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RESEARCH

Anticoagulant rodenticides exposure status among wild pit vipers (*Protobothrops flavoviridis*) and green anoles (*Anolis carolinensis*) in two Japanese Islands

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Abstract
To investigate the exposure of wild reptiles to anticoagulant rodenticides (ARs), liver ARs concentrations were analyzed using LCMS in individuals captured within ARs application areas. A total of 185 pit vipers (*Protobothrops flavoviridis*) from Amami Oshima Island and 89 green anoles (*Anolis carolinensis*) from Ogasawara Island were captured. Among the pit vipers, approximately 9% of the samples showed detection of three ARs (warfarin (WF), diphacinone (DP), and coumatetralyl (CT), with a maximum concentration of 436.5 ng/g. Additionally, the WF metabolite 10-OH WF was detected in 4 out of 10 individuals where WF was detected. No correlation was found between ARs detection rates and sex or sampling seasons, and no hemorrhaging signs were observed in pit vipers with ARs detection. In green anoles, DP was detected in only one individual at a concentration of 51.9 ng/g. Although high ARs accumulation was not observed, careful monitoring is needed as lizards may serve as exposure resources for higher−tier consumers.

Keywords Anticoagulant rodenticides · Reptiles · Secondary poisoning

Introduction

Anticoagulant rodenticides (ARs), which are also known as vitamin K antagonists, are widely used to control pests such as invasive rodents. They cause chronic bleeding by inhibiting vitamin K epoxide reductase. Depending on their toxicities, ARs are classified into two main groups: first−generation (FGARs) and second−generation (SGARs). While SGARs, such as bromadiolone (BM), difenacoum (DF), and brodifacoum (BF), cause lethal effects in a single intake due to their high toxicity and long−acting time, the FGARs (warfarin (WF), diphacinone (DP),

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and chlorophacinone) need multiple ingestions for killing rodents (Damin-Pernik et al. 2017. Although ARs are recognized as an effective tool for controlling pests, concerns regarding their detrimental impacts on non-target wild species are growing. For example, according to 30 studies from 1998 to 2015, ARs were detected in 55% of liver samples from non-target species (2694/4891 samples) (Nakayama et al. 2019). However, the target organisms of these field surveys are primarily mammals and avian species, and there are few reports on the exposure status of reptiles in ARs application areas. Therefore, this study aimed to assess the ARs exposure status of wild reptiles living in ARs application sites.

Amami Oshima Island, located approximately 1,400 km southeast of Tokyo, was treated with toxic DP baits in 2017 and 2018 to eradicate the invasive Philippine mongoose (*Herpestes auropunctatus*) (Abe 2022). Pit vipers (*Protobothrops flavoviridis*) are common reptiles on the island and prey on rodents in the wild (Koba 1963; Mori and Moriguchi 1988); hence we chose them as ARs exposure models.

Ogasawara Islands, volcanic islands located approximately 1,000 km south of Tokyo, have implemented an ARs application program to combat the population of invasive black rats (*Rattus rattus*) or Norway rats (*Rattus norvegicus*). This program involves the aerial dispersion of DP via helicopters and the placement of toxic DP bait stations across the islands (Kawakami 2019). Green anoles (*Anolis carolinensis*), small to medium−sized lizards, are also famous alien species on the islands. These anoles can be exposed to ARs through accidental ingestion of toxic baits or secondary exposure via the consumption of ARs−contaminated insects. Moreover, they could potentially serve as a source of exposure for higher−tier predators. Therefore, we chose anoles as the second ARs exposure model for reptiles.

