The turtle samples were on average a little higher (759.4 nmol/mg/min), but this was not statistically significant. The only group that is significantly different from the others were the venomous snakes (*Crotalus* and *Agkistrodon*), which had on average about double (1170 nmol/mg/min) the activity of the other snake groups.

3.4 N-acetyltransferase activity

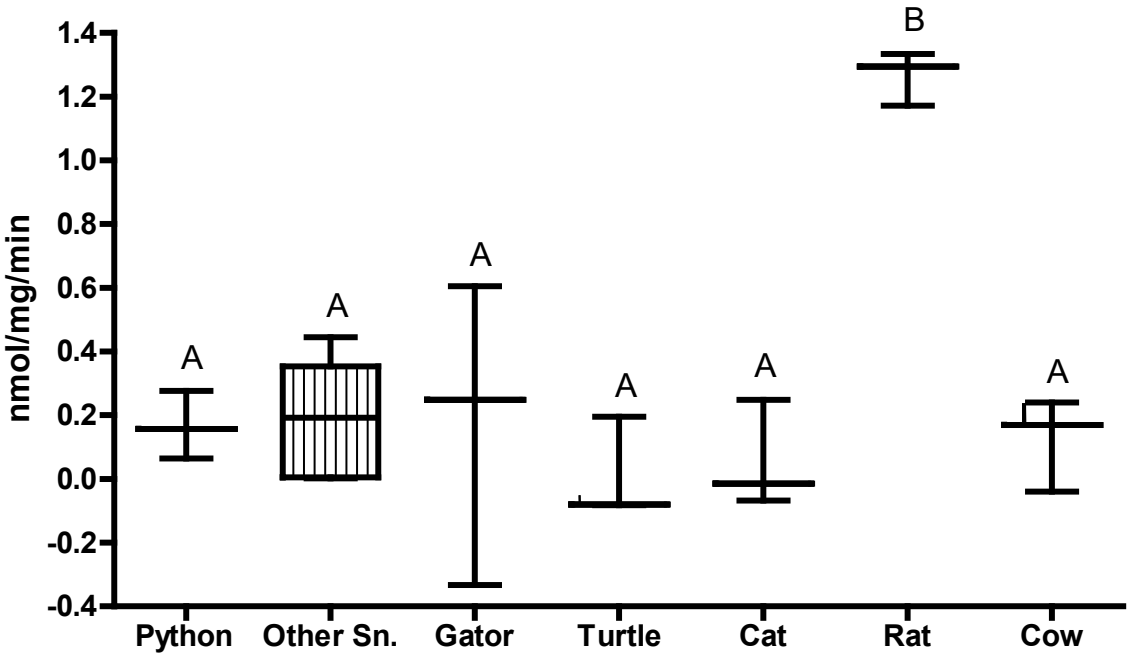

Figure 4. N-acetyltransferase activity in cytosol from liver homogenates of snakes and other vertebrates. For all groups n=3; except "Other Snakes" for which n=6. Box and whiskers indicate the median, 25-75th percentile and range of data. Groups not connected by the same letter are significantly different from each other ($p < 0.05$).

Activity of N-acetyl transferase was extremely low in most species tested (0.01 – 0.18 nmol/mg/min), except for the rat, which had an average activity of 1.27 nmol/mg/min (Figure 4). There were no statistically significant differences between any of the other taxa tested.

Genomic information on the UGT and NAT enzymes studied in the different species were obtained through comparison of published protein sequences (www.ncbi.nlm.nih.gov/Genbank). So far, complete genomes have been sequenced for 4 snake species: Burmese python (*Python bivittatus*), king cobra (*Ophiophagus hannah*), brown spotted pitviper (*Protobothrops mucrosquamatus*), and common garter snake (*Thamnophis sirtalis*). Apart from the king cobra, all other 3 species are reported to have a UGT 1A1 and a UGT2A1-like gene The two UGT isoforms in snakes have 83 – 90 % similarity, while the snake UGT1A1

has only 41 – 42 % similarity to the mammalian species we investigated (SI Figure 1). The snake UGT2A1 isoform has 65 -69 % similarity to the mammalian forms (SI Figure 2).

The American alligator (*Alligator mississippiensis*) genome showed, apart from the UGT1A1 and 2A1 isoforms that are also present in snakes, an additional UGT2C1 isoform. The genome that is taxonomically closest to the common snapping turtle (*Chelydra serpentina*) is the green sea turtle (*Chelonia mydas*). In addition to the UGT isoforms found in snakes (UGT1A1 and 2A1) it has genes that correspond to UGT1A6 and 1A8.

In rats and humans two basic forms of NAT are present, each with different polymorphisms that determine the enzymatic efficiency of the isoforms (SI Figures 3 and 4). Screening of published genome information for the four earlier mentioned snake species showed that *Python* and *Probothrops* have genes that code for an "arylamine NAT2-like" protein (SI Figure 4), but this NAT sequence is less than 40% similar to the mammalian forms (SI Figure 4). The other snake species, *Thamnophis* and *Ophiophagus*, do not have any genomic sequences that resemble NAT isoforms. For other reptilians, like *Alligator* and *Chelonia*, no arylamine NAT genes were found in their genomes. Screening the cow genome revealed that cows have only genomic information for the NAT1 isoenzyme, while the gene for NAT2 is missing. The similarities between bovine NAT1 and human and rat NAT1 are 83% and 81% respectively (SI Figure 3).

