Anticoagulant rodenticides are climbing the food chain to the top: a first proof of widespread positivity in grey wolves (*Canis lupus*)

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Abstract

Second-generation Anticoagulant Rodenticides (ARs) can be particularly critical for large carnivores, due to their widespread use and time-delayed impacts on their populations. While many studies explored the impacts of ARs on small and mesocarnivores, no study explored the extent to which they could contaminate large carnivores in anthropized landscapes of Europe.

We filled this gap by exploring spatiotemporal trends in grey wolf (*Canis lupus*) exposure to ARs in central and northern Italy, by relying on a dataset of dead wolves (n = 186) tested with standardized laboratory protocols. The determination of anticoagulants was carried out by means of a semiquantitative LC-MS/MS method.

Most wolves (n = 115/186, 61.8%) tested positive for ARs (1 compound, n = 36; 2 compounds, n = 47; 3 compounds, n = 16; 4 or more compounds, n = 16). Bromadiolone, Brodifacoum and Difenacoum, were the most common compounds, with Brodifacoum/Bromadiolone the combination of ARs that co-occurred the most (n = 61).

Both the probability to test positive for multiple ARs and the presence of Brodifacoum, and Bromadiolone in the liver, systematically increased in wolves that were found at more anthropized sites. Moreover, wolves became more likely to test positive for ARs through time, particularly after 2020.

Our results underline that rodent control, based on ARs baiting, increases the risks of unintentional poisoning non-target wildlife. However, this risk does not only involve small and mesocarnivores, but also large carnivores at the top of the food chain, such as wolves. Therefore, rodent control is adding one further conservation threat for endangered large carnivores in anthropized landscapes of Europe, whose severity could increase through time and be far higher than previously thought. Widespread monitoring schemes for ARs in European large carnivores should be devised as soon as possible.

Keywords:
Anticoagulant rodenticides; food chain; Italian wolf; rodenticide baits; rodents; top predator

1.Introduction

The long-term conservation of large mammals in anthropized landscapes is often said to depend upon a combination of legal protection, sustainable exploitation, the availability of suitable habitat and trophic resources, human tolerance, and infrastructure development (Apollonio et al., 2017; Di Marco et al., 2014; Di Minin et al., 2016; Kauffman et al., 2021; Wolf and Ripple, 2016). Moreover, many studies highlighted the risk posed by infectious or parasitic diseases, often from a One Health perspective (Cunningham et al., 2017).

However, exposure of large mammals to anthropogenic chemicals received proper attention only over the last few years (https://www.ewg.org/interactive-maps/pfas_in_wildlife/map/). This despite persistent, bioaccumulative and toxic (PBT) chemicals can enter the trophic chain and alter the physiology, behaviour, health, and reproduction of mammals (Torquetti et al., 2021; Saaristo et al., 2018; Zala and Penn, 2014; Köhler and Triebskorn, 2013), sometimes with temporally delayed dynamics which are detected only when it is too late to counteract their demographic impacts (Desforges et al., 2018). The impact of PBTs can be particularly critical for large carnivores (Rodríguez-Estival and Mateo, 2019), whose populations in many parts of the Global North, although recovering (Ingeman et al., 2022), are still relatively limited and potentially susceptible to strong shrinking.

Anticoagulant rodenticides (hereinafter ARs) are among the most problematic PBTs for predators, due to the possibility of secondary exposure through direct predation of rodents or the consumption of dead ones (Wright et al., 2022; Fernanez-de-Simon et al., 2018, 2022; Elmeros et al., 2019; López-Perea et al. 2018; Gedhun et al., 2015) given their long-term impact on the immune system of mammals (Serieys et al., 2018). This is especially true for second-generation ARs which are more effective against rodents than first-generation compounds and more persistent in the environment. Although the European Union over the years has adopted regulations that have progressively restricted the range and patterns of use of rodenticides, these normative changes failed so far to reduce exposure in non-target mammal predators (Elmeros et al., 2018), also due to different national laws and free trade between member states (Eisemann et al., 2018).

The grey wolf (*Canis lupus*) steadily expanded its distribution in Europe, over the last three decades, due to environmental change and increased legal protection (Cimatti et al., 2021), with previously disconnected populations becoming genetically connected for the first time after centuries (Fabbri et al., 2014). Despite exposure to ARs might be occurring among wolves in Europe, since *i*) rodents are part of the wolf diet (Newsome et al., 2016; Zlatanova et al., 2014) and *ii*) wolves are expanding into areas where rodent control is a routine activity, no study explored neither the occurrence and extent of this phenomenon, nor its spatio-temporal dynamics. This gap is surprising because ARs have been recently found in meso and large carnivores living near to human settlements, indicating that secondary exposure to these substances is not anymore restricted to small and mesopredators specializing on rodents (Serieys et al., 2018, 2019; McMillin et al., 2018; Rudd et al., 2018; Lestrade et al., 2021).

Here we want to fill this gap by exploring spatiotemporal trends in wolf exposure to ARs in central and northern Italy, by relying on a dataset of animals found dead between 2018 and 2022 and tested with standardized laboratory protocols.

2.Methods

2.1.Study area

The study area encompasses the Emilia-Romagna and Lombardy regions, as well as the northernmost portion of the Tuscany region (**Fig. 1**). This area is characterized by different ecosystems, from Mediterranean maquis on the coasts of Tuscany, to broad-leaved forests and sub-alpine grasslands in the Apennines, to alpine grasslands and glaciers in the Alps, to urbanized areas in the lowlands. The human population is estimated around 10.5 million people, across 46.039 km^2, with a density of 269.4 ± 167.6 inhabitants/km^2 (mean ± standard deviation).

