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ORIGINAL ARTICLE

Rodenticide contamination of cormorants and mergansers feeding on wild fish

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Abstract

Exposure of wildlife to anticoagulant rodenticides from sewer baiting and bait application is poorly understood. We analyzed residues of eight anticoagulant rodenticides in liver samples of 96 great cormorants, 29 common mergansers, various fish species, and coypu, in different German regions. Results show that hepatic residues of anticoagulant rodenticides were found in almost half of the investigated cormorants and mergansers due to the uptake of contaminated fish from effluent-receiving surface waters. By contrast, exposure of coypu to rodenticides via aquatic emissions was not observed. The maximum total hepatic anticoagulant rodenticide concentration measured in waterfowl specimens was 35 ng per g based on liver wet weight. Second-generation anticoagulant rodenticide active ingredients brodifacoum, difenacoum, and bromadiolone were detected almost exclusively, reflecting their estimated market share in Germany and their continuing release into the aquatic compartment. Overall, our findings reveal that second-generation anticoagulant rodenticides accumulating in wild fish are transferred to piscivorous predators via the aquatic food chain.

Keywords Biocides · Bioaccumulation · Biomonitoring · Persistence · Secondary poisoning

Introduction

Exposure of wildlife to anticoagulant rodenticides via the terrestrial food web is a well-known and documented environmental issue (van den Brink et al. 2018). Less documented, however, are anticoagulant rodenticide emissions to the aquatic environment and the likely transfer of persistent, bioaccumulative, and toxic second-generation anticoagulants such as brodifacoum along the aquatic food chain (Regnery et al. 2019a, 2020). Two recent studies from Germany (Regnery et al. 2024) and Pennsylvania, North America (Facka et al. 2024) clearly reinforced the relevance of previously neglected aquatic exposure pathways (Lemarchand et al. 2014). Both studies frequently detected residues of anticoagulant rodenticides in primarily piscivorous mammalian predators, Eurasian otter (*Lutra lutra*) and river otter (*Lontra canadensis*), despite the nowadays strictly regulated sale, supply, and use of rodenticides (Facka et al. 2024; Regnery et al. 2024).

As transfer and fate of anticoagulant rodenticides in the aquatic food web are not yet fully disclosed, our biomonitoring study aimed at providing further experimental evidence concerning the exposure of piscivorous predators to

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second-generation anticoagulant rodenticides via their prey in densely inhabited landscapes, such as Germany. Hence, we analyzed liver samples of 125 specimens of two exclusively piscivorous avian predators, great cormorant (*Phalacrocorax carbo*) and common merganser (*Mergus merganser*), as well as 41 liver samples of various freshwater fish species from different German regions (Bavaria, Rhineland-Palatinate, Saxony, Lower Saxony) regarding residues of all eight active ingredients used in biocidal anticoagulant rodenticides in Germany. Moreover, liver samples of 42 specimens of a semi-aquatic living, mammalian herbivore (coypu (*Myocastor coypus*)) from Lower Saxony, a region with previously documented rodenticide burden in otters (Regnery et al. 2024), were analyzed to compare their risk of exposure versus that of piscivores. We hypothesized that exposure of aquatic top predators to anticoagulant rodenticides is diet-driven, and coypu, unlike cormorants and mergansers, are thus less likely to be exposed. Chemical analyses were accompanied by post-mortem examinations of cormorant and coypu carcasses.

Experimental

Piscivorous waterfowl

The randomly investigated 96 great cormorants (*P. carbo*) from southern (Bavaria, $n = 50$), western (Rhineland-Palatinate, $n = 21$), north-western (Lower Saxony, $n = 1$), and eastern (Saxony, $n = 24$) parts of Germany (Fig. 1) belonged to the continental subspecies *P. carbo sinensis*. All cormorants had been shot near surface waters for nature conservation reasons based on state-specific species protection exception regulations between 2020 and 2023 (outside breeding season) and their carcasses were provided for post-mortem examination. In Germany, *P. carbo sinensis* inhabits the coastal areas as well as inland surface waters, with breeding occurrences in suitable habitats. Outside breeding season, encountered individuals can be sedentary birds, partial migrants, or migratory birds, respectively, as the Baltic Sea population generally migrates overland and winters from southern Germany to North Africa. Due to their vast foraging grounds and high mobility (cormorants may roam widely during the day and visit multiple feeding waters), exact origins of their fish prey cannot be determined with certainty.

Twenty-nine liver tissue samples of common mergansers (*M. merganser*) were received from an on-going

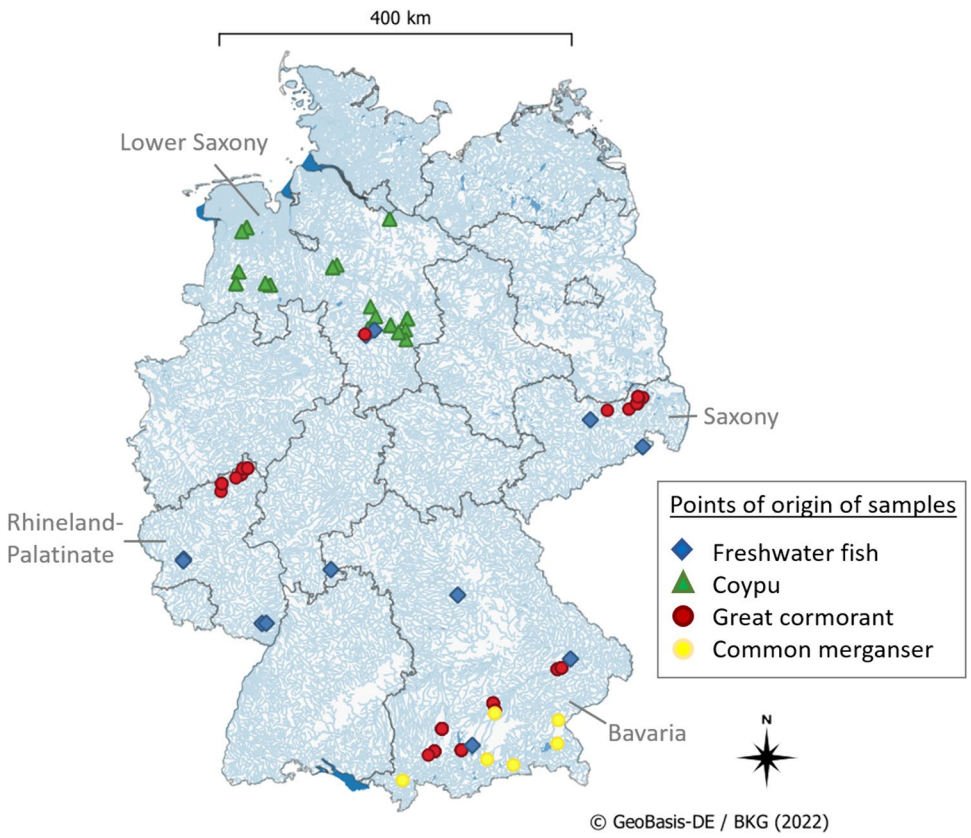

Fig. 1 Location of 208 samples of fish, coypu, cormorant and merganser analyzed for liver tissue. Please note that specimens originating from the exact same location are not illustrated by individual symbols

research project (FKZ A/20/03) about deterrence measures for nature conservation by Technical University of Munich, Wildlife Biology and Management Unit in collaboration with the Bavarian State Research Center for Agriculture, Institute for Fisheries. Adult birds had been culled at 6 selected stream sites in southern Germany (Fig. 1) in early spring 2023 (prior to the start of breeding season). In southern Bavaria, the common merganser lives as a sedentary bird year-round, with additional individuals passing through during winter months. Similar to cormorants, their prey consists primarily of small fish the size of 10–15 cm, which they hunt by diving in open surface waters. Thus, their foraging grounds generally overlap with those of great cormorants.

Freshwater fish

Freshwater fish sampling sites (Fig. 1) were in the broader vicinity of potential foraging grounds of analyzed cormorants and mergansers and included two streams each in Lower Saxony (Innerste, Leine) and Rhineland-Palatinate (Moselle, Queich), one stream in Saxony (Elbe), as well as one lake (Starnberger See) and three streams (Main, Isar, Pegnitz) in Bavaria. Individual ($n = 35$) and pooled ($n = 6$) liver tissue samples of species from different trophic levels such as common nase (*Chondrostoma nasus*), bleak (*Alburnus alburnus*), roach (*Rutilus rutilus*), chub (*Squalius cephalus*), brown trout (*Salmo trutta* f. *fario*), perch (*Perca fluviatilis*), pike (*Esox lucius*), pike-perch (*Sander lucioperca*), and European catfish (*Silurus glanis*) were kindly provided by the Bavarian Environment Agency, the Lower Saxony Water Management, Coastal and Nature Protection Agency, the Structural and Approval Directorate South (Upper Fisheries Authority) Rhineland-Palatinate, and the River Basin Community Elbe. The majority of liver tissue samples originated from fish caught between 2019 and 2023 during European Water Framework Directive biota monitoring campaigns.

Semi-aquatic living rodent

M. coypus, a semi-aquatic, invasive alien species with a plant-based diet, is classified as huntable game in most German federal states. A total of 42 coypu carcasses were obtained for post-mortem investigations from 17 different surface water locations in Lower Saxony (Fig. 1), at which coypu had been culled by hunters within the exercise of hunting rights between November 2020 and April 2021. Coypu are mainly nocturnal and crepuscular, respectively, and tend to stay along banksides during foraging.

Post-mortem investigation

Great cormorant carcasses from Saxony were examined according to routine procedures at the Museum of the Westlausitz Kamenz, whereas cormorant carcasses from Rhineland-Palatinate and Bavaria were handled at the Bavarian Environment Agency. Post-mortem examination of coypu carcasses and the single great cormorant from Lower Saxony was conducted at the Institute for Terrestrial and Aquatic Wildlife Research, University of Veterinary Medicine Hannover, Foundation. Recorded parameters for both species included biometric data, sex, estimated age, and nutrition status. For several specimens, the stomach content was also exemplarily recorded. Freezing of the carcasses prior to examination had prevented adequate blood sampling to screen for acute anticoagulant rodenticide poisoning characterized by coagulopathy. All sampled liver tissue was immediately frozen and shipped express on ice to the Federal Institute of Hydrology laboratory for chemical analyses.

Analytical methods and data analysis

Established analytical methods (Regnery et al. 2019b, 2024) were used for the quantitative chemical analysis of one pharmaceutical (phenprocoumon) and 8 biocidal (brodifacoum, bromadiolone, difenacoum, difethialone, flocoumafen, coumatetralyl, chlorophacinone, warfarin) anticoagulant active ingredients in liver tissue samples by liquid chromatography–tandem mass spectrometry. Method performance parameters for investigated species such as average recovery rates, method quantification limits, and estimated expanded measurement uncertainties are summarized in Tables S1–S3 (Supplementary Material) or already provided elsewhere (Regnery et al. 2019b, 2024). All reported analyte concentrations in liver tissue are based on wet weight. In addition, total hepatic lipid content of selected specimens was determined as described in Regnery et al. (2019b). Whenever total anticoagulant rodenticide concentrations are discussed in the following, residues of biocidal anticoagulants had been summed for each specimen, i.e., at least one of eight active ingredients detected above its respective method quantification limit, zero assigned for values below these limits. OriginPro, version 2021b (OriginLab Corporation, Northampton, MA, USA) was used for graphing and nonparametric Kruskal–Wallis analysis. Statistical difference was considered significant when $p < 0.05$.

Results and discussion

Age, sex, and body condition of examined specimens

The majority of investigated cormorants (i.e., 44 juveniles, 52 adults) was well nourished. Their determined total hepatic lipid contents were in the range of 2.7 ± 1.3% (in mergansers 5.0 ± 0.5%). The average body weights of female ($n = 34$) and male ($n = 61$) cormorants were 2182 ± 336 g and 2570 ± 321 g, respectively. Almost all cormorants had numerous nematodes in their gastrointestinal tracts. While stomach contents mainly consisted of small fish the size of 7–15 cm total length, a few larger fish up to 26 cm total length were also found. Identified ingested fish species were carp (*Cyprinus carpio*), chub, roach, and perch. The health condition of investigated coypu was predominantly good. Approximately two thirds were well nourished and observed stomach contents were considered typical for this herbivorous species. The average body weight of investigated coypu (i.e., 16 juveniles, 26 adults) was 3732 ± 1591 g for females ($n = 18$) and 4651 ± 1798 g for males ($n = 23$). Determined total hepatic lipid contents were in the range of 3.2 ± 0.6%.

Measured hepatic second-generation anticoagulant rodenticide residues

Overall, 46 out of 96 cormorants (47.9%) from all four regions exhibited quantifiable anticoagulant rodenticide residues in their livers, mostly from 1–2 second-generation anticoagulant rodenticide active ingredients with a maximum total anticoagulant rodenticide burden of 35.1 ng/g (Fig. 2). Concentrations measured in males and females indicated no statistical difference (Kruskal–Wallis test, $H(1) = 0.342$, $p = 0.559$). Brodifacoum was detected in 39 (max. concentration of 27.6 ng/g), difenacoum in 23 (max. 7.5 ng/g), and bromadiolone in 3 (max. 2.3 ng/g) specimens, respectively. Coumatetralyl was solely detected in one cormorant liver tissue sample at very low concentration (0.18 ng/g), corroborating the lesser bioaccumulation potential of first-generation

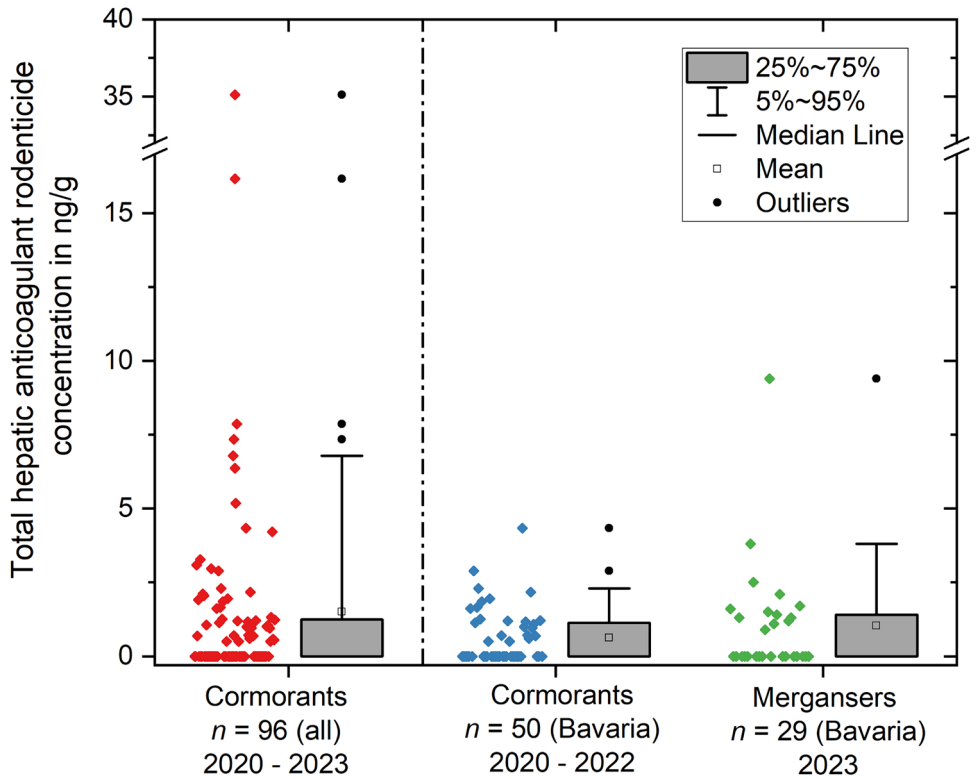

Fig. 2 Box plots of measured total anticoagulant rodenticide residue concentrations in liver tissue samples of investigated cormorants and mergansers from different German regions that had been shot near surface waters between 2020 and 2023. Residues of detected biocidal anticoagulants had been summed for each specimen, zero was assigned for values below the respective method quantification limits. Overall, 46 out of 96 cormorants (47.9%) and 13 out of 29 mergansers (44.8%) exhibited quantifiable anticoagulant rodenticide residues in their livers, mostly from 1 to 2 second-generation anticoagulant rodenticide active ingredients with a maximum total anticoagulant rodenticide burden of 35.1 ng/g based on wet weight. Rodenticide residue concentrations were not significantly different among groups, i.e., among all cormorants and cormorants and mergansers from Bavaria (Kruskal–Wallis test, $H(2) = 0.773$, $p = 0.679$)

anticoagulant rodenticides. In good agreement with findings from cormorants shot near Bavarian surface waters (Fig. 2), hepatic anticoagulant rodenticide residues were also detected in 13 out of 29 mergansers (44.8%), mostly from one second-generation active ingredient. Brodifacoum was detected in 12 specimens (max. concentration of 9.4 ng/g), bromadiolone in 2 (max. 1.6 ng/g), and difenacoum in one (0.5 ng/g), respectively. Residue levels of brodifacoum, difenacoum, and bromadiolone were not related to hepatic total lipid contents. Flocoumafen, difethialone, chlorophacinone, warfarin, and the pharmaceutical anticoagulant phenprocoumon were not detected above their respective method quantification limits in the analyzed waterfowl liver samples.

In contrast, solely one adult coypu exhibited elevated residues of 135.4 ng/g difenacoum in its liver, together with traces of a second active ingredient (1.1 ng/g brodifacoum). It should be emphasized that none of the biocidal and pharmaceutical anticoagulants were detected in any of the other 41 analyzed coypu. Thereof were 3 specimens that had been culled at the same location as the exposed one. In wild freshwater fish, measured total hepatic anticoagulant rodenticide concentrations (Fig. 3) matched previous records of rodenticides in fish from these effluent-receiving streams, e.g., Main, Isar (Regnery et al. 2019b), Elbe (Kotthoff et al. 2019), Moselle, Queich (Regnery et al. 2020), illustrating the continued emission of rodenticides from sewer baiting and outdoor surface baiting into the aquatic compartment. Their absence in fish from Starnberger See, an effluent-free lake, was also in good agreement with previous records (Regnery et al. 2019b). Highest total hepatic second-generation anticoagulant rodenticide levels in fish (mainly brodifacoum) of 74.5 ng/g (roach, 26 cm total length) and 95.6 ng/g (chub, 30.5 cm total length) were detected at two stream sites in Rhineland-Palatinate (Queich) and Lower Saxony (Innerste), respectively. At both sites, sewer baiting measures using baits deployed by wire in combined sewer systems had been carried out shortly before fish sampling campaigns, according to released public press communications.

Diet-driven exposure risk

As mentioned earlier, the exact origins of the waterfowl's ingested fish prey, and thus second-generation rodenticide residues, were unknown. Four cormorant individuals shot at surface waters in Bavaria had been tagged in Latvia, Finland, Switzerland, and Northern Germany, respectively. The limited and unforeseeable availability of biological tissue samples from protected species did not allow for strategic collection of corresponding predator and prey samples to ascertain full spatial and temporal overlap. Moreover, the prey composition of cormorants usually depends on what fish can be caught at all, or with as little effort as possible, rather than a strong preference for certain fish species (Keller 1998). Yet, the continuous presence of hepatic second-generation anticoagulant rodenticides in fish from effluent-receiving streams in the vicinity of foraging grounds of analyzed cormorants

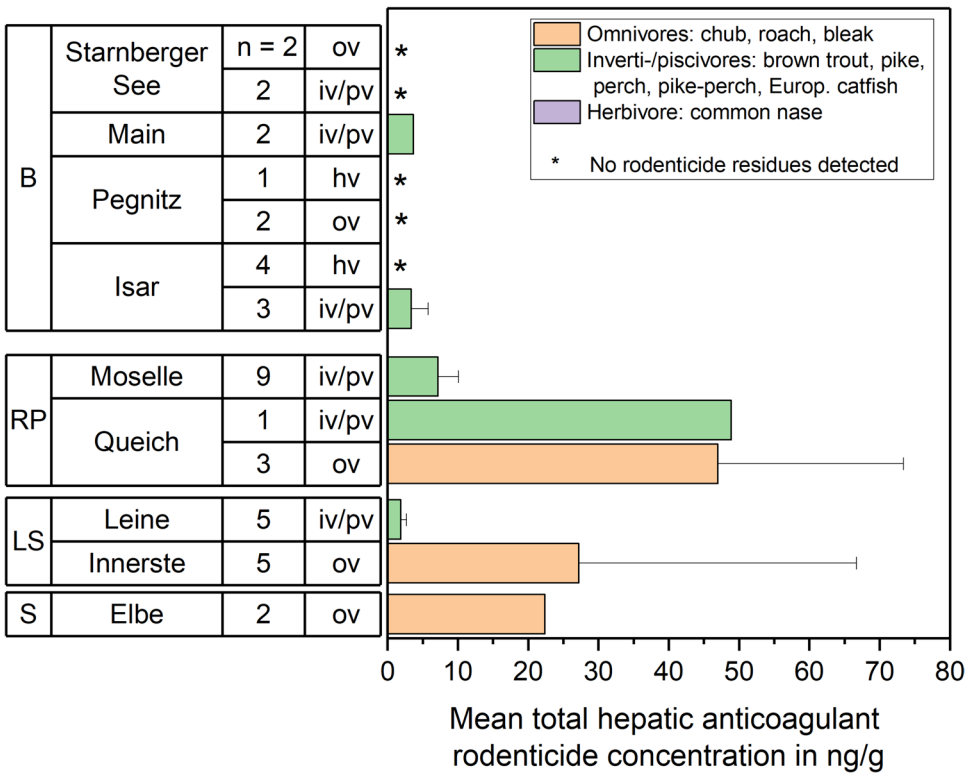

Fig. 3 Mean total anticoagulant rodenticide residue concentrations in liver tissue samples ($n = 41$) of different herbivorous (hv), omnivorous (ov), and inverti-/piscivorous (iv/pv) fish species from multiple surface water sampling sites located in Bavaria (B), Rhineland-Palatinate (RP), Lower Saxony (LS), and Saxony (S). Concentrations of detected biocidal anticoagulants, based on liver wet weight, had been summed for each specimen. Specimens were grouped by feeding-type, which presumably is a determining factor in second-generation anticoagulant rodenticide uptake. Where applicable, the relative standard deviation of mean values is shown. Highest total hepatic second-generation anticoagulant rodenticide levels in fish were observed at two stream sites (Queich, Innerste) with nearby sewer baiting

and mergansers demonstrates that exposure of piscivorous avian predators occurs via their fish prey. Residue levels in the analyzed waterfowl also clearly reflected current use patterns and the market dominance of brodifacoum, difenacoum, and bromadiolone containing biocidal products in Germany (Regnery et al. 2024). Another unequivocal indication was the absence of low-level anticoagulant rodenticide residues in coypu from Lower Saxony, a region previously known for pronounced anticoagulant rodenticide use and thus frequent detection in otters (Regnery et al. 2024). As pointed out in a recent review, including species from a diversity of trophic levels during biomonitoring is very helpful to comprehend exposure pathways (Keating et al. 2024). Primary exposure to difenacoum-containing bait was deemed most plausible to explain the elevated concentration detected in one adult coypu. Although their body size should prevent them from directly accessing tamper-resistant bait station, loose grain bait may be attractive for coypu when accessible. For instance, when baits are spilled from bait stations deployed near banks or deliberately offered.

Primary exposure of cormorants and mergansers to rodenticide bait, on the other hand, is considered extremely unlikely. The seemingly low hepatic rodenticide levels of investigated piscivorous waterfowl (Fig. 2) compared to reported secondary poisoning levels in predatory wildlife of the terrestrial food web (van den Brink et al. 2018) can most likely be explained by the absence of residues in fish from fish rearing ponds and surface waters without wastewater-borne rodenticide emissions (Regnery et al. 2019b; Kotthoff et al. 2019) that are frequently visited by cormorants during foraging (Keller 1998). Additional factors concerning piscivorous avian predators, such as the regurgitation of food if alarmed and a higher body temperature compared to mammals, may play a role too in terms of bioaccumulation and biotransformation (Kuo et al. 2022). The absence of second-generation anticoagulant rodenticides in 5 liver samples of common nase, a predominantly herbivorous fish species, also suggests that the foraging strategy is a determining factor in second-generation anticoagulant rodenticide uptake in the aquatic food web, e.g., such as the diversity and complexity of diets. Other fish caught at the same time at the Isar sampling site exhibited hepatic rodenticide residues in comparison (Fig. 3). However, more research (and data) will be required for a sound statistical assessment of such complex food web relationships.

