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The use of Liquid Chromatography-Mass Spectroscopy to Determine Specific Second-Generation Rodenticide Diastereomer Ratios Within Wildlife Liver Samples: The Pursuit Towards a Safer Bait Formula.

By

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

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of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2022

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Abstract

Second generation anticoagulant rodenticides (SGARs), developed first to exterminate rodent populations, have now become a means of accidental secondary wildlife intoxication. Utilizing highly potent bait formulations designed to overcome resistance, these SGARs now exhibit ongoing persistence in liver and other tissues. Particularly, SGARs persist antemortem within target consumer tissues allowing for non-target wildlife exposure. The aim of this research was to utilize bromadiolone's and brodifacoum's diastereomer configurations of cis and trans isomers to determine whether one isomer had less tissue persistence. To accomplish this, High Performance Liquid Chromatography paired with Mass Spectrometry (HPLC-MS/MS) was applied. Through partnership with the California Department of Fish and Wildlife, 45 non-target wildlife livers including coyote (*Canis latrans*), gray fox (*Urocyon cinereoargenteus*), and cougar (*Puma concolor*) were obtained and analyzed for diastereomer proportions of bromadiolone and brodifacoum. Current bromadiolone and brodifacoum baits were tested and isomer ratios were compared to ratios in primary consumer species and secondarily exposed non-target species. All 45 non-target samples contained brodifacoum and 41 had bromadiolone residues. For bromadiolone, trans-bromadiolone was shown to be more persistent in the food chain with 56% (23/41) having $\geq$ 91%. For brodifacoum, 35/45 cougar and coyote samples displayed the cis isomer as more persistent. However, gray foxes displayed the trans isomer of brodifacoum. Thus, brodifacoum's isomer pattern is variable, perhaps reflecting species differences in brodifacoum metabolism and elimination. Moreover, the benefits of reformulating brodifacoum to a certain isomer were not as clear. However, this research establishes that reformulation of bromadiolone bait to include less trans isomer will likely lower both food chain persistency and non-target wildlife exposure.

<u>Acknowledgments</u>

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1. Introduction

1.1 Anticoagulant Rodenticides

In order to successfully control a rodent infestation an individual may use a chemical from a group known as anticoagulant rodenticides or ARs. Categorized under pesticides, rodenticides are used specifically in the extermination of target species[1] such as squirrels, mice, rats, and other rodent species. Anticoagulant rodenticides are formulated as bait blocks for consumption by target species. ARs produced before 1970 were known as first-generation anticoagulant rodenticides or FGARs. Commonly used FGARs include warfarin, chlorophacinone, diphacinone, coumachlor, coumafuryl, coumatetralyl, and pindone (Berny et al., 2018). FGARs were short-lived in tissues after consumption. However, certain strains of rodents were found to be resistant to the chemicals. Due to the development of resistance by rodents to FGARs, second generation ARs (SGARs) were developed. SGARs including difethialone, brodifacoum, bromadiolone, and difenacoum are currently available for use in the United States (Horak et al., 2018). Brodifacoum and bromadiolone are the most commonly used rodenticides in the SGAR class. Brodifacoum grew in popularity due to its ability to exterminate bromadiolone-resistant house mice (*Mus musulus*) (Blažić et al., 2018). In recent decades bromadiolone has been the primary rodenticide for residential and commercial rodent control (Blažić et al., 2018). In the state of California, however; second generation rodenticides have been prohibited from sale through consumer outlets since September of 2020. The restriction was implemented to reduce possible exposure pathways to non-target[2] wildlife. Thus, SGARs can only be obtained by commercial and licensed pesticide companies to limit accidental exposure to these highly toxic chemicals (Messenger-Likes & Quinn 2020).

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[1] Target species are those that bait blocks are intended for.
[2] Non-target species are not the targeted species of AR bait, but may still experience secondary exposure.

Each SGAR formulation consists of a unique combination of stereoisomers commonly referred to as diastereomers. Diastereomers are characterized by an identical compound formula but differing spatial structures. These differences in spatial structure can be referred to as cis and trans isomers. The respective structures for bromadiolone and brodifacoum are provided in **Figures 1a & 1b**. Cis refers to the same side arrangement and trans to the opposite side arrangement of particular substituent groups (Libretexts, 2021).
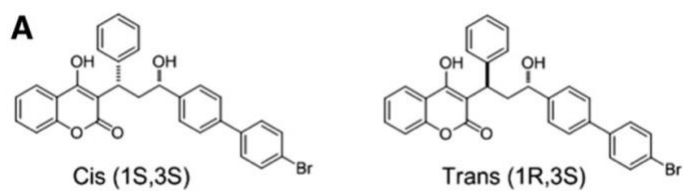
Figure 1a. Diagram of cis and trans isomers of bromadiolone displaying the alternating attachment of the middle phenyl ring. From "Management of Rodent Populations by Anticoagulant Rodenticides: Toward Third-Generation Anticoagulant Rodenticides" by Marléne et al., 2016, Drug Metabolism and Disposition, pg. 162
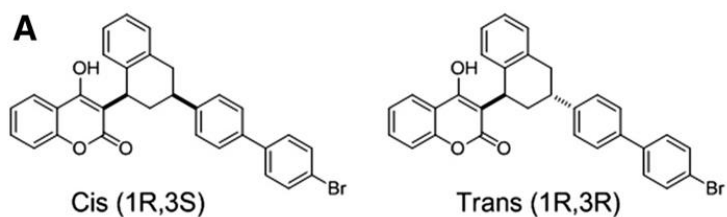
Figure 1b. Diagram of cis and trans isomers of brodifacoum displaying the alternating attachment of the tetralin group. From "Management of Rodent Populations by Anticoagulant Rodenticides: Toward Third-Generation Anticoagulant Rodenticides" by Marléne et al., 2016, Drug Metabolism and Disposition, pg. 162

Research has shown that there can be a change in diastereomer proportionality after bait consumption by target animals such as rodents (Damin-Pernik et al., 2016). Furthermore, the diastereomer ratio may fluctuate again after a non-target animal such as a cougar or fox ingests

the tissue of a target species.

Consumption of target animal tissue by non-target species is an unfortunate by-product of having such potent rodenticides and can lead to secondary intoxication. A study conducted on the persistency of bromadiolone within rodent tissues found that the risk of secondary exposure to non-target wildlife can occur 15-20 days after rodent AR consumption and possibly much longer (Sage et al., 2008). SGAR exposure has been identified in many non-target species such as birds of prey, canines, felines, insectivores, and many more (DuVall et al.,1989; Dowding et al., 20; Murray, 2011; Wiens et al., 2019) further identifying an unfortunate association between non-target species and anticoagulant rodenticide intoxication. A more sustainable solution to this issue is through the research and development of less persistent bait formulations that exterminate target species while still protecting various non-target wildlife.

2. Background

2.1 Development of Anticoagulant Rodenticides

In 1920s Wisconsin, a group of cattle experienced an increase in deaths and exhibited internal bleeding. Through investigation, it was determined that the cattle had consumed a fungal-infested sweet clover hay mixture. The combination of a naturally-occurring coumarin in the hay mixed with mold led to the formation of dicoumarol, which was determined to be the cause of the hemorrhage. FGARs were derived from the dicoumarol found in the hay (Hadler & Buckle 1992). The identification and chemical characterization of dicoumarol would be the key to the development of anticoagulant rodenticides across the globe.

Most notably, warfarin was derived from dicoumarol by the Wisconsin Alumni Research Foundation in the 1940s. Once created, warfarin quickly increased in popularity due to its potency and was the first anticoagulant available to consumer markets (Berny et al., 2018). Other

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first-generation rodenticides were also synthesized during this time including diphacinone and chlorophacinone. First generation rodenticides have a modest toxicity level, the lethal dosage of most first-generation chemicals is between 10-50 mg/kg for body weight. This low toxicity level requires the target species to consume bait repeatedly for death to occur. Specifically for warfarin, the lethal dosage is reported as 0.75 mg/kg for five consecutive feedings (Gupta, 2012). Due to this constant feeding, rodents began to develop resistance to the available bait formulations. To combat this resistance, SGARs were developed.

SGARs were designed with a few key factors in mind. The first was having a sufficiently high toxicity level to ensure that lethality could occur after a single feeding. Lethal dosages for bromadiolone in rats, mice, and rabbits are 0.65, 0.99, and 1.0 mg/kg, respectively (Gupta, 2012). The second is that by producing a group of rodenticides with such high toxicity levels, the chance of target species developing resistance would be less likely. Bromadiolone has shown efficacy against warfarin resistant target species such as *Rattus norvegicus* (Norway rats), *Rattus rattus* (black rat), and *Mus musculus* (house mouse) (Redfern & Gill, 1980). Similarly, brodifacoum has shown efficacy against a wide variety of species including *Rattus norvegicus* (Norway rats), and *Mus booduga Gray* (field mouse). Brodifacoum has also been used to control non-rodent species such as *Corvus ruficollis* (brown necked ravens) in Egyptian trade waterways. Lastly, SGARs were designed to be much more appetizing to target species than FGAR to ensure the consumption of bait (Gupta, 2012).

An unfortunate byproduct of SGAR use is the potential for secondary intoxication of wildlife and domestic animals. **Figure 2** below demonstrates the spread of AR residues through the food chain as they progress from primary exposure to secondary exposure (Regnery et al., 2018). For example, bromadiolone has been identified in *Mustela putorius* (polecats) in areas in

England where baits have been placed for rodents. Likewise, in a study done in and around New York brodifacoum was detected in 44 of 55 non-target wildlife animals suspected of anticoagulant poisoning (Gupta, 2012). These findings show that ARs can and are progressing through the food chain leading to secondary exposure and intoxication of wildlife (Berny et al., 1997; Fourel et al., 2017; Horak et al., 2018).
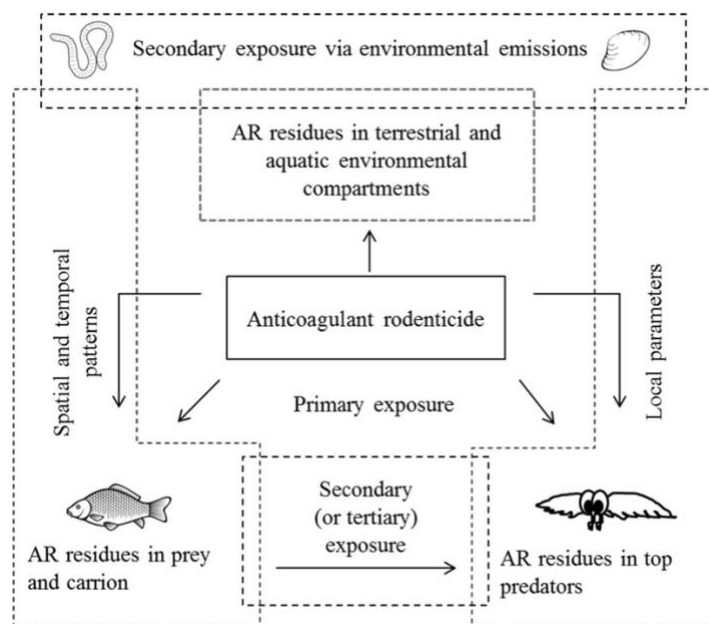
Figure 2. A demonstration of common exposure pathways of AR residue within target species and potential for secondary toxicity to non-target wildlife. Reprinted by permission from Springer Nature Customer Service Centre GmBH: Springer [Environmental Chemistry Letters] [Rating the risks of anticoagulant rodenticides in the aquatic environment: a review] [Regnery, J., Friesen, A., Geduhn, A., Göckener, B., Kotthoff, M., Parrhysius, P., Petersohn, E., Reifferscheid, G., Schmolz, E., Schulz, R. S., Schwarzbauer, J., & Brinke, M] [COPYRIGHT] 2018. https://link.springer.com/article/10.1007/s10311-018-0788-6

2.2 Properties of Bromadiolone and Brodifacoum

Bromadiolone (3-(3-(4'-bromobiphenyl-4-yl)-3-hydroxy-1-phenylpropyl)-4-hydrocoumarin) has two different structural configurations that exist in bait. These racemic ratios are at a fixed proportion chosen by governing agencies (Fourel et al., 2017). The different configurations can be attributed to having two chiral centers causing differences in functional group attachments, therefore producing a set of diastereomers known as cis and trans structures.

Further bromadiolone cis and trans separation can be classified as cis (1S,3S) and trans (1R, 3S). For reference see **Figure 1a** above for opposing arrangements of stereoisomers. The only difference is the placement of the benzene ring attached to the second carbon in the branched chain. In the cis structure, the benzene ring is attached to the backside of the carbon pointing outward, with the corresponding hydrogen molecule pointed forwards. The trans structure has these attachments in the opposite locations. The benzene ring is now attached to the front side of the carbon and the hydrogen is in the back (Damin-Pernik et al., 2016).