In the Tuscany region, approximately 107 - 110 packs of wolves were estimated between 2014 and 2016 (Apollonio et al., 2016), while in the Emilia-Romagna region, approximately 42 packs occurred between 2000 and 2009 (Caniglia et al., 2014). These originated from two distinct sub-populations, that had subsequently merged, around 2013, as the species expanded its distribution (Apollonio et al., 2013). In the Lombardy region, wolf expansion occurred mostly from the Western Alps (Marucco et al., 2022) and in the Po Plain, along the Ticino River (Dondina et al., 2020).

As wolves are territorial, and many individuals are forced to disperse and settle down into unoccupied habitats, the species progressively colonized the whole study area, starting from the more undisturbed habitat patches to the more disturbed agricultural and peri-urban environments in lowlands (Bassi et al., 2015; Zanni et al., 2023).

Moreover, the Emilia-Romagna and Tuscany regions host among the highest densities of wild ungulates in Europe (Apollonio et al., 2010). Available evidence indicates that in the study area, wolves rely mostly on wild ungulates, such as the roe deer (*Capreolus capreolus*) and the wild boar (*Sus scrofa*) (Bassi et al., 2017, 2020; Ferretti et al., 2019; Mori et al., 2017; Milanesi et al., 2012; Mattioli et al., 2011; Capitani et al., 2004), although they can also regularly include other preys, such as an invasive alien coypu (*Myocastor coypus*, Ferretti et al., 2019).

As elsewhere, in the study area rodent control is authorized according to provisions from the Regulation n. 1062/2014 from the European Commission (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32014R1062), which have been translated into provisioning from the Ministry of Health (Cabella et al., 2015). In Italy, both first-generation (Chlorophacinone, Coumatetralyl) and second-generation ARs (Brodifacoum, Bromadiolone, Difenacoum, Difethialone, Flocoumafen) have been authorized. In **Table 1**, the number of anticoagulant rodenticide formulations available on the market in Italy, based on active ingredient is given. Rodent control primarily - and almost exclusively - target synanthropic species, i.e., the house mouse (*Mus musculus*), the brown rat (*Rattus norvegicus*) and the black rat (*Rattus rattus*), while more sporadic interventions are also made against voles in agriculture, although these are now much less frequent than in the past (Capizzi & Santini, 2007). By being a non-selective method, rodenticides are not allowed for the control of coypus. Rodent management is performed mostly by pest control operators (hereinafter: PCO), which often include cleaning companies contracted to also carry out rodent control. According to Regulation n. 1062/2014 from the European Commission, PCOs can purchase any active ingredient, with concentrations up to 50 ppm of the active principle (Sinergitech, 2020). Noteworthy, the use of rodenticides is also allowed for amateurs, but these can only purchase small packages with concentrations of the active principle below 30 ppm (https://www.izs.it/IZS/Engine/RAServeFile.php/f/pdf_normativa/Biocidi-Rodenticidi/Biocidi_IZSTeramo.pdf). Rodent control interventions are carried out both at relatively large spatial scales (e.g., house blocks, inhabited areas, large private factories) as well as in form of localized interventions, such as those carried out in private houses or shops. However, rodent control interventions through rodenticides mainly target outdoor areas, since in several industrial and commercial settings rodents are controlled mostly indoors through trapping. Unlike other European countries (e.g., the United Kingdom), in Italy there are no restrictions to the use of the most powerful active principles in rodent control operations carried out outdoors.

2.2.Data collection and laboratory analysis

Our final sample of wolves included 186 individuals (**Fig. 1**), which had been collected between 2018 and 2022 by local authorities/people encharged and subjected to necropsy investigation by the University of Bologna and the Experimental Zooprophylactic Institute of Lombardy and Emilia-Romagna. They were subjected to toxicological examinations and had the coordinates of their site reported by local authorities. The age of the animal was estimated on the basis of dental development, body size and weight (Mòrner et al., 2005). Individuals had a quite balanced sex ratio (53.6 % were males), and our sample included either young or adult wolves (1st year of age = 27.9%; 2nd year of age = 34.6%, 3rd year of age or higher = 37.7%).

The determination of anticoagulants (Coumafuryl, Warfarin, Coumatetralyl, Coumachlor, Bromadiolone, Difenacoum, Brodifacoum, Flocoumafen, Difethialone) was carried out by means of a LC-MS/MS method (Vandenbrouke et al., 2008; Fourel et al., 2017, Bertero et al., 2020). In detail the sample (typically 40 g) was extracted by vigorous stirring with acetone (100 mL); after filtration on paper, an aliquot (2 mL) was dried under gentle nitrogen flow at 40°C. The residue was reconstituted with 2 mL of 2% ammonia solution in acetonitrile. Three defatting steps with n-hexane (2 mL) followed. Finally, an aliquot (1 mL) was stripped to dryness and reconstituted with 0,4 mL of acetonitrile. A 1 µL volume was injected into an LC-MS/MS system (Agilent QQQ 6460, equipped with an Agilent 1290 Infinity II UPLC). Chromatographic column was Zorbax Eclipse Plus C18 (2,1x50 mm, 1,8 µm). Column temperature was set at 40° C. Chromatographic separation was performed through a linear gradient using as aqueous phase a 0,1% formic acid solution and as organic phase 0,1% formic acid solution in acetonitrile. Flow rate was set at 0,4 mL/min. Run time was 11 min, with a post-time reconditioning of 2 min. Quantification was carried out by the external standard method in MRM mode (ESI negative) acquiring two proper and typical transitions, quantifier, and qualifier, for each analyte (**Tab. S1**). MS/MS parameters were set as follows: capillary 4000 V, gas temperature 300°C, gas flow 10 L min–1, nebulizer 35 psi, sheath gas temperature 300°C, sheath gas flow 12 L/min.