Conclusion

Extensive knowledge and understanding of actual exposure pathways of biocidal anticoagulant rodenticides is essential to improve environmental exposure and risk assessments, and consequentially risk mitigation measures for the aquatic environment. Our biomonitoring study demonstrated that piscivorous avian predators in anthropogenically influenced landscapes are exposed to second-generation anticoagulant rodenticides via their fish prey. Transfer of second-generation active ingredients along the aquatic food chain was thus confirmed. Without doubt, future improvements of regulatory measures concerning biocides will be required to mitigate the yet unknown consequences for aquatic wildlife from the nowadays almost exclusive application of second-generation anticoagulant rodenticides during chemical rodent control.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10311-024-01762-y.

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Author contributions Julia Regnery: Conceptualization, Validation, Formal analysis, Investigation, Writing—original draft, Funding acquisition; Hannah Schmieg: Data curation, Investigation, Writing—review & editing; Hannah Schrader: Data curation, Investigation, Writing—review & editing; Olaf Zinke: Data curation, Writing—review & editing; Friederike Gethöffer: Data curation, Investigation, Writing—review & editing; Sarah-Alica Dahl: Data curation, Writing—review & editing; Mario Schaffer: Data curation, Writing—review & editing; Julia Bachtin: Investigation, Writing—review & editing; Christel Möhlenkamp: Investigation, Writing—review & editing; Anton Friesen: Conceptualization, Writing—review & editing, Supervision.

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Data availability Data will be made available on request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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SAFE RODENT CONTROL: REAL-LIFE SOLUTIONS

Got Rats or Mice?

Don't turn to toxic, dangerous rodenticides to get rid of rodents. Instead, use exclusion and sanitation tactics to make sure they never enter your home or business in the first place. Sealing entry points helps prevent rodents from accessing buildings, and eliminating food, water, and shelter heads off a full-scale invasion.

Keep reading to learn about safe, cost-effective ways to thwart rodent infestations without using poisons that could hurt your family, your pets, and rare wildlife.

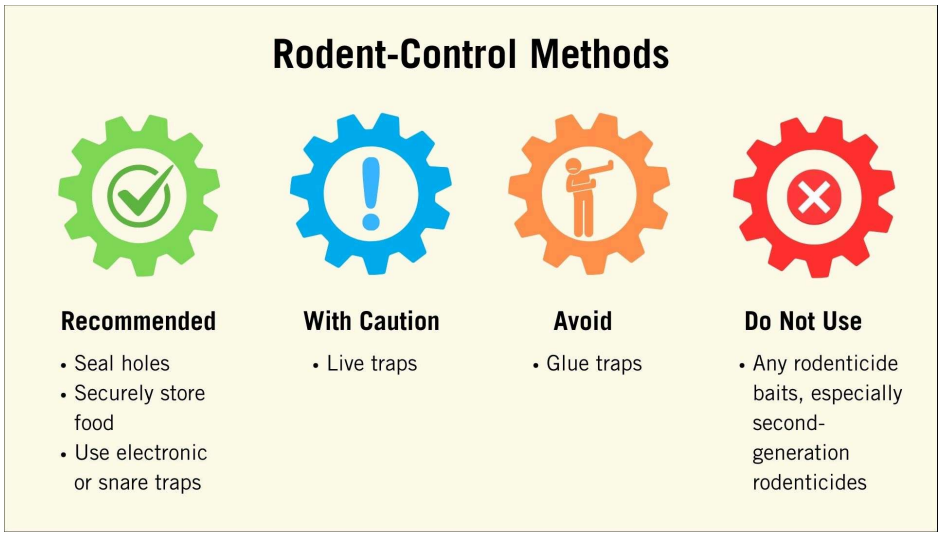
(The tips on this page are specifically for rats and mice, but we also have tons of ideas for rodenticide-free gopher, mole, and vole control.)

The Basics of Rat and Mouse Control

Rats and mice aren't just a nuisance — they can also damage property and spread diseases. So if you find signs of rodents, you want them gone forever, right? Poisons won't help with that —you'll have to keep poisoning rodents (and disposing of their bodies) over and over.

Instead, follow our three guiding principles for maintaining a rodent-free, rodenticide-free home: *prevent, identify, treat.* Many pest-control companies can help, but make sure they avoid rodenticides.

Prevent	Identify	Treat
Seal entry points to prevent rodents from entering your home or business. Be sure to use 1/4" x 1/4" metal mesh, steel wool, or insulating foam with a bittering agent to seal off existing entry points.	Look for signs of rats and mice: rodent droppings around food, in kitchen corners, inside cabinets, and under sinks; smudges and dark marks near entry points or nests; and chew or bite marks on food, wood, cardboard, or plastic.	Install rodent fertility-control bait stations. Remove existing rodents with snap or electronic traps.
Remove rodent attractants like consumables and shelter by ensuring that food and water are secure and surroundings are clean.	Also look for nesting material, such as shredded paper or fabric.	Install barn owl nesting boxes to draw owls who'll control rodents naturally.



Prevent

Eliminate Rodent Attractants and Entry Points

To keep rats and mice away permanently, prevent access to the building by sealing all possible entry points. It's equally important to eliminate rodent attractions like food and water by keeping food tightly sealed and repairing leaks. Periodically do a sweep of the exterior and interior of your building(s) to make sure your property isn't appealing for rodents.

Outdoor recommendations:

- Don't plant ivy — it provides shelter and harbors snails and slugs, a food source for rodents. Ivy on walls can form "rat ladders" to windows, attics, and other interior spaces.
- Keep compost piles as far away from structures as possible and grass cut to no more than 2 inches tall.
- Don't leave pet food or water out overnight.
- Maintain at least a 2-foot space between bushes, shrubs, fences, and buildings and remove tree limbs within 3 feet of a structure or roof.
- Avoid bird feeders, since they can also provide food for rodents.
- Keep outdoor grills and cooking areas clean.
- Keep firewood off the ground and as far away from structures as possible to mitigate shelter opportunities.
- Use trash bins that close securely to keep out rodents. If a bin is cracked or missing a lid, contact the Department of Sanitation for a replacement.

Indoor recommendations:

- Eliminate food and water sources:
  - Food in unsealed containers, like opened bags of chips, rice, cereal, crackers, flour, and other nonperishables
  - Pet food left out overnight or in an open bag instead of a secure container
  - Fruits or vegetables in bowls left outside of refrigerator
  - Leaky pipes or faucets
  - Open trash and compost containers
- Opt for garbage bins and compost containers with tops that seal tightly.
- Rinse food and beverage containers before discarding or recycling them.
- Clean your garbage and recycling bins frequently.
- Keep stovetops clean and free of food scraps.
- Maintain attic, crawlspaces, and cabinets near sinks clean and free of moisture.
- Declutter your home of papers, fabric, and other materials that rodents use for nesting.
- Use steel wool, caulk, foam with a bittering agent, or 1/4" x 1/4" metal mesh to seal these common entry points:
  - Holes near cabinets, closets, or doors leading outside or to crawlspaces and attics.
  - Holes around sink or appliance pipes
  - Cracked foundations in the basement or unscreened ventilation holes in the attic, especially in older structures
  - Gaps or cracks in baseboards
  - Holes in and around windows and doors
  - Missing screens in vents or crawlspaces under buildings

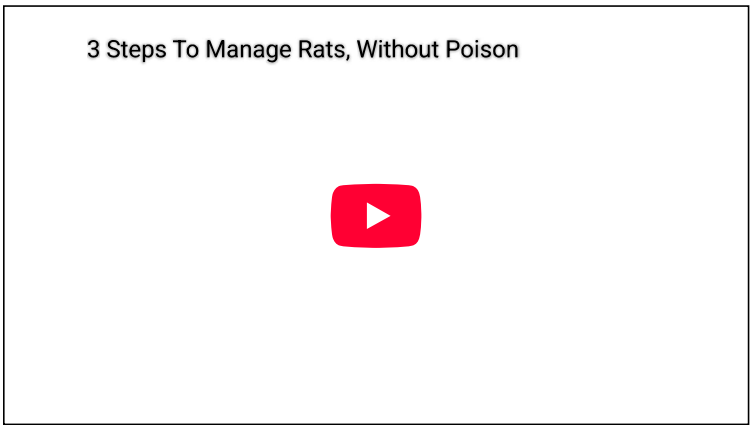
For even more rodent-deterrent tips, visit Raptors Are the Solution's Got Rats? webpage and Humane Pest Control's In Buildings webpage.

Promote Natural Predators

What's more of a deterrent to rodents than getting eaten?

Wild predators like snakes, hawks, and owls can help control rodent populations by gobbling up rats and mice before they get into your home. Barn owls are especially efficient hunters — a single family of barn owls can eat as many as 3,000 mice every year. To encourage barn owls to nest and hunt in your area, consider installing one or more nesting boxes. Strategic placement of nesting boxes combined with the use of traps and other preventative measures will go a long way toward managing your rodent problems.

For more information on installing *and* maintaining nesting boxes, visit the Hungry Owl Project. It's crucial to note that the Hungry Owl Project strongly urges you to use norodent poisons *at all*, either indoors or outdoors, while encouraging owls to spend time on your property. Rodent poisons could kill an owl who eats a poisoned rodent.



Identify

Look Out for Signs of Rodents

It might take some practice to master prevention methods, and of course sometimes new rodent access points and attractants will pop up before you notice them. Or maybe rodents got into your home before you discovered this webpage.

If any clever rats or mice have already invaded or ever sidestep your deterrent efforts, you'll know they've arrived if you're observant.

Stay alert to identify rodent stowaways by these telltale signs:

- Droppings, especially near a food source
- Shredded fabric or paper, which rodents use to nest
- Gnaw marks on food packaging or the building structure
- Scratching noises coming from the walls or ceiling, especially at night
- A musty, stale odor
- Greasy marks where rats have rubbed against a wall or doorway
- Unusual pet behavior

Treat

Decide How to Get Rid of Any Existing Rodents

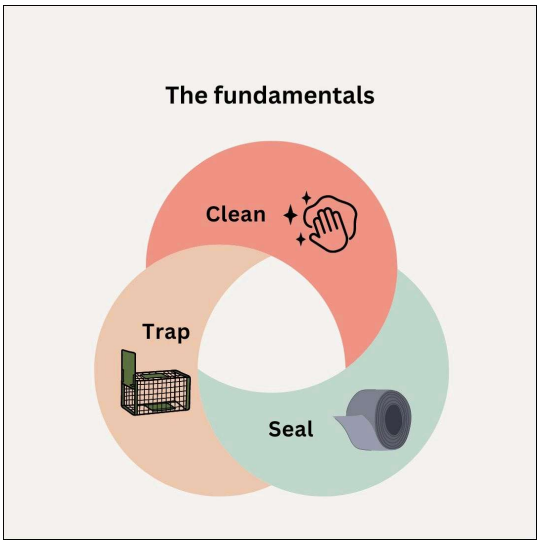
If you do see signs of rats or mice, we recommend a combination of continuing preventative measures and cautiously using the right kinds of traps or other treatment options, like hiring a professional.

Review all your options before deciding on a treatment plan. If you decide to work with a pest control professional, be sure the company is certified by Ecowise or GreenShield and familiar with integrated pest-management techniques. Integrated pest management is an environmentally sensitive approach focusing on long-term solutions by relying on common-sense practices and current, comprehensive information on pests' lifecycles and how they interact with the environment.

Consider Fertility Control

Rodent fertility control is an effective method to reduce rodent infestations in an environmentally friendly way. In a 2022 study examining the effectiveness of rodent-fertility products in reducing roof rat populations on a California poultry farm, rat activity was reduced by 94% with the incorporation of fertility control into existing integrated pest management. Place rodent birth control stations near locations with rodent infestations to eliminate the prolific reproductive cycle of rodents, which leads to infestations.

Even when using traps, remember: Using a multi-tactic approach to managing rodents — including fertility control — decreases the risk of dealing with future infestations.



Types of Traps

Traps are far from a perfect solution. Still, they're much better than rodenticides. Besides eliminating the dire threats that rodenticides pose to people, pets, and wildlife (including natural predators), using traps instead of poisons gives you clear confirmation of captured rodents, letting you better gauge treatment effectiveness. Traps also let you dispose of rodents immediately instead of dealing with rotting poisoned rodents who may die in out-of-reach places. For a guide on how to select and place traps, watch this video by the New York State Integrated Pest Management Program and check out our overview below.

Trap	Description
Snap trap	This is the oldest type of trap and uses a spring-loaded bar to kill a rodent on contact. Many modern snap traps prevent risk to children and pets by enclosing the device in a plastic box — which also hide the dead rodent, make for easier rodent disposal, and can be reused. Watch this instructional video on how to safely set a snap trap.
Electronic trap	This battery-powered trap delivers an electric shock that kills rodents quickly. It's a newer type of trap, and models are available for both rats and mice.
Live animal trap	This catch-and-release system that avoids killing a rat or mouse. Just note that some states prohibit releasing rodents into the wild, and the CDC warns that captured rats or mice might urinate, increasing the risk of disease spread.
Multiple-catch live mouse trap	This is a catch-and-release system that allows for capture of multiple mice. (See our warnings about live animal trap above.)
Glue trap	We *don't* recommend glue traps because the adhesive plate they use to capture rodents can also trap birds, baby animals, lizards, and even pets. Plus, these traps cause rodents undue suffering — and while they're waiting to die, they may also urinate and increase the risk of spreading disease.

Trapping Rodents Safely

It's still crucial to use traps safely and then properly dispose of dead rodents to reduce the risk of getting sick or spreading disease.

Trap-use tips:

- Always read and follow the label instructions on traps.
- Place traps out of children's and pets' reach or inside safety enclosure boxes.

Tips for rodent and nesting-material disposal:

- Use gloves when disposing of dead rodents, nests, or nesting material.
- Spray the dead rodent or nesting material with a disinfectant solution and allow it to soak for 5 minutes before disposing of the rodent or material in a secure plastic bag.
- Spray and wipe up the area surrounding the dead rodent or nesting material with a disinfectant.
- Place the plastic bag with the rodent or nesting material into another plastic bag, along with any wipes or rags you used to sanitize the area.
- Be sure to wash your hands thoroughly with soap and water when you're done.

Needs more tips? Check out these step-by-step tips on cleaning up after rodents — dead rodents from traps, rodent urine and droppings, and rodents in old vehicles — from the Centers for Disease Control and Prevention.

If You Use Rodenticides Despite the Consequences

Some people choose to use rodenticides despite their massive dangers and drawbacks, including their high risk of causing suffering and death in children and nontarget animals (and the fact that they'll probably leave dead rodents in your walls). If you or someone you know resort to this option, at least follow these guidelines to reduce the risks of using such potent poisons:

- Always read and follow the label instructions on the pesticide product. The label is the law, and you could be liable for any damage resulting from not following the label instructions.
- Use only products approved by the U.S. Environmental Protection Agency that are sold and used with tamper-resistant bait stations to help reduce poisonings of children, pets, and wildlife. Here's a list of EPA-approved rodenticide bait station products.
- Indoors, only put rodenticide bait stations in places completely inaccessible to children and pets: inside walls, under heavy appliances, or in enclosed crawlspaces.
- To protect wildlife, don't use any rodenticides containing anticoagulants (brodifacoum, bromadiolone, difenacoum, difethialone, diphacinone, warfarin, and chlorophacinone) as active ingredients.
- Once all signs of rodents are gone, remove bait stations promptly by placing them in a secure plastic bag.

More Resources

- Raptors Are the Solution: Got Rats?
- Northwest Center for Alternatives to Pesticides: Controlling Rats Without Poisons
- Poison-Free Malibu: Earth-friendly management techniques so people can make informed decisions about pesticides usage
- University of Florida's IFAS Extension
- U.S. Environmental Protection Agency: Identify and Prevent Rodent Infestations

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Turkey Vultures (Cathartes aura) from Southern California are Exposed to Anticoagulant Rodenticides Despite Recent Bans

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Turkey Vultures (*Cathartes aura*) from Southern California are Exposed to Anticoagulant Rodenticides Despite Recent Bans

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ABSTRACT.—Secondary poisoning with anticoagulant rodenticides (ARs) has been identified as an important threat for raptor conservation worldwide. In 2019, the California State Legislature passed Assembly Bill 1788 (made effective in 2020), which prohibits or limits the use of second-generation anticoagulant rodenticides (SGARs) in the state, as a follow-up to the California Department of Pesticide Regulation's ban on SGARS implemented in 2014. Currently, the adherence to these recent restrictions on ARs in southern California is unknown. To assess whether these bans prevented exposure of raptors and other wildlife to ARs, we investigated (1) the prevalence of exposure to eight different ARs in the blood of Turkey Vultures (*Cathartes aura*) before and after the 2019 ban, and (2) the distribution of resighted (encountered) wing-tagged Turkey Vultures included in this study to assess where exposure might occur. Of 27 Turkey Vultures tested for eight ARs, one out of 11 sampled in 2017 had detectable (trace) but not quantifiable levels of difethialone, and two out of 16 (12.5%) sampled in 2021 had detectable levels of diphacinone (one had 8 ppb; another indicated as positive without quantification). Overall, the prevalence of exposure to ARs was 11.1% (3 of 27), 7.4% for diphacinone and 3.7% for difethialone. Based on 93 resightings of 20 of the wing-tagged Turkey Vultures, all but one remained within the areas of Los Angeles, San Bernardino, Orange, Riverside, and San Diego Counties of southern California. Our study suggests that the exposure risk of Turkey Vultures to ARs persisted despite recent restrictions. Our small sample size and reliance on blood in live vultures rather than liver tissue in dead ones may be underestimating true ARs exposure in our study population. We propose a continued and integrated monitoring approach that includes measurements of ARs in both free-ranging (blood samples) and deceased (liver samples) Turkey Vultures for effective large-scale monitoring. This approach will assess compliance with current and future bans and regulations regarding the use of these poisons in California.

KEY WORDS: *ban*; *Cathartidae*; *difethialone*; *diphacinone*; *monitoring*; *prevalence*; *rodenticide*; *scavengers*.

CATHARTES AURA EN EL SUR DE CALIFORNIA ESTÁN EXPUESTOS A RODENTICIDAS ANTICOAGULANTES A PESAR DE RECIENTES PROHIBICIONES

RESUMEN.—La intoxicación secundaria con rodenticidas anticoagulantes (RA) ha sido identificada como una amenaza importante para la conservación de las aves rapaces en todo el mundo. En 2019, la Legislatura del Estado de California aprobó el Proyecto de Ley 1788 de la Asamblea Legislativa, efectivo en el 2020, que prohíbe o limita el uso de rodenticidas anticoagulantes (RA) de segunda generación (RASG) en el estado,

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como seguimiento a su prohibición, implementada en 2014, por parte del Departamento de Regulación de Pesticidas de California. Actualmente se desconoce el cumplimiento de estas recientes restricciones sobre los RA en el sur de California. Para evaluar si estas prohibiciones evitaban la exposición a estos RA en aves rapaces y animales silvestres, investigamos (1) la prevalencia de exposición a ocho RA en la sangre de *Cathartes aura* antes y después de la prohibición de 2019 y (2) la distribución de los encuentros visuales de *C. aura* marcados con bandas alares incluidos en este estudio para evaluar dónde podría producirse la exposición. De los 27 *C. aura* analizados para ocho RA, uno de 11 *C. aura* muestreados en 2017 tuvo niveles detectables (trazas) pero no cuantificables de difetialona, mientras que dos de los 16 (12.5%) *C. aura* muestreados en 2021 tenían niveles detectables de difacinona (uno tenía 8 ppb; otro indicado como positivo sin cuantificación). En general, la prevalencia de exposición a los RA fue del 11.1% (3 de 27), del 7.4% para la difacinona y del 3.7% para la difetialona. Sobre la base de 93 encuentros visuales de los 20 *C. aura* marcados todos, menos uno, permanecieron en los condados de Los Ángeles, San Bernardino, Orange, Riverside, y San Diego en el sur de California. Nuestro estudio sugiere que el riesgo de exposición de los *C. aura* a los RA persistió a pesar de las recientes restricciones. Aunque se basa en un tamaño de muestra pequeño y con las limitaciones de analizar únicamente la sangre en aves vivas en lugar de los hígados de aves muertas, nuestro estudio sugiere que el riesgo de exposición de *C. aura* a los RA persistió incluso después de que se implementaran las recientes restricciones. Proponemos un enfoque de seguimiento continuo e integrado que debe incluir la investigación de RA tanto en *C. aura* de vida libre utilizando muestras de sangre como en individuos muertos por medio de muestras hepáticas para un seguimiento efectivo a gran escala y para evaluar el cumplimiento de las prohibiciones y regulaciones actuales y futuras con respecto al uso de estos venenos en California.

[Traducción de los autores]

INTRODUCTION

Secondary poisoning with anticoagulant rodenticides (ARs) has been identified as an important threat for raptor conservation worldwide (Rattner et al. 2014, Elliott et al. 2016, Gómez et al. 2022). These compounds interfere with the synthesis of vitamin K-dependent coagulation factors in the liver of raptors and other animals who ingest them through the prey or carrion they feed upon (Hindmarch and Elliott 2018, Nakayama et al. 2019, Oliva-Vidal et al. 2022). Depending on the type, amount, and frequency of AR ingestion, raptors may show variable degrees of coagulopathy, hemorrhage, and blood loss, and eventually die as result of circulatory collapse and hypovolemic shock (Murray 2017, 2018, 2020).

As a measure to reduce the impact of ARs on raptors and other wildlife species, the California State Legislature passed the California Ecosystems Protection Act of 2019 (Assembly Bill 1788; entered into effect in 2020), prohibiting or limiting the use of second-generation ARs (SGARs) in the state (Quinn et al. 2019), and as a follow up to the California Department of Pesticide Regulation's ban on SGARS implemented in 2014. Currently, in California, products containing SGARs (e.g., brodifacoum, bromadiolone, difenacoum, and difethialone) can be purchased and used only by certified pest control companies and operators under very specific circumstances and are no longer sold or approved for consumer use. Furthermore, the California State Legislature recently passed a moratorium on diphacinone (Assembly Bill 1322), a first-generation AR (FGAR) still available to consumers. The ban became effective in January 2024. The effectiveness of these regulations and their enforcement remain unknown and will certainly depend on effective enforcement and political will. Despite this legislation, the use of rodent baits with ARs appears to be a persistent and common practice in natural, urban, and suburban areas of California (e.g., Kelly et al. 2014, Gabriel et al. 2018). This causes concern about the population impact on sensitive southern California raptors, including those now considered extirpated in certain areas, such as the breeding Burrowing Owl (*Athene cunicularia*; Bloom 2023), which is known to be affected by ARs in Arizona (Justice-Allen et al. 2017) or the White-tailed Kite (*Elanus leucurus*), a small mammal specialist (Dunk 2020) that is currently suffering from unexplained population declines in our study area (P. Bloom unpubl. data).

Several studies have reported variable prevalence of exposure of California raptors to ARs in the past (Lima and Salmon 2010, Kelly et al. 2014, Krueger et al. 2015, Franklin et al. 2018, Gabriel et al. 2018). Recently, scavengers, like the critically endangered California Condor (*Gymnogyps californianus*) and the non-threatened Turkey Vulture (*Cathartes aura*) have also had a high prevalence of exposure to ARs (Herring et al. 2022, 2023). These findings indicate ARs as a persistent, pernicious threat for raptors that may contribute an additive mortality factor for raptor populations (Roos et al. 2021).

Raptors have proven reliable indicators of environmental toxicological risk (Redig and Arent 2008, Gómez-Ramírez et al. 2014), thus serving as sentinel

species. Continued monitoring of the use of ARs in natural, rural, urban, and suburban areas, and studies aimed at quantifying the likelihood of secondary poisoning of raptors, are needed to assess the effectiveness of recent regulations and help reduce threats to raptors and other wildlife (Quin et al. 2019). These studies are usually based on the identification and quantification of ARs in the livers of raptors that have been admitted to rehabilitation centers and subsequently died or were euthanized because of their injuries or medical conditions (Slankard et al. 2019, Gómez et al. 2023, Elliott et al. 2022). Mortalities and other specimens from rehabilitation centers can provide a robust number of samples to assess environmental prevalence of ARs. Nevertheless, estimates of AR prevalence from rehabilitated and dead birds may be biased, as reliance on birds admitted to rehabilitation centers overestimates prevalence of exposure and dose received (Gómez et al. 2022).