Brodifacoum (3-[3-[4-(4-bromophenyl) phenyl]-1,2,3,4-tetrahydronaphthalen-1-yl]-4-hydroxychromen-2-one) also has two different spatial arrangements due to chiral centers that contribute to the racemic ratio. The cis (1R,3S) and the trans (1R, 3R) can be seen in **Figure 1b** above. Located in the middle of the molecule is a tetralin ring ($C_{10}H_{12}$), a derivative of the aromatic functional group naphthalene. In cis brodifacoum, the tetralin ring is attached to carbons in a front-facing position on both the 3-hydroxycoumarin and 4-bromobiphenyl. In the trans arrangement, the tetralin group is only attached in the front-facing position on the 3-hydroxycoumarin and the back position of the 4-bromobiphenyl group (Damin-Pernik et al., 2016). Even though diastereomers have identical compound formulas, the chemical properties can differ slightly from cis and trans substrate attachments allowing for the separation of individual diastereomers when using chiral HPLC chromatographic columns and specific chromatographic conditions.

2.3 Potential Routes of Non-target Species Exposure

Bromadiolone and brodifacoum rodenticides are categorized as "restricted use" and can only be used legally by a contractor or licensed professional. California prohibited the use of SGARs for rodent control due to their high risk of secondary wildlife exposure. However, the

bill outlines exceptions of use including protecting public health, waterways, offshore islands, many agricultural facilities, and production sites, and for research purposes (A.B. 1788, 2020). Though this new law is a step forward in the protection of wildlife, these exceptions make SGAR exposure a continuous and widespread issue (Messenger-Likes & Quinn, 2020).

For many non-target species their prey of choice are rodents such as rats, squirrels, and rabbits, which are target species exposed to SGAR baits. Similarly, animals such as opossums and raccoons will consume bait and are identified as primary consumers though not directly targeted groups. Once ingested by these species, the AR chemicals will accumulate in the liver and possibly lead to exposure and secondary intoxication of non-target wildlife (Elliott et al., 2018). SGARs are found in a large number of non-target species. A study conducted on 43 poisoned rabbits (*Oryctolagus cuniculus*) identified brodifacoum concentrations from 0.05-11.7 ppm with 41 of 43 having greater than 0.05 ppm (Rammell et al.,1984). In Denmark SGARs were detected in 90% of liver tissues from weasels and stoats with bromadiolone present at the highest concentrations (Elmeros et al., 2011). In addition, a study in Spain identified nocturnal raptors and mammalian carnivores commonly exposed to SGARs (Hernandez-Moreno et al., 2013).

In addition to consuming target species, exposure to SGARs can also occur due to contaminated dietary items such as insects. Swift foxes (*Vulpes velox*) in South Dakota were examined for their dietary habits and showed insects to be 27% of their diet (Uresk & Sharps, 1986). With a diet consisting of insects, the possibility of secondary poisoning due to the consumption of contaminated invertebrates is a possibility (Seljetun et al., 2019). For example, slugs (*Deroceras reticulatum*) were exposed to brodifacoum bait in the wild for 15 days and 23 were collected for examination of AR residues. Brodifacoum was detected in 91% of the slugs

collected. This suggests that slugs and possibly other insects can pose a considerable risk of non-target wildlife exposure (Alomar et al., 2019). Furthermore, a study on the diet of urban foxes (*Vulpes vulpes*) in Switzerland showed 17.5% of invertebrate species in stomach contents further validating the potential for secondary exposure (Contesse et al., 2004).

2.4 Mechanism of Action: Bromadiolone and Brodifacoum

Bromadiolone and brodifacoum cause coagulopathy by depleting concentrations of vitamin K_1, resulting in cessation of production of vitamin K-dependent clotting factors II, VII, IX, and X (Gupta, 2012). These clotting factors must bind to calcium ions (Ca^{2+}) to participate in clot formation. To bind correctly, the glutamyl residues located on those clotting factors must be converted to γ-carboxyl glutamyl (gamma carboxyl glutamyl). This conversion process is called carboxylation. Carboxylation uses a cofactor of vitamin K_1 hydroquinone to covert the glutamyl residues. In this reaction carboxylase, in the presence of vitamin K, converts vitamin K_1 hydroquinone to an epoxide known as vitamin K_1 2,3-epoxide. Under normal conditions vitamin K_1 epoxide is reduced to vitamin K_1 by epoxide reductase (VKOR) and reutilized. However, under conditions where ARs are present the enzyme epoxide reductase loses its functionality and thus is not able to reduce vitamin K_1 2,3-epoxide to vitamin K_1. With the lack of vitamin K_1 present, the carboxylation process cannot occur, and subsequently, no active clotting factor can be generated (Gupta, 2012). SGARs inhibit vitamin K_1 2,3-epoxide reductase more effectively than FGARs, have increased half-lives through elevated lipid solubility, undergo continuous hepatic absorption through recirculation, leading to accumulation in the liver (Gupta, 2012).

2.5 Clinical Signs and Postmortem Lesions of SGAR Intoxication

The clinical features of SGAR poisoning are the same for all bait formulations. Once a species is exposed, important systems of the body begin to experience significant hemorrhaging due to the loss of clotting factors (Gupta, 2012). Clinical hemorrhaging will occur 2-3 days after the initial exposure. On physical examination, clinical signs can include tachycardia, hematomas, lethargy, depression, hemorrhagic shock, and lameness (Hovda et al., 2016)

Gross pathological findings from AR exposure reveal significant internal hemorrhaging. For example, in 43 New Zealand rabbits exposed to brodifacoum varying degrees of abdominal, thoracic, stomach, kidney, and muscle hemorrhaging were found (Gupta, 2012).

In the instance that AR consumption is caught soon after the initial exposure, emesis is induced followed by administration of activated charcoal treatment with cathartic to decrease AR absorption. The next form of treatment for severe exposure is the administration of vitamin K_1. Once administered orally or subcutaneously the vitamin K_1 can begin the production of new clotting factors. Coagulopathic animals are treated with fresh frozen plasma to provide clotting factors and/or whole blood to restore blood volume.

2.6 Second Generation Anticoagulant Rodenticide Research

2.6.1 Non-Target Wildlife Studies

Protecting the lives of non-target species from secondary rodenticide poisoning is a common goal among the environmental scientific communities. Research has been published that investigated the persistence of trans and cis isomers of common SGARs. Many of these studies examined a single animal species and its unique isomer proportionality postmortem relative to bait isomer proportions.

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A study conducted in France examined diastereomer ratios of both bromadiolone and brodifacoum within the livers of nineteen wild rats (*R. norvegicus)* and then compared that to bait ratios. Commercial bait was assessed using LC-MS/MS instrumentation and bromadiolone was found to be 70-90% trans and 30-10% cis isomers. Brodifacoum was 48-58% cis and 52-42% trans isomers. Bromadiolone and brodifacoum were found in 31.6% and 36.8% of the rat livers evaluated. Of those with bromadiolone present, five out of the six livers had fully metabolized the cis isomer form leaving 100% trans isomer. Of those with brodifacoum present, all seven livers had relatively equal proportions of each isomer and were close to the proportions seen in the bait. Only two of the livers showed a decrease in cis isomer proportions compared to bait. Overall, this study identified that the trans isomer of bromadiolone had a greater persistency in liver postmortem possibly due to animals metabolizing and eliminating the cis isomer (Fourel et al., 2016).

Similarly in France, researchers investigated the occurrence of trans and cis isomers of bromadiolone in red foxes (*Vulpes vulpes*). Forty-eight livers were collected and 39 were positive for bromadiolone. Most significantly, 35 of the 39 livers had 100% of the trans isomer present and no detectable cis isomer. The other four samples had trans isomer of 64% or greater and cis of 36% or less. The bromadiolone liver diastereoisomers ratios from the 35 livers were significantly altered from bromadiolone bait, which is regulated to contain 70-90% trans in its formulations (Fourel et al., 2017).

In Spain, wild boars (*Sus scrofa*) are commonly exposed to SGARs through primary bait ingestion or through ingesting of target species containing SGARs. A study conducted in 2020 examined the isomer ratios within wild boar liver and compared them to that of the bait blocks (Alabau et al., 2020). This study found that out of 51 wild boars collected in Barcelona City

25.5% had bromadiolone and 47.1% had brodifacoum present. Bait blocks were analyzed to obtain isomer ratios for comparison to boar liver ratios. Twelve bromadiolone bait formulations were averaged and found to have 64% trans and 36% cis. Four brodifacoum bait formulations were averaged and found to have 46% trans and 54% cis. For bromadiolone, the wild boar livers showed a more balanced proportionality than that of the bait blocks. The boars were shown to have 54% trans isomer and 46% cis isomer. For brodifacoum, the isomer percentage was 45% trans and 55% cis which is essentially identical to that of the bait (Alabau et al., 2020).

In a study of 58 postmortem Réunion harrier raptor *(Circus maillardi)* livers, evidence of shifting diastereomer isomer proportions relative to bait was identified (Fourel et al., 2021). Forty-one out of the 58 livers had bromadiolone present and 40 of 41 (97.6%) had 100% trans isomer. For brodifacoum, 29 livers showed exposure, 20 of which showed cis brodifacoum in greater proportion (Fourel et al., 2021).

Both isomer configurations have their own unique elimination kinetics that affect their half-lives. Bromadiolone trans and cis half-lives are 75 and 27 hours, respectively (Lattard & Benoit, 2018). Brodifacoum trans and cis half-lives are 69 and 121 hours, respectively. The conclusion can be made that the isomer present in the wildlife livers of various species is usually the isomer with a longer half-life (Lattard & Benoit, 2018).

These research findings, which include various wildlife species and urban/rural locations, show consistent cis and trans isomer proportionalities (summary in **Table 1**). The cis isomer of bromadiolone tends to be present at lower concentrations in species with secondary exposures whereas the trans isomer is present at higher concentrations. These findings for bromadiolone also show a significant difference in isomer proportionality compared to the bait. For brodifacoum, only a small number of combined samples from these studies showed a difference

in isomer proportions from bait. These findings suggest that only certain isomer forms of SGARs persist longer in non-target wildlife. By decreasing the proportion of the more persistent isomer used in bait formulations, the potential for secondary intoxication of wildlife could be reduced since persistence in tissues would be less.

Table 1. Summary of Prior Diastereomer Research with associated species

Studied Species	Sample Size (n)	Contained Bromadiolone	Contained Brodifacoum	Isomer Analysis	
				Bromadiolone	Brodifacoum
Wild rats *R. norvegicus* (Fourel et al., 2016)	N=19	N=6/19	N=7/19	83.3% (5/6) contained 100% trans isomer	All seven were similar to bait proportions
Red foxes Vulpes vulpes (Fourel et al., 2017)	N=48	N=39/48	N/A	89.7% (35/39) contained 100% trans isomer	N/A
Wild boar Sus scrofa (Alabau et al., 2020)	N=51	N=13/51	N=24/51	All 13 were similar to bait	All 24 were similar to bait
Réunion harrier raptor *Circus maillardi* (Fourel et al., 2021)	N=58	N=41/58	N=29/58	97.6% (40/41) contained 100% trans isomer	70.0% (20/29) Accumulation of cis isomer proportion >62%

3. Research Objectives

The purpose of this research was to identify which isomeric forms of bromadiolone and brodifacoum show a greater persistency in both primary bait consumers and select secondarily exposed species. This objective was met by examining the isomer proportion within bait blocks and comparing that to the proportions found in primary consumer liver samples. To assess the isomer persistency through the food chain, secondary consumer wildlife liver samples were evaluated to identify the isomer proportions compared to bait formulations and primary consumer species. The goal was to determine if changes in isomer ratios might lead to the formulation of SGAR baits that decrease the potential for secondary intoxication of exposed non-target wildlife as a result of less tissue persistency.

4. Materials and Experimental Methods

4.1 California Animal Health and Food Safety Laboratory Pre-Existing Method

An existing method for the extraction and detection of ARs in liver using HPLC-MS analysis was modified for this project. The HPLC-MS method was validated in conjunction with the California Animal Health and Food Safety Laboratory System, Toxicology Laboratory, University of California, Davis, California and the University of Kentucky Veterinary Diagnostic Laboratory, Toxicology Laboratory, University of Kentucky, Lexington, Kentucky (Smith et al., 2017).

4.2 Reagents

HPLC grade acetonitrile, methanol, submicron filtered water, and Optima LCMS grade formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Dispersive solid phase extraction (dSPE) prepackaged 15 mL centrifuge tubes containing 175 mg of magnesium sulfate ($MgSO_4$), 50 mg florisil PR, and 50 mg Alumina B PSA were obtained from United Chemical Technologies (Bristol, PA, USA).