The limit of quantification (LOQ) was 1 µg /Kg for all analytes. A concentration found ≥ 1 µg /Kg did indicate a positive sample, while a concentration < 1 µg /Kg denoted a negative sample. Therefore, this method gives a result positive or negative for the presence of anticoagulants; in a healthy animal, anticoagulants should not be present. Even if the concentration of anticoagulants above the LOQ is evaluated by the method this data is not reported as a result because the meaning of different levels of principles in wolves as for other non-targeted species for the baits is not clear due to the lack of information. In particular, there is a lack of information about; species sensitivity, consequences of sublethal effects, effects of different-level exposure to rodenticides, the relation among residues of multiple ARs, their relative potency and combined effect at the level of the individual, quantitative estimates of mortality, identification of the occurrence of sublethal effects and long-term ecological consequences and the effects of multiple low-level AR exposures (Rattner et al., 2014), toxicokinetic aspects, absorption, distribution, excretion/elimination and especially all factors affecting metabolism (such as; differences in breed, sex, age, physiological state or disease states, nutritional states, individual genetic aspects, presence of enzyme inducers or inhibitors in the diet and so on).

Regarding the evaluation of the analytical data, it is emphasized that the latter is only one part that makes up the story of "each case" examined; the other components include not only lesions and the anatomopathological picture but also the anamnesis and recent symptoms. The latter data referring to wild animal carcasses found in the territory are not available. Therefore, we are only able to classify as poisoned animals those in which the toxicological examination was positive (presence of anticoagulants) in association with an anatomopathological picture indicative of acquired coagulation disorders.

In all other cases, only positivity on toxicological examination could be reported without the characteristic alterations of coagulation caused by ARs, on subjects in good body condition. One factor that can complicate the interpretation of the data is the state of preservation of the carcass, which when suboptimal, can alter the anatomopathological pictures and interfere with the finding of the ARs.

Taking into account all these considerations we put the data of quantitative level found in the livers in relation to the area of recovery of the carcasses with the purpose to see if there were higher levels of anticoagulants in different areas.

2.3.Statistical analyses

We modelled how the effect of landscape characteristics, measured at the sites where wolves had been found, affected *i*) their probability of testing positive to 1, 2, 3 or more ARs (among Brodifacoum, Bromadiolone, Coumatetralyl, Difenacoum, Difethialone, Flocoumafen), *ii*) the presence of Brodifacoum and Bromadiolone, the two most common ARs (see below), detected in their livers.

Rodent control in the study area is associated with urban areas, farms, and animal husbandry. In these environmental conditions, we expected wolves to be positive to a higher number of ARs, due to the higher exposure to contaminated rodents, which would be scavenged or hunted. Moreover, as exposure to ARs is expected to occur in these areas with a higher frequency than in natural habitats, we expected wolves from anthropized areas to have a higher presence of ARs in their livers.

Environmental characteristics of the site where each wolf has been found were quantified by aggregating important environmental attributes with Partitioning Around Medoids cluster analysis (Kassambara, 2017). Rather than using only the presence of human infrastructures, we opted for creating a composite index, reflecting both human presence and other important topographic and land cover characteristics of the study area. These included *i*) the presence of human infrastructures, by using the Human Footprint Index (Venter et al., 2009) as a proxy, *ii*) the percentage of tree cover and iii) croplands at a 250 m scale, measured through the MODIS/Terra Vegetation Continuous Fields (https://lpdaac.usgs.gov/products/mod44bv006/), *iv*) the elevation, *v*) the roughness of each point and the *vi*) topographic position index, indicating if a certain point was on a mountain top or on the bottom of a valley (Wilson et al., 2007). Environmental variables were calculated as median values in a buffer with a radius of 6 km around the point. This size corresponded to an area of approx. 113 km^2, reflecting the most recent estimates for the home range of the species reported in Italy (Mancinelli et al., 2018; Mattioli et al., 2018).

The silhouette method, the elbow method, and the gap statistic method supported the existence of two different environmental conditions (**Fig. S1**). By overlaying them with a satellite imagery of the study area, and by exploring the distribution of environmental characteristics in the two groups (**Fig. S2**), we noticed that the first group corresponded to relatively undisturbed areas on hills and mountains, with high levels of tree cover and terrain roughness, and low presence of human infrastructures. On the other hand, the second group corresponded to lowland areas with a high presence of human infrastructures and croplands.

We modelled the probability of testing positive to multiple ARs through a Bayesian ordered-logit formulation (Bürkner and Vuorre, 2019). On the other hand, we used a zero-altered Gamma regression (Zuur et al., 2017) to model the presence of Brodifacoum and Bromadiolone detected in the liver of tested wolves.

In our models, we also controlled for the sex and age class of each individual, two potentially confounding variables that were measured as ordered variables with polynomial contrasts. Anthropization was deemed to be a potentially important predictor of positivity to ARs, as rodent control in the study area is mostly concentrated around urban areas and in farms and animal husbandry. Moreover, in anthropized landscapes, wolves could face a higher exposure to anticoagulants as their diet might rely more on rodents, due to the lack of their main prey such as ungulates. Finally, young male wolves were assumed to be more at risk of exposure from ARs, as this group is the most involved in dispersal (Ausband, 2022; Morales-González et al., 2021; Caniglia et al., 2014), when individuals cannot rely on group hunting, thus shifting to smaller preys, like rodents. We used bivariate thin-plates splines to measure the spatial correlation of observations and a cyclic cubic spline to measure cyclic variations, accounting for the temporal correlation of observations, in the temporal distribution of recoveries, between January 2018 and December 2022 (Wood, 2017). Exploratory analyses indicated that predictors did not have any association between them, nor any spatial, or temporal pattern.