Studies of the prevalence of AR exposure in free-ranging raptor populations are rare. This has been recently accomplished using liver samples from culled Barred Owls (*Strix varia*) and Barred/Spotted Owl hybrids in the western USA, as part of a program aimed to reduce the impact of these birds on the Spotted Owl (*Strix occidentalis*; Gabriel et al. 2018, Hofstadter et al. 2021). Another approach has been the use of whole blood (or plasma/serum) for AR testing, yielding variable prevalence of exposure (Kwasnoski et al. 2019, Herring et al. 2022, Oliva-Vidal et al. 2022). The nonlethal, random sampling of free-ranging birds also avoids the killing of animals for investigating AR exposure, which is particularly valuable in regards of animal welfare and in declining and/or endangered species. Blood collection also enables the repeated sampling of recaptured birds. Unfortunately, a major caveat of this approach is an apparent lower sensitivity of testing blood compared with liver samples (Murray 2020, Herring et al. 2022, Oliva-Vidal et al. 2022). This may be a result of the short half-lives of ARs in blood, which only indicates recent exposure, from days to weeks (Murray 2020, Herring et al. 2022, Gómez et al. 2022); conversely, liver samples usually indicate chronic, longer-term AR exposure, persistence, and bioaccumulation (Gómez et al. 2022). Recently, Herring et al. (2022) compared liver and blood AR values in California Condors and Turkey Vultures and found that the prevalence of ARs in blood was much lower (10%) than in the liver (93%) of Turkey Vultures. More studies are needed to better understand the pharmacokinetics and toxicokinetics of ARs in non-target animals, and the value of blood, liver, and other tissues for surveillance, as they clearly differ among species and for the specific compound (Horak et al. 2018). Nevertheless, the detection of ARs in blood confirms the presence of these compounds in the environment and sheds light on the recency of exposure in natural populations (Oliva-Vidal et al. 2022).

The Turkey Vulture is an obligate scavenger, feeding on the carcasses of a wide variety of dead animals commonly found in urban, suburban, and natural areas (Kirk and Mossman 2020). Many coastal southern California Turkey Vultures are resident birds (Garrett and Dunn 1981, P. Bloom unpubl. data). As obligate scavengers, Turkey Vultures exploit multiple types of carrion, including dead rodents and domestic and wild carnivores such as bobcats, foxes, coyotes, and weasels, making them susceptible to exposure and bioaccumulation of many environmental poisons and pollutants (Kirk and Mossman 2020). Throughout their extended home range and varied landscapes where they find food, Turkey Vultures can be easily captured and in relatively large numbers (Bloom et al. 2019). Due to their broad distribution, resident status and extensive home range, Turkey Vultures may be useful avian sentinels for ARs, lead, and other pollutants available over large areas (Kelly et al. 2014, M. Saggese unpubl. data), allowing us to assess compliance to the recent state restrictions on the use of ARs and the risk of AR exposure to raptors.

During the past 7 yr, Turkey Vultures from southwestern California, the largest urban and suburban area in the state, were live captured, tagged and released during a collaborative research program aimed at assessing their potential as environmental sentinels for the presence of spent lead ammunition, characterizing their breeding ecology and movements, investigating their exposure to pathogens, and evaluating their population genetics (P. Bloom, M. Saggese, A. Bonisoli-Alquati, A. Koedel, and A. Eagleton unpubl. data). Several studies have reported exposure of Turkey Vultures to ARs (Kelly et al. 2014, Herring et al. 2022, 2023) in California. Our objective in this study was to investigate the prevalence of exposure to eight different ARs in Turkey Vultures from southern California. We hypothesized that the recent state bans on the use of ARs would reduce exposure to ARs in birds captured after the ban (2014 for FGARS and 2019 for SGARs) compared to before the ban. We also investigated the distribution of the Turkey Vultures included in this study to assess where exposure might occur.

METHODS

We trapped Turkey Vultures using a walk-in trap, as previously described (Bloom et al. 2007, 2019) at

Anaheim Lake (33.867116°N, 117.851124°W), Orange County, southern California, USA, between 2016 and 2021. Once trapped, birds were physically examined, measured, aged, sampled, wing-tagged, and weighed. We accessed Turkey Vulture nests (all nests located in Orange County, P. Bloom unpubl. data) and wing-tagged and sampled five nestlings. Blood (<1% of body weight) was collected from the basilic vein with heparinized syringes. Blood was kept refrigerated until arrival to the laboratory, where plasma was separated by centrifugation at 2500 G × 10 min and saved in cryovials kept at −80°C.

Plasma samples (1.2 mL) from 27 Turkey Vultures were shipped overnight to the California Animal Health and Food Safety Laboratories (CAHFSL; Davis, CA, USA) for AR testing and quantification by liquid chromatography-tandem mass spectrometry for four FGARs (chlorophacinone, warfarin, coumachlor, and diphacinone), and four SGARs (brodifacoum, bromadiolone, difethialone, difenacoum). Quality control samples included both unfortified and fortified bovine calf serum (Sigma-Aldrich). Two fortified serum samples were included at 2.5 ppb and 25 ppb levels of ARs. The lower concentration of 2.5 ppb was used for the reporting limits of all of the reported ARs with the exception of difethialone, which had a reporting limit of 25 ppb. An internal standard, d4-diphacinone, was included with all samples including quality control, and it was verified present for each analysis. We reported an AR as "trace" if detected, but not quantified (when an AR was identified at a concentration below the reporting limit). To test for a difference in prevalence of ARs before and after the 2019 ban, we used Fisher's exact test applied to detection of any of the four SGARs.

We also assessed the movements of the patagial-tagged Turkey Vultures, largely considered resident in the area (southwestern California, west of the Mojave Desert), by mapping all sightings reported to the Bird Banding Laboratory (US Geological Survey, Maryland, USA; retrieved October 2023) to assess and infer where these birds forage and may become exposed to rodenticides. Most observation records included exact encounter coordinates or provided the name of the location (i.e., a city park or nature preserve); in those cases we used approximate coordinates based on the descriptive details provided by the observer.

RESULTS

Of 27 Turkey Vultures tested for eight different ARs, 11 before and 16 after the 2019 bill came into effect, the overall prevalence of exposure to ARs was 11.1% (3 of 27). The overall prevalence of exposure to difethialone was 3.7% (one of 27), whereas the prevalence of exposure to diphacinone was 7.4% (two out of 27). For the 16 Turkey Vultures sampled in 2021, the prevalence of exposure to diphacinone was 12.5% (two out of 16).

Three out of 11 and two out of 16 Turkey Vultures, were nestlings; the remaining birds were all >6 mo old. We did not detect ARs in any of the nestlings. Among the non-nestlings, only one Turkey Vulture sampled in 2017 had detectable (trace) but not quantifiable levels of difethialone, the only SGAR detected. Two Turkey Vultures sampled in 2021 had detectable levels of diphacinone, an FGAR (one had 8 ppb; another indicated positive without laboratory quantification). The prevalence of exposure to the four SGARs among non-nestlings before (one out of 8) and after the ban (zero out of 14) did not differ significantly (odds ratio = 0.00, 95% CI = [0.00, 22.29], $P = 0.364$; Adjusted Cramer's V = 0.19, 95% CI = [0.00, 0.69]).

Twenty of the 27 Turkey Vultures we tagged and sampled were encountered (a total of 93 sightings) between November 2017 and May 2023. Except for one outlier (not shown but observed in San Jose, Santa Clara County), all the marked Turkey Vultures for which we have encounter data (19 out of 27 birds) remained and foraged within the scope of five different counties (Los Angeles, San Bernardino, Orange, Riverside, and San Diego) in southern California (Fig. 1).

DISCUSSION

Results of this study indicate that at least three out of 27 Turkey Vultures (or 11%) were exposed to FGARs and SGARs in a large area of southern California. Although the sample size was small and a larger sample size may have better detected exposure, our study suggests that the exposure risk of Turkey Vultures to ARs persisted after the recent bans were implemented. Such risk may extend to other raptor species. This was not surprising, given that considerable quantities remain in homes for private use and as of this date may still be available on store shelves (P. Bloom unpub. data, M. Saggese unpubl. data) and online, with potential unauthorized use in different urban, suburban, and rural settings. Furthermore, there are still legal exemptions to the recent bans (e.g., agricultural use).

Prevalence of exposure to ARs was generally low, with only one FGAR (diphacinone) and one SGAR (difethialone) detected. Overall, it was also in line with a recent estimate of 10% prevalence of AR exposure in Turkey Vultures' blood (Herring et al. 2022).

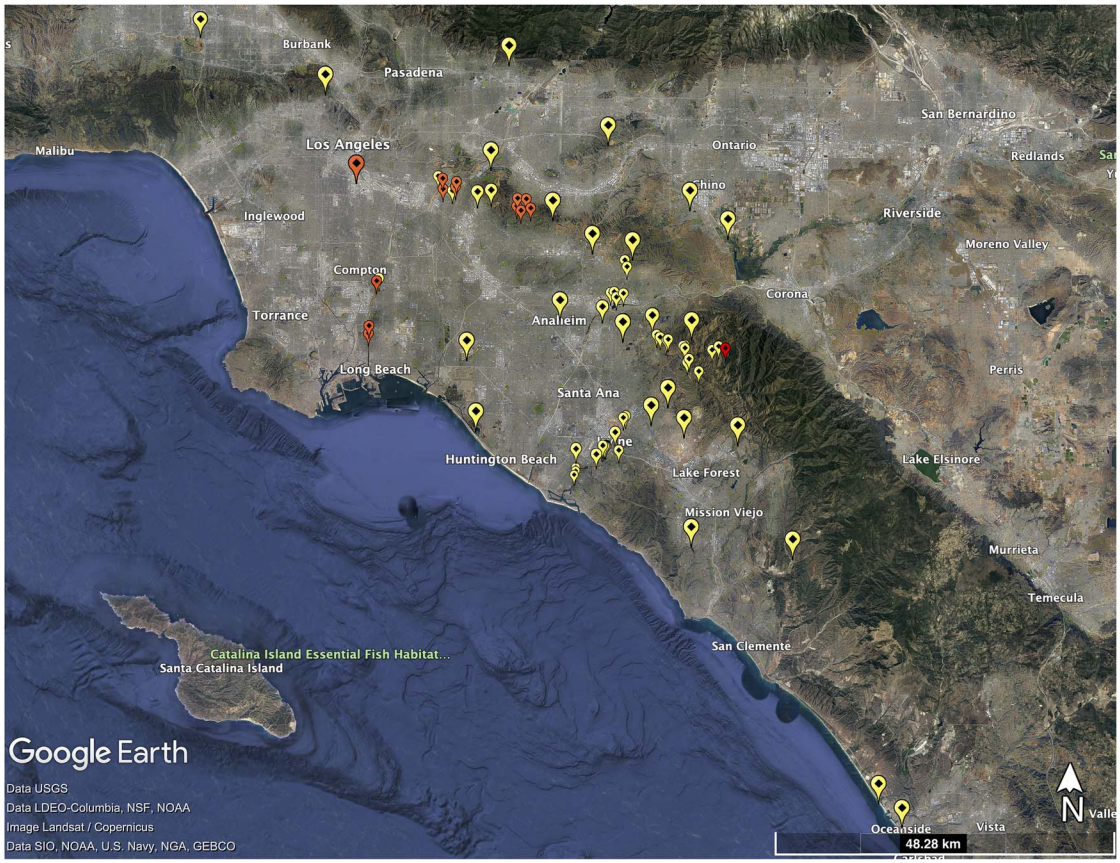

Figure 1. Visual sightings for wing-tagged southern California Turkey Vultures. Each icon in the figure corresponds to a sighting location of one of 19 different Turkey Vultures. Each icon indicates one sighting, except for a few areas in which sighting locations were identical. Larger icons indicate a clearly distinguishable sighting, and smaller icons indicate multiple sightings that were close to each other. Yellow icons indicate birds that were negative for ARs and red icons indicate those that tested positive.

The presence of ARs is of concern, especially for difethialone, an SGAR that since 2014 has been restricted to professional pest control agents and county agencies, for both indoor and outdoor use in California (Elliott et al. 2016, California State Legislature Bill AB 1788). Since 2020, its use has only been allowed under specific circumstances, with the goal of reducing the risk of exposure for nontarget wildlife (Riley et al. 2007, Moriarty et al. 2012, Cypher et al. 2014, Benson et al. 2019). However, we note that the one SGAR detection in our study occurred prior to the 2019 ban.

The other AR detected, diphacinone, is an FGAR offered for public use and under fewer restrictions than SGARs. FGARs are still available for professional use in California for different types of rodent control, and they require multiple exposures to kill rodents. However, the frequent detection of diphacinone in non-targeted wildlife and in baits has resulted in the recent passage of a bill in California placing new restrictions on the use of diphacinone starting on 1 January 2024 (California State Legislature Assembly Bill 1322). Continued monitoring of prevalence of AR exposure in Turkey Vultures may help monitor the efficacy of this ban in reducing environmental concentrations of diphacinone.

We acknowledge that the small sample size of 27 birds analyzed in this study implies that our estimates of prevalence of exposure to ARs should be interpreted with caution. Nonetheless, our results based on blood samples may underestimate the prevalence of exposure to ARs in Turkey Vultures from southern

California. ARs have a limited half-life in blood, and their detection in blood can only indicate recent exposure (Gómez et al. 2022). Kelly et al. (2014) found a 95% prevalence of exposure to ARs in the liver of 19 Turkey Vultures submitted from different rehabilitation centers in California. However, studies comparing the prevalence of AR exposure in blood and liver are few. For example, in central California, Herring et al. (2022) found a several fold higher prevalence of exposure to ARs in the liver than in the blood of Turkey Vultures. Assuming a similar relationship and based on the high prevalence values found in previous studies, we cannot rule out higher prevalence of exposure in the southern California Turkey Vulture population.

Our goal was to assess whether Turkey Vultures continue to be exposed to ARs after the recent bans, which they do. Although the extent of this exposure is probably higher than the 12.5% we report here for birds sampled after these bans, the ability to sample free-ranging birds of prey suggests that live-captured Turkey Vultures could be useful to assess recent exposure to ARs in a wide environmental range where a pathway of exposure to these highly toxic compounds occurs. We suspect that Turkey Vultures are exposed to ARs by ingesting the liver and potentially the gastrointestinal tract of scavenged animals (Hindmarch and Elliott 2018, Nakayama et al. 2019, Oliva-Vidal et al. 2022).

As changes in California's legislation regarding the use of ARs continue and existing California Legislature bills are enforced, it will be important to monitor the effectiveness and adherence of both the public and professional pest control companies. Raptors are one of the groups more widely studied for the purpose of contaminant surveillance. However, some raptor species have relatively limited home ranges and a large-scale evaluation of AR use in a particular region such as southern California may not be possible by sampling individuals on a broad spatial and temporal scale. However, Turkey Vultures are widely distributed, cover large foraging areas, and can be trapped relatively easily at multiple locations. Their large size allows adequate volumes of blood to be collected, and the broad spectrum of carrion consumed exposes them to multiple prey species potentially contaminated with ARs. These aspects make Turkey Vultures good sentinels for AR exposure and toxic effects in raptors and the environment in general.

The ecological and toxicological significance of the AR levels in blood of Turkey Vultures, as for many other raptors, have not yet been fully determined. The use of blood for evaluating exposure to ARs in scavengers has shown variable, sometimes contrasting, results (Herring et al. 2022, Oliva-Vidal et al. 2022). Further studies comparing blood and liver AR concentrations (paired samples) in birds that die or are euthanized at rehabilitation centers may prove useful to better understand these reported differences through comparative testing. Additionally, using liver tissue from recently deceased vultures to test for ARs will better elucidate the occurrence and intensity of bioaccumulation. The use of blood clotting assays has been recommended (Hindmarch et al. 2019) and could constitute another useful and complementary approach to assess AR exposure and effects in Turkey Vultures. Thus, identifying, refining, and validating methodologies for future studies on AR exposure in this species is key to implementing a monitoring program that will be reliable, effective, and inexpensive. Meanwhile, we propose an integrated monitoring approach that should include both free-ranging and deceased Turkey Vultures for effective large-scale monitoring of AR in southern California.

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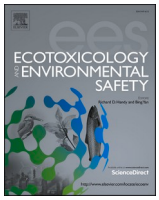

Brodifacoum causes coagulopathy, hemorrhages, and mortality in rainbow trout (*Oncorhynchus mykiss*) at environmentally relevant hepatic residue concentrations

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ABSTRACT

Widely used second-generation anticoagulant rodenticides like brodifacoum are classified as persistent, bioaccumulative, and toxic. Widespread exposure of terrestrial and avian non-target species is well-known and recently hepatic anticoagulant rodenticide residues have been detected in wild fish. However, no sufficient data exist to interpret the effects of these findings on fish health. In order to assess the potential impact of rodenticide residues on fish, we exposed rainbow trout (*Oncorhynchus mykiss*) to brodifacoum-spiked feed. In a first experiment, individually kept trout (body weight ca. 200 g) were exposed to a single dose of brodifacoum and observed for 15 days. In a second experiment, fish (body weight ca. 330 g) were kept in groups and fed every 7 or 8 days with brodifacoum-spiked feed for up to 60 days. Sampling of trout every 15 days over the 60 days period allowed monitoring of brodifacoum concentrations in serum, liver, and muscle tissue, as well as occurring effects over the course of the experiment. In both experiments, brodifacoum doses of $\geq$ 75 µg/kg body weight caused prolonged or non-measurable blood coagulation times. Disturbed hemostasis led to hemorrhages and anemia with significantly decreased albumin levels. In the 60 days-experiment, brodifacoum doses $\geq$ 100 µg/kg body weight caused additionally discoloration, apathy, and anorexia, resulting in reduced weight gain, and ultimately mortality. The delay until the onset of overt symptoms (14–17 days) highlights the importance of test duration while investigating effects of anticoagulant rodenticides in fish. The lowest hepatic brodifacoum concentration associated with effects in trout was on average 122.6 ng/g liver wet weight, which is in the range of previously reported brodifacoum residues in wild fish. These findings illustrate the risks associated with the use of anticoagulant rodenticides for freshwater fish and reinforce the need to stipulate all available and appropriate risk mitigation measures to prevent emissions at source.

1. Introduction

Since the middle of the 20th century, the mainstay of rodent control measures has been anticoagulant rodenticides (ARs) (Berny et al., 2014; Buckle and Eason, 2015), of which potent second-generation anticoagulant rodenticides (SGARs) such as brodifacoum are classified as persistent, bioaccumulative, and toxic (PBT; Regnery et al., 2019a; van den Brink et al., 2018). Unintentional poisoning of non-target wildlife occurs via direct consumption of AR-containing bait (primary poisoning), feeding of intoxicated animals or carcasses (secondary poisoning), or via environmental emissions (López-Perea and Mateo, 2018; Regnery et al., 2019a; Shore and Coeurdassier, 2018). As detailed in an extensive review by Rattner et al. (2014b), ARs inhibit the vitamin K epoxide reductase (VKOR), causing a deficiency of vitamin K. Vitamin K deficiency results in a lack of functional vitamin K dependent proteins like the clotting factors II, VII, IX, and X. After intoxication with ARs, already produced functional clotting factors are used up, which is why the onset of coagulopathy takes several days. Appearing symptoms, apart from spontaneous or trauma related hemorrhages, are anemia, pale mucosa, lethargy, and anorexia. Animals can die of blood loss or

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succumb to relatively minor hemorrhages causing localized ischemia, hypoxia, and cell death at vital sites (Rattner et al., 2014b; Rattner and Mastrota, 2018; Valchev et al., 2008). To monitor wildlife exposure to ARs, most studies focused on mammals and birds (López-Perea and Mateo, 2018; Shore and Coeurdassier, 2018). However, linking hepatic AR concentrations with lethal or sublethal effects remains challenging as toxicity varies considerably amongst species and individuals (Rattner et al., 2014b; Rattner and Mastrota, 2018; Thomas et al., 2011). In a probabilistic analysis, Thomas et al. (2011) calculated toxicity thresholds for different predatory bird species. Hepatic SGAR residues as low as 80 ng/g wet weight were associated with a 20 % chance of becoming symptomatic (Thomas et al., 2011).

In recent years, awareness regarding the presence of aquatic AR exposure pathways and associated risks emerged (Regnery et al., 2024, 2019a and references therein). SGAR residues have been frequently detected in the ng/g concentration range in liver tissue of fish caught alive (Cavanagh and Ward, 2014; Kotthoff et al., 2019; Masuda, 2014; Regnery et al., 2024, 2020, 2019b). Furthermore, the presence of VKOR and the vitamin K dependent clotting factors have been demonstrated in fish (Beato et al., 2020; Hanumanthaiah et al., 2001; Tavares-Dias and Oliveira, 2009). Yet, detailed studies on effects of SGARs on fish are scarce and have limited informative value, especially with reference to associated hepatic residue levels. It is well known that the effects of warfarin, a pharmaceutical and first-generation anticoagulant rodenticide (FGAR), on fish are comparable to mammals (e. g., Fernández et al., 2014; Granadeiro et al., 2019; Jung and Kawatsu, 1995). Reported LC_{50} (lethal concentration for 50 % of the test organisms) values for rainbow trout (*Oncorhynchus mykiss*) exposed to ARs for 96 h via the water phase range from 40 μg/L for brodifacoum to 65,000 μg/L for warfarin (eCA, 2016a, 2016b; Regnery et al., 2019a). Furthermore, Wu et al. (2023) reported sublethal effects of 90 h exposure to brodifacoum in zebrafish (*Danio rerio*) at concentrations ≥ 200 μg/L and a significant effect on survival at a concentration of 800 μg/L. Considering the low water solubility of SGARs, it seems more likely that environmental exposure of fish to SGARs occurs via dietary uptake rather than aqueous exposure (Regnery et al., 2019a). It has been assumed that carcasses of poisoned animals or terrestrial invertebrates feeding on rodenticide bait can transfer ARs to aquatic organisms (Regnery et al., 2020, 2019a and references therein). According to biomonitoring data, feeding-type appears to be a determining factor in SGAR uptake in fish (Regnery et al., 2024, 2020). One study assessed the acute toxicity of diphacinone, chlorophacinone, and brodifacoum to multiple fish species in relation to hepatic concentrations (Riegerix et al., 2020). After difficulties with oral uptake of bait, capsulated AR solution or AR-spiked feed, fish were exposed via intraperitoneal injection (IP), followed by a 72 h observation period. Beside mortality, the Russell's viper venom time of exposed fish was evaluated and was prolonged depending on the AR dose. The most toxic AR for fish in the experiment by Riegerix et al. (2020) was brodifacoum with a median lethal dose (LD_{50}) range between 36,000–96,000 μg/kg body weight (bw) and a corresponding average liver concentration of 38,100 ng/g brodifacoum based on wet weight. In another study, the whole-body brodifacoum residues of wild fish found dead after airborne bait distribution during rat eradication measures, indicating a lethal intoxication, ranged between 58–1160 ng/g wet weight, whereas other fish caught alive days afterwards exhibited whole-body brodifacoum residues up to 315 ng/g wet weight (Pitt et al., 2015). Apart from that, to our knowledge, no experimental data exists to interpret the effects of AR residues in wild fish in the context of environmentally relevant concentrations and routes of exposure.

To evaluate the potential impact of ARs on wild fish, it is key to understand the relationship of liver concentrations of the substances as observed in biomonitoring studies and the effects on fish health associated with these hepatic residues. Linking defined oral doses of the SGAR brodifacoum with resulting hepatic brodifacoum concentrations and occurring effects in rainbow trout was the main objective of the present study. Such experimentally determined links are crucial for regulators in assessing the relevance of AR residues detected in wild fish, and in their decisions to implement risk mitigation measures to minimize AR exposure for fish. In order to account for different exposure situations, we conducted two separate experiments, covering an acute and a chronic/sub-chronic primary poisoning event. In a first experiment, trout were fed a single dose brodifacoum-spiked feed and observed for 15 d. In a second experiment, trout were exposed every 7 or 8 days to brodifacoum up to 60 d and samples were taken every 15th d. Given that vitamin K acts as an antidote to AR intoxication, its oversupplementation in commercial feed can limit the comparability to wild organisms (Rattner and Harvey, 2021). Hence, we used customized menadione-free fish feed in our study that contained naturally sufficient vitamin K_1 and K_2 to enable a physiological blood coagulation. Commonly applied tests to investigate the clotting ability of blood, such as prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT; Bates and Weitz, 2005; Tavares-Dias and Oliveira, 2009), were applied to fish and endpoints related to anemia were assessed.