4.3 Preparation of Standards

An internal standard mix for LC-MS analysis was used containing 50 μg/ml of deuterated diphacinone (D-4) and 5 μg/ml of deuterated bromadiolone (D-5) diluted in methanol. These deuterated rodenticides were purchased from CDN Isotopes (Quebec, Canada) and had a purity of 99%. To prepare calibration curves throughout the project, two different standard solutions were used: 1.0 μg/ml and a fifty μg/ml anticoagulant mix both diluted in methanol. These mixtures included the anticoagulant rodenticides chlorophacinone (ChemService Inc., USA), diphacinone (ChemService Inc., USA), brodifacoum (ChemService

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Inc., USA), dicumarol (Sigma, USA), difenacoum (ChemService Inc., USA), bromadiolone (ChemService Inc., USA), coumachlor (Aldrich, USA), warfarin (ChemService Inc., USA), and difethialone (U.S. EPA, Washington, DC). The laboratory routinely uses these anticoagulant mixes for daily analytical testing. For this reason, the standards contain other rodenticides not examined in this project. These solutions were made by laboratory analysts throughout the project and kept at $10\degree C$ until the time of use.

4.4 Sources of Samples

Control chicken livers were purchased from a retail store in Sacramento and used for check samples and calibration curve in matrix. The livers were blended whole until homogenized and placed in a Fisher Isotemp freezer (Fair Lawn, NJ, USA) at $-20\degree C$. To ensure the integrity of analytical testing the chicken livers were verified by LC-MS methods. This was done to ensure that the control matrix was free of any interfering analytes.

Commercially available bromadiolone and brodifacoum bait were obtained for analysis of diastereomer ratios. Brodifacoum baits included Havoc-XT Bloks Lot#201619 and Lot#201619 (Neogen, MI, USA) and Jaguar All Weather Bait Chux Lot #11499 (Motomco, WI, USA). Bromadiolone baits included Just One Bite II Bar Lot#2JB009 (Phoenix, AZ, USA) and Contrac All Weather Blox Lot#K48698-12392 (Bell Laboratories, Inc. WI, USA).

Liver samples containing bromadiolone and brodifacoum were obtained from primary consumers and non-target species (**Table 2**). Primary consumer species samples included opossums (*Didelphidae* spp.), skunk (*Mephitidae* sp.), raccoons (*Procyon lotor* spp.), and squirrel (*Sciuridae* sp.). Non-target species included coyotes (*Canis latrans*), gray foxes (*Urocyon cinereoargenteus*), and cougars (*Puma concolor*). All samples were obtained in collaboration with the California Department of Fish and Wildlife. All liver samples for target

and non-target species were previously analyzed by CAHFS, Toxicology Section as part of routine case investigation.

Table 2. Primary Consumer and Non-target Species Liver Tested

Species	Total Samples (n)	Contained Bromadiolone	Contained Brodifacoum
Opossum	n=3	n=3	n=3
Skunk	n=1	n=1	n=1
Raccoon	n=3	n=3	n=3
Squirrel	n=1	n=1	n=1
Coyote	n=15	n=13	n=15
Gray Fox	n=15	n=13	n=15
Cougar	n=15	n=15	n=15

4.5 Anticoagulant Rodenticide Extraction Method

Liver tissue used as calibrators, matrix blank, spikes, and wildlife liver samples were weighed out at 1.0 ± 0.2 grams and placed within a 50mL centrifuge tube. Five µL of internal standard containing deuterated bromadiolone and diphacinone was added to all tubes. Calibration curves in matrix were then prepared and new curves were made each time a set of wildlife livers were analyzed. The preparation of calibration curves used an anticoagulant mixture at 1.0 µg/ml and 50 µg/ml diluted in methanol fortified into one gram aliquots of liver (**Table 3**). The liver spike contained 70 µL of the 1.0 µg/ml anticoagulant mixture and the check only contained internal standard.

Stainless steel beads were placed in all the tubes and then shaken on a GenoGrinder for 5 minutes at 750 RPM (SPEX Sample Prep 2010 GenoGrinder, Metuchen, NJ, 08840). Then 6 mL

of 10% methanol in acetonitrile was added and the tubes were again placed in a GenoGrinder for 5 minutes at 750 RPM. Using a magnet, the ball bearings were removed, and samples were then shaken for 30 minutes on a wrist action shaker at max setting (VWR Vx-2500 multi-tube vortexer). After the samples were centrifuged for 5 minutes at 2500 RPM at 20°C (Beckman Coulter Avanti J-E centrifuge, Indianapolis, IN, 46268) the supernatant was collected into 15 mL QuEChERS tubes and vortexed briefly (Fisher Vortex Genie 2 Scientific Industries). QuEChERS tubes were then placed on a wrist action shaker for 30 minutes at max setting. The supernatant was removed and poured into 16x125 mm disposable glass tubes and placed on an N-Evap using nitrogen gas and kept at 30-50°C (Meyer Organoation N-Evap Analytical Evaporator, West Berlin, MA, 01503). To reconstitute samples, 1 mL of methanol was added, and samples were vortexed and sonicated. Pasteur pipets along with 1 mL syringes with Millex 0.22 μm, 13 mm diameter filters attached were used to transfer samples to new 16x125 mm glass tubes. Then 100 μL of the filtered sample with 900 μL of HPLC methanol was placed in autosampler vials and vortexed lightly to mix. The samples were then ready for HPLC-MS analysis.

Table 3. Preparation of Calibrators Ranging from 20-2000 ppb Using Both Anticoagulant

Mixtures.

Calibrator	Concentration of Calibrant (ppb)	Spiked Solution
1	20	20 μL of 1.0 $\mu g/ml$ mix
2	50	50 μL 1.0 $\mu g/ml$ mix
3	70	70 μL 1.0 $\mu g/ml$ mix
4	100	100 μL 1.0 $\mu g/ml$ mix
5	500	10 μL 50 $\mu g/ml$ mix
6	1000	20 μL 50 $\mu g/ml$ mix
7	2000	40 μL 50 $\mu g/ml$ mix

4.6 Rodenticide Bait Extraction Method

Bait blocks had to be crushed with a mallet for analytical testing to be completed. Using a

16x125 mm disposable glass tube, 1.0 ± 0.2 grams of rodenticide bait was added with 10mL of

HPLC grade methanol. The test tubes were rotated for 30 minutes (Heldolph Reax 2) and

centrifuged at 2000 RPM for 5 minutes. One hundred μL of supernatant was transferred into an

autosampler vial and mixed with 900 μL of methanol. Once capped the samples were ready for

HPLC-MS analysis using the same analytical method as wildlife samples.

4.7 HPLC-MS Analysis Method

Agilent Technologies 1290 Infinity II High Performance Liquid Chromatograph (Agilent

Technologies, Santa Clara, CA, USA) paired with a Sciex 7500, QTRAP with Optiflow Pro Ion

Source (Sciex, Framingham, MA, USA) was utilized for analysis. Diastereomer separation was

done with the Agilent ZORBAX RRHD Eclipse Plus C18, 95Å, $2.1 \ x \ 100$mm, 1.8 μm, column.

The flow rate was held at 0.350 mL/minute and 2 μL of sample was injected. The mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient chart is shown in **Table 4** below. The analytical method had a run time of 17 minutes with divert valve parameters of 0-1 minutes to waste, 1-16 minutes to instrument, and 16-17 to waste.

Table 4. Solvent ratio for chromatographic separation at given time in analytical run. A. solvent is 0.1% formic acid in water & B. is 0.1% formic acid in acetonitrile.

Time (mins)	A%	B%
0.00	99.0	1.0
1.50	99.0	1.0
9.50	2.0	98.0
13.50	2.0	98.0
13.60	99.0	1.0
17.40	99.0	1.0
17.50	99.0	1.0
18.00	99.0	1.0

For mass spectrometry analysis, cis and trans bromadiolone and cis and trans brodifacoum were monitored. To accomplish this the analytes were split into two multiple reaction monitoring (MRM) experiments using electrospray ionization in negative ion monitoring mode. Cis and trans bromadiolone were acquired in experiment 1 while brodifacoum cis and trans were acquired in experiment 2. Sheath gas was set at 50 psi, auxiliary gas 70 psi, curtain gas 40 psi, and collisionally activated dissociation (CAD) was set at 10 psi. The ion

source temperature was 400 ℃ and a spray voltage of 4500 V was implemented. Specific mass

spectrum parameters for the analytes such as precursor ion, product ion, collision energy, and

retention times are shown in **Table 5**.

Table 5. MS optimization parameters for analytes: quantifier ions, confirmation ions, CE, and

HPLC retention times. Experiment 1 MRM bromadiolone cis and trans and experiment 2 MRM

cis and trans brodifacoum.

Analyte of interest	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)	Retention time (mins)
Bromadiolone Quantifiler (trans)	525.0	250.0	-50.0	9.05
Bromadiolone Confirmation (trans)	525.0	219.0	-60.0	9.05
Bromadiolone Quantifiler (cis)	525.0	219.0	-50.0	9.15
Bromadiolone confirmation (cis)	525.0	219.0	-50.0	9.15
Brodifacoum Quantifiler (trans)	521.0	187.0	-50.0	10.2
Brodifacoum Confirmation (trans)	521.0	135.0	-50.0	10.2
Brodifacoum Quantifiler (cis)	521.0	187.0	-50.0	10.1
Brodifacoum Confirmation (cis)	521.0	135.0	-50.0	10.1

4.8 Data analysis

As shown in **Table 3**, calibration curves were created for each set of primary consumer and non-target wildlife liver samples. Throughout the project obtaining adequate and reliable calibration curves was unsuccessful, which precluded the determination of individual diastereomer concentrations. Alternatively, peak area ratios of cis and trans isomers were calculated for baits, primary species, and non-target wildlife samples. Data was processed using quantitation software from Sciex analytical OS version 2.1.6. 59781. The target analytes were identified through specific MRM transition scans. To calculate average isomer ratios and standard deviation values Excel statistical software was used. Peak area calculations were performed if retention times matched within ± 0.20 minutes, quantifier ion signal was present, and if percent differences in standard curve isomer peak area were <20%.

Statistical evaluation of data was completed in partnership with the UC Davis Statistical Laboratory. The methodology included a One-way Analysis of Variance (ANOVA) that analyzed source populations. For this project there were 6 sources: bait, gray foxes, cougars, coyotes, raccoons, and opossums. The skunk and squirrel sample could not be included in this ANOVA due to having only one sample for each. Normality of residuals was assessed using Shapiro-Wilk's test and equal variances among the sources was assessed using Levene's test. For both proportions, the arcsine transformation was chosen to satisfy both assumptions. Post hoc analyses were performed with Tukey's procedure for multiple comparisons. Furthermore, individual plots showing the mean values of cis and trans bromadiolone and brodifacoum in each population source was also done.

5. Results Summary

5.1 Quantification Method Selection

Calibration curves were attempted throughout the entirety of the project. Curves were evaluated for four analytes of interest: bromadiolone cis and trans and brodifacoum cis and trans. The calibration ranges were from 20-2000 ppb see **Table 2**. For acceptable calibration curves, the R^2 values needed to be at or above 0.985 (usual weighting of $\frac{1}{x^2}$ used). Curves were bracketed around liver samples, having a curve before samples ("pre") and a curve after samples ("post"). The curves obtained for the four analytes in this research did not meet the passing requirements on a consistent basis.

A possible contributor to the curve inconsistency was the lack of manufacturer isomer proportion data for analytical standards. When purchased, bromadiolone and brodifacoum standards do not come with information regarding the amount of cis and trans present in the given formula. To work around this, a prepared standard for each calibrator value was analyzed for its isomer percent proportions using peak area. Once calculated, those percentages were applied to the calibration curve concentrations in the quantification process to account for the isomer ratios present. For example, bromadiolone 1000 ppb was found to have 82% trans isomer and 18% cis isomer. Thus, the calibrator concentration would change to 820 ppb trans and 180 cis. Without having the true manufacturer proportions, the values obtained can only be estimated isomer ratios in standards.

Attempts to mend the calibration curves were made and these included narrowing the calibration curve range from 20 ppb to 1200 ppb, using a methanol rinse after each injection, and preparing new anticoagulant mixes from original standards. Overall, the choice to do quantitative analysis through isomer peak area percentages was more dependable and appropriate.