2.4.Comparison with other recovered wildlife

To have a more complete understanding of temporal trends in wildlife exposure, we compared our findings about wolves, with positivity to ARs in other wildlife that has been recovered and tested for these compounds in the Emilia-Romagna region. Contrary to wolves that have always been tested for AR, in the case of these species, only those individuals that showed signs of acquired coagulopathies on pathological examination were tested for AR. This dataset (n = 176), included recoveries of multiple species, that could prey or consume dead individuals of rodents, which occurred between 2018 and 2022, mostly red fox (*Vulpes vulpes*, n = 67), common buzzard (*Buteo buteo*, n = 23), Eurasian badger (*Meles meles*, n = 13), wild boar (*Sus scrofa*, n = 9), European hedgehog (*Erinaceus europaeus*, n = 9), coypu (n = 7), house mouse and rats (n = 7), stone and pine marten (*Martes* sp., n = 4), and other diurnal (n = 15) and nocturnal (n = 8) raptors. Individuals were subjected to the same laboratory analyses that were used for wolves, and we modelled the temporal trends of positivity to Coumatetralyl, Brodifacoum, Bromadiolone, Difenacoum, Difethialone and Flocoumafen. This dataset was used as a “control”, to detect any temporal change in the use of rodenticides, at least for part of the study area. A Bayesian Generalized Additive model, with a cyclic cubic spline, and a Bernoulli distribution of the response, was used to model temporal fluctuations in the probability that a recovered animal was positive to rodenticides.

Model selection for both wolves and recovered wildlife followed a stepwise approach, starting from a null model, and then evaluating the effect of each covariate on the predictive accuracy of candidate models, through leave-one-out cross-validation (Vehtari et al., 2017). Statistical analyses were carried out with the statistical software R (R Core Team, 2022) and with STAN (Carpenter et al., 2018), through the ‘brms’ package (Bürkner et al., 2017). A reproducible dataset and software code is available at the following link: https://osf.io/yqv4n/

3.Results

Our findings indicate that most wolves (n = 115/186, 61.8%), analyzed between 2018 and 2022, tested positive for ARs (1 compound, n = 36; 2 compounds, n = 47; 3 compounds, n = 16; 4 or more compounds, n = 16). The most common compounds were Bromadiolone (n = 97), Brodifacoum (n = 93) and Difenacoum (n = 26, **Fig. S3**), which often occurred in the same individual (**Fig. S4)**. Overall, Brodifacoum/Bromadiolone was the combination of ARs that co-occurred the most (n = 61), followed by a mix of Brodifacoum/Difenacoum (n = 20; **Tab. S2**).

Of the 115 wolves who tested positive for ARs, 19 presented an anatomopathological picture attributable to acquired coagulopathies with evident coagulation alterations (i.e., macro, and microscopic hemorrhages) while 96 died of other causes such as vehicle collision, gunshot, intraspecific aggression, diseases, presenting laboratory positivity to AR, even if in the absence of characteristic pathological lesions.

Leave-one-out cross-validation retained anthropization and the time when wolves were found as meaningful covariates. Wolves from more anthropized areas had a lower probability than wolves from less anthropized areas of being negative to ARs or testing positive for a single compound, but they had a higher probability to test positive for 2, or more, ARs (**Fig. 2**). Moreover, wolves had a higher chance of testing positive for ARs from late summer to late winter, and this probability became higher after 2020, particularly the probability of testing positive to 3, or more, ARs (**Fig. 3**). Model selection indicated that wolves from more anthropized areas had also a higher concentration of Brodifacoum in their liver (**Fig. 4**). However, the concentration of Bromadiolone was not significantly higher.

As for other wildlife species, positivity to ARs was found to be particularly high for the red fox, where 60 individuals out of 67 (89.6%) showed traces of rodenticides. Moreover, also 18 buzzards out of 23 (78.3%) were positive. However, when considering the temporal distribution of positivity to ARs, among all the various wildlife species, no clear trend emerged (**Fig. S5**).

The Bayesian ordered-logit GAM modelling the number of ARs, and zero altered Gamma GAM modelling Brodifacoum and Bromadiolone presence in the liver of wolves and our Bernoulli GAM modelling presence/absence of ARs in recovered wildlife, converged, and showed a good fit to the data. No spatial correlation was detected, as the inclusion of coordinates with a thin-plate spline did not improve model fitness. A complete overview of model selection is given in **Appendix S1**.

4.Discussion

The grey wolf is now widespread in Italy, with an estimated population of 2,945 – 3,608 individuals (La Morgia et al., 2022), and a conservation status that changed from “Vulnerable” to “Near Threatened” during the last decade (Rondinini et al., 2022). Nevertheless, our findings highlight a concerning situation regarding the exposure of this species to both first and second-generation ARs. In our opinion our findings should raise a concern about *i*) our true understanding of wolf ecology in human-dominated landscapes, *ii*) the extent to which grey wolves in Italy, and more generally in Europe, might be subjected to secondary exposure to ARs, altogether with the long-term consequences of this phenomenon, *iii*) the lack of selectivity of rodent control through ARs and the need to update regulations about their use.

4.1.Understanding of wolf ecology in anthropized landscapes

More than half wolves in our sample tested positive for one, or more, ARs, particularly after 2020. While we expected some individuals to show traces of rodenticides (Di Blasio et al., 2020), due to the trophic flexibility of the species, a similar magnitude was largely unforeseen. Moreover, both the number of ARs and the presence of Brodifacoum in the liver of wolves, increased in wolves that had been found in anthropized environments.