2. Material and methods

2.1. Test organism

Rainbow trout (*O. mykiss*) were cultivated at the Bavarian Environment Agency under disease-controlled conditions. In the 15 d-experiment, ca. 1-year old trout (198 ± 9 g, 26 ± 1 cm; Table A.1) were used, in the 60 d-experiment ca. 1.5-years old trout (326 ± 19 g, 30 ± 1 cm; Table A.1). A few weeks prior to the start of experiments, blood samples were collected from the vena cava caudalis under anesthesia (100 mg/L tricaine methanesulfonate for 5 min; Tricaine Pharmaq®, Pharmaq, Bergen, Norway). For sex determination, heparinized blood was centrifuged (1320 rcf, 10 min, 4 °C; plasma stored at −80 °C) and vitellogenin (Vtg) was analyzed in the plasma using a rainbow trout Vtg ELISA Kit (Prod. No. V01004402, Biosense Laboratories AS, Bergen, Norway; dilution 1:20). Additionally, a passive integrated transponder (PIT; AL-VET ID Minitransponder, Dechra, Germany) was injected into the muscle to mark individuals. The experiments were approved by the regional government of upper Bavaria (authorization number ROB-55.2–2532.Vet_02–20–192, 10. March 2021) and performed according to German legislation.

2.2. Test substance and preparation of spiked feed

Preceding chemical analyses revealed high levels of artificial vitamin K_3 (menadione) in commercially available fish feed. To avoid oversupply of fish with vitamin K during experiments, extruded feed without artificially added menadione was custom-made for this study by Simplyfish AS (Stavanger, Norway). The custom-made feed (3 mm) was designed to meet the nutritional requirements of growing rainbow trout by use of high-quality raw materials and contained naturally 3.15 μg/100 g vitamin K_1 and 3 μg/100 g vitamin K_2 (MK-4 and MK-7; Tables A.2 and A.3). Fish were fed the menadione-free feed a minimum of 60 d prior to the experiments. The hepatic vitamin K_1 level in the liver decreased from 130 ng/g in trout fed with commercial fish feed to 59 ng/g in trout fed menadione-free feed. Pretests indicated no adverse effects of the menadione-free feed on blood coagulation times.

To prepare the spiked feed, frozen (–80 °C) menadione-free feed was milled (Retsch ZM 200 with 500 μm sieve, Haan, Germany) and 40 g (15 d-experiment) or 300 g (60 d-experiment) feed were mixed with 10 % gluten as binding agent. Subsequently, the test substance was added. Homogenized, larval, red chironomid midges were filtered and the liquid thoroughly blended with the dough to increase attractiveness of the feed. The dough was coated with peanut oil during pelletization (in-house production). Afterwards, feed pellets (6 mm) were dried for 24 h at 37 °C. Control-feed without addition of test substance was prepared in the same way using separate equipment. In addition to strict cleaning

protocols, mostly disposable products were used to prevent contamination/carry-over of test substance during feed preparation.

Analytical grade brodifacoum (CAS: 56073–10–0; purity: 98.9 %) was purchased from LGC Standards GmbH (Wesel, Germany). For the 15 d-experiment, a stock solution (6.0928 mg brodifacoum dissolved in 100 mL acetone (purity ≥ 99.8 %; VWR Chemicals, Darmstadt, Germany)) was used to prepare feed concentrations up to 5.714 μg/g feed, whereas solid brodifacoum was added directly to the dough, when preparing higher concentrations. For the 60 d-experiment, a stock solution of 22.7 mg brodifacoum dissolved in 100 mL acetone was used. Chemical analysis ensured that acetone was completely evaporated from self-prepared feed pellets before they were fed to trout (Text A.1). Moreover, nominal brodifacoum concentrations in spiked and control-feed were verified by analytical measurements (Text A.2). As the deviation of nominal and measured brodifacoum concentration was less than 20 % in all of the prepared feed concentrations (Table A.4), nominal doses were used to report results in accordance with OECD recommendations (OECD, 2019a).

2.3. Experimental setup and sampling

Experiments were performed in a flow-through system supplied with a blend of reverse-osmosis permeate and aerated spring water (conductivity: 200 μs/cm, hardness: 6.3 °dH). The light/dark cycle was 12/12 h with 30 min twilight in the mornings and evenings. Tanks were regularly cleaned by removing feces and remaining feed to maintain water quality. Trout were acclimatized to water conditions for 48 h before they were randomly distributed to tanks, in which they were accustomed for at least 9 d. The sex ratio was comparable in all tanks and treatment groups. Tanks were randomly assigned to treatment groups in both experiments. One day before each experiment started, fish were anesthetized (anesthesia protocol provided in Text A.3), weighed, and measured, allowing for accurate calculation of brodifacoum doses to be administered (calculated according to Equation A.1). During experiments, the time until administered spiked-feed pellets were consumed by fish was measured for each tank. In addition, each regular feed uptake was assessed semi-quantitatively in 4 levels ascending from score 1–4 'no', 'severely reduced', 'reduced', 'good' for each fish (15 d-experiment) or as mean value for all fish per tank (60 d-experiment). Additionally, in the 60 d-experiment anorectic fish were separately documented. In accordance with OECD Guideline 203 (OECD, 2019b), the occurrence of clinical symptoms such as abnormal ventilation, abnormal swimming behavior (e. g., apathy), loss of equilibrium, hemorrhages, and other visible abnormalities was scored three times a day in fish from the 15 d-experiment and at least once a day in fish from the 60 d-experiment using a 3-level scoring system, ascending from score 0 'no symptom' to score 3 'high-grade'. At termination of exposure, sampling involved the collection of blood from the caudal vein (vena cava caudalis) of anesthetized fish (for details refer to Text A.3). After complete withdrawal of blood, death was ensured by cutting the spine. The body condition (external and internal) of each fish was evaluated and tissue samples were taken. Collected blood samples were immediately processed. Serum, liver, and muscle (homogenized filet) tissue samples for chemical analysis of brodifacoum residues were stored at −80 °C until processing.

Trout deceased during experiments were removed from tanks and sampled if conditions allowed. Moribund fish (i.e., loss of ability to swim coordinately, loss of equilibrium, or high-grade apathy) that met previously defined termination criteria were euthanized to reduce the period of suffering, and sampled accordingly. Dead fish and euthanized moribund fish were summarized in the parameter mortality (Table A.5).

2.3.1. 15 d-experiment (single dose administered)

Trout were housed individually in 80 tanks. Each tank (27 L water volume) was equipped with an in- and outflow (flow rate: 20 L/h), and aeration. Physico-chemical water parameters at the inflow (temperature: 11.5 ± 0.1 °C, pH: 7.3 ± 0.0, conductivity: 197.7 ± 1.3 μS/cm) were monitored continuously (IQ Sensor Net: System 2020 3 G; Xylem, Weilheim, Germany). Oxygen (O_2: average concentration: 8.6 ± 0.4 mg/L), nitrite (NO_2^-: < 49 μg/L), and ammonia (NH_3: max. 1.0 μg/L, mean 0.3 ± 0.2 μg/L; calculated based on measured total ammonium concentration according to Hobiger 1996) levels were checked once a week in each tank. Each of the eight treatment groups (referred to as groups 1_{15}–8_{15} in the following) comprised of ten individually housed trout. Fish were administered a single dose of brodifacoum-spiked feed (groups 1_{15}–8_{15}: 0; 0.64; 3.2; 16; 80; 400; 2000; 10,000 μg/kg bw) at day 0 of the 15 d-experiment. The dose was mixed in the feed equivalent to 1.4 % bw and administered in two portions. To exclude leaching of test substance from spiked feed, pellets were fed one by one. Starting day 1, control-feed (1 % of bw, divided in two portions) was fed daily over the runtime of the experiment. At sampling on day 15, coagulation times, hematocrit, and albumin level, as well as brodifacoum residues in serum and liver were assessed in every fish, and exemplarily in 2 muscle tissue samples per treatment group.

2.3.2. 60 d-experiment (multiple doses administered)

Trout were kept in groups ($n = 20 + 2$ additional fish per tank) in 16 aerated tanks filled with 600 L water (flow rate: 100 L/h). Water quality parameters were continuously monitored in the inflow (temperature: 11.7 ± 0.4 °C, pH: 7.4 ± 0.0, conductivity: 198.0 ± 1.2 μS/cm) and outflow (oxygen saturation: 92 ± 3 %, min. 80 %). Ammonia and nitrite levels were determined twice a week (calculated NH_3: max. 3.2 μg/L, mean 1.5 ± 0.6 μg/L; NO_2^-: < 49 μg/L). One control group and seven treatment groups were assessed in duplicate. Starting at day 0, trout were administered multiple doses of brodifacoum-spiked feed (1 % of bw, divided in two portions) every 7 or 8 days, i.e., fish were administered a total of 8 brodifacoum doses over 60 d (Table 1). In-between administered brodifacoum doses, fish were fed daily with menadione-free, unprocessed feed (1 % of bw, divided in two portions). Self-prepared control-feed was solely fed the day before administration of brodifacoum doses to prevent feed refusal by fish (i.e., due to its change in pellet size and taste). Every 15 d, all fish were measured and weighed under anesthesia and five randomly selected trout per tank were removed for sampling as detailed in Text A.3. Based on the weight measurements, the daily feed quantity was recalculated prior to the next administration of brodifacoum-spiked feed to consider growth of trout (Equation A.1).

2.4. Blood and tissue analyses

2.4.1. Blood coagulation times

Citrated blood (S-Monovette® Citrat 9NC 0.106 mol/L 3.2 %, 1.4 mL, Sarstedt, Nümbrecht, Germany) was centrifuged (1160 rcf, 10 min, 4 °C) and the plasma stored on ice prior to analyses. PT, aPTT and TT were measured with a BFT II Analyzer (Siemens Healthcare, Erlangen, Germany). For PT and aPTT, manufacturers instructions had to be modified as follows to analyze fish plasma. 360 μL Thromborel®S was mixed with 90 μL 0.05 mol/L $CaCl_2$ directly before the PT assay. 50 μL plasma were incubated for 60 s (37 °C). Following, 200 μL of the $CaCl_2$-Thromborel®S mixture was added and the time it took the sample to clot was measured. To analyze aPTT, 50 μL plasma were mixed with 50 μL pathromtin and incubated for 120 s (37 °C). 100 μL 0.05 mol/L $CaCl_2$ was added and aPTT determined. TT was assessed according to manufacturers instructions. All reagents were purchased from Siemens Healthcare. For each assay, samples were analyzed in duplicate and the measurement interrupted at 1200 s. Only values classified as valid were used for subsequent data analysis (maximal relative deviation of the individual values PT: 11.5 %, aPTT: 10.5 %, TT: 10 %; RiliBÄK, 2022).

2.4.2. Hematocrit and albumin level

Two hematocrit sodium heparinized capillaries (Hirschmann

Table 1
Cumulative nominal brodifacoum doses in μg/kg body weight of the treatment groups and investigated endpoints at the four samplings during the 60 d-experiment. NA = not applicable (no remaining fish in the treatment group).

	Period 1 (day 0 – 15)		Sampling 1	Period 2 (day 16 – 30)		Sampling 2	Period 3 (day 31 – 45)		Sampling 3	Period 4 (day 46 – 60)		Sampling 4
	1. dose day 0	2. dose day 8	Day 15	3. dose day 16	4. dose day 23	Day 30	5. dose day 31	6. dose day 38	Day 45	7. dose day 46	8. dose day 53	Day 60
Group 1_{60}	0.00	0.00	Coagulation times[a], hematocrit, chemical analysis	0.00	0.00	Coagulation times, hematocrit, albumin level, chemical analysis	0.00	0.00	Coagulation times[b], hematocrit, chemical analysis	0.00	0.00	Coagulation times, hematocrit, albumin level, chemical analysis
Group 2_{60}	0.78	1.56		2.34	3.13		3.91	4.69		5.47	6.25	
Group 3_{60}	1.56	3.13		4.69	6.25		7.81	9.38		10.94	12.50	
Group 4_{60}	3.13	6.25		9.38	12.50		15.63	18.75		21.88	25.00	
Group 5_{60}	6.25	12.50		18.75	25.00		31.25	37.50		43.75	50.00	
Group 6_{60}	12.50	25.00		37.50	50.00		62.50	75.00		87.50	100.00	
Group 7_{60}	25.00	50.00		75.00	100.00		125.00	150.00		NA	NA	
Group 8_{60}	50.00	100.00		150.00	200.00		NA	NA		NA	NA	

[a] Only analyzed in 1 replica (5 fish) per treatment group.
[b] Only analyzed in 1 replica (5 fish) per treatment group except group 1_{60} and group 6_{60} (all fish).

Laborgeräte, Eberstadt, Germany) per sample were filled with lithium-heparin (lithium heparin LH, 1.3 mL, Sarstedt, Nümbrecht, Germany) whole blood, one end sealed, and centrifuged (11,696 rcf, 10 min, room temperature). The hematocrit values were derived with the Hawksley microhematocrit reader (Hawksley & Sons, Lancing, UK). To analyze the albumin level, heparinized blood was centrifuged (1320 rcf, 10 min, 4 °C). Albumin level was determined in the plasma with a fully automatic clinical chemical analysis system (diagnosis II, element RC3X, scil animal care company, Viernheim, Germany).

2.4.3. Residues of brodifacoum

Chemical analysis of brodifacoum in liver and muscle tissue samples was done by liquid chromatography-tandem mass spectrometry (4500 QTrap, Sciex, Darmstadt, Germany) after ultra-sound assisted solvent extraction and dispersive solid phase extraction clean-up as detailed in Regnery et al. (2019b). The clean-up and enrichment steps were omitted during extraction of tissue samples from treatment groups that had received high brodifacoum doses (i.e., groups 5_{15}–8_{15} in the 15 d-experiment, groups with a cumulative dose ≥ 6.25 μg/kg bw in the 60 d-experiment). Serum samples were extracted following the method by Dong et al. (2015). In all samples, brodifacoum was quantified using an isotope-labeled internal standard (brodifacoum-d4, TRC, North York, Ontario, Canada). Extracts of samples with residual brodifacoum concentrations that exceeded the linear 9-point calibration standard range of 0.05–40 ng/mL (correlation coefficient $r > 0.99$) were appropriately diluted prior analysis. Previously established and validated method quantification limits of brodifacoum were 0.9 ng/g in liver tissue, 0.3 ng/g in muscle tissue, and 0.8 ng/mL in serum (Dong et al., 2015; Regnery et al., 2019b). All reported brodifacoum concentrations in liver and muscle tissue are based on wet weight.

2.5. Credibility of data and statistic

The reporting criteria for ecotoxicity studies (CRED) proposed by Moermond et al. (2016) are provided in the Appendix A, supplementary file 2. Six fish were excluded due to causes unrelated with the experiment itself (Table A.6). Statistical analyses were performed with R (version 4.3.1, R Core Team, 2023). The α-level was set to 0.05. If necessary, data were transformed to gain normal distribution and homogeneity of variances. If possible, (mixed) linear models (package "lme4"; Bates et al., 2015) were performed including "tank" as a random factor in the 60 d-experiment. Treatment groups with ≤ 3 fish were excluded from statistical analyses. Furthermore, no statistical analysis of brodifacoum residues in serum was performed for a total dose of 25 μg/kg bw brodifacoum, because group 4_{60} comprised values below the quantification limit. Sex was included as a fixed factor in the analyses of the coagulation times in the 15 d-experiment. For pairwise comparisons with the control group (all analyses except comparisons of the same total dose of different groups at different samplings), the Dunnett`s test was used. Treatment groups with the same cumulative dose at different samplings were compared with Tukey contrasts. If the data distribution did not allow to perform a mixed linear model, a Kruskal-Wallis rank sum test followed by a Wilcoxon rank sum exact test, adjusted with Benjamini-Hochberg for pairwise comparisons, was used. However, it was not possible to consider the pseudo-replication of the 60 d-experiment in the non-parametric test. The coagulation data are censored at 1200 s. In the 15 d-experiment, an analysis of the coagulated versus not coagulated PT was performed with Firth regression (package "logistf"; Heinze et al., 2023) with a Benjamini-Hochberg post-hoc correction. Subsequently, the coagulated data were analyzed with a linear model. The endpoint mortality was not analyzed with a statistical model because of the differing total brodifacoum doses. For an overview, hepatic residue concentrations of both experiments were plotted against the normalized cumulative dose. A polynomial regression was performed as a simple model to compare exposure and hepatic residue concentrations between the two experiments. No data were extrapolated based on the simplified model. Additional information regarding statistical analyses is provided in Tables A.7 and A.8.

3. Results

3.1. Uptake of brodifacoum-spiked feed

All trout consumed their administered complete dose of brodifacoum-spiked feed. No significant differences in the measured feeding duration of the complete daily doses of brodifacoum-spiked feed occurred in both experiments (15 d-experiment: $F = 1.655$, $d.f. = 7|72$, $p = 0.133$; 60 d-experiment: $F = 0.934$, $d.f. = 7|107$, $p = 0.484$). Feeding of one pellet in the 15 d-experiment and one portion of brodifacoum-spiked feed in the 60 d-experiment took on average 2.5 s and 102 s,

respectively. As the time until almost complete uptake of brodifacoum-spiked feed in group 7_{60} amounted to several hours on day 38 (6th feeding) these data were excluded from statistical analysis.

3.2. Observed effects during the experiments and mortality

In the first 15 d of both experiments, none of the fish died and no clinical symptoms were evident with exception of a severe hemorrhage from the gills of one trout (group 7_{15}, 2000 µg/kg bw) after 14 d. The hemorrhage caused no dyspnea. Furthermore, no mortality occurred in group 1_{60} to group 6_{60} during the entire exposure period. However, in group 8_{60} the first fish died after 17 d and in group 7_{60} after 28 d at a cumulative dose of 150 µg/kg bw and 100 µg/kg bw, respectively. 5 surviving fish of group 8_{60} were sampled after 30 d and 2 surviving fish of group 7_{60} after 45 d. Mortality of group 7_{60} and group 8_{60} is summarized in Table 2.

Most deceasing fish showed increasing signs of discoloration, apathy, anorexia, and tumbling movement, often lasting over several days. Apathy was solely observed in group 7_{60} (days 32–45, up to 100 % with score 3) and group 8_{60} (days 18–30, up to 100 % with score 2 and 50 % with score 3). Likewise, reduced feed intake occurred in group 7_{60} (onset on day 32, mean score 3.2 ± 0.52, period 3, day 32–45) and group 8_{60} (onset on day 24, mean score 3.0 ± 1.12, period 2, day 17–30) with up to 100 % of anorectic fish completely refusing feed uptake (all other groups$_{15/60}$ with mean score 4.0 ± 0.0–0.2). Anorexia was solely in group 8_{60} reflected by a significantly reduced weight gain of 42 ± 21 g between 15 d and 30 d of the 60 d-experiment compared to a mean increase of 75 ± 12 g in the control group ($F = 3.677$, $d.f. = 7|7.8386$, Dunnett: $p < 0.001$; Tables A.9 and A.10). Additionally, exophthalmos or hemorrhages were observed in some fish. However, other fish showed no clinical symptoms and died within a few hours. Moribund fish had pale gills, lost control of equilibrium, and were not able to swim coordinately.

3.3. Blood coagulation times

In the 15 d-experiment, the PT of fish exposed to 80 µg/kg bw brodifacoum (group 5_{15}) was significantly prolonged and more than twice as long as in the control group (Fig. 1 and Table A.11; $F = 5.645$, $d.f. = 4|44$, $p < 0.001$). Higher doses of brodifacoum (groups 6_{15}–8_{15}) in the 15 d-experiment led to a mean PT > 1000 s. Likewise, doses of ≥ 400 µg/kg bw brodifacoum significantly prolonged aPTT by 41–54 % (Table A.11; $F = 16.034$, $d.f. = 7|71$, $p < 0.001$). In contrast, TT was not affected by exposure to brodifacoum (Table A.11; $F = 0.985$, $d.f. = 7|71$, $p = 0.449$). Sex had no influence on blood clotting of fish with a measurable coagulation time (PT: $F = 0.004$, $p = 0.948$; aPTT: $F = 0.622$, $p = 0.433$; TT: $F = 0.497$, $p = 0.483$). However, slightly more female fish had a PT > 1200 s (Fig. A.1; $X^2 = 54.012$, $p = 0.047$).

Exact values for PT, aPTT, and TT of the 60 d-experiment are provided in Table A.12. After 15 d of the 60 d-experiment, 80 % of the analyzed fish in group 8_{60} (100 µg/kg bw) had a PT > 1000 s (Fig. 2.1). The corresponding values for aPTT and TT were not affected. In the second sampling (Fig. 2.2) , no PT was measurable in all surviving fish of group 8_{60} (200 µg/kg bw) and 9 of 10 fish of group 7_{60} (100 µg/kg bw).

Table 2
Cumulative mortality [%] of group 7_{60} and group 8_{60}during the 60 d-experiment. NA = not applicable (no fish remained in the treatment group).

	Mortality 16–22 d (dose)	Mortality 23–30 d (dose)	Mortality 31–37 d (dose)	Mortality 38–45 d (dose)
Group 7_{60}	0 % (75 µg/kg bw)	13 % (100 µg/kg bw)	77 % (125 µg/kg bw)	91 % (150 µg/kg bw)
Group 8_{60}	21 % (150 µg/kg bw)	85 % (200 µg/kg bw)	NA	NA

Furthermore, the mean aPTT was significantly increased by 36 % in group 7_{60} and considerably prolonged or not measurable in group 8_{60} ($X^2 = 35.064$, $d.f. = 7$, $p < 0.001$). On the contrary, TT values indicated that fibrin was formed significantly faster in group 8_{60} compared to controls ($F = 3.431$, $d.f. = 7|8.4482$, $p = 0.049$). After 45 d (Fig 2.3), the PT was not measurable in both surviving fish of group 7_{60} (150 µg/kg bw), and their aPTT was increased by 72 %. While one fish of group 6_{60} (75 µg/kg bw) had a PT above 1200 s, PT was significantly prolonged by on average 40 % in all other fish of group 6_{60} ($F = 10.173$, $d.f. = 1|17$, $p = 0.005$). At the end of the experiment (Fig. 2.4), out of 14 fish of group 6_{60} (100 µg/kg bw) the PT of 4 was not measurable, the PT of 3 trout was more or less prolonged, while the PT of 7 fish was within the range of the control group ($F = 6.484$, $d.f. = 5|5.7436$, $p = 0.023$). The aPTT of group 6_{60} was significantly prolonged by 20 %, whereas no difference occurred in the TT of all treatment groups (aPTT: $X^2 = 14.608$, $d.f. = 5$, $p = 0.012$).

3.4. Hematocrit and albumin level

In the 15 d-experiment, the mean hematocrit value of all fish was 35 ± 4 %. Except for the fish with gill bleeding (group 7_{15}, hematocrit of 12 %), both the hematocrit and albumin level (total average 2.7 ± 0.2 g/dL) were not affected by the single dose brodifacoum after 15 d (Table A.13). However, at the second sampling of the 60 d-experiment, hematocrit values had decreased slightly to 33 ± 12 % in group 7_{60} and significantly to only 4 ± 4 % in group 8_{60} (Table A.14; $F = 13.655$, $d.f. = 7|8.318$, $p = 0.001$) compared to the control group (hematocrit of 41 ± 4 %). Furthermore, the albumin level of group 8_{60} was 1.4 ± 0.4 g/dL and, thereby, significantly lower than the control group (2.7 ± 0.1 g/dL) after 30 d of exposure to brodifacoum (Table A.14; $F = 10.251$, $d.f. = 7/67.493$, $p < 0.001$). In moribund fish the average hematocrit was 2.7 ± 1.0 % (max. 4.5 %), and the mean albumin level was 1.0 ± 0.3 g/dL. In groups 2_{60}–6_{60}, neither hematocrit nor albumin levels were affected by exposure to brodifacoum during the entire experiment.