5.2 Method Performance

Successful and reliable chromatographic separation of diastereomers was achieved throughout the project. The peaks obtained were identified as major and minor diastereomers based on previously reported values in commercial bait and then confirmed with applied LC-MS/MS instrumentation (Fourel et al., 2016). Bromadiolone demonstrated a major isomer form of trans and a minor form of cis whereas brodifacoum has a major isomer form of cis and minor isomer form of trans. In terms of chromatographic peaks, the first diastereomer peak to elute was labeled with the specific major isomer form **(Figure 3a-b).**
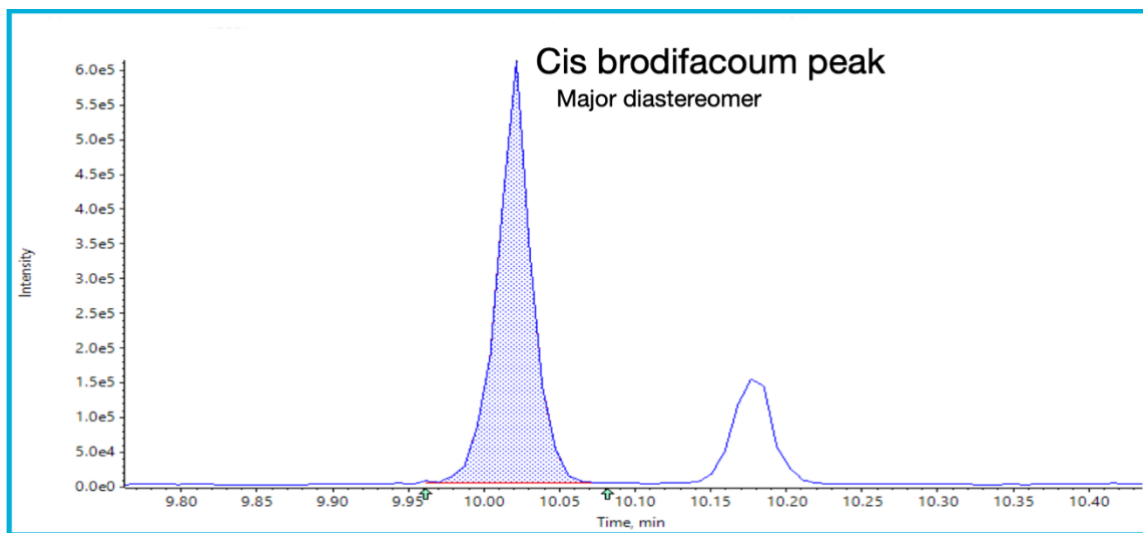

Figure 3a. Example cougar liver utilized in this project that contained brodifacoum taking from MRM transition 521.0/135.0..
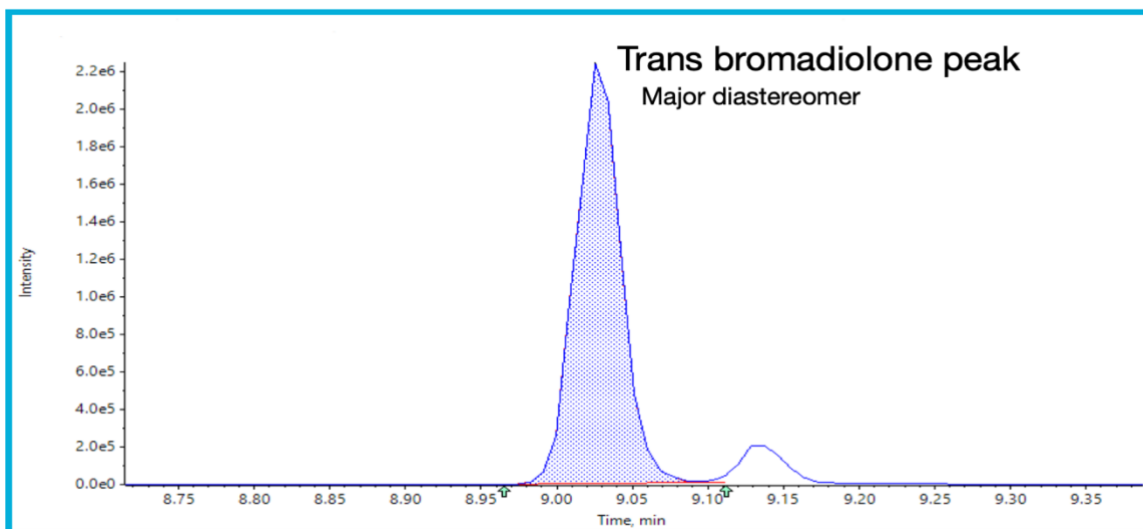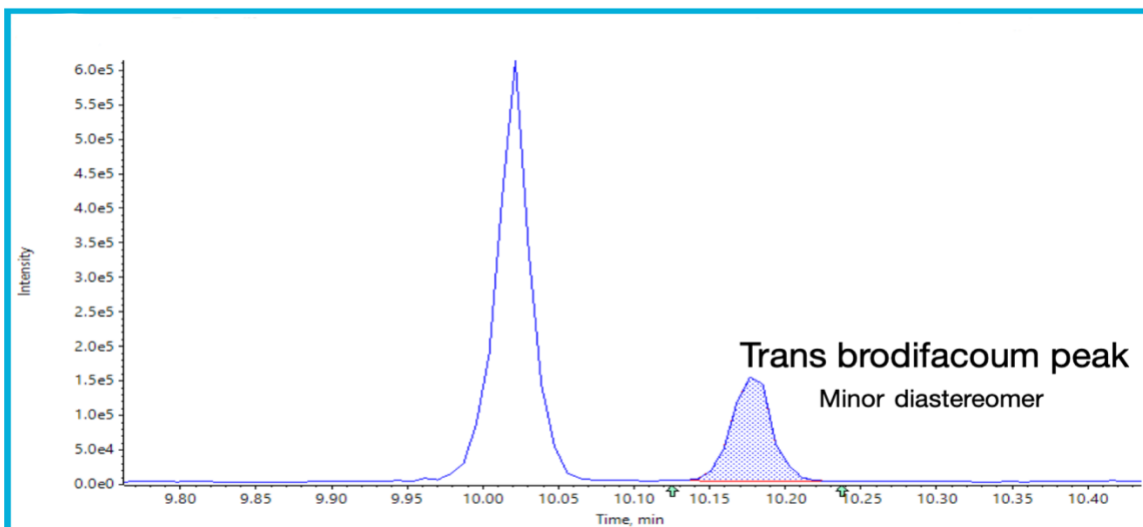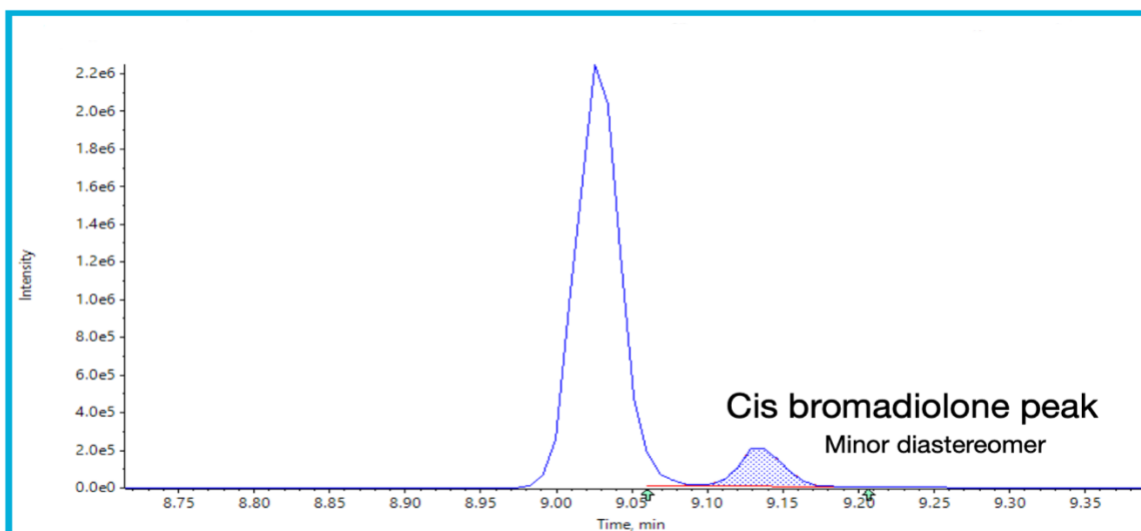*The cis (major) and trans (minor) peak separation is clearly exhibited.*

Figure 3b. Example gray fox liver utilized in this project that contained bromadiolone taken from MRM transition 525.0/250.0. The trans (major) and cis (minor) peak separation is clearly exhibited.

The AR extraction method implemented for non-target and primary consumer liver samples has a method accuracy of $\pm20\%$ (Smith et al.,2017). Since the method was only modified in the last step, which was to dilute all extracted samples by ten, the accuracy was applied to this project.

The standard curves, run with control chicken liver, were used as verification that proportions of isomers in the samples were not changing throughout runs. All curves successfully passed accuracy and precision results with an isomer proportion fluctuation $<20\%$. During analytical examination of primary species for bromadiolone, it was found that four samples had a strong signal for the quantitative ion. However, the samples lacked a signal for qualitative ion. The results are still reported due to the quantitative ion being present **(Figure 5).**

5.3 SGAR Isomer Proportions in Bait

Five different formulations of SGAR bait were evaluated. Each sample of bait was evaluated at 10x dilution and was run three consecutive times. The average cis and trans percentages were taken from these three runs and used as a reference point for commercial bait isomer proportions (**Figure 4**). For bromadiolone, Just One Bite II Bar trans: cis (68.7%:31.3%)

and Contrac All Weather Blox bait trans: cis (65%:35% and (65.2%:34.8%) two blocks from the same lot were assessed. For brodifacoum, bait proportions were: Jaguar All Weather Bait Chux trans: cis (38.1%:61.9%), Havoc-XT Bloks lot#201619 (39%:61%), Havoc-XT Blocks lot#201619 (42.2%:57.8%).
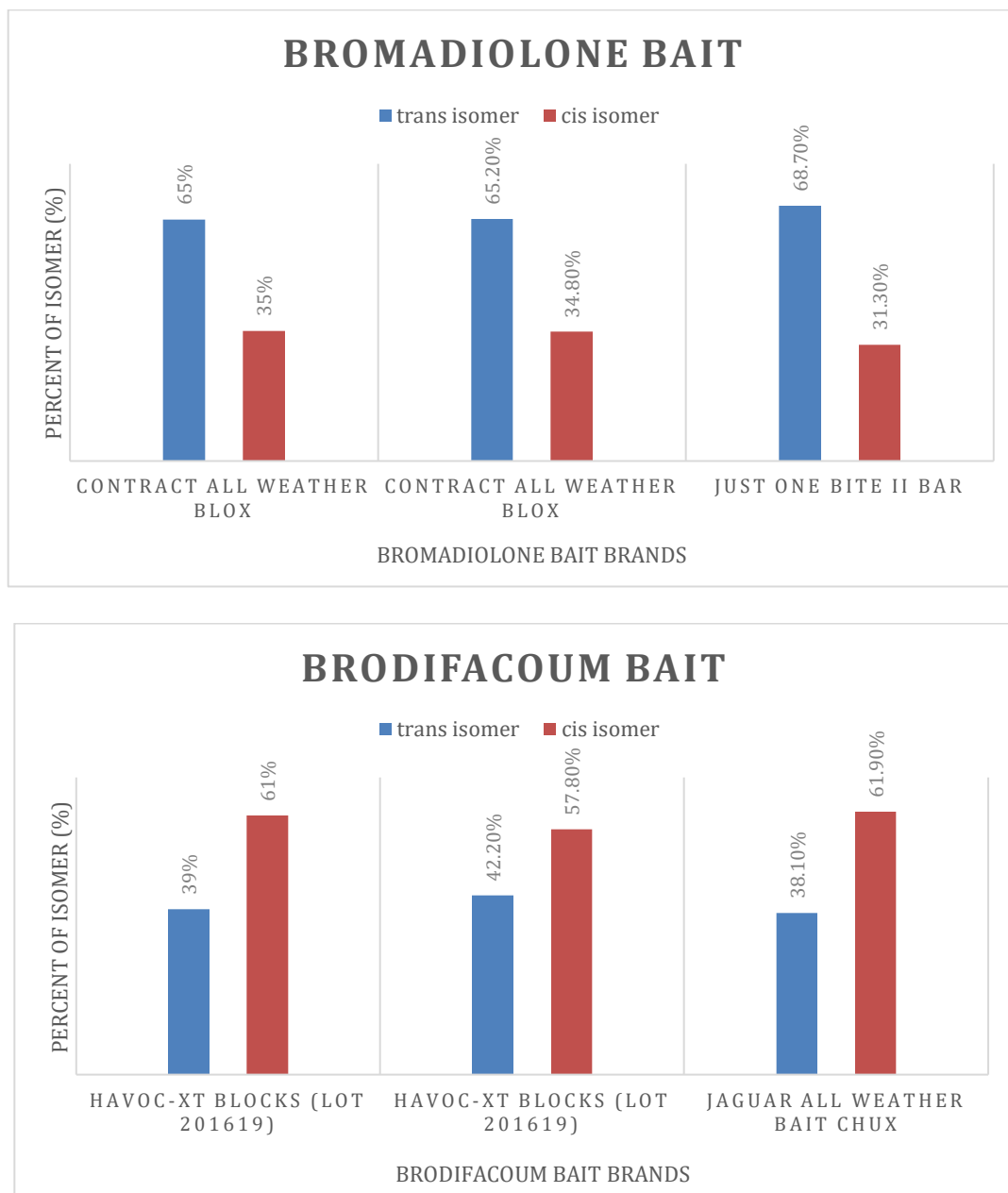

Figure 4. Ratio of SGAR isomers present in various bromadiolone and brodifacoum bait samples. Bromadiolone bait exhibits a predominant trans isomer, whereas brodifacoum showed the cis isomer as dominant.