4. Discussion

In this study we investigated if snakes species have different detoxification pathways for phenolic compounds like acetaminophen than other vertebrate species, which could explain the observed unusual toxicity of acetaminophen in snake species like the brown treesnake and the Burmese python (Savarie et al., 2000; Mauldin & Savarie, 2010). In humans and other mammalian species, acetaminophen metabolism has been well studied, and the activities of different enzymes involved have been described (Bertolini et al., 2006). Under low dosing scenarios most of the acetaminophen is glucuronidated in the liver, with a small amount being sulfated (Figure 5). The conjugated metabolites are then excreted in urine or bile. However, during an overdose event, these two pathways can get saturated, which leads to an accumulation of acetaminophen that can then be metabolized by CYP2E1 into N-acetyl-p-benzoquinoneimine (NAPQI), a highly reactive intermediate. The NAPQI intermediate is a substrate for glutathione S-transferase (GST), which conjugates the electrophilic metabolite to glutathione, and is then excreted. When the GST enzyme gets saturated, or glutathione reserves are rapidly depleted, an accumulation of NAPQI can occur, which leads to acute liver and kidney damage (Hart et al., 1991).

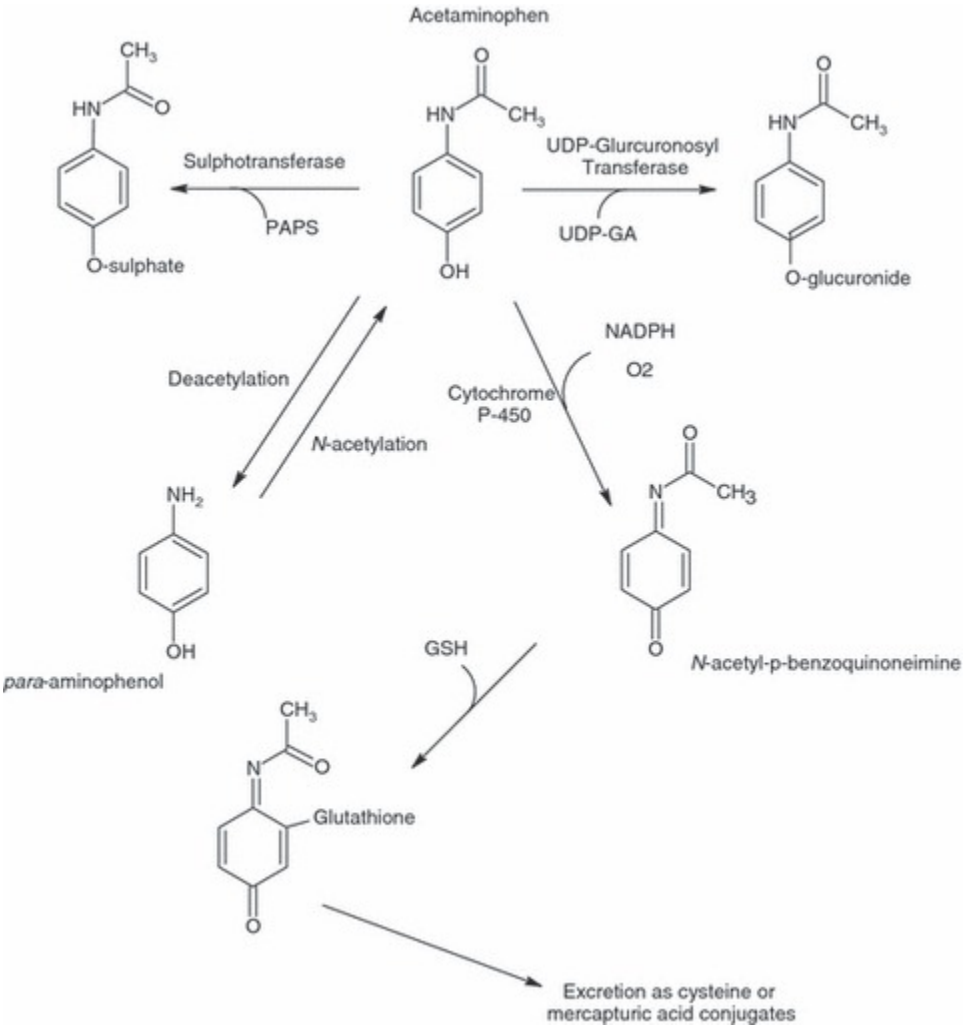

Figure 5. Metabolism of acetaminophen, after McConkey et al. (2009)

To compare the activities of the enzymes involved in acetaminophen metabolism, snake liver samples were analyzed, together with samples from 2 other reptiles (American alligator and snapping turtle) and 3 mammalian species (cat, rat, cow). The results showed that none of the snake samples had any glucuronidation activity, while sulfation and glutathione conjugation activities were comparable to the other species. A limitation of this study is that we did not use acetaminophen (or aminophenol for NAT) as a substrate in the enzyme assays, but used comparable substrates that were better suited for the chosen methods, and therefore the conclusion about severe acetaminophen toxicity in snakes is indirect.