3.5. Chemical analyses

No residues of brodifacoum were detected above the respective quantification limits in serum, liver, and muscle tissue samples of fish from control groups in either of the two experiments (Table 3). In all other treatment groups of both experiments, brodifacoum was detected in every analyzed liver tissue sample. Hepatic concentrations followed a second-order polynomial regression against the normalized cumulative dose ($\log_{10}(x + 1)$ with a coefficient of determination of $R^2 = 0.8$ (Fig. A.2)). In livers of euthanized and deceased fish, brodifacoum concentrations ranged between 71 and 826 ng/g (wet weight) with an average concentration of 370 ± 178 ng/g. Exposure of fish of different treatment groups to the same cumulative dose in the 60 d-experiment led to on average similar mean hepatic brodifacoum concentrations (Fig. 3 and Table A.15; 6.25 µg/kg bw: $F = 0.274$, $d.f. = 2|30$, $p = 0.762$; 12.5 µg/kg bw: $F = 0.2406$, $d.f. = 2|2.617$, $p = 0.802$; 25 µg/kg bw: $F = 6.233$, $d.f. = 2|2.528$, $p = 0.105$). However, higher cumulative brodifacoum doses resulted in more variable residues in the liver with significant differences at a cumulative dose of 100 µg/kg bw brodifacoum (50 µg/kg bw: $F = 8.200$, $d.f. = 2|2.857$, $p = 0.066$; 100 µg/kg bw: $X^2 = 8.300$, $d.f. = 2$, $p = 0.016$). In groups 1_{15}–4_{15} and groups 1_{60}–4_{60} no brodifacoum was detected in the serum of trout (Table 3). In the 60 d-experiment, the highest observed brodifacoum concentration in blood was 32 ng/mL, whereas up to 1860 ng/mL brodifacoum were detected in trout of group 8_{15} in the 15 d-experiment. In the 60 d-experiment, the brodifacoum concentration in serum of fish from different treatment groups exposed to the same cumulative dose decreased with increasing number of lower single doses (Fig. 4 and Table A.15; 50 µg/kg bw: $F = 3.886$, $d.f. = 2|30$, $p = 0.032$; 100 µg/kg bw: $X^2 = 20.67$, $d.f. = 2$, $p < 0.001$). In both experiments, low residues of brodifacoum were found in exemplarily analyzed muscle tissue at a cumulative dose of

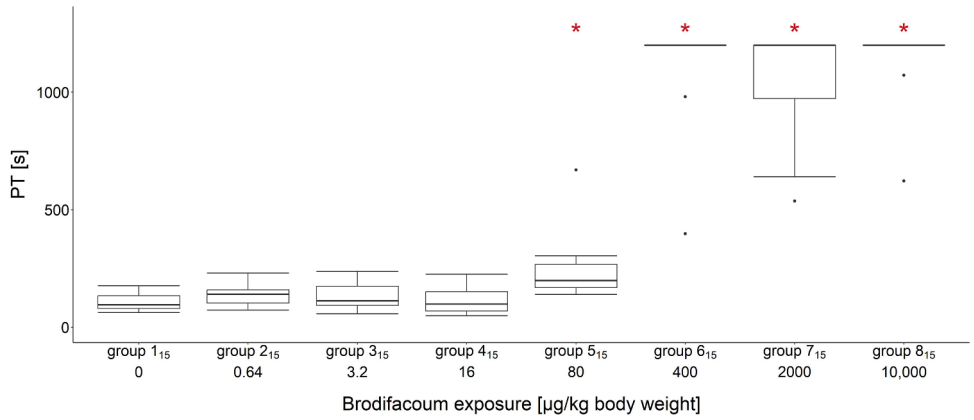

Fig. 1. Prothrombin time (PT) in 15 d-experiment (all groups $n = 10$). The boxplots depict the 25th and 75th percentile, the median, as well as the minimum and maximum values (whiskers). Outliers are displayed as dots. Asterisks highlight significant differences compared to the control group ($p < 0.05$, exact values see Table A.11).

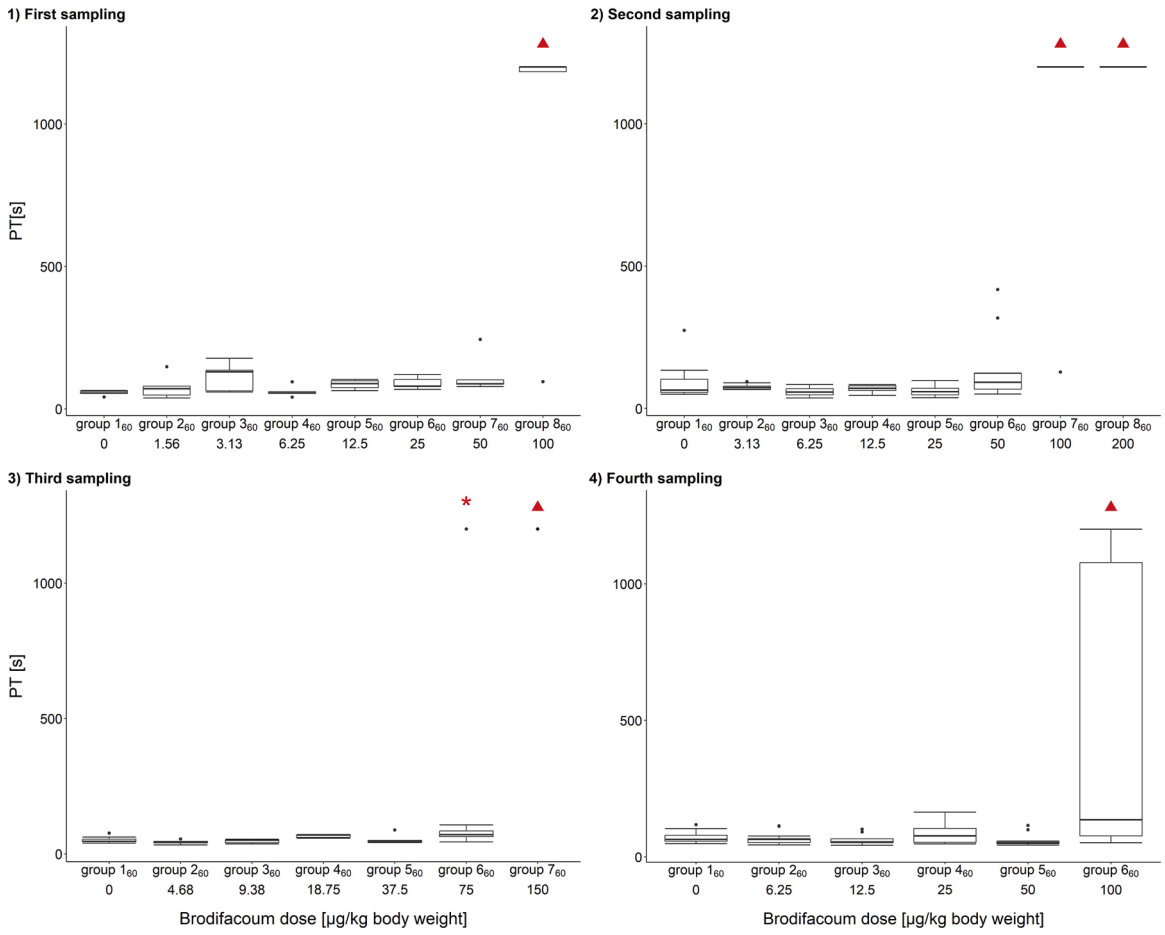

Fig. 2. Prothrombin time (PT) at each of the four samplings in the 60 d-experiment. 1): First sampling (15 d; all groups $n = 5$). 2): Second sampling (30 d; group 1_{60}–7_{60} $n = 10$, group 8_{60} $n = 5$). 3): Third sampling (45 d; group 1_{60} and group 6_{60} $n = 10$, group 2_{60}–5_{60} n = 5, group 7_{60} $n = 2$). 4): Fourth sampling (60 d; group 1_{60}–3_{60} and group 5_{60} $n = 13$, group 4_{60} and group 6_{60} $n = 14$). The boxplots depict the 25th and 75th percentile, the median, as well as the minimum and maximum values (whiskers). Outliers are displayed as dots. An asterisk highlights significant differences compared to the control group ($p < 0.05$; exact values see Table A.12). Triangles highlight mean PT of more than fivefold increase compared to the control group.

$\geq$ 3.13 µg/kg bw (Table 3). The highest observed concentration in a euthanized trout (150 µg/kg bw brodifacoum) was 8.5 ng/g muscle.

4. Discussion

Conducting aquatic toxicity experiments with SGARs is demanding because of their specific mode of action and challenging substance properties. The strong adsorption and low water solubility of brodifacoum suggest oral uptake as the primary route of wild fish exposure. Successful oral administration of the test substance is therefore a prerequisite to testing effects of brodifacoum under environmentally relevant conditions. In our study, rainbow trout consumed spiked pellets

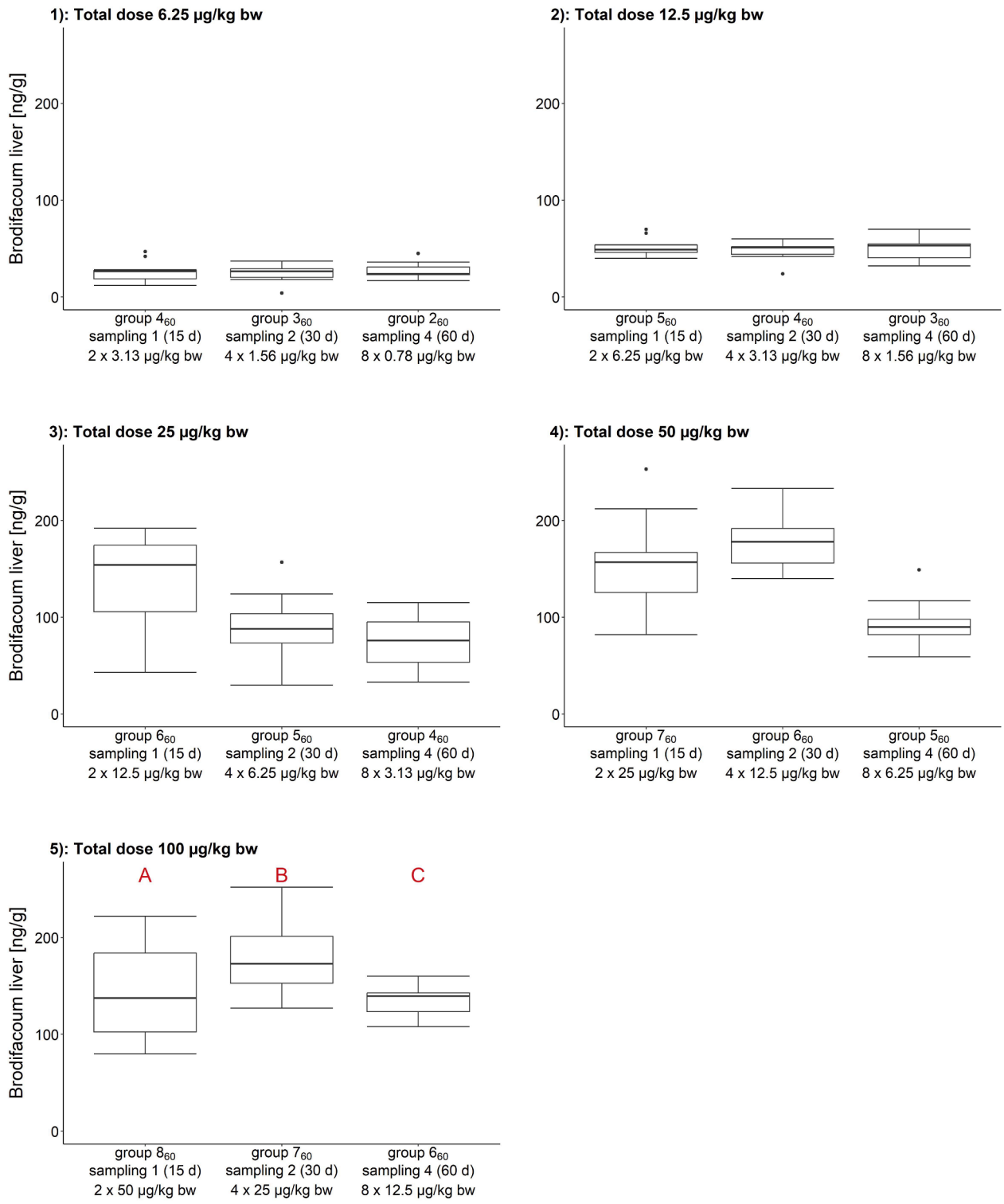

Fig. 3. Comparison of residues in the liver of different treatment groups with the same cumulative brodifacoum dose at different samplings (sampling 1: 2 doses of brodifacoum, sampling 2: 4 doses of brodifacoum, sampling 4: 8 doses of brodifacoum). The boxplots depict the 25th and 75th percentile, the median, as well as the minimum and maximum values (whiskers). Outliers are displayed as dots. 1): total dose of 6.25 µg/kg bw brodifacoum. 2): total dose of 12.5 µg/kg bw brodifacoum. 3): total dose of 25 µg/kg bw brodifacoum. 4): total dose of 50 µg/kg bw brodifacoum. 5): total dose of 100 µg/kg bw brodifacoum. Different capital letters depict significant differences between the groups. Further statistical information is provided in Tables 3 and A.8.

within seconds and no effect on palatability was observed. The delay of 14–17 d until onset of first overt symptoms of a coagulation disorder in trout (cold water fish) was noticeably longer compared to usually 2–10 d previously reported in terrestrial species (Rached et al., 2020; Rattner et al., 2014b; Rattner and Mastrota, 2018). This clearly emphasizes the importance of the testing duration (length of observation period) when exposing aquatic organisms to slow acting SGARs. It also highlights the benefits of preliminary testing prior to experiments to optimize the study design for such challenging substances, preventing potential mis-interpretations in terms of sensitivity (e.g., due to overdosing) and thus

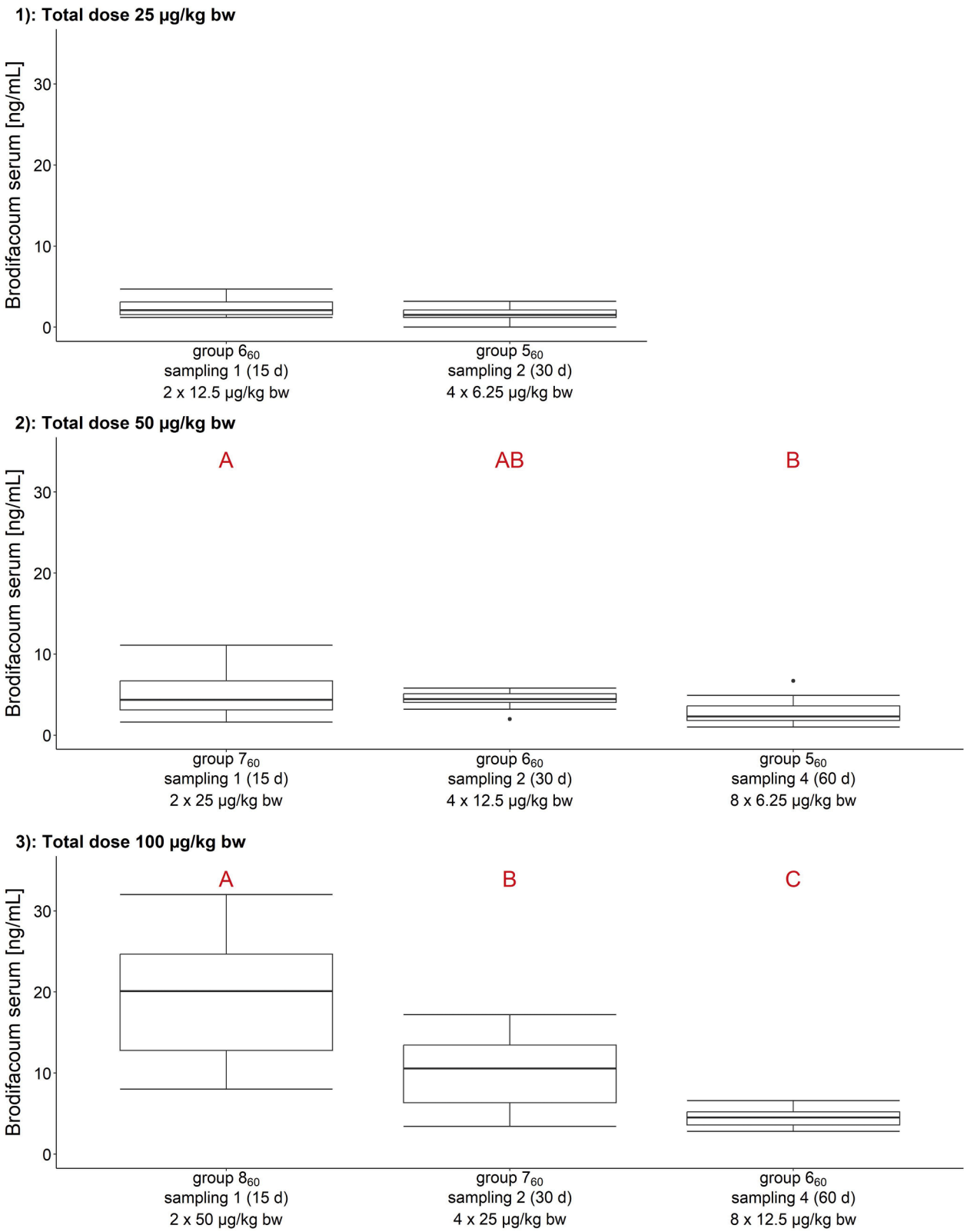

Fig. 4. Comparison of residues in the blood serum of different treatment groups with the same cumulative brodifacoum dose at different samplings (sampling 1: two doses of brodifacoum, sampling 2: four doses of brodifacoum, sampling 4: eight doses of brodifacoum). The boxplots depict the 25th and 75th percentile, the median, as well as the minimum and maximum values (whiskers). Outliers are displayed as dots. 1): total dose of 25 µg/kg bw brodifacoum (sampling 4 of group 4_{60} is not depicted as all values were < LOQ). 2): total dose of 50 µg/kg bw brodifacoum. 3): total dose of 100 µg/kg bw brodifacoum. Different capital letters depict significant differences between the groups. Further statistical information is provided in Tables 3 and A.8.

Table 3
Overview of measured brodifacoum residues in liver and serum (mean ± standard deviation, individual values or if some values < LOQ (limit of quantification) range of values) and muscle (individual values) of fish; LOQ liver = 0.9 ng/g; LOQ serum = 0.8 ng/mL; LOQ muscle = 0.3 ng/g; NA = not applicable (no remaining fish in the treatment group); Cum. brod. dose: Cumulative brodifacoum dose.

	Sampling	Cum. brod. dose [µg/kg bw]	*n* liver | serum | muscle	Brodifacoum concentration		
				Liver [ng/g]	Serum [ng/mL]	Muscle [ng/g]
Group 1_{15}	15 d	0	10 | 10 | 2	< LOQ	< LOQ	< LOQ; < LOQ
Group 2_{15}	15 d	0.64	10 | 10 | 2	6.2 ± 1.1	< LOQ	< LOQ; < LOQ
Group 3_{15}	15 d	3.2	10 | 10 | 2	16.4 ± 4.4	< LOQ	< LOQ; < LOQ
Group 4_{15}	15 d	16	10 | 10 | 2	130.8 ± 32.7	< LOQ	< LOQ; 1.1
Group 5_{15}	15 d	80	10 | 10 | 2	279.6 ± 103.2	4.8 ± 2.1	2.1; 2.1
Group 6_{15}	15 d	400	10 | 10 | 2	359.3 ± 56.5	68.4 ± 20.1	3.8; 4.4
Group 7_{15}	15 d	2000	10 | 10 | 2	535.5 ± 235.0	334.6 ± 102.1	16; 17
Group 8_{15}	15 d	10,000	10 | 10 | 2	866.0 ± 377.2	1145.3 ± 404.0	29; 46
Group 1_{60}	15 d	0	11 | 11 | 2	< LOQ	< LOQ	< LOQ; < LOQ
	30 d	0	10 | 10 | 2	< LOQ	< LOQ	< LOQ; < LOQ
	45 d	0	10 | 10 | 2	< LOQ	< LOQ	< LOQ; < LOQ
	60 d	0	13 | 13 | 2	< LOQ	< LOQ	< LOQ; < LOQ
Group 2_{60}	15 d	1.56	10 | 10 | 2	10.3 ± 2.3	< LOQ	< LOQ; < LOQ
	30 d	3.13	10 | 10 | 2	18.1 ± 5.4	< LOQ	< LOQ; < LOQ
	45 d	4.68	10 | 10 | 2	17.5 ± 1.5	< LOQ	0.4; 0.6
	60 d	6.25	13 | 13 | 2	26.9 ± 8.0	< LOQ	0.5; 1.0
Group 3_{60}	15 d	3.13	10 | 10 | 2	15.7 ± 4.6	< LOQ	0.4; 0.7
	30 d	6.25	10 | 10 | 2	24.2 ± 9.1	< LOQ	0.5; 0.6
	45 d	9.38	10 | 10 | 2	41.8 ± 6.3	< LOQ	1.1; 1.8
	60 d	12.5	14 | 14 | 2	49.6 ± 11.9	< LOQ	0.9; 1.1
Group 4_{60}	15 d	6.25	10 | 10 | 2	26.6 ± 11.0	< LOQ	0.4; 0.7
	30 d	12.5	10 | 10 | 2	47.7 ± 10.1	< LOQ –1.4	0.6; 0.8
	45 d	18.75	10 | 10 | 2	58.0 ± 12.3	< LOQ	1.3; 1.5
	60 d	25	14 | 14 | 2	73.1 ± 25.5	< LOQ	1.4; 1.7
Group 5_{60}	15 d	12.5	10 | 10 | 2	51.7 ± 9.5	< LOQ –1.6	1.7; 1.9
	30 d	25	10 | 10 | 2	89.2 ± 36.0	< LOQ –3.2	0.7; 1.7
	45 d	37.5	10 | 10 | 2	97.8 ± 23.0	< LOQ –5.6	1.2; 1.3
	60 d	50	13 | 13 | 2	93.7 ± 22.7	2.8 ± 1.6	0.9; 1.3
Group 6_{60}	15 d	25	10 | 10 | 2	136.0 ± 49.0	2.5 ± 1.3	2.5; 2.7
	30 d	50	10 | 10 | 2	178.7 ± 28.9	4.3 ± 1.1	2.9; 4.4
	45 d	75	10 | 10 | 2	122.6 ± 27.1	3.1 ± 1.0	2.1; 3.0
	60 d	100	14 | 13 | 2	135.6 ± 15.3	4.5 ± 1.1	3.0; 3.4
Group 7_{60}	15 d	50	10 | 10 | 2	155.9 ± 50.1	5.1 ± 3.0	3.0; 3.8
	30 d	100	10 | 10 | 2	181.5 ± 39.2	9.9 ± 4.5	3.8; 4.2
	45 d	150	2 | 2 | 2	192.8; 401.2	5.0; 9.7	2.2; 2.8
	60 d	NA	NA	NA	NA	NA
Group 8_{60}	15 d	100	10 | 10 | 2	143.6 ± 50.6	19.4 ± 7.8	3.3; 4.0
	30 d	200	5 | 5 | 2	146.0 ± 59.6	18.1 ± 4.8	4.8; 5.0
	45 d	NA	NA	NA	NA	NA
	60 d	NA	NA	NA	NA	NA

underestimation of effects. Our results demonstrate that testing effects of SGARs on rainbow trout, and most likely also on other fish species, requires species-dependent test durations and cannot be assessed using current standard biotests. In addition, the fact that commercial fish diet is generally highly supplemented with vitamin K, most likely not reflecting the environmental vitamin K intake of fish, requires attention. So far, no test guideline accounts for the very specific mode of action of SGARs and considers vitamin K in commercial diet, which acts as an antidote against AR intoxication.