5.4 SGAR Isomer Proportions in Primary Consumer Species

The primary consumer species included three opossums, three raccoons, one skunk, and one squirrel. All eight liver samples contained both bromadiolone and brodifacoum (**Figure 5**). For 2 of 3 opossums, the liver contained 100% trans bromadiolone and zero percent cis, the last opossum sample had a ratio of (82.9:17.1%). The brodifacoum trans and cis isomers were found to range from 2.6 to 43.2% and 56.8 to 97.4%, respectively. The three raccoons contained bromadiolone trans isomer from 37.7 to 47.4% and cis isomer from 52.6 to 59.2%. Brodifacoum raccoon isomers were trans from 35 to 67.9% and cis from 32.1 to 65%. The squirrel liver contained bromadiolone trans and cis isomers at 75 and 25%, respectively and brodifacoum trans and cis isomers at 30.6 and 69.4%, respectively. The skunk liver contained bromadiolone trans and cis isomers at 86.3 and 13.7%, respectively and brodifacoum trans and cis isomers at 50.4 and 49.6%, respectively.
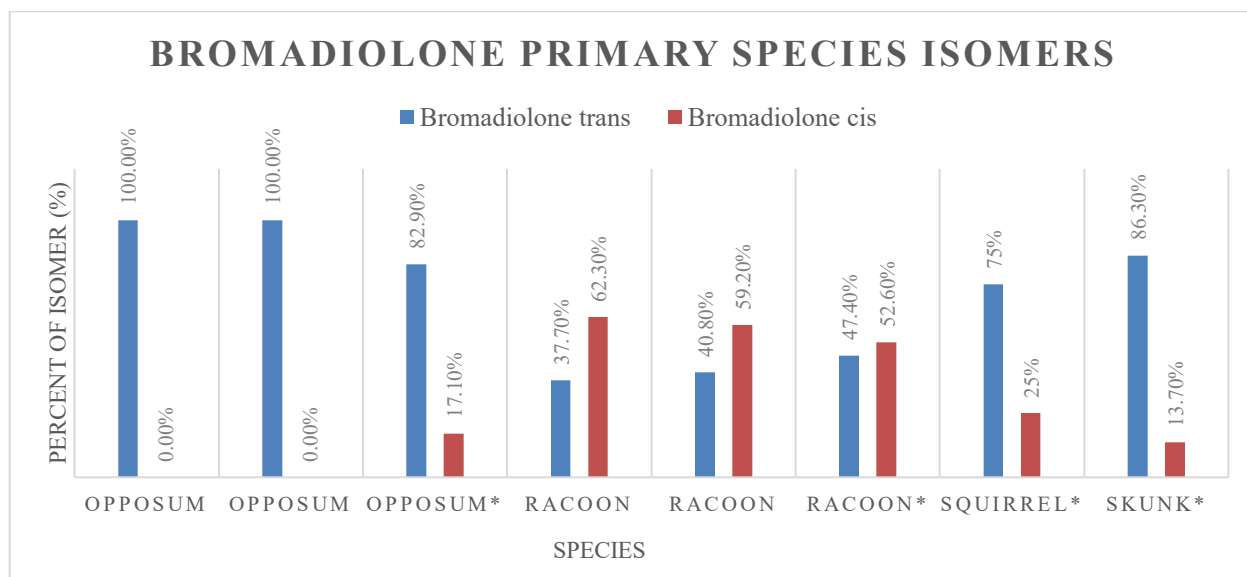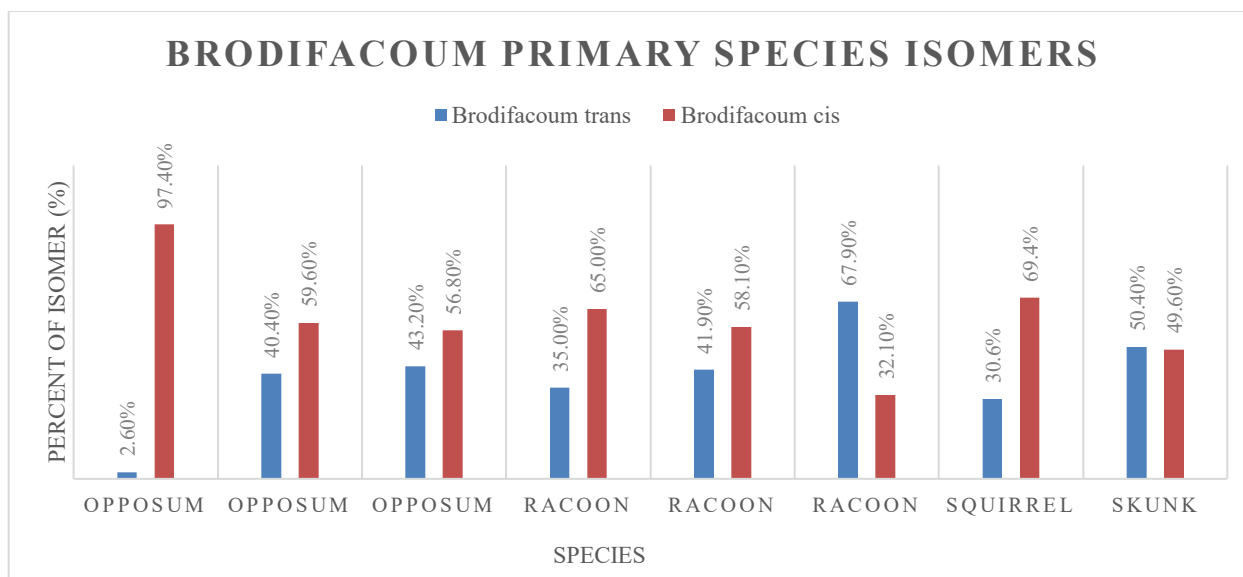
Figure 5. Ratio of bromadiolone and brodifacoum isomers present in primary species samples. Bromadiolone trans isomer was more predominant in 5/8 samples. The four bromadiolone samples with asterisks had high signal intensity for quantitative ion but lacked strong signal of qualitative ion. Brodifacoum cis isomer was predominant in 6/8 samples.

5.5 SGAR Isomer Proportions in Non-Target Species

Coyotes

Thirteen of the 15 coyote samples examined contained bromadiolone. Of these, 13/13 demonstrated trans isomer in much greater proportion over cis (**Figure 6**). Two samples contained 100% trans isomer and seven others contained trans isomer ≥ 91%. Two samples exhibited trans isomer dominance (≥ 77 %) and the last two samples had ratios of trans:cis of 60.5:39.5% and 61.8:38.2%, respectively,

All fifteen coyotes had brodifacoum present and 14/15 contained the cis isomer in larger proportion. Five of the samples had cis isomer ≥ 82 %, six had 70 to 78%, and three samples with cis from 58 to 69%. The last sample had a reversed isomer proportion containing more trans than cis at 60.8:39.2%.

COYOTE BROMADIOLONE ISOMER RATIOS

■ Bromadiolone trans   ■ Bromadiolone cis

PERCENT OF ISOMER (%)

COYOTE SAMPLES

COYOTE BRODIFACOUM ISOMER RATIOS

■ Brodifacoum trans   ■ Brodifacoum cis
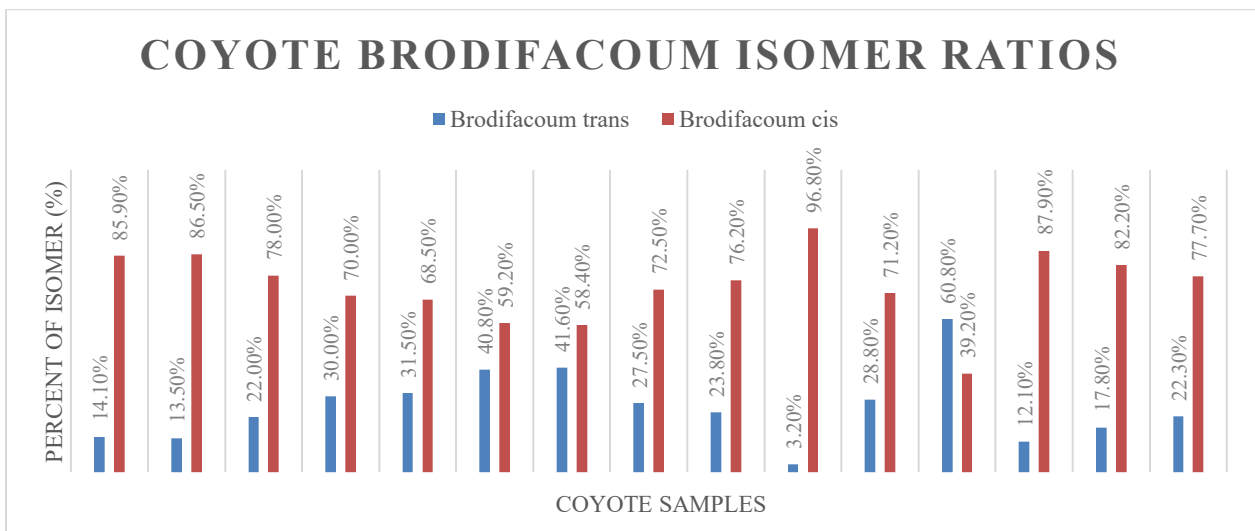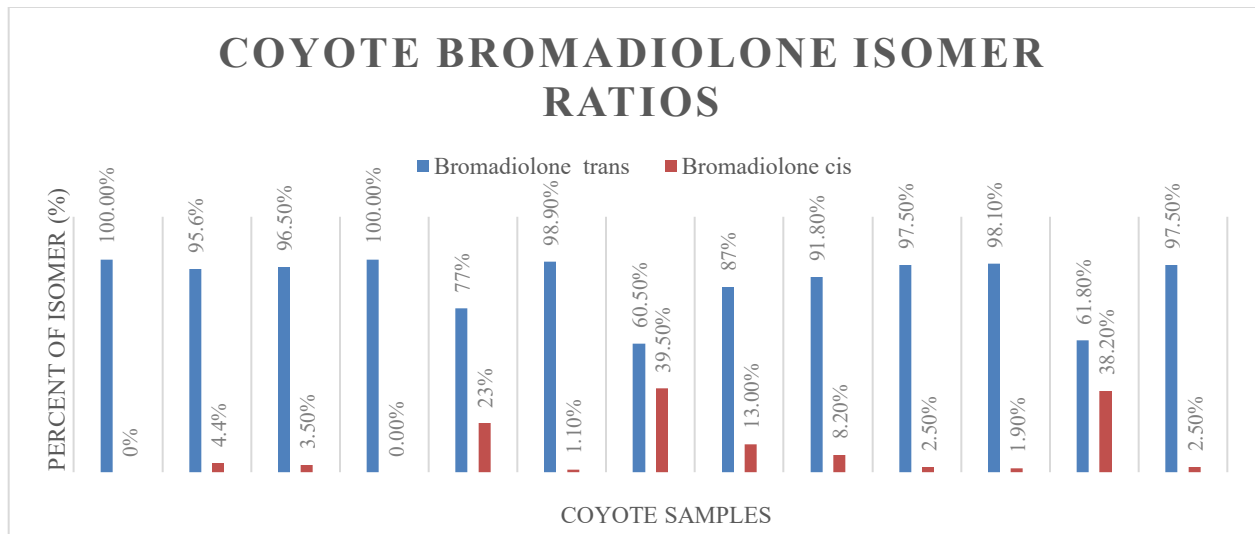
PERCENT OF ISOMER (%)

COYOTE SAMPLES

Figure 6. Ratio of bromadiolone and brodifacoum isomers present in coyote liver samples. Thirteen samples contained bromadiolone, whereas all fifteen samples contained brodifacoum.