In Europe wolves, although capable of exploiting many different preys, have traditionally been regarded as relying on wild ungulates or livestock (Zlatanova et al., 2014). Our findings indicate that rodents might be consumed regularly, and perhaps might also be an important food in certain seasons and environmental conditions, even where wild ungulates are abundant. Indeed, Ferretti et al. (2019) reported invasive alien coypu as an important prey, whose importance can locally be comparable to that of the roe deer. Although coypu cannot be controlled with ARs, it is a major pest in northern Italy (Cocchi and Bertolino, 2021), and it is likely subjected to illegal baiting with ARs. Empirical evidence indicates that wolves in the Po Plain regularly feed on coypu (*Myocastor coypu*), which may be somehow involved in the contamination with ARs.

Among wolves two categories of individuals could be more susceptible to contamination: the first is lone nomadic individuals moving in unfamiliar landscapes ("floaters", *sensu* Fuller et al., 2003). Floaters include different types of wolves, such as juveniles undergoing dispersal, adults that faced pack disruption or old individuals that left their pack (Mancinelli et al., 2019). These have two characteristics that could increase their exposure to ARs. First, by not being able to hunt large prey in groups (MacNulty et al., 2012), floaters could have shifted to smaller prey, like coypus, or rats (*Rattus norvegicus, Rattus rattus*). As floaters usually avoid contacts with resident packs, by moving between territories, to minimize the risk of aggressions (Cassidy et al., 2017) this makes them prone to move more around human settlements, or along anthropogenic landscape features (Mancinelli et al., 2019). In our study area, where packs started their colonization from the most undisturbed habitats (Bassi et al., 2015), floaters were forced to concentrate their movements in the most anthropized areas, where they could have sustained themselves by scavenging or hunting rodents. It is also plausible that this particular group of individuals could have further increased their frequentation of anthropized areas during COVID-19 lockdowns, due to decreased human disturbance. In turn, this would have increased their exposure to ARs and produced the marked increase in positivity observed after 2020. The second categories of wolves potentially more prone to contamination are those belonging to the packs that recently started to colonise plain areas with high levels of human presence and limited access to natural prey: this process is growing in importance in Italy where these last environments are more and more frequently hosting breeding pairs that exploit the rich anthropogenic food sources (e.g., Tuscany see Zanni et al., 2023). In both cases, wolves found themselves in environments where resource distribution was mainly determined by human activities as consequence garbage, slaughter remains, limited barrier livestock farms and small synanthropic mammals probably constitute the bulk of the food biomass available.

Thus, our study calls for a detailed assessment of wolf diet and movements in human-dominated landscapes, and how individuals undergoing different life stages could change their diet. Since our data is based on the opportunistic collection of dead individuals, in the near future it would also be important to set up methods, such as scat analysis (Prat-Mairet et al., 2017), which would allow us to assess exposure to ARs homogeneously across a wolf population.

4.2.Selectivity of chemical control of rodents

By having detected for the first time a significant level of contamination from anticoagulants in wolves, our study is a warning on on the penetration of anticoagulant rodenticides into the food chain of terrestrial ecosystems in Europe. Indeed, finding high frequencies of contamination in a species believed to prey mostly on ungulates raises serious concerns about the actual level of bioaccumulation that rodent control can determine, even in those species which are not specialized in rodents. The study reveals a relevant spatial spread, with significant temporal variations, in the use of rodenticides across the study area, affecting even some of the most persistent active ingredients.

Worldwide, rodenticides are the most widely used technique for rodent control (Capizzi et al., 2014). Empirical evidence suggests that rodenticides are used without adequate awareness and as a preventive measure, often resorting to so-called permanent baiting. Although permanent baiting is explicitly banned in official EU documents, it still finds application in the daily practices of many professionals and amateurs engaged in rodent control. There is a need to identify integrated approaches to rodent control that can limit the use of rodenticides to only those situations where they are truly needed, and which prioritize the use of trapping and environmental sanitation. Moreover, even when rodenticides are needed, the use of compounds with lower persistence and toxicity towards nontarget species should be preferred (e.g., cholecalciferol, Witmer, 2018).

Finally, in regulating the use of these substances, environmental risk must be balanced with the social benefits of synanthropic rodent control (e.g., Van den Brink et al., 2018).

4.3.Exposure to ARs in expanding wolf populations and potential consequences for conservation

The potential widespread positivity to ARs calls for the rapid creation of a pan-European surveillance network for toxic chemicals in recovering populations of large carnivores.

Our findings are not based on randomly sampled individuals, but rather on a convenience sample that probably included more individuals from anthropized areas and/or undergoing nomadic behaviour. Even if our level of exposure could hardly be taken as representative of the whole population in the study area, it reasonably indicates that exposure to ARs can involve a considerable number of individuals.

It is not easy to identify what causes and factors can explain these findings. The risk of ARs accumulation in predators depends on both the frequency with which they are present in their prey, as well as their concentration (Lopez Perea and Mateo, 2018). Confounding factors could include the unauthorized use of rodenticides against coypu (Cocchi and Bertolino, 2021), or occasional baiting against voles (*Microtus sp*.) in orchards, although the latter is not an activity that has seen a recent increase in the territory. But the discrepancy is inevitable between exposure to ARs and the finding of animals that died from other causes at a time subsequent to exposure even by many days, leading to mismatched data, especially in the case of species that make very large movements (Mancinelli et al., 2018; Mattioli et al., 2018) and very persistent active ingredients (Horak et al., 2018).