4.1. *Effects of brodifacoum exposure*

In the 60 d-experiment, doses of 100–200 µg/kg bw brodifacoum caused high mortality rates from day 17 onwards (Table 2). Although no mortality occurred in the 15 d-experiment even at much higher doses of 10,000 µg/kg bw brodifacoum, it is reasonable to conclude from the data of the 60 d-experiment that fish exposed to > 80 µg/kg bw brodifacoum would have died of coagulopathy during an extended observation period. The results of the experiments support the evidence that the mode of action of ARs in fish is comparable to that of mammals and birds (Fernández et al., 2014; Granadeiro et al., 2019; Jung and Kawatsu, 1995; Riegerix et al., 2020; Salte and Norberg, 1991). Brodifacoum-exposed fish showed dose-dependent typical symptoms of an intoxication with ARs such as prolonged clotting times, hemorrhages, discoloration, anemia, lethargy, and anorexia (Rattner et al., 2014b; Scott and Sloman, 2004; Valchev et al., 2008; Webster et al., 2015). Coagulation times were assessed with a method developed for human blood adapted for fish. In our study, the physiological magnitude and variability of PT in trout was higher than in other vertebrates (i.e., up to a twofold increase in mean PT in different control groups). Consequently, a 25 % increase of PT, as suggested in literature as an indication of AR intoxication (Rattner et al., 2014b), was not suitable in our experiments. However, the dose-dependent effects on the PT were clear and consistent in our study (Figs. 1 and 2). Clinical symptoms such as apathy, anorexia, hemorrhages and mortality caused by brodifacoum exposure occurred in test groups in which at least a fivefold increase of PT was observed compared to the corresponding control group. Therefore, significant differences of PT or a fivefold increase compared to controls, respectively, were interpreted as a pathological and thus biological relevant effect. In both experiments, 75 or 80 µg/kg bw brodifacoum caused a prolonged but still measurable PT. Higher doses of brodifacoum led in each case to a severely impaired hemostasis following a steep dose-response curve similar to those reported for other species (Rattner et al., 2014a; Rattner and Mastrota, 2018). As described for humans, the aPTT was also affected by exposure to brodifacoum, but less sensitive than the PT assay (Bates and Weitz, 2005). The unaffected TT excludes a deficiency of fibrinogen or a dysfibrinogenemia. The

physiological TT substantiates nonfunctional vitamin K dependent clotting factors as a cause of the observed coagulation disorder. The significant decrease of TT after 30 d in group 8_{60} has no pathological relevance and is likely due to accumulation of unused fibrinogen (factor I).

In both experiments, coagulopathy partially led to hemorrhages primarily in the gills and pseudobranchs. The hemorrhages caused a severe decrease of both the hematocrit value and the albumin level. In teleost fish, hematocrit values below 20 % indicate an anemia (Clauss et al., 2008). As described in literature for other organisms, anemia caused pale gills, discoloration, as well as behavioral changes in our study (Ludwig and Strasser, 2001; Rattner et al., 2014b). Erythrocytes are essential for the binding and transport of oxygen in vertebrates (Ludwig and Strasser, 2001). Interestingly, even in fish with severe anemia (hematocrit < 5 %), apathy but no dyspnea was observed. The high oxygen saturation in the flow-through system was probably sufficient for the relative low oxygen demand of these fish that were mostly inactive due to apathy. Anorexia and apathy in group 8_{60} (day 15–30) were related to a significantly reduced weight gain.

4.2. Brodifacoum residues in tissues

Our results implied that low doses of brodifacoum were bound almost completely in the liver of fish after gastrointestinal absorption into the blood stream (Table 3). Highest accumulation of SGARs in the liver compared to muscle and blood had also been shown in avian and mammalian species (Bachmann and Sullivan, 1983; Huckle et al., 1989, 1988). Liver brodifacoum concentrations in exposed trout from both experiments were dose-dependent and the non-linear concentration curve obtained from plotting measured hepatic concentration (wet weight) against the normalized cumulative dose administered fitted a second-order polynomial regression (Fig. A.2). A substantial decrease of brodifacoum liver concentration in fish over the course of the experiment was unlikely as brodifacoum showed no measurable hepatic *in vitro* clearance in rainbow trout (Regnery et al., 2022). Furthermore, in mammals, the resistance of brodifacoum to biotransformation and its persistence in liver (estimated half-life of > 300 d) is well documented, contrasting its more rapid elimination (several hours to multiple days) from blood (Horak et al., 2018 and references therein). However, ARs bind with high affinity to the blood transport protein albumin (André and Guillaume, 2004; Petitpas et al., 2001). Moreover, brodifacoum is assumed to undergo enterohepatic recirculation (Hauck, 2017; Horak et al., 2018). Elevated residual concentrations in the blood serum of trout exposed to the highest brodifacoum doses in the 15 d-experiment suggested that brodifacoum was particularly present in serum when liver tissue binding sites for 4-hydroxycoumarin anticoagulants were saturated (Mosterd and Thijssen, 1991; Thijssen and Baars, 1989). This can lead to increased hepatic concentrations as the liver is well supplied with blood, and remaining blood in the blood vessels is analyzed together with the hepatic tissue. On the contrary, reduced albumin levels due to blood loss will cause a loss of albumin-bound brodifacoum. This effect may partly explain the lower brodifacoum concentration detected in the liver of severe anemic fish of group 8_{60}, compared to group 6_{60} and 7_{60} at the second sampling. Furthermore, albumin is the most important protein to maintain the colloid oncotic pressure. An insufficient amount of albumin can lead to an excessive fluid loss from capillaries (Concannon, 1993). Given that brodifacoum concentrations were based on liver wet weight, a higher fluid content in the tissue will have an influence on calculated residue concentration, as described by Valverde et al. (2020) for bromadiolone concentrations in common kestrels (*Falco tinnunculus*).

4.3. Influence of housing conditions and multiple dosing

The study design of the two experiments differed in three main aspects: observation time (15 vs. 60 d), single vs. multiple administration of brodifacoum-spiked feed, and housing conditions (individual vs. groups). Exposure in groups compared to individual exposure had no effect on overt symptoms, mortality, and blood parameters during the first 15 d of the experiments. Therefore, a relatively uniform feed intake by all fish in a tank was assumed. Due to the chosen experimental design of the 60 d-experiment it is not possible to clearly separate the effects of exposure time and multiple feeding. Multiple feedings are known to especially influence the toxicity of FGARs in terrestrial species, whereas usually a single feed of SGARs delivers a toxic dose (Rattner and Mastrota, 2018; van den Brink et al., 2018). In the 60 d-experiment it was possible to analyze fish with the same cumulative dose of brodifacoum from treatment groups exposed to different multiple single doses at the first, second, and fourth sampling. Investigated effects of the same cumulative doses of brodifacoum were similar in the different treatment groups. Interestingly, the effect of the cumulative dose of 100 µg/kg bw brodifacoum on PT was slightly less pronounced in group 6_{60} after 60 d than in group 7_{60} and group 8_{60} after 30 d and 15 d, respectively. This might indicate some adaptive response to the chronic exposure of sublethal doses of brodifacoum. Though multiple feeding had no effect on corresponding hepatic concentration at lower total doses, we noticed that multiple feedings of high single doses of brodifacoum (i.e., 12.5, 25 and 50 µg/kg bw) led to more variable liver concentrations in fish compared to groups that received lower brodifacoum doses (Fig. 3). This increase of variation in hepatic residue concentrations at higher brodifacoum doses was also observed in the individually exposed trout in the 15 d-experiment (Fig. A.2). In good agreement with the above described pharmacokinetic of brodifacoum, hepatic concentrations were mostly driven by the total cumulative brodifacoum dose at sampling rather than a single administered dose, whereas serum concentrations in trout reflected the different administered single doses. Fish with the same cumulative dose revealed lower serum residues at sampling when total doses were administered in multiple smaller portions (Fig. 4).

4.4. Sensitivity of rainbow trout to brodifacoum

In the present study, coagulopathy was observed in rainbow trout ≥ 75 µg/kg bw and mortality in a range of 100–200 µg/kg bw brodifacoum (lowest observed adverse effect level – LOAEL). The corresponding no observed adverse effect level (NOAEL) in both experiments was ≤ 50 µg/kg bw brodifacoum. Compared to the LC_{50} of 40 µg/L provided in the European Union Competent Authority Assessment Report (eCA, 2016a), rainbow trout were less sensitive to brodifacoum when exposed via the diet. In the environment, however, a dietary exposure (e.g., via insects, poisoned carcasses, associated to particles) is more likely due to the hydrophobic properties of SGARs (Regnery et al., 2019a). Compared to the limited available data of other fish species, rainbow trout are very sensitive to brodifacoum. Riegerix et al. (2020) determined in four different fish species exposed via IP a median LD_{50} (72 h) range of 36,000 – 102,000 µg/kg bw brodifacoum. This considerable difference in sensitivity compared to our study might be due to the application method and the length of the observation period. The bioavailability and toxicity of substances can differ substantially between oral and IP application (Delistraty et al., 1998; Ning et al., 2015; Wang et al., 2015). Moreover, our results have shown that an observation period of 72 h is not sufficient to observe mortality caused by the AR-specific mode of action at concentrations of up to 10,000 µg brodifacoum/kg bw in rainbow trout. Thus, mortality observed in Riegerix et al. (2020) from 36,000 µg brodifacoum/kg bw could also be related to other factors, despite the observed coagulation disorder. In addition, the discrepancy in sensitivity could be caused by species-specific differences. Large species-specific variabilities in the susceptibility to SGAR are also known for birds (Thomas et al., 2011). Rattner and Mastrota (2018) reviewed the oral toxicity of ARs for mammals and birds. Rainbow trout are more sensitive to brodifacoum than most investigated bird species with an estimated LD_{50} of 250–11,600 µg/kg bw. In fact, in our study mortality of trout occurred at a dose comparable to that of barn owls (*Tyto alba*;

estimated lethal dose 150–182 μg/kg bw brodifacoum) – a species considered very sensitive towards SGAR intoxication – and sensitive mammals such as the Richardson's ground squirrel (*Spermophilus richardsonii;* LD_{50} estimate 130 μg/kg bw) and rabbits (*Oryctolagus cuniculus*; LD_{50} estimate 200–300 μg/kg bw) (Newton et al., 1990; Rattner and Mastrota, 2018).

4.5. Linking hepatic SGAR residues with effects in fish

The lowest mean hepatic brodifacoum concentration associated with effects was 122.6 ng/g (Table 3 and Table A.12). In livers of dead or moribund fish, residues between 71–826 ng/g brodifacoum were detected. These concentrations are within the range of previously detected environmental SGAR concentrations of almost 100 ng/g in livers of wild fish (Masuda, 2014; Regnery et al., 2024). When comparing hepatic AR residues detected in wild fish with the corresponding effects found in our study, it should be considered that the conditions during the experiments were designed to meet the requirements of rainbow trout to an optimal degree (OECD, 2019b). Furthermore, trout were prevented from other negative environmental stressors such as elevated water temperature, reduced dissolved oxygen levels, infectious diseases, predation, or contaminants. It is long recognized that captive birds may be less sensitive to toxicity of ARs than free-ranging individuals (Rattner et al., 2020). In the environment, adverse effects in fish induced by the SGAR-dependent coagulation disorder are likely to occur at even lower hepatic concentrations than reported here. The considerable time-delay of 17 days between brodifacoum exposure and onset of mortality resulted most likely in overdosing of the test fish fed every 7 or 8 days with brodifacoum-spiked feed.

5. Conclusion

To assess the environmental impact of rodenticide residues measured in wild fish, we exposed rainbow trout to brodifacoum-spiked feed. Brodifacoum caused coagulopathy, anemia, and mortality in rainbow trout at environmentally relevant hepatic concentrations. The presented data provide a solid reference for critical hepatic SGAR residues in fish and thereby help interpreting fish monitoring data in terms of potential adverse effects. The observed effects were dose-dependent and consistent with the mode of action known for other vertebrates. In comparison with other non-target organisms, rainbow trout are relatively sensitive to brodifacoum exposure. Depending on the species, the observation period is crucial for the assessment of the impact of brodifacoum on fish: after uptake of brodifacoum-spiked feed, the onset of overt symptoms took at least 14 d in rainbow trout. In both experiments, even the lowest tested brodifacoum dose led to detectable hepatic residues. Accordingly, fish exposed to SGARs are a potential source for secondary poisoning of piscivorous predators in the aquatic food chain. Considering the huge species-specific differences in susceptibility to AR intoxication and the very limited data on fish, further studies on the lethal and sublethal effects of different AR in fish are necessary. Overall, our study clearly indicates the risks associated with the use of AR for wild fish and reinforces the need to stipulate all available and appropriate risk mitigation measures to prevent emissions at their source, e.g., from urban rat baiting campaigns in sewers and near watercourses, or during rat eradication measures on islands and coast lines.

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Hermann Ferling: Writing – review & editing, Methodology, Investigation, Data curation. **Karina Annika Bucher:** Writing – review & editing, Methodology, Investigation. **Stefanie Jacob:** Writing – review & editing, Supervision, Conceptualization. **Julia Regnery:** Writing – review & editing, Validation, Methodology, Investigation, Funding acquisition, Conceptualization. **Hannah Schrader:** Writing – review & editing, Project administration, Methodology, Investigation, Conceptualization. **Julia Schwaiger:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Anton Friesen:** Writing – review & editing, Supervision, Conceptualization. **Hannah Schmieg:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.117629.

Data availability

Data will be made available on request.

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SENESTECH

NEWS RELEASES

SenesTech and Irvine Campus Housing Authority Announce Successful Field Trial of Evolve™ Rat Birth Control at UC Irvine

SURPRISE, Ariz., June 25, 2025 – SenesTech, Inc. (NASDAQ: SNES, "SenesTech" or the "Company"), a leader in fertility control for managing animal pest populations, today announce a nine-month field trial of Evolve™ Rat Birth Control at the University Hills (UHills) residential community at the University of California, Irvine. The project was conducted in partnership with the Irvine Campus Housing Authority (ICHA), the nonprofit corporation that manages University Hills, and reflects a growing commitment to ecologically responsible pest management.

SENESTECH

The initiative began in September 2024 and marked the exclusive use of Evolve Rat Birth Control across common areas of the UHills community. This deployment was part of a broader effort supported by residents interested in moving away from rodenticides in favor of integrated pest management (IPM) strategies that prioritize environmental safety and wildlife preservation. Located adjacent to a wildlife reserve, the community's shift away from rodenticides further supports the protection of local wildlife and ecosystems.

"This project highlights how community-driven action and science-based solutions can work together to support sustainability," said Joel Fruendt, President and CEO of SenesTech. "Evolve aligns with these goals by providing a non-lethal, poison-free solution to rodent control."

Evolve was deployed across 267 bait stations previously containing rodenticides and snap traps. According to field observations, rats consumed 98% of Evolve across all stations each month, indicating high acceptance and performance of the bait. As expected, consumption dropped as the rat population dropped.

The field trial also supported the efforts of the HOOT Group (Help Our Owls Thrive), a grassroots initiative formed by UHills residents to promote owl nesting and reduce rodenticide use. Together, the partners implemented a range of actions, including installation of enclosed snap traps, removal of rodent harborage areas, and installation of barn owl nesting boxes to encourage natural predation.

"The partnering with SenesTech in utilizing Evolve has supported ICHA's and the UHills community's heightened focus on IPM sensitivity to the local wildlife and raptor population," said Andrew Herndon, Vice President of Community Development at ICHA. "Evolve appears to offer a reduction of poisons in the environment while at the same time a reduction of the rodent population."

As a result of the collaboration, rodenticides have been eliminated from all 267 bait stations across common areas in UHills. SenesTech is pleased to contribute to this transition and to support communities like UHills and organizations like ICHA and HOOT that are leading the way in redefining effective, responsible rodent control.

ICHA plans to continue using Evolve in University Hills.

About SenesTech

We are committed to improving the health of the world by humanely managing animal pest populations through our expertise in fertility control. We invented ContraPest®, the only U.S. EPA-registered contraceptive for male and female rats, as well as Evolve™ Rat and Evolve™ Mouse, EPA-designated minimum risk contraceptives for rodents, reflecting our mission to provide products that are proactive, safe and sustainable. ContraPest and Evolve fit seamlessly into all integrated pest management programs, significantly improving the overall goal of effective pest management. We strive for clean cities, efficient businesses and happy households – with a product designed to be humane, effective and sustainable.

For more information visit https://senestech.com/.

Safe Harbor Statement

This press release contains "forward-looking statements" within the meaning of federal securities laws, and we intend that such forward-looking statements be subject to the safe harbor created thereby. Such forward-looking statements include, among others, our belief that the UHills project reflects a growing commitment to ecologically responsible pest management; our belief that this

project highlights how community-driven action and science-based solutions can work together to support sustainability; and our expectation for a multi-pallet restocking order for continued deployment. Forward-looking statements may describe future expectations, plans, results or strategies and are often, but not always, made through the use of words such as “believe,” “may,” “future,” “plan,” “will,” “should,” “expect,” “anticipate,” “eventually,” “project,” “estimate,” “continuing,” “intend” and similar words or phrases. You are cautioned that such statements are subject to risks, uncertainties and other factors that could cause actual results to differ materially from those reflected by such forward-looking statements. Such factors include, among others, the successful commercialization of our products, market acceptance of our products, regulatory approval and regulation of our products and other factors and risks identified from time to time in our filings with the Securities and Exchange Commission, including our Annual Report on Form 10-K for the fiscal year ended December 31, 2024. All forward-looking statements contained in this press release speak only as of the date on which they were made and are based on management's assumptions and estimates as of such date. Except as required by law, we do not undertake any obligation to publicly update any forward-looking statements, whether as a result of the receipt of new information, the occurrence of future events or otherwise.

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SenesTech, Inc. represents that Evolve™ qualifies as a minimum risk pesticide under FIFRA Section 25(b) and is exempt from registration by the U.S. Environmental Protection Agency.

ContraPest® is registered federally as a General Use Product but requires an RUP license if being deployed in Connecticut. Due to conditional registration status in California, the product is not allowed to be used in public schools or childcare facilities within the state of California at this time.

ContraPest® is a registered trademark of SenesTech, Inc. Rat Birth Control™, Elevate Bait System™, Elevate Bait Station™, ContraPest Elevate Bait™, Isolate Bait System™, and Evolve™ are trademarks of SenesTech, Inc. ContraPest is intended for professional use only. Read and follow all label instructions for target species Norway and roof rats.

ContraPest 400mL tanks and trays are designed to fit the **PROTECTA EVO Express® bait station. PROTECTA EVO Express is a registered trademark of Bell Laboratories, Inc. and SenesTech is not related or affiliated with Bell Laboratories, Inc.

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Ecotoxicology. 2015 May;24(4):844-62. doi: 10.1007/s10646-015-1429-5. Epub 2015 Feb 25.

Anticoagulant rodenticides in urban bobcats: exposure, risk factors and potential effects based on a 16-year study

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Abstract

Anticoagulant rodenticides (ARs) are increasingly recognized as a threat to nontarget wildlife. High exposure to ARs has been documented globally in nontarget predatory species and linked to the high prevalence of an ectoparasitic disease, notoedric mange. In southern California, mange associated with AR exposure has been the proximate cause of a bobcat (Lynx rufus) population decline. We measured AR exposure in bobcats from two areas in southern California, examining seasonal, demographic and spatial risk factors across landscapes including natural and urbanized areas. The long-term study included bobcats sampled over a 16-year period (1997-2012) and a wide geographic area. We sampled blood (N = 206) and liver (N = 172) to examine exposure ante- and post-mortem. We detected high exposure prevalence (89 %, liver; 39 %, blood) and for individuals with paired liver and blood data (N = 64), 92 % were exposed. Moreover, the animals with the most complete sampling were exposed most frequently to three or more compounds. Toxicant exposure was associated with commercial, residential, and agricultural development. Bobcats of both sexes and age classes were found to be at high risk of exposure, and we documented fetal transfer of multiple ARs. We found a strong association between certain levels of exposure (ppm), and between multiple AR exposure events, and notoedric mange. AR exposure was prevalent throughout both regions sampled and throughout the 16-year time period in the long-term study. ARs pose a substantial threat to bobcats, and likely other mammalian and avian predators, living at the urban-wildland interface.

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2020

Laboratory Evaluation of the Effectiveness of the Fertility Control Bait ContraPest® on Wild-Captured Black Rats (*Rattus rattus*)

Shane Siers

Robert T. Sugihara

Israel Leinbach

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Laboratory Evaluation of the Effectiveness of the Fertility Control Bait ContraPest® on Wild-Captured Black Rats (*Rattus rattus*)

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ABSTRACT: A non-toxic liquid fertility control bait for rats has recently become commercially available (ContraPest® from SenesTech, Inc.). This product contains two chemicals, both of which impair spermatogenesis in male and reduce ovulations in female rats. We tested the efficacy of this bait in wild-caught adult black rats from the island of Hawai'i in a short-term laboratory trial. A control group (n = 25) was offered placebo bait and the treatment group (n = 25) was offered fertility control bait, both *ad libitum*, during a 15-day introduction period and during the first of four breeding rounds, for a total of 58 days of exposure. After treatment, all rats were provided placebo bait for the remainder of the study and randomly paired with mates from within their treatment groups for two additional breeding cycles. Treatment and control groups comprised 10 breeding pairs each, with random re-pairings between breeding rounds. The treatment group produced no litters during the first and second breeding rounds, while 70% of the control females produced litters. In the third breeding round, 70 days after stopping treatment, the treatment group produced three litters (six pups) compared to seven litters (24 pups) in the control group. During a fourth and final breeding round, control rats were crossed with treated rats, producing six litters (27 pups) from treated dams and nine litters (40 pups) from control dams, indicating no apparent infertility effect 99 days after cessation of treatment. This study demonstrates that the reproduction rate of wild-caught black rats can be chemically suppressed if provided *ad libitum* access to the fertility control bait under laboratory conditions.

KEY WORDS: 4-vinylcyclohexene diepoxide, black rat, contraception, fertility, laboratory efficacy, *Rattus rattus*, reproductive inhibition, triptolide, VCD

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INTRODUCTION

Invasive mammals impose great environmental and economic costs through damage to agricultural crops, natural resources, and human health and safety (Bergman et al. 2000, Pimentel et al. 2005). Invasive mammals can be particularly devastating on islands and have led to considerable species decline and extinctions (Courchamp et al. 2003, Doherty et al. 2016), with rats being one of the most damaging taxa of invader (Jones et al. 2008, Varnham 2010). In addition to the damage they cause in natural environments, invasive pest rodents have caused severe human and economic costs through damage to agriculture contributing to famine, vectoring zoonotic diseases, and mechanical damage resulting from gnawing and nesting behavior (Singleton et al. 2010, Himsworth et al. 2013). In Hawai'i, invasive black rat (*Rattus rattus*) populations damage crops and food stores, kill native flora and fauna, and are reservoirs and vectors of human disease (Meerburg et al. 2009, Shiels et al. 2014), including leptospirosis and rat lungworm disease (Jarvi et al. 2014, 2015, 2017).

In addition to traditional components of integrated pest management such as sanitation (removal of food sources), habitat management, and physical exclusion, large-scale rat control in protection of agriculture, human heath, and natural resources has typically involved the use of rodenticides: lethal toxicants formulated into an attractive and palatable bait matrix (Hadler and Buckle 1992, Buckle 1999, Witmer et al. 2007, Witmer and Eisemann 2007, Buckle and Smith 2015). However, shifting societal values (e.g., in opposition to perceived risk of poisoning of nontarget wildlife, animals and children) are increasing the demand for non-toxic, non-lethal alternatives for resolution of human-wildlife conflicts. Traditional anticoagulant rodenticides have aroused concern over poisoning of non-target species, environmental contamination, and humaneness (Mason and Littin 2003, Eason et al. 2010). Wildlife fertility control has been considered as a potential long-term management approach for reducing pest populations and the damage they cause (Miller at al. 1998). Fertility control has been predicted to prevent the rebound of rodent populations seen after rodenticide application (Gao and Short 1993) by reducing the rate of reproduction following temporary release from density-dependent population regulation (Jacob et al. 2008).