Gray Foxes

Gray fox liver isomer ratios followed a similar SGAR pattern to that of the coyotes, (**Figure 7**). Out of the 13 samples containing bromadiolone, all of them demonstrated a significant prevalence of the trans isomer. Notably, cis bromadiolone was not detected in four samples leaving 100% trans isomer. Six samples contained trans bromadiolone ≥ 91% and the last three were ≥ 79% trans isomer.

Brodifacoum isomer proportions varied for the 15 samples collected. Six of the 15 (40%)

samples demonstrated an increase in cis brodifacoum isomer. Of those six, proportions of the cis present had a range of 51.7 to 88.3%. The other nine samples had trans brodifacoum as the predominant isomer form. Four of the nine samples exhibited proportions within the range of 50.5 to 59.8%, another four had 64.3 to 76.1%, and one sample had 99.4% trans isomer.
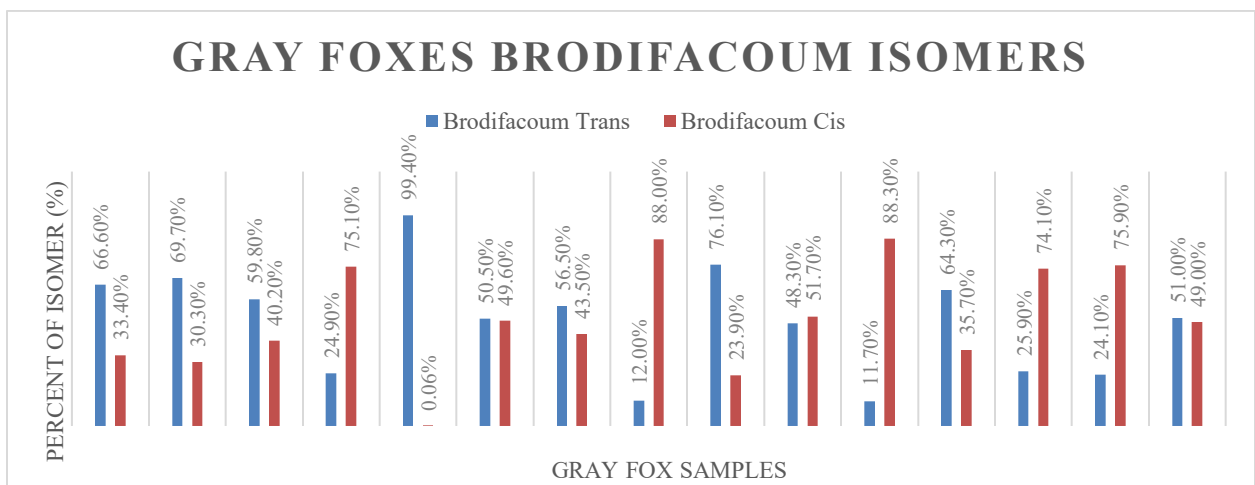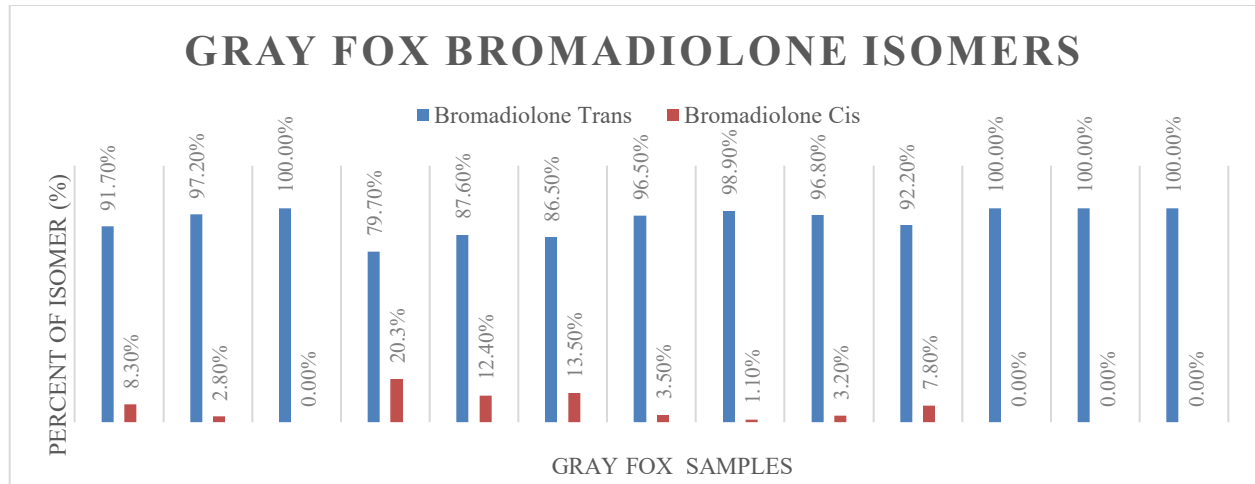

Figure 7. Ratio of bromadiolone and brodifacoum isomers present in gray fox liver samples. Thirteen samples contained bromadiolone, whereas all fifteen samples contained brodifacoum.

Cougars

Bromadiolone trans isomer was dominant in all 15 cougar samples (**Figure 8**). Three samples had 100% trans isomer present, a common occurrence in the three varied species. Ten

samples had high proportions of trans isomer (92.1 to 99.2%), and the last two samples

contained 89.8% and 84.0%, respectively.

Brodifacoum isomer proportions for all 15 cougars had higher cis isomer percentages.

Four of the 15 samples were approximately 50:50% trans:cis and 10 ranged from 65.1 to 87.4%

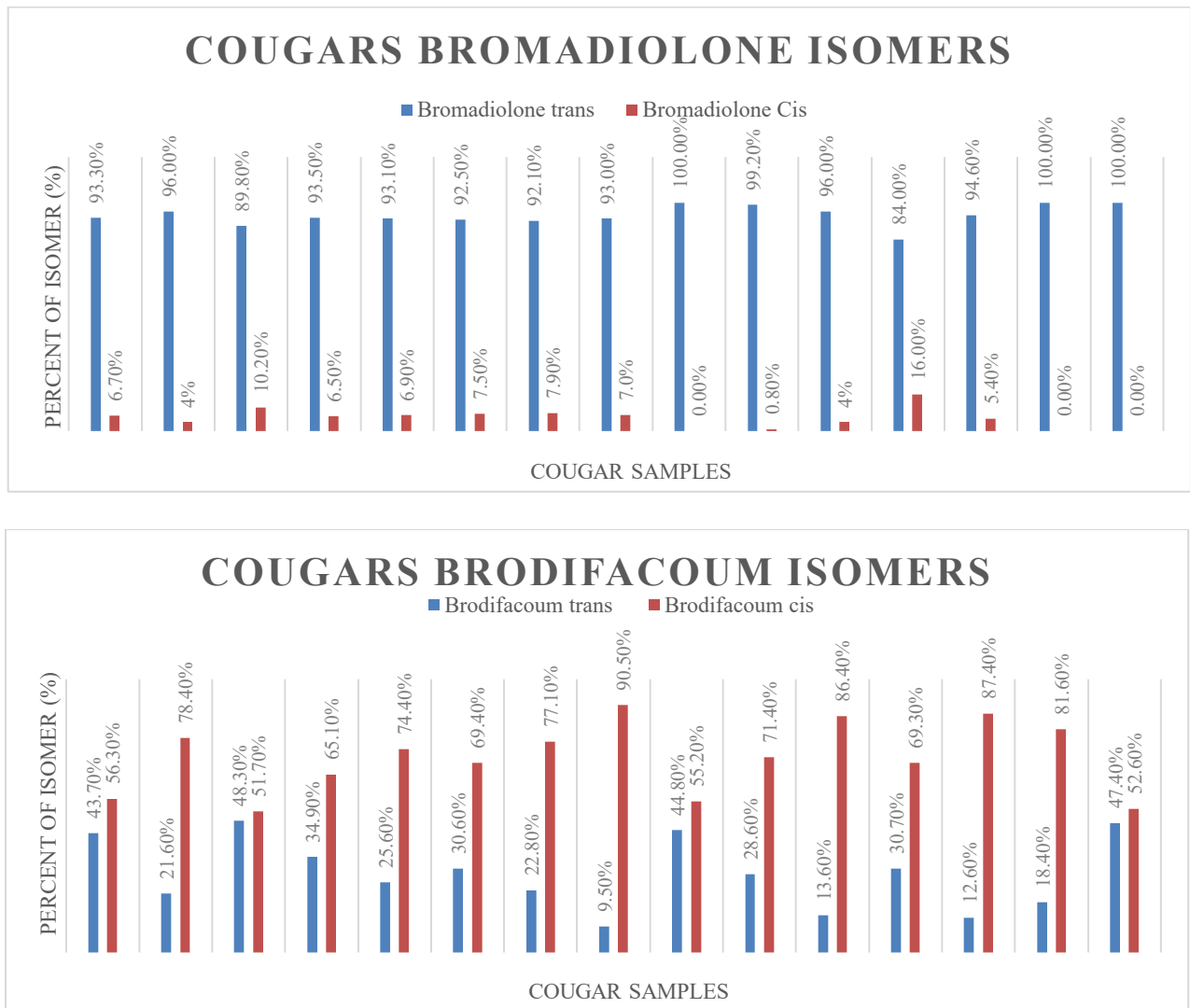cis isomer. The last sample had a cis isomer concentration of 90.5%.

Figure 8. Ratio of bromadiolone and brodifacoum isomers present in cougar liver samples

5.6 Statistical Analysis Results

Coyotes

Cis and trans isomer proportions of brodifacoum and bromadiolone were plotted against the bait and primary species proportions. Results suggest that for brodifacoum the trans isomer is lower than bait proportions and lower in primary consumers. Thus, cis is greater than bait and shows greater persistency in the coyote samples. Two samples are close to bait and one sample has the proportions reversed, favoring trans brodifacoum. For primary consumers there is a lack of consistent isomer persistence, which makes primary to secondary wildlife interpretations difficult.

For bromadiolone, the trans isomer is significantly higher than bait and displayed a greater persistency in coyote's livers. The two samples that deviate from the pattern of ≥ 90% trans isomer still had greater trans concentrations of approximately 61%. For primary consumers, most samples displayed a higher trans isomer as well.
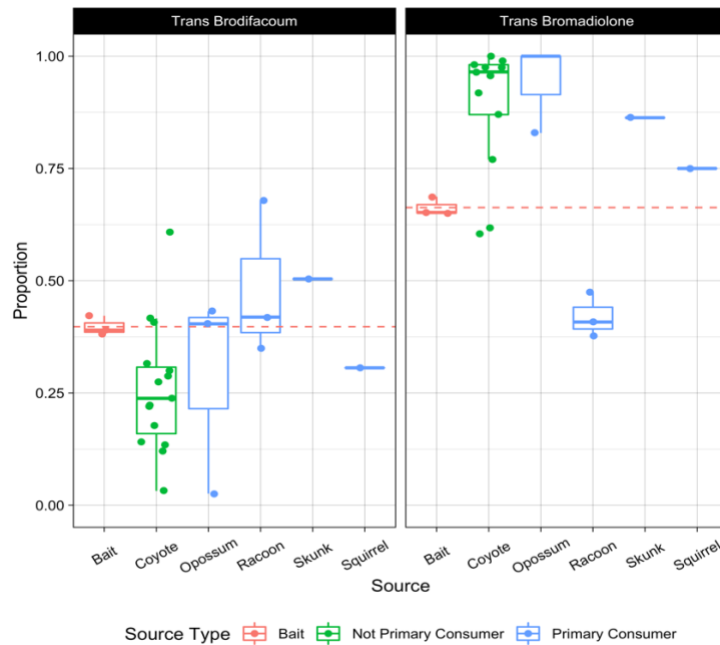
Figure 9. Coyote bromadiolone and brodifacoum isomer proportions plotted against bait and primary consumer species. The red dotted line signifies the mean value of the bait blocks.

Gray Foxes

Gray fox samples containing brodifacoum were found to have trans isomer proportions greater than bait samples. Nine of the 15 gray fox samples had greater proportions than bait. As seen in Figure 10 there is a large variability in the spread of the samples. The highest proportion of trans was nearly 100% and the lowest was 51%.