The results indicated that snakes in general do not have a functional UGT isoform that can conjugate phenolic compounds, like acetaminophen. While the activity of a sulfotransferase isoform could compensate for this lack of UGT activity by conjugating phenolic compounds with a sulfonate group, it is generally accepted that sulfotransferases have a high substrate affinity, but relatively low activity compared to glucuronosyltransferases, presumably because of a slow, rate-limiting synthesis of the sulfonate donating cofactor PAPS (James, 2014). This means that that snakes exposed to acetaminophen would rapidly accumulate this compound in their liver. As in mammals, this could lead to the production of the highly reactive NAPQI metabolite by CYP isoforms, followed by liver damage. However, when brown treesnakes were exposed to acetaminophen they appeared to die from methemoglobinemia and not from liver damage (Clark et al., 2018), which indicates that another process is likely responsible for the sensitivity in snakes.

A pathway that may explain the occurrence of methemoglobinemia is the accumulation of aminophenol as a metabolite of acetaminophen. Aminophenol is a known inducer of methemoglobinemia, but generally does not appear to occur as a result of acetaminophen exposure. In humans the deacetylation process of acetaminophen by amidase has been observed, and can cause toxicity in kidneys, but is usually rapidly reversed by the activity of N-acetyltransferase (NAT) (Nicholls et al., 1995), although in rare cases of severe overdose methemoglobinemia has been observed (Kanji et al., 2013). If in snakes this process is inhibited or non-existent, it would lead to an accumulation of aminophenol, which could then reduce hemoglobin in red blood cells, and induce methemoglobinemia. We therefore measured NAT activity in the liver samples, and indeed found that the snake livers have no significant NAT activity, which would explain the observed hypoxia in acetaminophen exposed brown treesnakes (Clark et al., 2018).

The same phenomenon of methemoglobinemia after acetaminophen exposure has been observed in cats and other felines, who also lack an active UGT isoform that can metabolize acetaminophen, and in addition, as shown in our results and has been reported by others, cats also lack NAT enzyme activity, which leads to the accumulation of aminophenol and the consequent occurrence of methemoglobinemia (Nash et al., 1984; McConkey et al., 2009).

The low SULT activity towards phenolic compounds in cats has been described before, but is not entirely absent because sulfated metabolite is excreted by cats when dosed with acetaminophen (Gregus et al., 1983; Watkins et al., 1986; Savides et al., 1984).

The total lack of UGT activity in snakes appears not to be universal in reptiles; the results presented here demonstrate that the samples of the American alligator and the snapping turtle did have measurable UGT activity compared to the snakes, but this activity was much lower than the rat and cow samples we tested. This would imply that alligators and turtles would be

less sensitive to acetaminophen toxicity, but it has been reported that the Nile monitor lizard, another reptilian species, is still sensitive to acetaminophen (Mauldin & Savarie, 2010), which may be explained by a slow UGT conjugation rate combined with a lack of NAT activity, as was demonstrated in this study. Little is known about conjugating enzymes in turtles, but given the broad spectrum of ecological niches that the taxonomical order of Testudines occupy and the variety of food sources they use, it would be very interesting to investigate the expression of conjugating enzymes in a broad variety of turtles.

Interestingly, while some amphibians also appear to lack UGT activity towards phenolic compounds, and predominantly use sulfation as a biotransformation pathway, other amphibians have active UGT-like glucusidation enzymes, that use glucose instead of glucuronic acid as cofactor (Ueda et al., 2011). The use of glucose as a cofactor for UGT enzymes is also seen in invertebrates, and therefore considered a more primitive process than the use of glucuronic acid, which is found in higher vertebrates (Mackenzie et al., 1997)

Now that more and more complete annotated genomes are available, the enzymatic activity results obtained in this study can be compared to genomic information on the presence of specific genes that code for these enzymes in different species. As of now, complete genomes have been sequenced for 4 snake species: Burmese python (*Python bivittatus*), king cobra (*Ophiophagus hannah*), brown spotted pitviper (*Protobothrops mucrosquamatus*), and common garter snake (*Thamnophis sirtalis*). Apart from the king cobra, all other 3 species are reported to have a UGT 1A1 and a UGT2A1-like gene (www.ncbi.nlm.nih.gov/Genbank). For the king cobra only a partial UGT 2A2 like gene was reported. In mammals the UGT1A1 isoform is mostly involved with conjugating bilirubin, while UGT2A1/2A2 is active in conjugating bile salts and steroid hormones. The lack of other UGT isoforms in snakes, especially UGT1A6 which has simple phenolic compounds as preferred substrates, would explain the lack of activity seen in our experiments towards 2-naphthol, and as a consequence towards acetaminophen. A protein BLAST revealed that the two UGT isoforms in snakes have 83 – 90 % similarity, while the snake UGT1A1 has only 41 – 42 % similarity to the mammalian species we investigated (SI Figure 1). The snake UGT2A1 isoform has 65 -69 % similarity to the mammalian forms (SI Figure 2). Even if the mammalian UGT isoforms are promiscuous and accept other substrates than their preferred substrate, the considerable structural differences between the mammalian and the snake isoforms may be an additional reason why snakes cannot glucuronidate simple phenols like naphthol and acetaminophen.