SenesTech, Inc. (Flagstaff, Arizona) markets a U.S. Environmental Protection Agency-registered, commercial liquid bait formulation, ContraPest®, for fertility control in rats. It contains two active ingredients that target both follicle development and spermatogenesis, blocking reproduction in both sexes. The active ingredient 4-vinylcyclohexene diepoxide (VCD) causes primordial follicle depletion leading to premature ovarian failure (Hoyer et al. 2001, Mayer et al. 2002, Mayer et al. 2004, Mark-Kappeler 2011). Follicular maturation progresses from the primordial stage to primary, secondary, antral and preovulatory in preparation for ovulation (Mayer et al. 2002). VCD targets the finite pool of primordial follicles;

once depleted, and after the remaining follicle types have been eliminated by atresia or ovulation, ovarian function is terminated (Hoyer et al. 2001, Mayer et al. 2004, Jacob et al. 2008, Mauldin 2013). VCD causes primordial follicle loss by interfering with KIT signaling, a key cellular growth and survival pathway within the oocyte (Mark-Kappeler et al. 2011). Atresia is a natural process in the ovary to eliminate follicles not destined for ovulation. VCD greatly accelerates this natural process (Hoyer at al. 2001). The second active ingredient in ContraPest is triptolide, a diterpene triepoxide purified from the traditional Chinese medicinal plant *Tripterygium wilfordii*. Triptolide stops growing follicles in the ovary and sperm production in the testes (Lue et al. 1998, Huynh et al. 2000, Xiong et al. 2011, Zeng et al. 2017). ContraPest has very low concentrations of both actives, VCD at 0.09% and triptolide at 0.001%. The combination of these two active ingredients acts synergistically to suppress reproduction in both sexes. Witmer et al. (2017) recently tested the palatability and efficacy of ContraPest fertility control bait in both Sprague-Dawley laboratory rats and in wild-caught Norway rats (*R. norvegicus*). Sprague-Dawley rats were provided *ad libitum* access to the liquid bait, along with *ad libitum* chow and water for 21 days. Rats that took treatment bait were placed in breeding pairs, as were control rats that took bait without active ingredients. Rats that received treatment bait had no offspring, while 100% of control rats had litters after one breeding round. Similar results of no offspring were found in breeding pairs of wild-caught Norway rats, tested in the laboratory, which took treatment bait and then completed two breeding rounds (Witmer et al. 2017).

ContraPest has yet to be tested on black rats, the species with the most widespread impacts on island ecosystems (Jones et al. 2008, Shiels et al. 2014). To date, there are no reports on the impact of the combination of ContraPest's two active ingredients on black rats, nor has the impact of VCD on their fertility been reported. Of the two active ingredients, only triptolide's impact on male black rat fertility has been studied (Singla et al. 2013, Singla and Challana 2014). In Singla and Challana (2014), the reproductive toxicity of triptolide was examined in no choice feeding at 0.1%, 0.2%, or 0.3% for 5 days with wild-caught male black rats. After 15 days post-dosing, the treated male rats were mated with healthy, untreated and cyclic female rats for 15 days. Only the male rats that took 0.2% triptolide sired no pups which may have been due to significantly reduced sperm motility and viability (Singla and Challana 2014). When the concentrations of triptolide taken by wild-caught brown rats versus wild-caught black rats are compared, taking into account the difference in percent concentrations in the two different baits, average consumption for each male rat, and difference in body weights between the larger brown versus black rats, the male black rats were exposed to 1,376 times the amount of triptolide taken by choice by the male brown rats. No mortalities were reported for either black or brown male rats in these two studies (Dyer et al. 2013, Singla and Challana 2014). Given the large difference in triptolide concentration that induced infertility in male black versus male brown rats, we sought to determine what impact ContraPest would have on the fertility of wild-caught black rats. Perhaps ContraPest would be ineffective in black rats due to their apparent insensitivity to triptolide, as over a 1000-fold greater triptolide was needed to suppress male rat fertility. Because ContraPest is presented in bait stations which both male and female black rats would visit, we presented ContraPest to both sexes to determine the impact on their fertility and fecundity.

METHODS

Animal Acquisition, Preparation, and Disposition

Wild black rats were live-trapped in forests and other conservation areas near Hilo and Volcano, County of Hawaii. In addition to trapping at our own USDA Hilo facility, permissions for trapping were granted by the Environmental Office of the Keaukaha Military Reservation, the site manager of Mauna Loa Orchards, and private owners of residential properties in the village of Volcano. Captured rats were transported to the testing facility and dusted with Drione® insecticide (Bayer, Research Triangle Park, NC) to treat for ectoparasites before being housed. Fifty rats of equal sex ratio were housed individually in numbered metal laboratory cages in a climate- and lighting-controlled laboratory space at the testing facility (20-22°C, ambient humidity, and 12 hr on/off light cycle). Cages (22 cm × 57 cm × 19 cm) were furnished with PVC refuge tubes sized for one or two rats (isolation or breeding events) and commercially purchased shredded paper bedding, replaced as needed. All rats had unrestricted access to a maintenance diet of Purina® rodent chow pellets (Nestle Purina PetCare Company, St. Louis, MO), and water was provided *ad libitum* in 250-ml inverted glass bottles with stainless steel sipper tubes throughout the duration of the study. Rats also received wood chew sticks with replacement as necessary.

All rats were individually housed for a minimum quarantine period of 3.5 weeks to ensure that no females were pregnant at the outset of the study phase. Rats were weighed at the beginning of the quarantine period, prior to pairing, and again at the end of the trial phase. All young born during the study were removed upon parturition and euthanized via an overdose of inhalant anesthesia (isoflurane) with subsequent carbon dioxide (CO_2) immersion. Adult rats were euthanized via CO_2 overdose at the end of the study. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and within the terms of the study protocol approved by the Institutional Animal Care and Use Committee of the U.S. Department of Agriculture National Wildlife Research Center (QA-2570).

Statistical Analyses

All statistical analyses and data visualization were performed in the R language for statistical computing (R Core Team 2016). Specific functions and tests are described within the methods subsections below.

Bait Consumption

Liquid ContraPest, containing the active ingredients VCD and triptolide (i.e., active bait), or an identical formulation lacking the active ingredients (i.e., placebo bait), was offered *ad libitum* in identical 250-ml inverted

glass bottles, as were the bottles providing water. Daily bait consumption was estimated by measuring the bait level within the bottles with a graduated scale. While co-housed for breeding we continued to record bait consumption, though we were unable to determine how much bait was consumed by each individual. To test for an effect of the active ingredients on bait consumption (i.e., palatability effects of VCD and triptolide), data from the initial exposure and first breeding cycle phases (the period during which the treatment group received the active bait) were subjected to a linear mixed effects model with bait type (active vs. placebo), sex, and study phase (active bait exposure vs. pre- and post-exposure periods) as fixed effects and individual identification (ID) as a random effect (1|ID) to account for multiple repeated measures for each individual. Modeling was conducted using the function "lmer" in the package "lme4" (Bates et al. 2015), with the model specified as:

$$\text{consumption} \approx \text{bait} + \text{sex} + \text{phase} + (1|\text{ID})$$

To obtain a p-value for the effect of bait type on consumption, we performed a likelihood ratio test comparing this model to a null model without the bait term in an analysis of variance (ANOVA); the p-value for the χ^2 comparison of the two models is reported as the statistical significance of the bait effect.

Reproductive Inhibition Trials

Prior to pairing for breeding, all rats were pretreated with the placebo bait formulation for a five-day conditioning period to ensure that rats were familiar with the bait prior to the treatment period (trial Days -6 to -1). Within each sex group, 13 rats were randomly assigned to the active bait treatment group and 11 females and 13 males were assigned to the placebo bait control group. After the conditioning period, the treatment group was administered the active bait for 15 days while the control group continued to receive the placebo bait (Days 0 to 15). Weight, sex, cage number, and treatment group assignment of each pair was recorded before the initiation of the breeding cycles.

During the first of four breeding rounds (Round 1, Days 15 to 35), the treatment and control groups continued receiving active and placebo bait, respectively. Ten females were randomly paired with ten males within their respective study groups (treated females paired with treated males, control females paired with control males) and the males were placed within the females' cages for mating, with individual IDs recorded for each pairing. The remaining rats in each group continued to be housed individually, to be substituted for rats found to be unfit for breeding due to poor condition, injury, or rejection of a male by the female partner during the course of the breeding cycle.

Males were paired with females for 21 to 23 days. If a male was rejected by the female within 24 to 48 h, one of the spare males from the same study group (treatment or control) was substituted for breeding. Females and/or cage papers were examined daily for discarded vaginal plugs as an indication that they had been inseminated. After the pairing period, males were removed and returned to their individual cages. Females were monitored for parturition daily for 23-28 days following removal of males. Within 24 h of birth, pups were removed, counted, and euthanized.

At the completion of the first breeding cycle, the active bait provided to the treatment group was withdrawn and replaced with the placebo bait to determine the persistence of a reproductive inhibition effect (Day 58). At this time, the treatment group had been continuously exposed to the active bait for 58 days. All rats were provided the placebo bait for the remainder of the study.

For a second and third breeding cycle (Rounds 2 and 3, Days 58 to 79 and 105 to 127), pairings within study groups were re-randomized without replacement so that males were placed with different females than in previous breeding cycles. For a fourth and final breeding cycle (Round 4, Days 156 to 177), females from the treatment group were crossbred with males from the untreated control group, and treated males were paired with un-treated females in order to assess whether treatment of a single sex suppressed reproduction.

After the last round of breeding, all animals were euthanized and body weights recorded. Liver, kidneys (combined), spleen, adrenal glands (combined), and reproductive organs were excised, cleaned of fats and/or connective tissues, and weighed for future comparative analysis.

Statistical differences between counts of litters for treatment and control groups, per breeding round, were tested with Fisher's exact tests Wilcoxon rank tests, with α set at 0.05 and two-tailed p-values reported. These methods were not intended to distinguish between a contraceptive effect on males or females.

RESULTS

Bait Consumption

Daily bait consumption by sex, study group, and study phase is depicted in Figure 1. All rats readily consumed the bait. The median rat (ranked by consumption) consumed an average of 74 ml of bait per day; individual means ranged from 66 to 83 ml per day. There was no effect of inclusion of the active ingredients in the bait formulation on bait consumption (i.e., there was no apparent negative effect of active ingredients on palatability; study phase term $p = 0.739$).

During this project, males (which grow larger than females) gained significant weight, but there was no discernible difference in weights between test groups of males or females receiving placebo or active-ingredient baits during the treatment phase (Figure 2).

Reproductive Inhibition Trials

During pairings, there were only two occasions when males were removed and replaced due to incompatibility/ aggression by females. Pairings and litter size details for all four breeding rounds are tabulated in Siers et al. (2017).

Numbers of litters and pups per litter for each of the ten breeding pairs per round are summarized in Table 1. During the first breeding round, when the treatment group had been exposed to active ContraPest bait for 15 days and was continuing to consume the active bait, there were no litters within the treatment group, while seven litters, totaling 32 pups, were born to the 10 control pairs (70% breeding success). During the second breeding round, which began simultaneously with the replacement of the

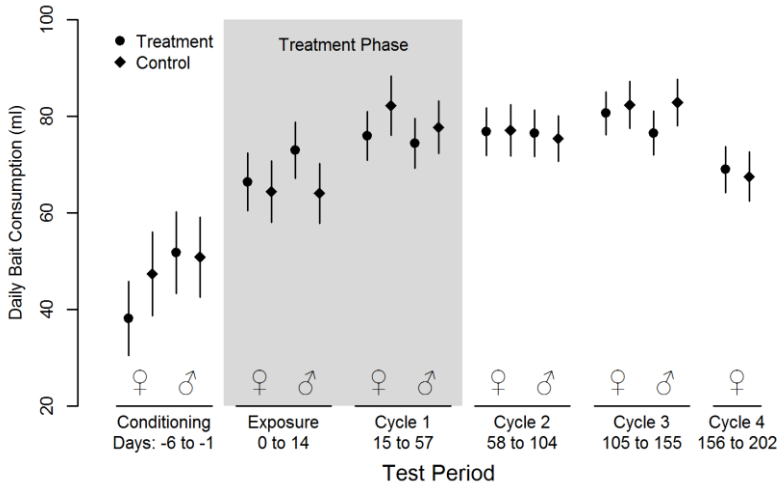

Figure 1. Bait consumption. Mean daily bait consumption and 95% confidence intervals (1.96*SE) by sex, study group, and study phase. Bait including the active ingredient was only offered during the Treatment Phase. Individual consumption data were only available while rats were individually-house (not while paired for breeding). There is no Cycle 4 consumption data for males because they were euthanized immediately following breeding. N = 11 for the female control group, N = 13 for all other groups.

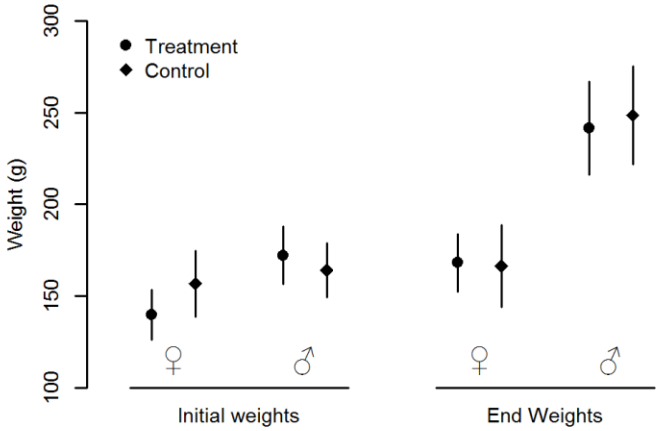

Figure 2. Body weights. Mean rat body weights and 95% confidence intervals (1.96*SE) by sex and study group. Within sexes, there were no significant weight differences between treatment and control groups (α = 0.05). N = 11 for the female control group and N = 13 for all other groups.

Table 1. Litter count and litter size results for female rats (N = 10 per study group). "Bait" indicates whether the treatment group was provided either the active ingredient ContraPest product or the placebo version during the breeding cycle. "Mating" denotes whether females were matched to males by study group (treatment-treatment/control-control) or crossed with males of the opposite study group (treatment-control/control-treatment). "Mean Litter" size is calculated only from females with litters (zeroes not included in the average); however, the Wilcoxon rank test for difference in litter size is based on a litter size of zero for dams without litters. * p ≤ 0.05; ** p ≤ 0.01.

Breeding Round	Days Paired	Bait	Mating	Litters		Pups		Mean Litter ± SD	
				Ctrl	Trt	Ctrl	Trt	Ctrl	Trt
1	15-35	Active	Matched	7	0**	32	0	4.57 ± 1.05	0**
2	58-79	Placebo	Matched	7	0**	27	0	3.86 ± 2.17	0**
3	105-127	Placebo	Matched	7	3	24	6	3.43 ± 1.18	2.00 ± 1.41*
4	156-177	Placebo	Crossed	9	6	40	27	4.44 ± 2.91	4.50 ± 2.22

treatment group's active bait with placebo, there continued to be no reproduction in the treatment group pairs and 70% breeding success (seven litters, 27 pups) in the control group. During both first and second breeding rounds, the difference between seven control litters and zero treatment litters was statistically significant ($p = 0.004$). By the beginning of the Round 3, the active bait had been replaced by placebo for 47 days. During this round, control group reproduction remained at 70%, while treatment group reproduction increased to 30%; though continued reproductive suppression is apparent, the difference between control and treatment group litters was not statistically significant at $\alpha = 0.05$ ($p = 0.178$). Considering only females that produced litters, the litter sizes in the treatment group (four, one, and one) were smaller than those in the control group ($p \leq 0.05$). By the fourth round of breeding, commencing 99 days after removing active bait, treatment group females paired with control group males reproduced at a rate indistinguishable from control group fecundity in previous rounds. There was no apparent suppressive effect when mating control group females with treatment group males, with this group producing the most litters (90% breeding success) and bearing litter sizes indistinguishable from previous control female breeding rounds.

DISCUSSION

These results demonstrate complete reproductive inhibition for wild-caught black rats exposed to ContraPest bait containing the active ingredients VCD and triptolide, *ad libitum*, under laboratory conditions, for at least 15 consecutive days prior to mating and throughout a 43-day breeding cycle. The inhibitive effect persisted through the second breeding cycle. When paired a third time, 47 days after cessation of treatment, a partial suppressive effect was apparent but not statistically significant, though litter sizes were significantly smaller for the few treatment females that did reproduce. By 99 days post-treatment (Round 4) there was no apparent effect of reproductive inhibition. Given that fertility was rebounding by the third breeding cycle, we are unable to draw any useful inference from the cross-breeding of treated and control animals, and the ability to detect any potential sex-specific effect is confounded by the dissipation of the treatment effect. The impact of the two active ingredients of ContraPest caused infertility for a length of time that exceeded the spermatogenic cycle reported by Singla et al. (2013); however, our study design did not allow for discrimination between a contraceptive effect on males or females. In practice, both sexes will receive the bait, and based on prior research cited in the introduction we expect that both sexes are affected to some degree.

Reproduction within our control group was not 100%. During the first three breeding cycles, three (30%) of the control group pairings did not resulted in litters, and one (10%) of the parings in the fourth cycle did not produce a litter. This could potentially be a result of failure of wild-caught rats to fully adjust to captivity in a relatively short timeframe, or an indication of incomplete fertility in the source population from which these individuals were drawn. While differences in litters and litter sizes were statistically significant during the first two breeding rounds, without 100% reproduction in the control group it is difficult to argue that the lack of any litters in the first two breeding rounds was solely attributable to the ContraPest treatment and with no influence of sub-fertility in the treatment group.

By design, ContraPest is a contraceptive and not a sterilant. As a result, no evidence of permanent infertility was detected following the 58-day active bait exposure period. Whether more prolonged exposure to ContraPest would lead to permanent sterilization cannot be inferred from our study. Further studies would be needed to assess the effect of long-term exposure on fertility.

Refinements of this or other fertility control baits might afford non-toxic and non-lethal alternatives for protection of agriculture, human health and safety, and natural resources under some management scenarios. For instance, ContraPest could complement conventional use of toxicants that have caused a rapid knockdown of the pest population; following up with the use of ContraPest could prevent the well-known rebound of a poisoned rat population (Andrews 1977). Timing the use of ContraPest would be key to diminishing rat population expansion due to seasonal weather changes and/or abundance of food sources. In Hawai‘i, for example, ContraPest could be considered to suppress the marked increase in rat population that follows the peak of strawberry guava fruiting by two to three months (Shiels 2010).

ContraPest has also undergone successful trials for reducing brown rat population levels in the New York City Subway system in 2013 (Klein 2017). Because brown rats in subway trash rooms chose liquid when given choice between solid and liquid matrices to deliver the active ingredients, ContraPest is currently formulated as a liquid. For treatment of black rats in challenging tropical terrains, where deployment of bait boxes with liquid tanks is not feasible, it will be necessary to develop and evaluate a solid form of ContraPest that can be aerially deployed and is durable in the tropical environment. This study demonstrates that the active ingredients in ContraPest, when ingested *ad libitum* in the current liquid formulation, can profoundly reduce black rat reproductive output.

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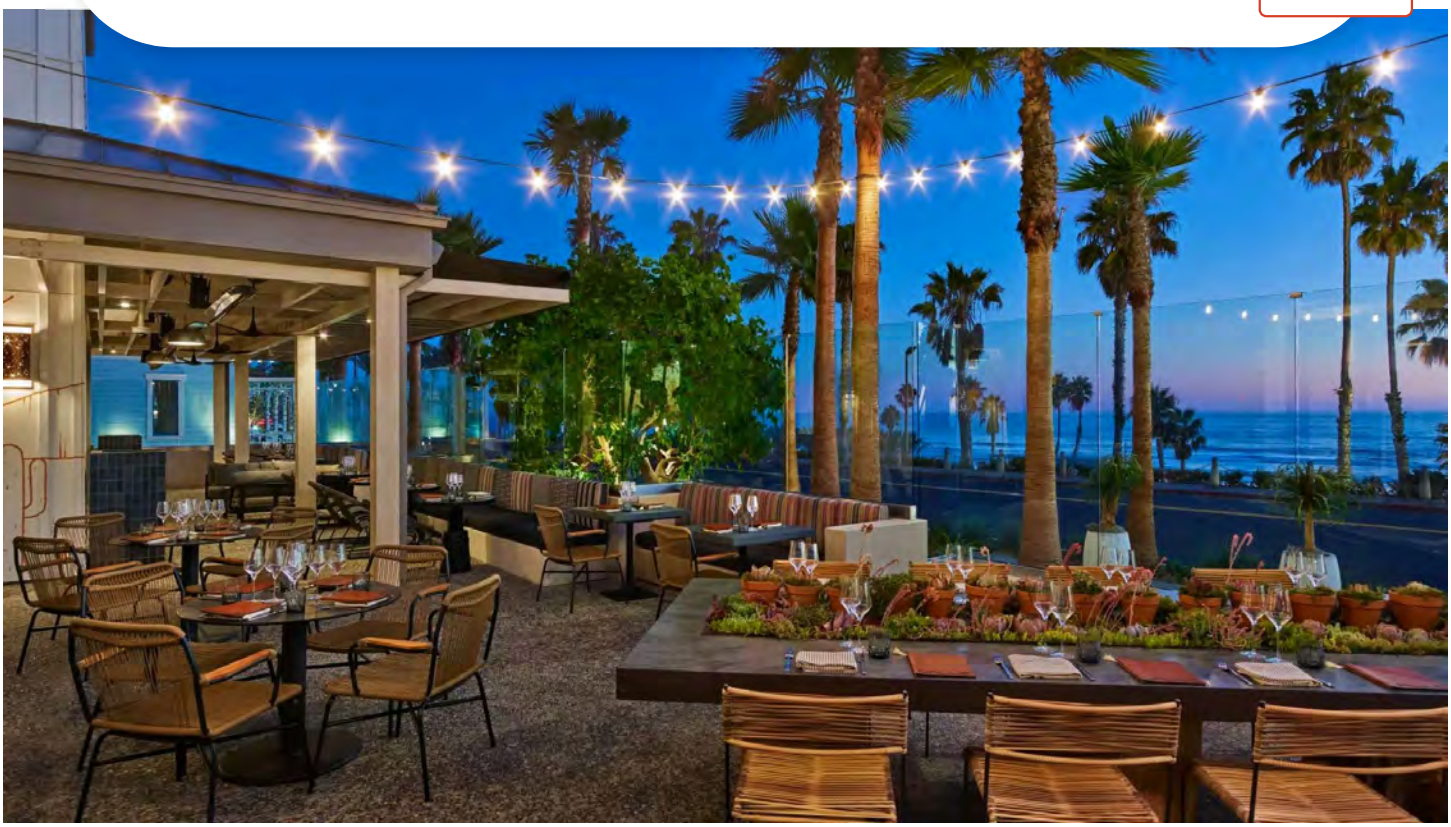


How Many Restaurants Are In California – Report

Thomas Campbell

Updated March 17, 2025

There are **98,156 restaurants in the state of California** according to data which we compiled using Google's Maps Data. This number represents all restaurants listed on Google Maps in California and includes bars, coffee shops and other restaurant categories.

The data in this article is a representation of the number of restaurants in California and the various key findings we

Table of Contents

1. 98,156 Restaurants in California

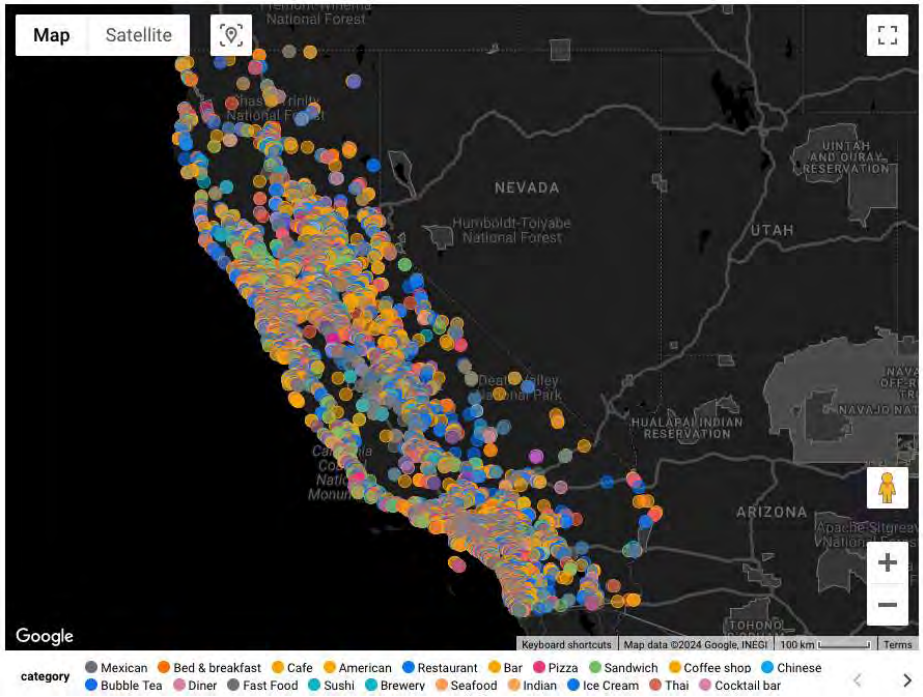
discovered.