For bromadiolone, the trans isomer was greater in all samples than what was found in bait. Furthermore, trans showed greater persistency in the gray fox tissues. Gray fox livers with bromadiolone are much tighter together with less sample spread around the mean.
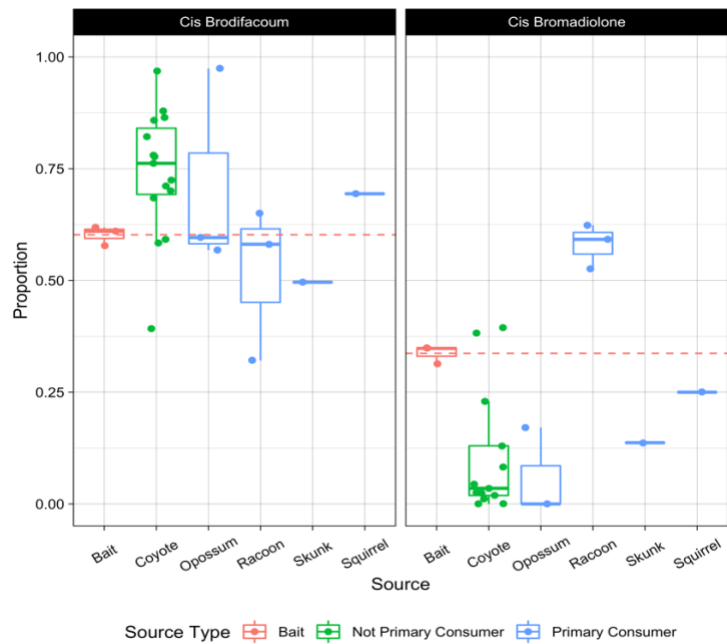
Figure 10. Gray fox bromadiolone and brodifacoum isomer proportions plotted against bait and primary consumer species. The red doted line signifies the mean value of the bait blocks.

Cougars

For brodifacoum, cougar samples follow a similar pattern to that was seen in coyotes. All

cougar samples had lower trans isomer and higher cis isomer concentrations compared to the

bait. The cis isomer of brodifacoum proving to be more persistent in all cougar liver samples. The four samples that stand out on the plot all have cis as more dominant with percentages of 51 to 56%.

For bromadiolone, all cougar samples had the trans isomer proportion significantly increased from bait. Following the same pattern as coyote and gray foxes, cougars also displayed a higher persistency of trans isomer. Trans bromadiolone for cougars had the most consistent proportion with all 13 samples ≥ 84%.
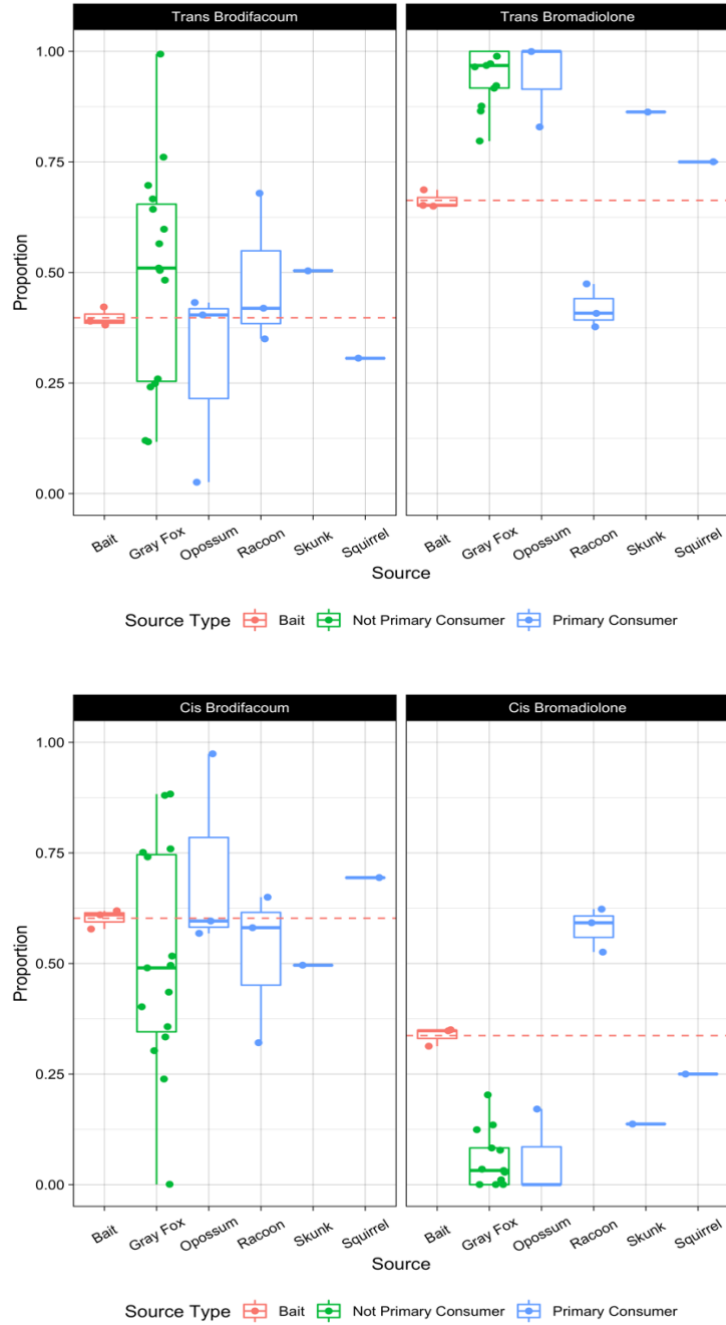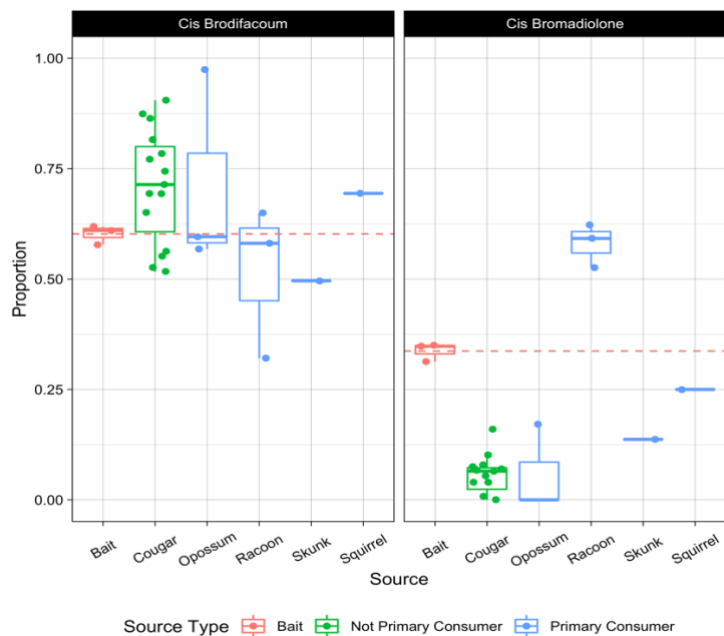
Figure 11. Cougars bromadiolone and brodifacoum isomer proportions plotted against bait and primary consumer species. The red dotted line signifies the mean value of the bait blocks.
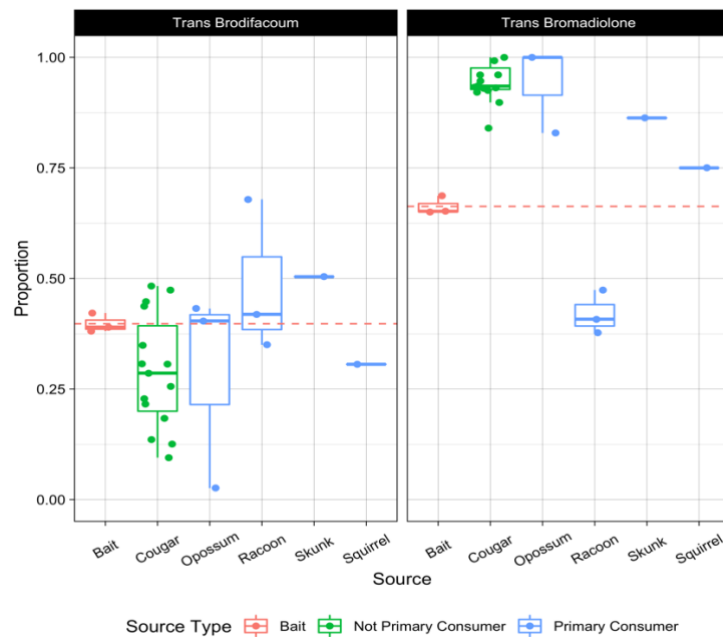
ANOVA Pairwise Plot

The ANOVA plot was done for the trans isomer of bromadiolone and brodifacoum of all sample sources. Since the trans and cis proportions add up to 100%, applying symmetry allows for only one isomer to be accessed in ANOVA testing. A significance level of $p < 0.05$ was applied. The stars represent significance, and the number of stars represents to what level of significance, the more stars the more significance. The "NS" means that there was no significant difference between the groups examined. The skunk and the squirrel sample could not be added to this testing since only one data point was available for each.

For brodifacoum, there is no significance ($p < 0.05$) across all six sample sources. Most notable is the lack of significance in the non-target wildlife liver proportions to bait proportions. In addition, there is no significance between primary species and non-target wildlife. Thus, these findings show no significant isomer difference between bait, primary species, and secondary species.

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For bromadiolone, the results show statistical significance and differences between certain sample populations. The most significant discovery is the difference between bromadiolone bait and the three secondary wildlife species. Cougar and gray foxes' proportions are considered highly significant from bait, denoted with the two stars. Opossum samples had a higher persistency of trans, which follows the same pattern seen in secondary consumer samples. Due to this, there was no significant difference between opossums and secondary consumers. However, due to raccoon samples favoring the cis isomer of bromadiolone there is remarkable significance seen between the secondary consumer samples and raccoons.
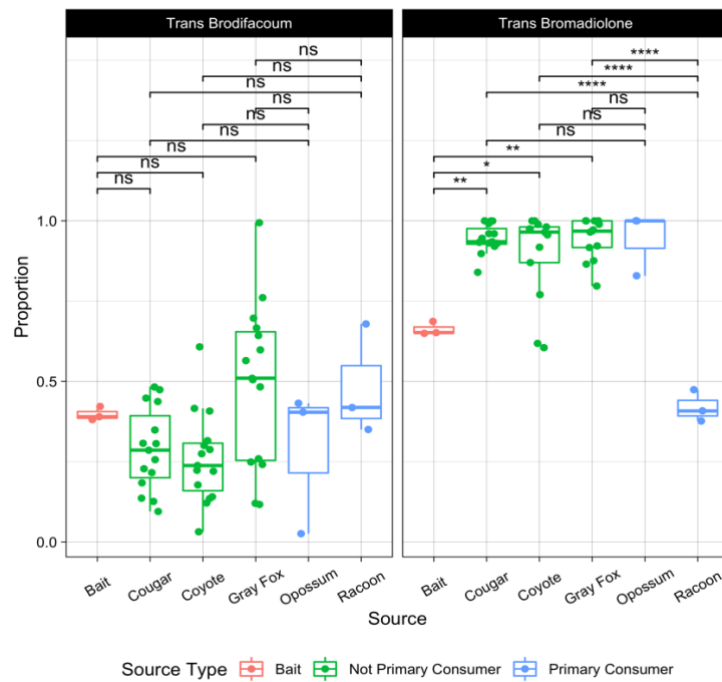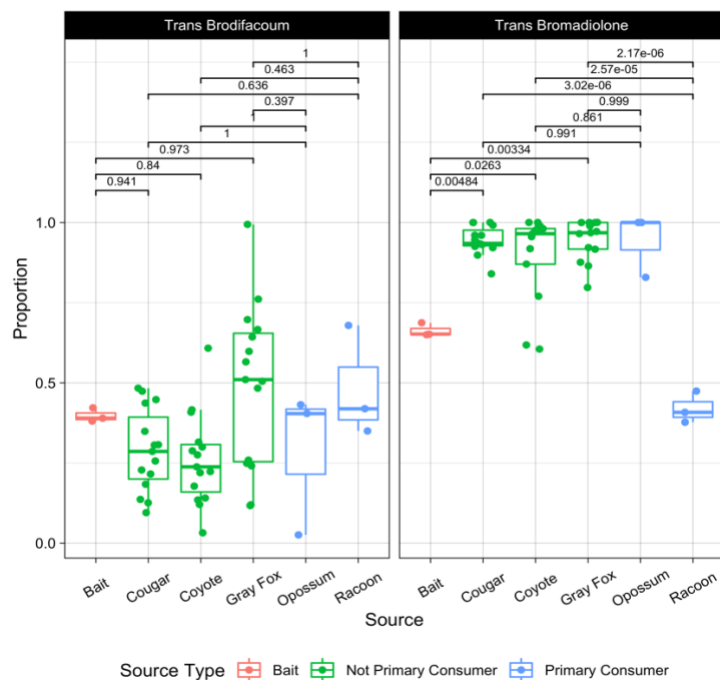
Figure 12. ANOVA pairwise plot of trans bromadiolone and brodifacoum in all samples ($p < 0.05$). Significance is indicated by a star and NS is not significant. The second plot is the calculated p values obtained in testing.