Materials and methods

Detailed information on the analysis was presented in supplementary information. In brief, 185 pit vipers (male: 114, female: 71) captured in the Amami Oshima Island under the Habu Capture Encouragement and Purchase Project from February to October 2023 were obtained from the Naze Public Health Center (Kagoshima, Japan). 89 green anoles (male: 53, female: 32, unknown: 4) captured in the Ogasawara islands (Ani-jima, Chichi-jima, and Haha-jima islands) from July 2022 to September 2023 were obtained from the Kanto Regional Environment Office and the Ogasawara Wildlife Research Society. For both species, body length (from cloaca to snout tip) and body weight as well as head length for pit vipers were measured, and sex was determined. Livers, kidneys, intestines, muscles, and stomach and intestine contents were collected and frozen before being sent to Hokkaido University (Sapporo, Japan). Samples were kept at 20 ºC until use. ARs were extracted from livers using a combination of the QuEChERS approach and dispersive solid phase extraction method described by Lawal et al. (2018). Quantifications of ARs were performed by liquid chromatography coupled to mass spectrometry (1290 Infinity II LC system, 6495B Triple Quad LC/MS; Agilent Technologies, Santa Clara, CA, USA) using a Poroshell 120 column (EC-C18, φ1.9 µm, 3.0 × 100 mm; Agilent Technologies, Santa Clara, CA, USA).

Results

Table 1 shows the number of pit vipers in which ARs were detected. Of the 145 pit vipers surveyed, 13 individuals (approximately 9%) showed ARs residue levels above the limit of quantification (LOQ) (1.6 ng/g). the detected ARs were all FGARs (WF, DP, and CT), but no SGARs were found. In order to understand the metabolic profiles of

Table 1 ARs residual status in wild pit viper livers on the Amami Oshima Island [LOQ: limit of quantification]

2023		> LOQ number / Sample number									
		February	March	April	May	June	July	August	September	October	Total
FGARs	WF	0/25	2/20	1/20	0/20	1/20	0/20	3/20	2/20	1/20	10/145
	DP	1/25	1/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	2/145
	CT	0/25	0/20	0/20	0/20	1/20	0/20	0/20	0/20	0/20	1/145
SGARs	BF	0/25	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/145
	BM	0/25	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/145
	DF	0/25	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/145
Total ARs		1/25	3/20	1/20	0/20	2/20	0/20	3/20	2/20	1/20	13/145
WF metabolites	4'-OH	0/25	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/145
	6-OH	0/25	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/145
	7-OH	0/25	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/145
	8-OH	0/25	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/145
	10-OH	0/25	0/20	0/20	0/20	1/20	0/20	2/20	1/20	0/20	4/145

ARs in reptiles, we also analyzed the concentrations of five hydroxylated forms of WF (4'-OH, 6-OH, 7-OH, 8-OH, and 10-OH WF). One of the warfarin metabolites i.e., 10-OH WF was also detected from certain pit vipers. There was no correlation between ARs detection rate and sampling seasons (month (individual numbers in which ARs were detected); February (1), March (3), April (1), May (0), June (2), July (0), August (3), September (2), and October (1)). In green anoles, only one (out of 89 individuals) showed a DP residue level above LOQ.

Table 2 provides detailed information about the 13 pit vipers and one green anole in which ARs were detected. In pit vipers, no correlation was observed between sex and ARs residue status (6 males and 7 females). WF was detected in 10 pit vipers at concentrations ranging from 2.19 to 436.48 ng/g, with 10-OH WF also found in 4 of them at concentrations ranging from 14.75 to 167.37 ng/g. DP and CT were also detected in 2 and 1 individual, respectively (concentrations of 32.20 and 36.97 ng/g, 30.3 ng/g, respectively). One male green anole showed a DP residue level of 51.9 ng/g.

Discussion

In this study, various concentrations of FGARs were detected in livers of pit vipers regardless of season, indicating a year−round application of ARs on the island. Although no hemorrhaging signs were observed in individuals where ARs were detected, it remains uncertain whether this absence of symptoms was due to low toxicity or the potential disappearance of signs caused by the prolonged freezing time. Weir et al. (2016) investigated the toxicity of ARs on western fence lizards (*Sceloporus occidentalis*) and reported low toxicity since LD_{50} values were near 1750 mg/kg for DP and above 1750 mg/kg for CT. Moreover, Mauldin et al. (2020) observed no death or hemorrhaging signs in boa constrictors (*Boa constrictor*) despite the liver residual level of DP reaching 890 ng/g. Since this concentration is higher than the maximum level observed in our study (436.5 ng/g of WF), it is likely that none of the 13 pit vipers with ARs residue in livers experienced acute adverse effects. The variation of blood coagulation chemistry between reptiles and poikilotherms may be a key factor in determining susceptibility to ARs. AROCHA-PISIANGO et al. (1981) reported very high concentrations of plasma fibrinogen in *Caiman crocodilus*. This high fibrinogen level in reptiles could potentially lead to resistance against ARs.