However, considering that both the number of ARs and the presence of Brodifacoum in the liver of wolves increased with the level of anthropization at the sites where these had been found, the most likely hypothesis is that an increased frequentation of peri-urban areas (Zanni et al., 2023) raised wolf exposure to ARs through two different mechanisms. These included mostly the predation, or scavenging, of contaminated rodents as well as perhaps the consumption of some poisonous baits, made with ARs and targeting wolves (intentional poisoning). If wolf positivity to ARs had arisen mostly from poisonous baits, we would have expected some spatial or temporal clustering, deriving from the constraints that offenders would face to deploy baits (Faulkner et al., 2018). We did not find any evidence for a similar clustering. On the other hand, both the red fox and diurnal raptors had widespread positivity to ARs across the Emilia-Romagna region, without any temporal trend. Taken together, these two findings indicate that the high prevalence of ARs among recovered wolves derived mostly from the widespread use of these substances for pest management and the positivity found in the wolves object of this study, could be understood as accidental poisoning.

Considered that rodent control is common in many other parts of Italy and Europe (Eisemann et al., 2018), where it already affects raptors (Gomez et al., 2022; Nakayama et al., 2017), smaller carnivores (Wright et al., 2022; Fernanez-de-Simon et al., 2018, 2022; Elmeros et al., 2018, 2019; López-Perea et al. 2018; Gedhun et al., 2015) and domestic pets (Calzetta et al., 2018; Berny et al., 2010) we believe that secondary exposure to ARs might be an overlooked phenomenon for European wolf populations.

This could bear two consequences for wolves. The first one is toxicosis, which is suspected to be a relevant source of mortality for urban coyotes (*Canis latrans*) in North America (Poessel et al., 2015). This scenario might be realistic only for those wolves whose diet is largely based on rodents, but it is hard to make predictions about its impacts, as we currently do not have threshold values for ARs in the grey wolf.

On the other hand, there is evidence that ARs can amplify immune dysfunctions in carnivores, increasing their impact on mortality. For example, Serieys et al. (2015) found that bobcats (*Lynx rufus*), that had been exposed to ARs had a higher probability of having a severe level of mange. Subsequent studies (Fraser et al., 2018; Serieys et al., 2018) showed that this was due to multiple impacts of ARs on the immune system, including on gene expression, that compromised the immune response of bobcats against mange. Moreover, ARs are suspected to affect pregnancies in domestic dogs (Fitzgerald et al., 2017) and their impacts can also be exacerbated by simultaneous exposure to multiple compounds (Serieys et al., 2015).

It should be noted that 83.8% of the positive wolves did not show an anatomopathological picture indicative of coagulation disorders. This could lead one to think that many positive wolves had sublethal concentrations which could have been a contributing cause of death. In facts, chronic exposure to ARs would have compromised the hepatic metabolism, coagulation, and behavior of wolves, undermining their capacity to react to dangerous situations (Fournier-Chambrillon et al., 2004). Moreover, poisoned individuals, due to behavioral alteration and the incapacity to effectively hunt could approach anthropized landscapes more easily, remaining victims of car collisions or direct persecution (Musto et al., 2021). This may be somewhat of a shortcoming in the sampling strategy, but it is nonetheless something that is inevitably present in ecotoxicology studies based on the analysis of animals found dead, not affecting the consistency of the findings (Schwartz et al., 2020).

Although we still need to understand the extent to which ARs can affect the immune response in the grey wolf, their populations in Europe regularly experience infectious and parasitic diseases (Millán et al., 2016; Kołodziej-Sobocińska et al., 2014) and sometimes have low genetic variability (Hindrikson et al., 2016), two threats whose demographic impact could be magnified by sublethal exposure to ARs. Although it is unlikely that ARs affected wolves living in undisturbed areas, increased wolf mortality in anthropized landscapes could generate widespread and unpredictable source-sink dynamics. These scenarios are particularly concerning, given the increasing pressure in some areas of Europe for the lethal control of wolves, a practice whose long-term impact on wolf populations is still uncertain (Lennox et al., 2018; Treves et al., 2016), and the difficulties in monitoring wolf populations at a temporal and spatial resolution that would allow for adaptive management (Merli et al 2023).

5. Conclusion

This study emphasizes the need for national and international coordination in the collection of carcasses of large carnivores in wild and anthropized ecosystems. This study also wants to encourage researchers to integrate multiple sources of information about the presence and mortality of wolves, and more generally large carnivores, in Italy and across Europe, (*i*) to answer relevant questions about illegal killing and cryptic conflicts with human activities, which can seriously affect the conservation status of their populations despite their increasing abundance; (*ii*) understand environmental phenomena of bioaccumulation and disproportionate use of anticoagulants, with repercussions on the entire food chain of terrestrial ecosystems.

Finally, our study underlines that animal and bait poisoning, a widespread practice in urban and rural areas, is a public health concern (DiBlasio et al., 2020), in particular, because it is potentially harmful to humans and the environment including non-targeted domestic and wild species. These results underline that controlling rodents by anticoagulants baits includes risks of unintentional poisoning of non-target animals both primary poisoning through ingestion of baits (intentional poisoning) and secondary exposure consuming issues from animals which carry anticoagulants (accidental poisoning) leading to a cumulative number of animals affected over time, in particular those at the top of the food chain, as previously reported (Rattner et al., 2014; Fisher et al., 2019).

This work wants to give a contribution to the lack of formal estimates of a number of wolves affected by anticoagulants including animals killed by these poisons and animals in which the anticoagulants were present in the livers, but another primary cause of death was seen/demonstrated.

CRediT authorship contribution statement

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Carmela Musto, Jacopo Cerri, Maria Cristina Fontana, Silva Rubini, Annalisa Santi, Arianna Rossi, Alessandro Bianchi, Alberto Biancardi, Chiara Garbarino: **Data curation;**
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Alberto Biancardi, Laura Uboldi, Alessandro Moretti: **Laboratory Analysis**;
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Giuseppe Merialdi, Marco Apollonio, Mauro Delogu, Chiara Garbarino: **Supervision.**

Supplementary materials

Supplementary data associated with this article can be found in the online version at: https://osf.io/yqv4n/

Data availability

Data available via Open Science Framework Digital Repository: https://osf.io/yqv4n/

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICAL APPROVAL

Not applicable.