Bel
vie
Los

7.6 Restaurants Per
4. 0.63 Restaurants Per Square Mile
5. 4,596 Fast-food Restaurants
6. Fast Food Price Dissatisfaction Up 31%
7. Mexican Cuisine Has 14,457 Options
8. 88 Michelin Star Restaurants
9. 6,254 Bars
10. 13,547 Pizza Restaurants
11. 7,618 Coffee Shops
12. 1,837 Hamburger Restaurants
13. Conclusion
14. FAQ

98,156 Restaurants in California

There are 98,156 restaurants in the the state of California according to Google's data. The most popular restaurant categories (in no particular order) in California include: Mexican, Cafe, American, Pizza, Bar, Sandwich, Chinese, Bubble Tea, Fast Food and others.



7.6 Restaurants Per Capita

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Restaurants Per 1000 People

Los Angeles Has The Most Restaurants

Los Angeles has the most restaurants in California with 6,682. San Diego is not far behind Los Angeles and has 3,695 total restaurants while San Francisco has 3,234. Rounding up the top five are San Jose with 2,158 and Fresno with 1,403.

3.	CA	San Franci...	3,234
4.	CA	San Jose	2,158
5.	CA	Fresno	1,403
6.	CA	Oakland	1,282
7.	CA	Long Beach	1,276
8.	CA	Sacramento	1,199
9.	CA	Bakersfield	1,122
10	CA	Riverside	852

0.63 Restaurants Per Square Mile

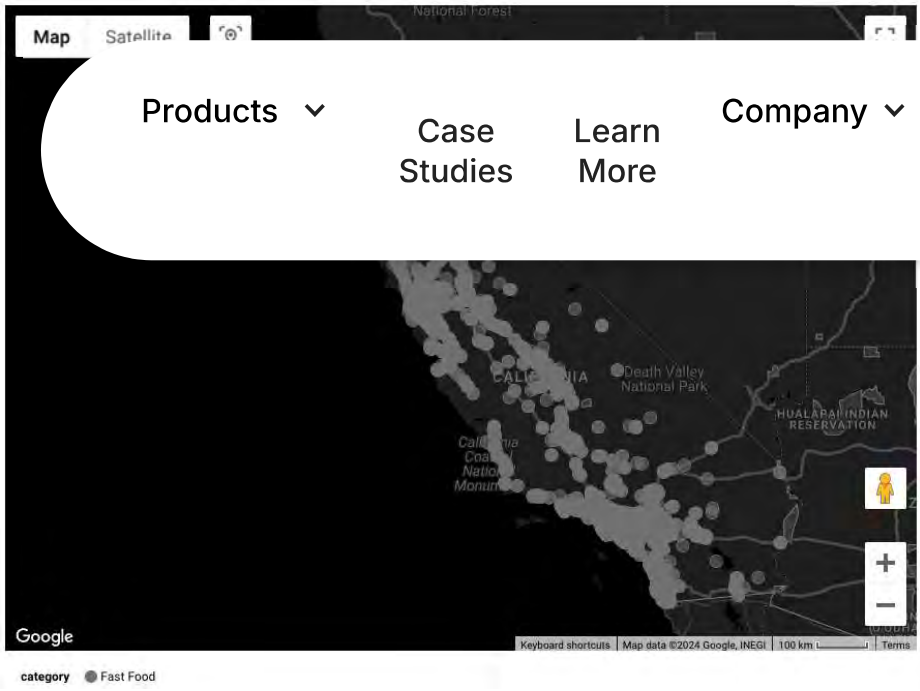
With land area of 155,812.8 square miles according to United States Census Bureau, there are 0.63 restaurants per one square mile in California.

0

Restaurants Per Square Mile

4,596 Fast-food Restaurants

4,596 restaurants are fast food which makes up about 5% of all restaurants in California. Most popular chains include McDonald's, Carl's Jr., Wendy's and others.



Fast Food Price Dissatisfaction Up 31%

Due to California's new minimum wage state law, we also tracked Google Maps reviews for the keyword 'expensive' in fast-food restaurant listings from November, 2023 up until now.

Prior to the minimum wage increase for fast food workers coming into effect, there was an average of 1,332 Google reviews that contained the keyword word 'expensive' for fast food restaurants. After the law came into effect, there was an average of 1,740 Google reviews for fast-food restaurants that had the keyword 'expensive' in it. This is an increase of 31% since the law came into effect.

+0%

More Fast Food Restaurant 'Expensive' Reviews

Among a variety of cuisine options throughout California, Mexican cuisine has the highest number of restaurants with 14,457. This is followed by the following cuisine focused restaurants: Chinese (3,532), American (2,683), Japanese (1,905), Italian (1,772) and Indian (986).

88 Michelin Star Restaurants

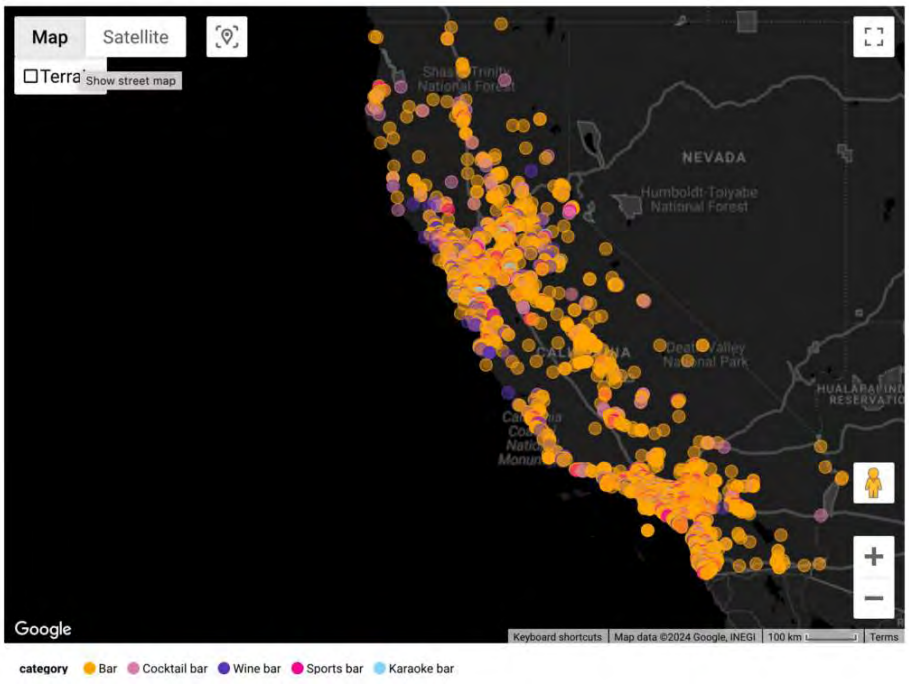
California is home to 88 Michelin Star restaurants that range from one to three stars. 66 restaurants in California have one Michelin star distinction, 12 have two Michelin star distinction and six have three star distinction. The six restaurants with three Michelin star distinction are: Atelier Crenn (San Francisco), Addison (San Diego), Quince (San

Francisco), Single Thread (Healdsburg), Benu (San Fran

Michelin Star Restaurants

6,254 Bars

California has a lot of bar options throughout the state which includes 6,254 options. Popular bar categories include: bar, sports bar and cocktail bar.



13,547 Pizza Restaurants

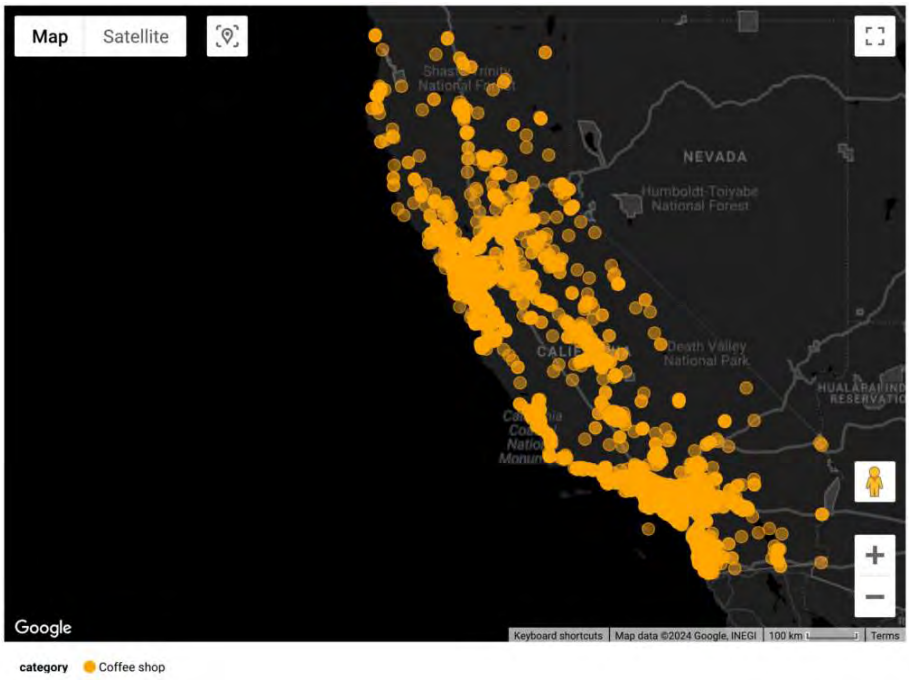
California has plenty of pizza restaurants with 13,547 resta
Litt

0

Pizza Restaurants

7,618 Coffee Shops

California has 7,618 coffee shops across the state with popular chains including Starbucks, Peet's Coffee, Dunkin and others.



1,837 Hamburger Restaurants

California also boasts a significant number of hamburger resta

Hamburger Restaurants

Conclusion

California's restaurant scene is one of the largest in the United States and offers an enormous variety of restaurants to food lovers across the state. From Mexican cuisine to fast food, there is something for everyone in California and finding a restaurant shouldn't be a problem with an average of almost two restaurants per square mile in the entire state.

Get The Latest Restaurant Trends, Guides & Tips

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FAQ

- **Which city in California has the best restaurants?**
- **How many 3 star restaurants are in C**
- **What food is LA known for?**

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Toxicological responses to sublethal anticoagulant rodenticide exposure in free-flying red-tailed hawks

February 10, 2023

View Data Release

An important component of assessing the hazards of anticoagulant rodenticides to non-target wildlife are observations in exposed free-ranging individuals. The objective of this study was to determine if environmentally realistic, sublethal first-generation anticoagulant rodenticide exposures via prey, can result in direct or indirect adverse effects to free-flying raptors. We offered black-tailed prairie dogs (Cynomys ludovicianus) that had fed on Rozol® Prairie Dog Bait (0.005% active ingredient chlorophacinone) to six wild-caught red-tailed hawks (RTHA, Buteo jamaicensis), and uncontaminated black-tailed prairie dogs to two wild-caught RTHAs for 7 days. On day 6, blood was collected from all eight RTHAs to determine chlorophacinone's effects on blood clotting time. Russell's viper venom time and prothrombin time of chlorophacinone-exposed RTHAs exceeded mean values of reference RTHAs by more than 5 standard deviations. The observed coagulopathy confirmed that the RTHAs in the treated group were exposed to and adversely affected by chlorophacinone via secondary exposure through diet. On day 7, the RTHAs were fitted with tail-mounted VHF radio telemetry transmitters. Red-tailed hawks were release eir fate

(survival, activity, and overt physiological condition) was monitored for 33 days. Four of these six chlorophacinone-exposed free-flying RTHAs exhibited ptiloerection, an indication of thermoregulatory dysfunction due to chlorophacinone toxicity, but no signs of intoxication were observed in the reference hawk. It is noteworthy that prothrombin time values were associated with the duration and frequency of ptiloerection. These findings demonstrate that sublethal chlorophacinone exposure can directly or indirectly evoke adverse effects in wild birds. Furthermore, our anecdotal observations suggest that duration and frequency of ptiloerection from low level CPN exposures may be modulated by territoriality and mate presence. Although our sample sizes were small, this study is a first to relate coagulation times to adverse clinical signs in free-ranging birds.

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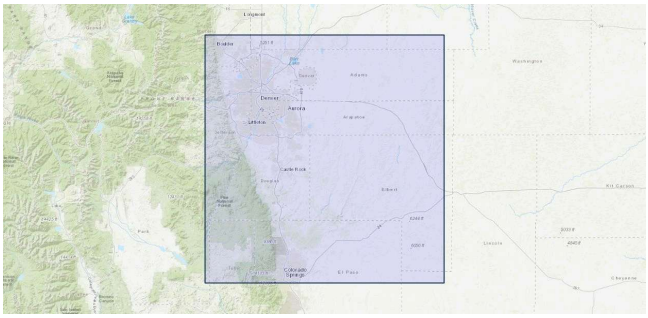
Study Area



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A Comprehensive Guide for Grocery Store in California 2025

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Table Of Content

How Many Grocery Stores are in California?

Services Offered by Grocery Stores in California

California's grocery sector is a dynamic marketplace integral to the state's economy, facilitating the flow of goods from agricultural producers to a varied consumer base.

> *"Food for the body is not enough. There must be food for the soul." — Dorothy Day*

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Price Range of Different Grocery Stores in California

Distribution of Grocery Stores Across California's Districts

Price Range of Grocery Stores Across California's Districts

Most Visited Grocery Stores in California's Districts

Top Rated Grocery Stores in California's Districts

Diverse Establishments Featuring Grocery Stores in California

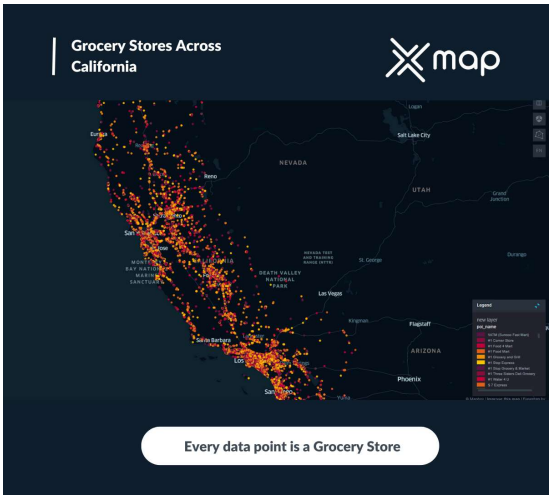
How xMap Grocery Store Data Can Help Businesses

Conclusion

Reflecting on Dorothy Day's words, California's grocery stores do more than just feed the body; they cater to the soul by connecting people with quality food from diverse cultures and origins.

How Many Grocery Stores are in California?

California boasts a vast network of 30,132 grocery stores, offering an extensive range of options from large supermarkets to niche specialty stores. This vast array ensures accessibility to diverse food options contributing to the diverse grocery stores landscape in the USA and the state's eclectic tastes and dietary needs.



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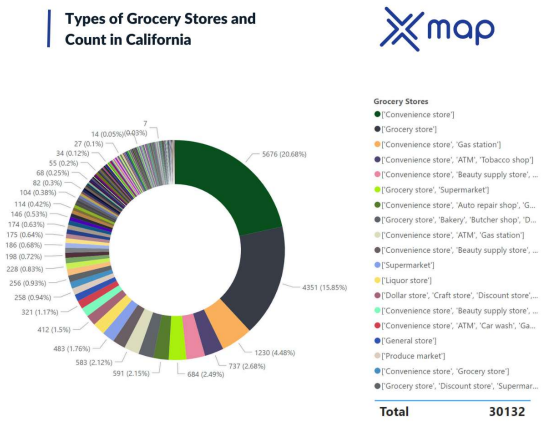
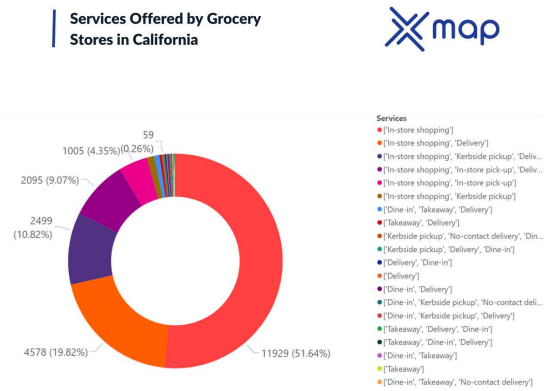


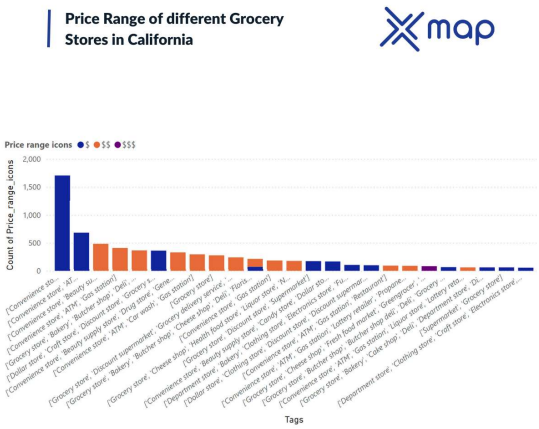
Services Offered by Grocery Stores in California

The grocery stores across California have adapted to modern consumer demands by offering various services beyond traditional shopping. A significant portion provides in-store shopping combined with delivery and pickup options, reflecting a shift towards more flexible and convenient shopping experiences.



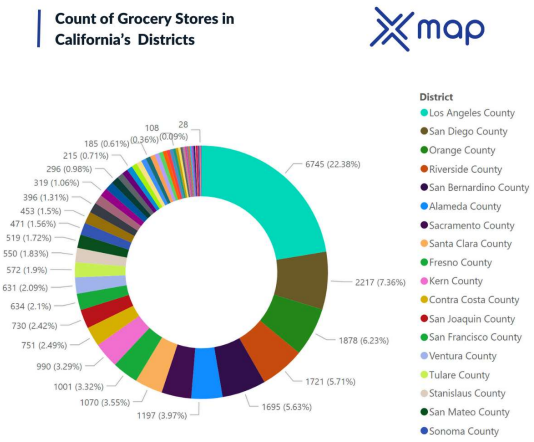
Price Range of Different Grocery Stores in California

The pricing within California's grocery sector varies widely. Stores range from budget-friendly supermarkets to high-end specialty outlets offering gourmet and organic products, ensuring that diverse consumer demographics across the state can access shopping experiences aligned with their income levels and lifestyle preferences.



Distribution of Grocery Stores Across California's Districts

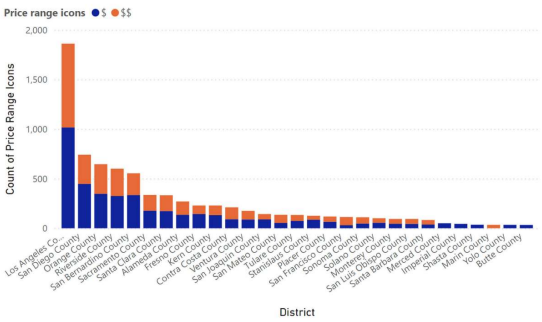
With a strategic spread across the state, the highest concentration of grocery stores is found in populous counties like Los Angeles and San Diego, ensuring optimal accessibility for all residents, regardless of their location.



Price Range of Grocery Stores Across California's Districts

The price range varies notably across different districts. In wealthier neighborhoods, consumers often find premium grocery stores and luxury food markets, while in less affluent areas, retailers offer affordable grocery options to cater to budget-conscious shoppers. This localized pricing strategy helps businesses effectively align with community-specific economic profiles.

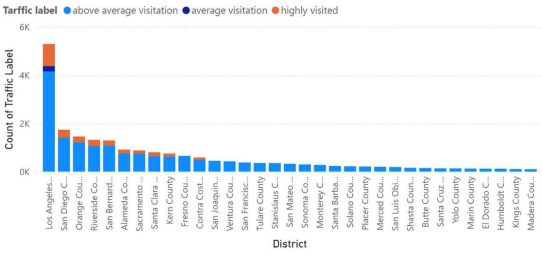
Price Range of Grocery Stores in California's Districts



Most Visited Grocery Stores in California's Districts

Los Angeles County leads with the highest foot traffic in grocery stores, making it a critical area for grocery businesses due to its vast consumer base and high demand.

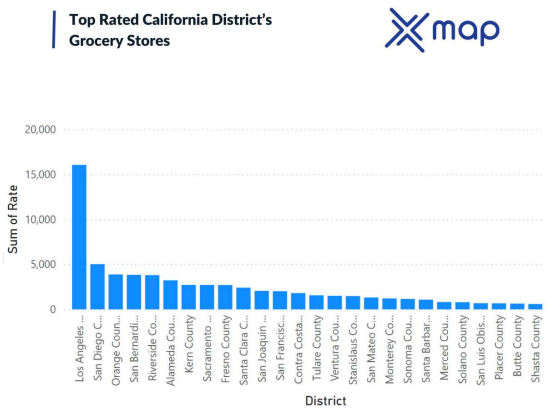
Most Visted California District's Grocery Stores



Top Rated Grocery Stores

in California's Districts

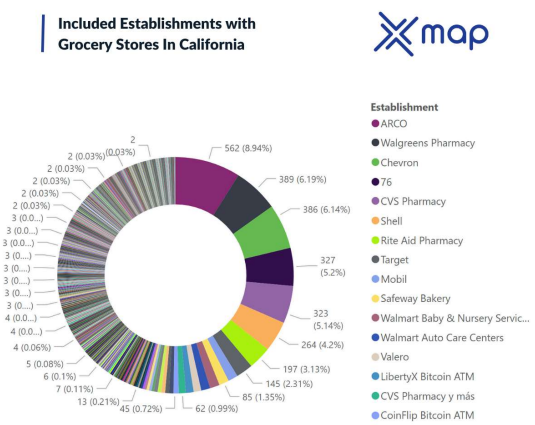
Stores in Los Angeles County are not only the most frequented but also rank highest in customer satisfaction, emphasizing the importance of quality and customer service in retaining and attracting consumers.



Diverse Establishments Featuring Grocery Stores in California

California's market is notable for its variety of establishments incorporating grocery stores, such as major pharmacies and gas stations, offering groceries alongside their primary goods. This trend highlights the evolving nature of retail,

blending different services to provide comprehensive solutions to consumer needs.



How xMap Grocery Store Data Can Help Businesses

xMap provides invaluable data on California's grocery stores, aiding businesses in refining their operational strategies or entering new market segments. Here's how xMap can support your business decisions:

Strategic Site Selection: Use demographic and competitor analysis data to identify the best locations for new outlets.

Price Optimization: Adjust pricing strategies based on thorough local

market data and competitor pricing to ensure competitiveness.

Enhancing Customer Experience: Leverage customer feedback and service trend data to enhance satisfaction and loyalty.

Market Expansion: Pinpoint underserved areas for potential expansion, utilizing targeted marketing strategies.

Conclusion

xMap's robust insights into the grocery store sector equip businesses with the necessary tools to navigate and succeed in California's competitive market. For personalized strategies and deeper insights, reach out to xMap at sales@xmap.ai. Utilize the power of xMap to drive your business forward in California, where data-driven decisions and in-depth local understanding pave the way for success in the grocery industry. Businesses exploring international retail strategies can also benefit from insights like those found in Sazka stores in the Czech Republic, which highlight how

localized data can guide smart decision-making.

Get in Touch

Whatever your goal or project size, we will handle it.
We will ensure you 100% satisfication.

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> *"We focus on delivering quality data tailored to businesses needs from all around the world. Whether you are a restaurant, a hotel, or even a gym, you can empower your operations' decisions with geo-data."*

Mo Batran
CEO & Founder @ xMap

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