In the other reptilians we tested for UGT activity towards 2-naphthol, more UGT coding genes are found. The American alligator (*Alligator mississippiensis*) genome is also available, and shows apart from the UGT1A1 and 2A1 isoforms that are also present in snakes, an additional UGT2C1 isoform, which may explain the slightly higher observed UGT activity in our alligator

samples. The completely sequenced genome that is taxonomically closest to the common snapping turtle (*Chelydra serpentina*) that we investigated, is the green sea turtle (*Chelonia mydas*). This species has a greater variety of UGT isoforms; in addition to the ones found in snakes (UGT1A1 and 2A1) it has genes that correspond to UGT1A6 and 1A8. This makes it plausible that the common snapping turtle also has more UGT isoforms than snake species.

Based on what is known about the role of N-acetyltransferase (NAT) in causing methemoglobinemia in cats, it is relevant to investigate the presence of NAT genes in the animal models used in this study. In rats and humans two basic forms of NAT are present that conjugate an acetyl group to arylamines like aminophenol, each with different polymorphisms that determine the enzymatic efficiency of the isoforms (SI Figures 3 and 4). In cats only one isoform (NAT1X2) is found (www.ncbi.nlm.nih.gov/Genbank), and because we did not measure any significant activity towards p-aminobenzoic acid in the cat samples, this one isoform is probably not functional towards aminophenols, but may be able to metabolize other substrates (McConkey et al., 2009).

The lack of NAT activity in our snake samples would imply that snakes could also be missing the genes for NAT isoforms. Screening of published genome information for the four snake species for which complete genomes are available showed that *Python* and *Probothrops* have genes that code for a "arylamine NAT2-like" protein (SI Figure 4), while *Thamnophis* and *Ophiophagus* do not have any genomic sequences that resemble NAT isoforms. Given the lack of NAT activity towards p-aminobenzoic acid in the snake samples we tested, it can be concluded that the NAT2-like gene that is found in some snakes is not coding for a functional enzyme, although it may be able to other types of substrates. The sequence in GenBank is less than 40% similar to the mammalian forms (SI Figure 4). In addition, for other reptilians, like *Alligator* and *Chelonia*, no arylamine NAT genes are found in their genomes (www.ncbi.nlm.nih.gov/Genbank), which further supports the assumption that functional forms of these genes towards arylamine substrates are absent in reptilians.

While the rat samples had good NAT activity in the samples tested in this study, it was initially concerning that no activity was found in the cow samples. However, screening the cow genome revealed that cows have only genomic information for the NAT1 isoenzyme, while the gene for NAT2 is missing (Glenn et al., 2010). In other mammals, like rat and human, both NAT1 and NAT2 can use p-aminobenzoic acid as substrate, although the polymorphisms in these genes result in a wide variety of actual enzymatic activities (Sim et al., 2008). The absence of NAT activity in our cow samples may be explained by both these factors: the lack of NAT2 enzyme, and a slow, or non-functional NAT1 isoform. The similarities between bovine NAT1 and human and rat NAT1 are 83% and 81% respectively (SI Figure 3).

From a phylogenetic point of view, it is very interesting to analyze why certain taxa do not have functional genes for the conjugation of phenolic compounds. It is assumed that UGT isozymes have evolved as a result of the herbivore-plant arms race in which selective pressure benefitted plants with potentially toxic phenolic compounds, and increasing the fitness of herbivores with effective detoxification pathways for these chemicals (Bock, 2016). This coevolution of plants and herbivores presumably resulted in plants rich in poor tasting, toxic phenolic deterrents and animals with a wide variety of detoxifying enzymes. In cats and other felines the phenol-type UGT gene is present, but has suffered several point mutations which made the gene non-functional (Court & Greenblatt, 2000). The investigations presented here indicate that snakes do not seem to have a coding sequence at all for a phenol-type UGT isoform. These two different genomic origins for the sensitivity to acetaminophen are most likely a result of evolutionary processes related to feeding strategies. Both felines and snakes are top predators, with limited or no exposure to plant derived phenolic compounds (Shrestha et al., 2011). This would mean that there is no selective pressure on having, or maintaining, a functional enzyme system that detoxifies phenolic compounds.

5. Conclusions

Based on the investigations presented here, snake species do not have a functional phenol-type glucuronidation enzyme, and they are also lacking acetylation activity. These observations were in concordance with the lack of genomic coding sequences for these enzymes. The lack of these enzymes make snakes very susceptible to toxicity of phenolic compounds like acetaminophen, and explains the observed methemoglobinemia observed in brown treesnakes exposed to acetaminophen. While the toxic effects of acetaminophen in snakes appear to be the same as has been observed in cats and other felines, the underlying mechanism is different in that snakes appear to be missing the gene for a phenol-type UGT, whilst in felines this gene has mutated into a pseudogene. It is unclear if snakes ever had a functional phenol-type UGT gene, and further comparisons of genomic information and enzyme activities with other reptilians could shed more light on the phylogenetic history of these enzymes.