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Figures

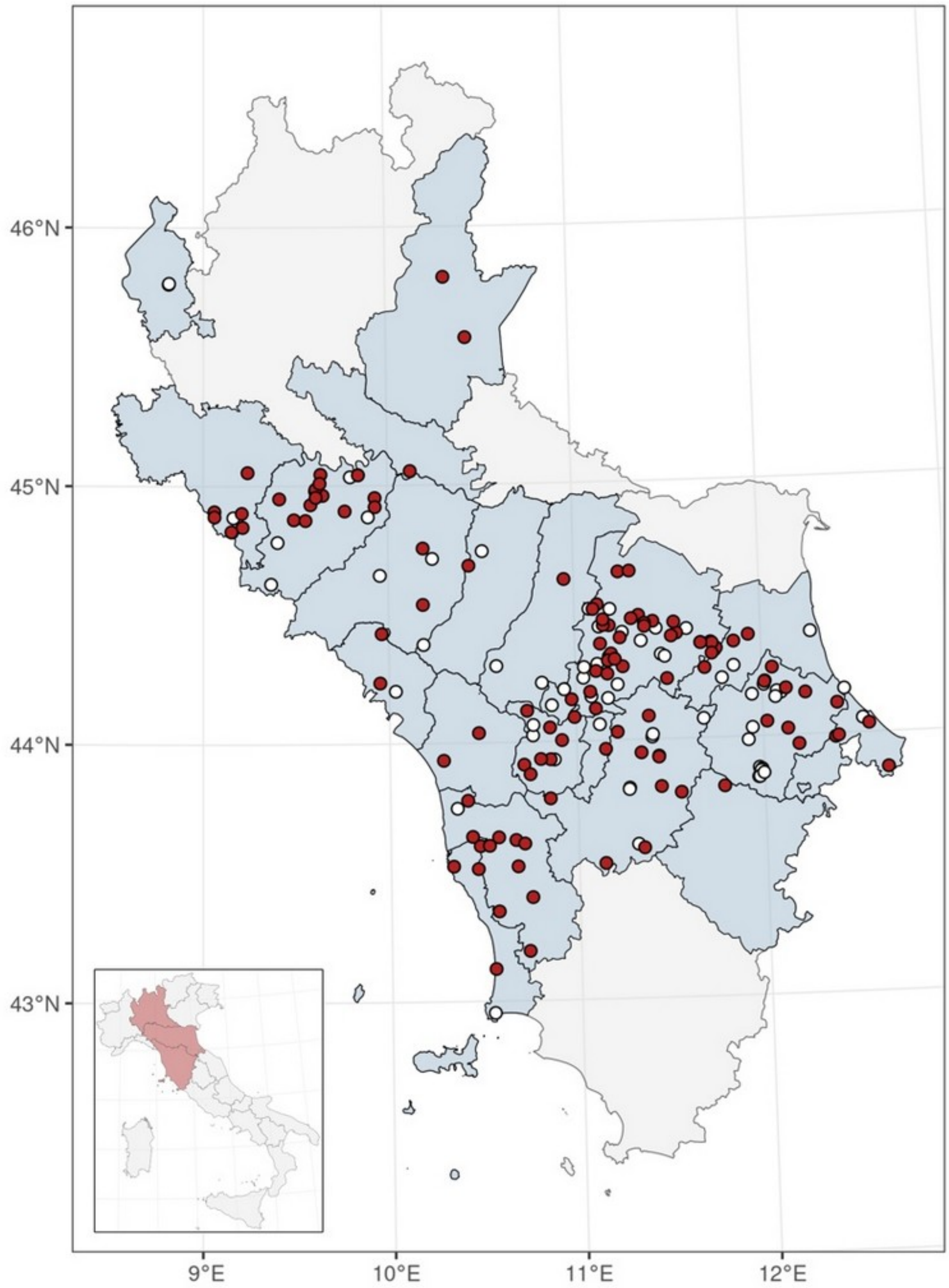

Fig. 1. Distribution of wolves that were found dead in the study area and were negative (white dots) or positive (red dots) to anticoagulant rodenticides (ARs). Provinces in the Emilia-Romagna, Lombardy, and Tuscany regions, that were covered by data collection are highlighted. The position of the study area in Italy is shown in the figure in the lower-left corner.