Pit vipers eat not only frogs or lizards but also rodents such as house mice in the wild (Koba 1963; Mori and Moriguchi 1988). Therefore, secondary ARs poisoning may occur throughout the food chain. In our study, fur believed to be from rodents was found in the intestine of one pit viper (individual number: 9–12, WF concentration: 320.4 ng/g). Watanabe et al. (2010) demonstrated in vitro experiment using liver microsomes and reported that 4'-OH WF was the most dominant metabolite (70–80%) in avians, while in rats, it accounted for 50% of all metabolites. In addition, Khidkhan et al. (2024) confirmed that birds administered WF via gavage predominantly exhibited 4'-OH WF in their blood, while 10-OH WF was also detected in some avian species. In turtles, 4'-OH WF seemed to be the major metabolite (Yamamura et al. 2021). Interestingly, our study found that the sole metabolite detected in the livers of pit vipers was 10-OH WF, suggesting a unique WF metabolic system in pit vipers which is distinct from mammals, birds and some reptilian species.

In New Zealand, Wedding et al. (2010) recorded the first observation of native lizards eating baits containing ARs. Although only one specimen in our study showed a DP level above LOQ, it is plausible that green anoles in the

Table 2 Individual information of pit vipers and a green anole in which ARs were detected

Species	Individual ID	Body weight (g)	Sex	Concentration (ng/g)			
				WF	10-OH WF	DP	CT
Pit viper	2–4	546	F			32.2	
	3–4	701	M	12.9			
	3–10	583	F	23.9			
	3–19	463	F			37.0	
	4–5	690	F	5.8			
	6 – 5	635	F				30.3
	6–15	792	M	48.5	20.4		
	8 – 4	637	M	45.6	14.8		
	8–8	703	M	436.5	149.4		
	8–20	867	F	2.8			
	9–11	425	F	9.4			
	9–12	722	M	320.4	167.4		
	10–10	771	M	2.2			
Green anole	2–37	5.3	M			51.9	

LOQ were 1.6 ng/g for pit vipers and 3.0 ng/g for green anole

Ogasawara islands could inadvertently ingest ARs through toxic baits. Several studies have shown that invertebrates such as insects are exposed to ARs in their natural environments (Alomar et al. 2018; Dowding et al. 2010). Given that green anoles are insectivores (Stehle et al. 2017), food chain transfer could be another possible route for ARs exposure. The concentration of DP in our study was 51.9 ng/g in one green anole and it is lower than 127 ng/g observed in giant ameivas (*Ameiva ameiva*), which showed no clinical signs (Mauldin et al. 2020). Therefore, it is highly probable that this anole experienced no acute toxic effects. According to Kato and Suzuki (2005), Ogasawara buzzards, an endemic subspecies in the Ogasawara islands, primarily prey mainly on rodents (50.9%) and green anoles (32.7%). Although no significant accumulation of ARs in green anoles was observed in this study, continued monitoring of ARs exposure in anoles is crucial as they may serve as exposure sources for higher−tier consumers.

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Author contributions Y.Y and S.N. made research concenpt, analyzed data and wrote the main manuscript text. M.K., S.S., R.D., M.K., Y.B.Y., and Y.I. analyzed data. M.I. and S.M.M.N. made research concenpt, acquired fundings and supervised. All authors reviewed the manuscript.

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Data availability The data is available upon requested to the corresponding author.

Declarations

Ethical approval All the animal samples used in this manuscript were collected under the permission obtained from the Kanto Regional Environment Office and the Ogasawara Wildlife Research Society.

Competing interests There is no Competing Interests in this manuscript.

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