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Supplemental Information

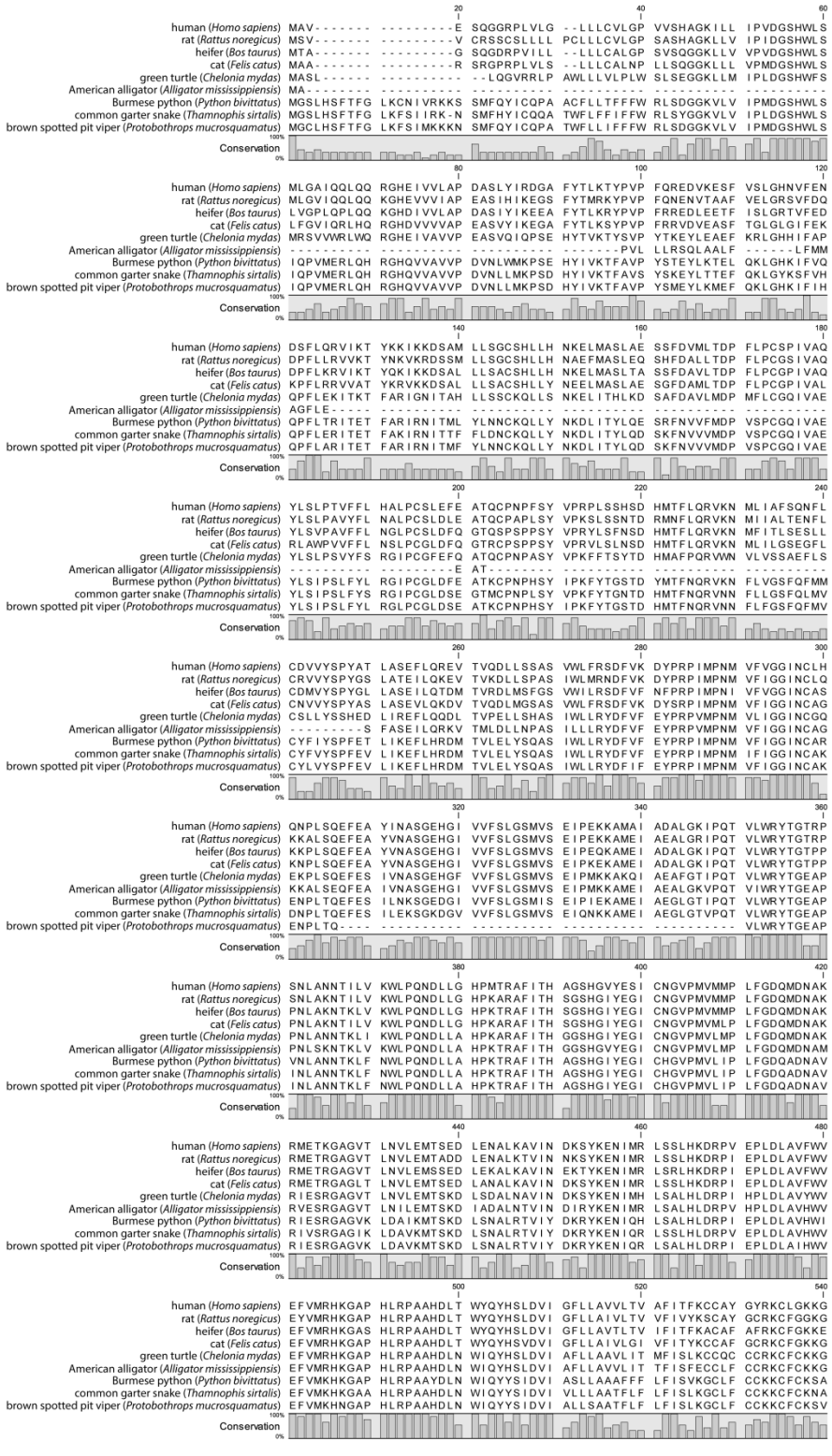
human (Homo sapiens)
rat (Rattus noregicus)
heifer (Bos taurus)
cat (Felis catus)
green turtle (Chelonia mydas)
American alligator (Alligator mississippiensis)
Burmese python (Python bivittatus)
common garter snake (Thamnophis sirtalis)
brown spotted pit viper (Protobothrops mucrosquamatus)
Conservation

Figure 1. Alignment of human (Uniprot: P22309), rat (Uniprot: Q64550), Heifer (Uniprot: A7YWD3), cat (Uniprot: BAA24692), American alligator (Uniprot: A0A151N9S0), Burmese python (NCBI: XP_007434974), common garter snake (NCBI: XP_013913257) and brown spotted pit viper (NCBI: XP_015678725) UDP-glucuronosyltransferase 1A1 protein.