6. Discussion

The advancement of SGAR isomer research has been a driving force in the pursuit of non-target wildlife protection. Even though steps have been taken to limit secondary exposure of wildlife, continued presence of SGARs within the food chain is occurring (López-Perea et al., 2019). Therefore, this study demonstrates once more the differing ratios of isomers and their unique persistency in coyotes (*Canis latrans*), gray foxes (*Urocyon cinereoargenteus*), and cougars (*Puma concolor*). Furthermore, isomer proportions in non-target species were compared to bromadiolone and brodifacoum bait to evaluate isomer ratio fluctuation.

6.1 Bromadiolone Diastereomer Ratios

In total, 45 non-target wildlife samples of coyotes (*Canis latrans*), gray foxes (*Urocyon cinereoargenteus*), and cougars (*Puma concolor*) were tested and 41 contained bromadiolone.

From those containing bromadiolone 38/41 or 92.7% exhibited a trans isomer that was

significantly different from bait proportions. The bromadiolone bait obtained for this project

showed 65 to 68.7% trans isomer, the major isomer form, which agrees with prior bait isomer

proportions (Fourel et al. 2016, Fourel et al., 2017, Alabau et al., 2020). The range of trans

isomers found within the liver samples was 84 to 100%. Eight of the non-target samples had

bromadiolone trans isomer at 100% proportionality, thus zero cis isomer form detected. To

assess possible isomer fluctuation across the food chain, eight primary species were examined.

Most notably, three possums and one skunk also demonstrated an accumulation of trans isomer.

Moreover, two of the three possums had 100% trans isomer demonstrating that cis may be more

easily metabolized and excreted.

A small percentage (n=4,12.2%) of samples did not follow the same isomer proportion

pattern. Three of these came from coyotes, which had trans isomer percentages either close to the

bait ratios or significantly below (<62%). Lastly, one gray fox liver had 79.7% trans isomer.

Though 79.7% is above the bait ratios, the difference is not as substantial as the other 38 samples

mentioned above.

6.2 Brodifacoum Diastereomer Ratios

Brodifacoum did not show as extensive a difference from bait to postmortem sample. All

45 samples contained brodifacoum and 78% (35/45) contained cis isomer as the predominant

diastereomer. The bait blocks tested had 57.8 to 61.9% cis isomer form. With these proportions

in mind, 9/35 (25.7%) of samples were found to be within the isomer bait range. The other 26

samples were in the range of 63 to 96.8% cis isomer with 17/26 (65%), falling between 63 to

79%. Though this range is higher than bait, the deviation is not as drastic. Notably only two

samples, a cougar and coyote, had cis isomers in the 90% range.

The remaining 10 samples (22%) had the trans isomer form of brodifacoum as the dominant diastereomer. Most interestingly, nine of the 10 samples came from grey foxes and one from a coyote. The range of trans isomer was 50.5% to 99.4% with 9 of 10 falling within 50.5 to 76.1%. The last sample had a trans isomer proportion of 99.4%, leaving the cis isomer nearly gone.

This variability in brodifacoum isomer ratios is clearly displayed in **Figure 12**. There is no significance difference between the bait proportions and the proportions found in coyote, gray fox, and cougar. The statistical findings paired with the fluctuation in isomer percentages make brodifacoum challenging to interpret.

6.3 Specific Non-Target Species Diastereomer Analysis

Coyotes and Cougars

Coyotes with bromadiolone have a consistent pattern of trans isomer dominance. Ten samples had trans isomer of 87 to 100%, leaving the last three samples with ratios comparable to bait. Similarly, cougars containing bromadiolone demonstrated the most significant elimination of cis isomer out of the 3 species with trans isomer range of 84-100% (3 livers at 100% trans and 10 samples with > 91%).

Coyotes with brodifacoum displayed an overall cis isomer dominance (14/15). Interestingly, one liver sample had the trans isomer in greater proportion. For coyotes, only one liver sample showed a substantial cis isomer percentage of 96.8%. Most samples fell within 70 to 78% cis isomer. Similarly, cougars had cis isomer in greater proportion. However, four of the 15 samples were consistent with bait proportions. One cougar sample did show significant cis isomer with 90.5% cis isomer. Overall examination of brodifacoum diastereomer pattern does reveal a cis isomer accumulation and a trans isomer elimination. However, 24% (n=45, 11/45) of

all species livers examined for brodifacoum were like bait proportions. In contrast only 7% (n=41, 3/41) of all species samples had bromadiolone ratios similar to bait.

The prior SGAR research relevant to these two species is based on SGAR exposure, not specific diastereomer ratios. There is currently no published literature regarding diastereomer patterns for bromadiolone and brodifacoum within coyotes and cougars. Though there is no species-specific research, the findings are consistent with Réunion harrier raptors (*Circus maillardi*), Red foxes (*Vulpes vulpes*), Red kit (*Milvus milvus*) and wild rats (*Rattus norvegicus*) (Fourel et al., 2021, Fourel et al., 2017, Fourel et al., 2017, Fourel et al., 2016). All of these research findings identified that bromadiolone residuals present in liver and fecal samples were significantly different from bait and that trans bromadiolone was predominant.

Gray Foxes

All gray foxes' livers, examined for bromadiolone, exhibited a consistent trans isomer dominance similar to what has been reported in red foxes (*Vulpes vulpes*) (Fourel et al., 2017). The findings of Fourel et al., 2017, identified that cis-bromadiolone was not present in red fox livers after bromadiolone use and that it lacks persistency in the food chain. Hypothetical conclusions about the primary consumer, water voles, were also discussed in their research. Their conclusions suggested that water voles had consumed bromadiolone bait and thus eliminated the cis isomer, leaving only trans isomer when they died. The water vole results of their project agree with the findings of this project where 4/8 livers from primary consumers demonstrated trans isomer in a larger proportion (range 82.9 -100%) with two opossum livers having 100% trans isomer.

Brodifacoum diastereomers within gray foxes were varied and showed minimal consistency with a specific isomer dominance. Nine of the 15 samples had brodifacoum trans

isomer in greater proportion, which is noticeably different from the cis dominance seen in coyotes and cougars. The prior research done on brodifacoum diastereomer research has concluded that in wild boars the isomer ratio is not different from bait and in wild rats the proportions varied indicating the need for further research to be done to make a conclusion (Alabau et al., 2020, Fourel et al., 2016).

6.4 Proposed Pathway of Secondary Intoxication

Bromadiolone

The bromadiolone bait examined in this study was found to have 65 to 68.7% trans isomer form. The trans isomer percentage in primary consumers in this project (opossum, skunk, and squirrel) was significantly different from bait. This pattern follows the results from Fourel et al., (2016) that wild rats (*Rattus norvegicus*), a target species, were able to limit or fully eliminate the cis isomer while still accumulating the trans isomer. Moreover, this pattern of trans isomer dominance continues into secondary species as noted in this study.

The ability of the trans isomer to persist within tissues longer may be due entirely to its differences in metabolism and elimination. Trans bromadiolone has a half-life three times the length of the cis isomer, taking 75 hours to eliminate half its initial concentration. The cis isomer only takes 25 hours (Damin-Pernik et al., 2017). The proposed pathway for secondary intoxication involves a primary species consuming bait which is followed by metabolism and elimination of cis while trans isomers persist in the animal. When a secondary consumer ingests a primary consumer, it is proposed that within the tissues is a significant amount of trans isomer. Thus, when the secondary species ingest the tissues, the main form is trans bromadiolone with little to no cis isomer. Results of this study support the conclusion that secondary consumers are exposed primarily to the trans isomer via the food chain. Specifically, skunks, squirrels, and opossums were noted to have elevated concentrations of trans isomer ($\geq 82\%$).

The above manner of secondary intoxication is supported by five of the eight primary consumers evaluated. However, all three raccoons exhibited a higher level of cis bromadiolone isomer. The reason for this apparent species difference is unclear, although it is possible that species differences in metabolism and elimination occur. Nonetheless, based on these findings it is possible that secondary species who consume tissue rich with cis isomer may also more readily metabolize and eliminate it. This is suggested since only 62.5% of the primary consumers had cis-isomer elimination, but secondary species like gray foxes and cougars had all samples with little to no cis isomer.

Pharmacokinetic differences between the cis and trans isomers is hypothesized as the main contributor to their relative concentrations in tissues. This is due to a study provided by Damin-Pernik et al., (2017) who investigated tissue persistency of each isomer. This is based on the ability cis and trans bromadiolones to inhibit vitamin K epoxide reductase (VKOR), which inhibits vitamin K_1 leading to lack of clotting factors. The VKOR results for bromadiolone cis was 31 ± 2 and trans 26 ± 2 (Damin-Pernik et al., 2017). The results showed no difference in bromadiolone cis and trans isomer's ability to inhibit vitamin K_1.

Brodifacoum

The cis isomer form of brodifacoum demonstrated a greater persistency in postmortem tissue for cougars and coyotes. For cougars 11/15 and coyotes 12/15 liver samples had cis isomer in greater proportion. Like bromadiolone, each isomer of brodifacoum has significantly different half-lives. Cis brodifacoum is nearly double that of trans isomer, taking 121 hours. Furthermore, based on additional findings from Damin-Pernik et al., (2017), brodifacoum cis and trans abilities to inhibit VKOR were comparable. Overall, cis isomer persistency in cougars and coyotes suggests half life being the largest contributor.

Gray foxes proved to be unique in their isomer proportions, straying away from coyote and cougar patterns. In total 10/15 samples had trans isomer proportions larger than cis. This is quite different from the cis isomer dominance seen in the other two species of this study. A study evaluating brodifacoum isomer residuals within Réunion harrier livers found that 9/29 livers had a greater trans isomer form (Fourel et al., 2021). Similar to the findings of this project, the rest of the Réunion harrier livers (n=20) had cis as the dominant isomer.

From the primary species examined, only one skunk and one raccoon had a greater trans isomer with the rest having cis. These findings suggest that not all primary/target species will follow the cis isomer persistency and trans elimination pattern. Additionally, if a secondary species has trans isomer dominance they may have received that from the primary/target species tissues. However, further research needs to be done to identify the specific reason for this isomer variability.

6.5 Assumptions and Limitations of Study

Assumptions in the intoxication pattern of primary and secondary species. For primary species analysis, the assumption that bromadiolone and brodifacoum residues were from consuming bait and not other exposed animals was applied. It was assumed that bait ingestion was the predominant route of exposure in primary consumers. However, some primary consumers could conceivably ingest both bait and exposed prey due to their omnivorous nature. For secondary species, the assumption was that the SGAR residues were from consuming target/primary species tissues and not bait directly.

Limitations of this study include a limited number of bromadiolone and brodifacoum bait formulations and a limited primary species sample size. Some variability within a given species could be due to differences in when an individual was exposed to an SGAR and when it ultimately died. The longer the period of time, the more metabolism and/or elimination of the

diastereomers could have occurred thus affecting the percentages detected. Lastly, some

diastereomer fluctuation could have occurred naturally over the time between necropsy and

analytical testing.

7. Conclusion

This research successfully identified isomer persistency of bromadiolone and brodifacoum in bait, primary consumers, and non-target wildlife samples. HPLC-MS allowed for adequate chromatographic separation of the diastereomers and confirmation through mass spectra data. Bromadiolone trans isomer proportions in primary and secondary species were significantly different from bait ratios. Moreover, in non-target wildlife trans bromadiolone was found to be more persistent in postmortem liver tissue. The analysis of primary consumers found that three opossums and one skunk also demonstrated a greater trans isomer form ($82.9 - 100\%$). Thus, it is possible that secondary animals are accumulating bromadiolone trans isomer through consumption of primary tissue that already contains most or all trans isomer form. Bromadiolone had a continuous pattern of trans isomer accumulation and cis elimination. Moreover, statistical testing provided further confirmation of highly significant differences between bait and secondary wildlife species. This research proposes that current bromadiolone bait should be reformulated to include less trans isomer. New bait favoring the less persistent isomer may reduce secondary intoxication of wildlife that occurs through consumption of primary tissues.

Brodifacoum results found that coyotes and cougar samples favored cis isomer persistency, while gray foxes favored trans isomer. With these isomer fluctuations it is difficult to make a firm argument of bait reformulation for brodifacoum. This is also reflected in statistical testing, which displayed no significance found between the bait, primary, and secondary isomer proportions.

Future work on this project could examine the reapplication of calibration curves allowing for concentrations of each isomer to be quantified in the liver samples. Also, obtaining more primary and target species livers to assess ratios across the food chain would be useful.

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