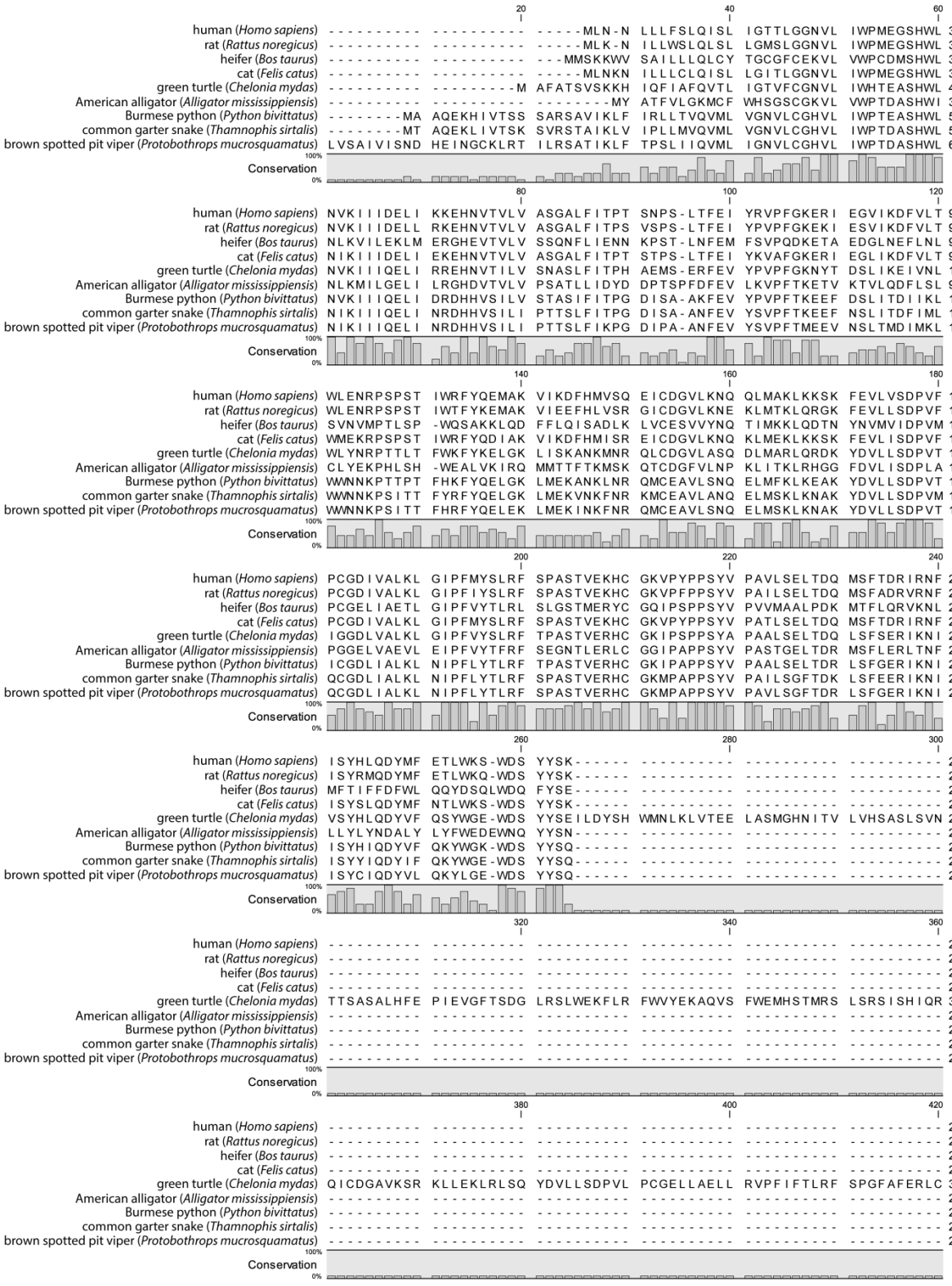

Figure 2. Alignment of human (Uniprot: Q9Y4X1), rat (Uniprot: P36510), Heifer (NCBI: NP_001092414), cat (NCBI: XP_003985357), American alligator (Uniprot: A0A151MIX4), Burmese python (NCBI:

XP_015745339), common garter snake (NCBI: XP_013929149) and brown spotted pit viper (NCBI: XM_015815164) UDP-glucuronosyltransferase 2A1 protein.

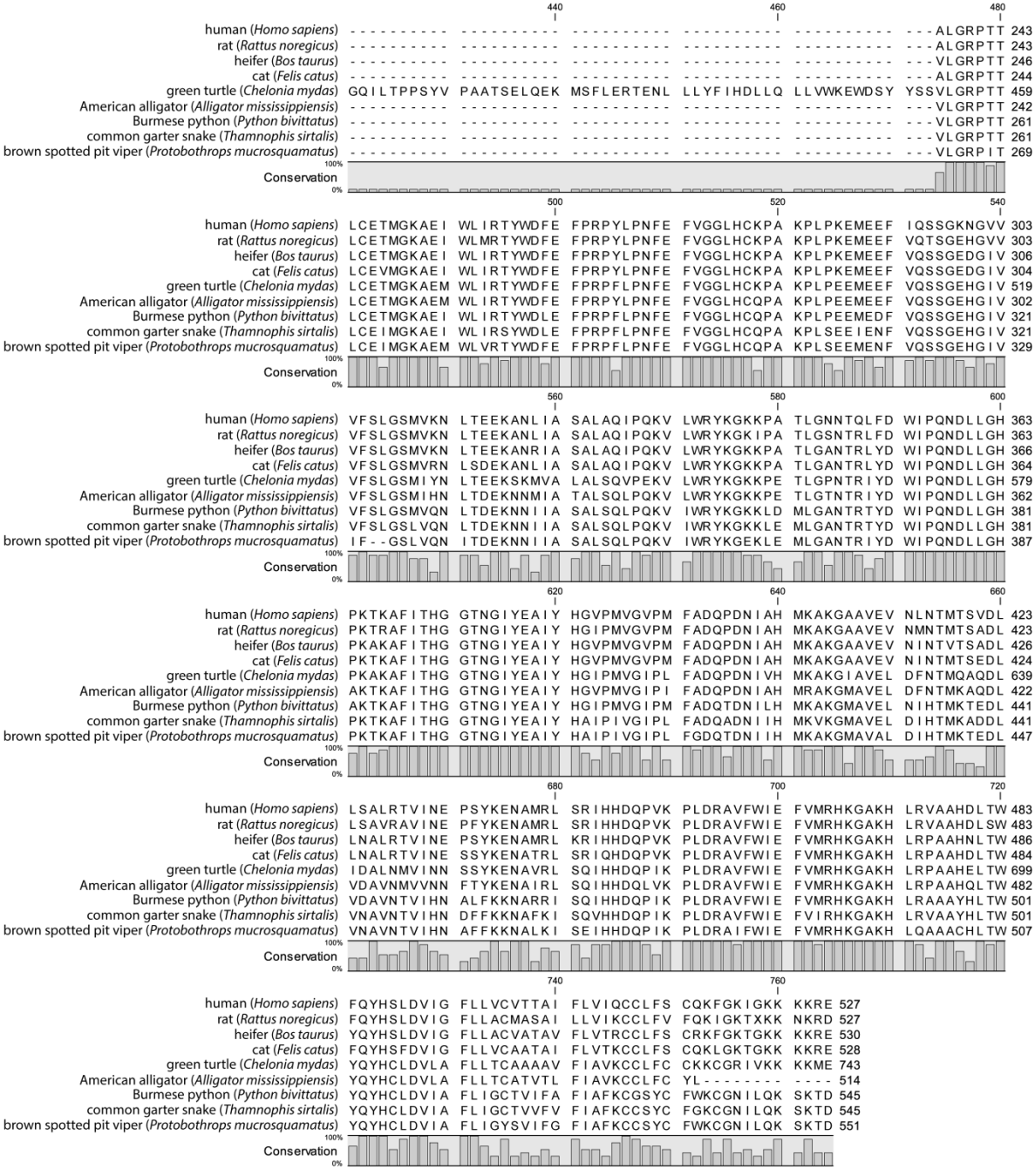

Figure 2 (continued). Alignment of human (Uniprot: Q9Y4X1), rat (Uniprot: P36510), Heifer (NCBI: NP_001092414), cat (NCBI: XP_003985357), American alligator (Uniprot: A0A151MIX4), Burmese python

(NCBI: XP_015745339), common garter snake (NCBI: XP_013929149) and brown spotted pit viper (NCBI: XM_015815164) UDP-glucuronosyltransferase 2A1 protein.

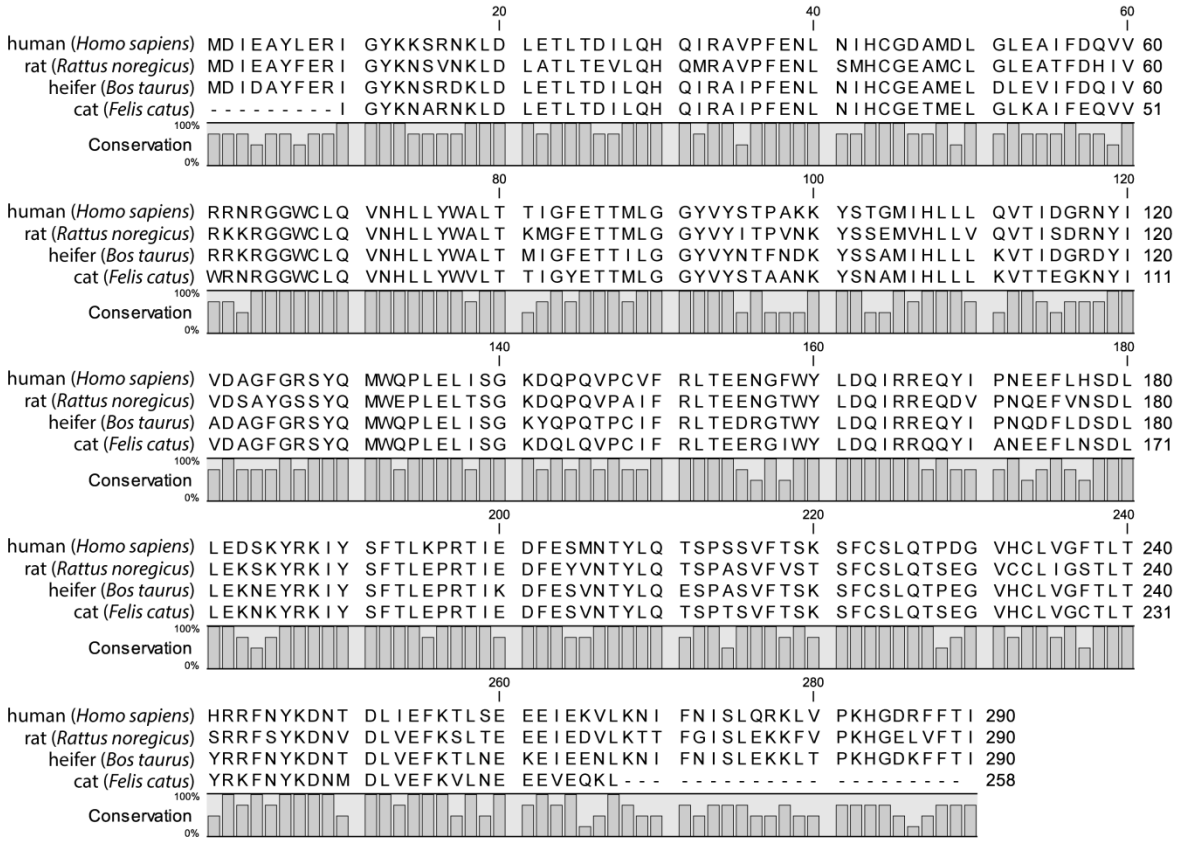

Figure 3. Alignment of human (Uniprot: CAA34905), rat (Uniprot:P50297), heifer (Uniprot: Q1JPA6) and cat (Uniprot: O62696) arylamine N-acetyltransferase 1 protein.

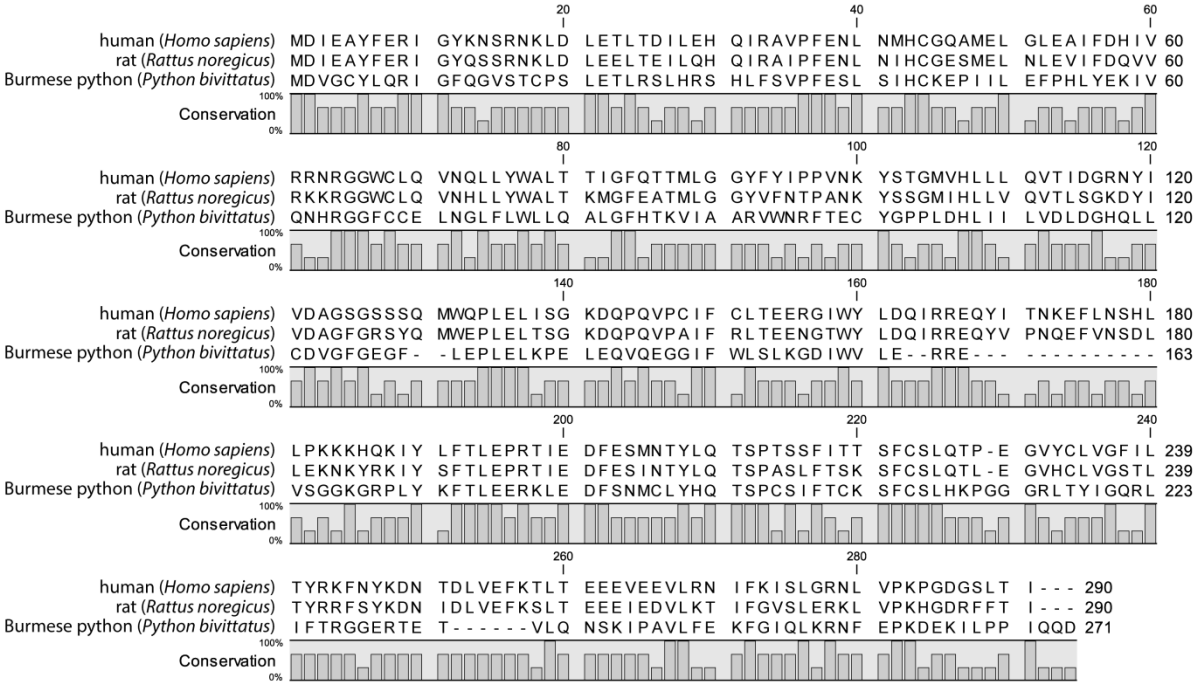

Figure 4. Alignment of Human (Uniprot: P11245), rat (Uniprot: P50298) and python (NCBI: XP_007442853) the arylamine N-acetyltransferase 2 protein.