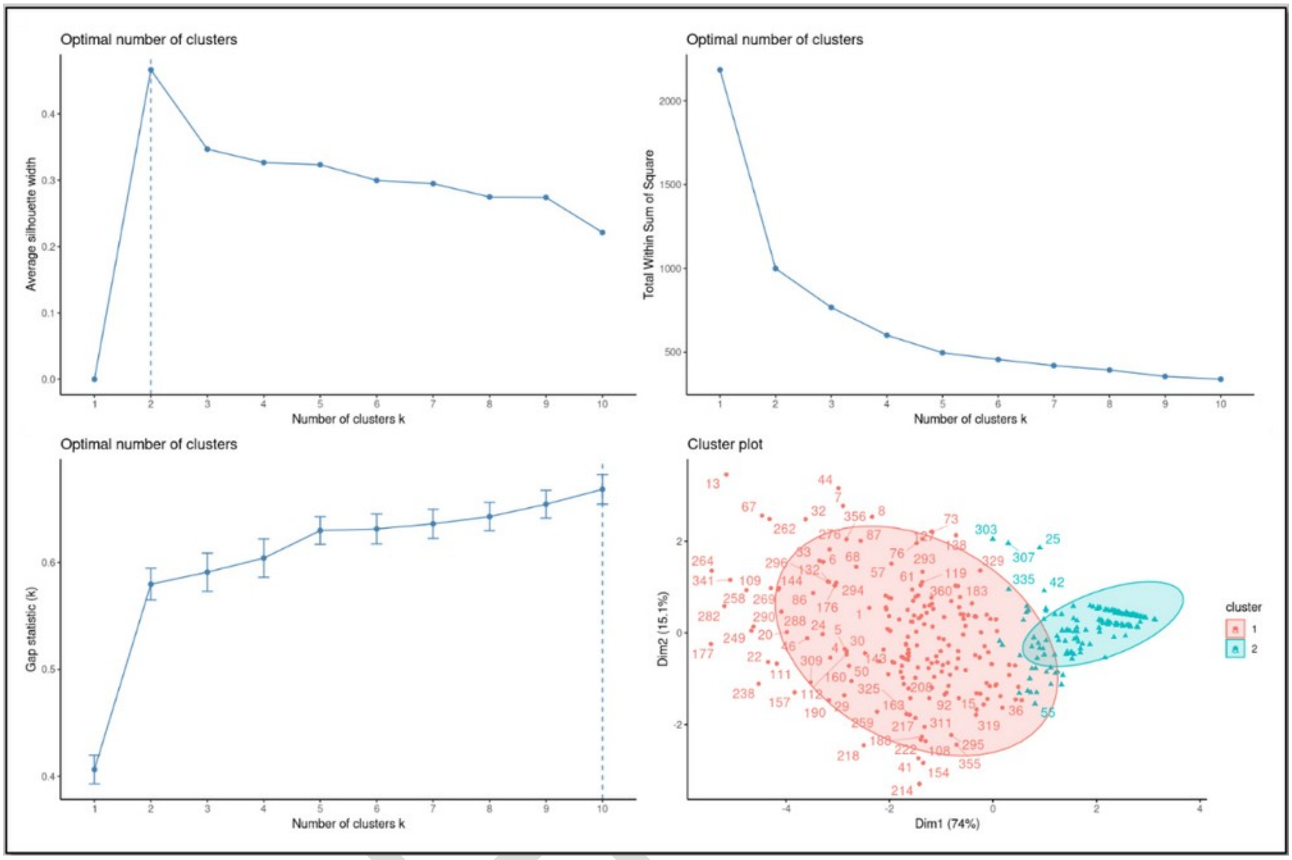

Fig. S1. Optimal number of clusters, according to the silhouette width method, the within sum-of-squares and the gap statistics method. And overall cluster plots (lower-right figure) representing the distribution of observations between the two clusters.

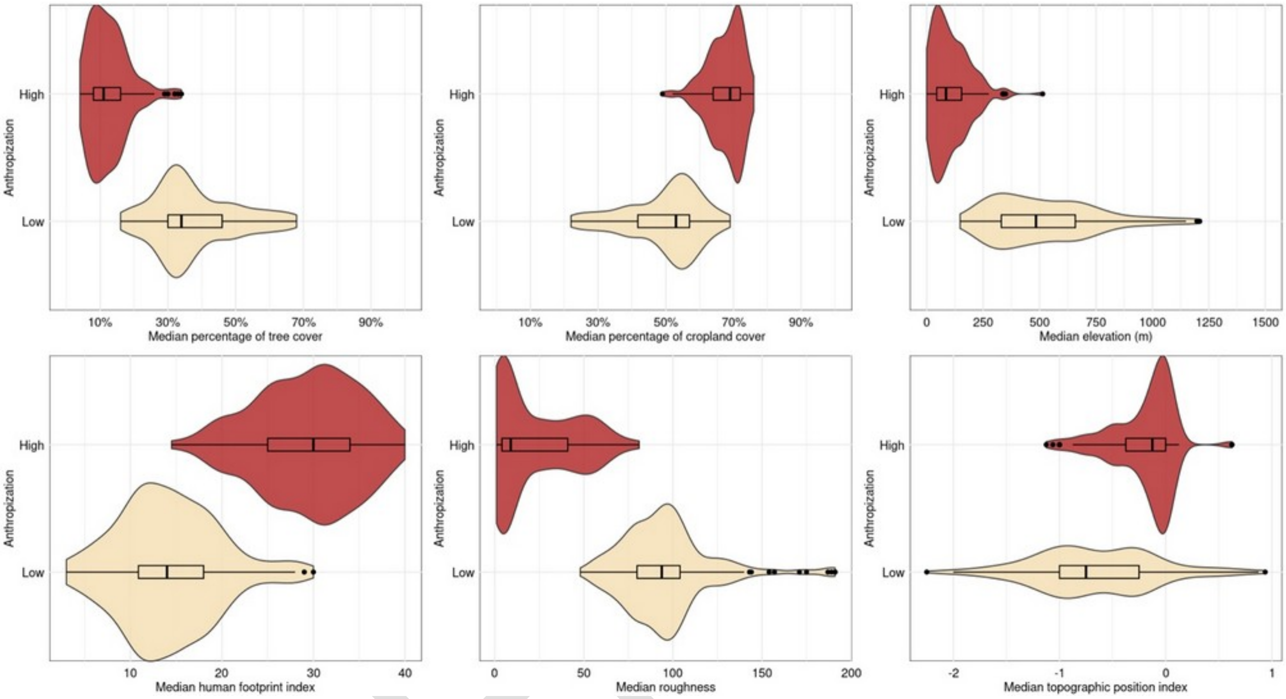

Fig. S2. Characteristics of areas categorized, through PAM cluster analysis, as having low or high anthropization: median percentage of tree cover, median percentage of cropland cover, median elevation, median human footprint index, median roughness, and median topographic position index.

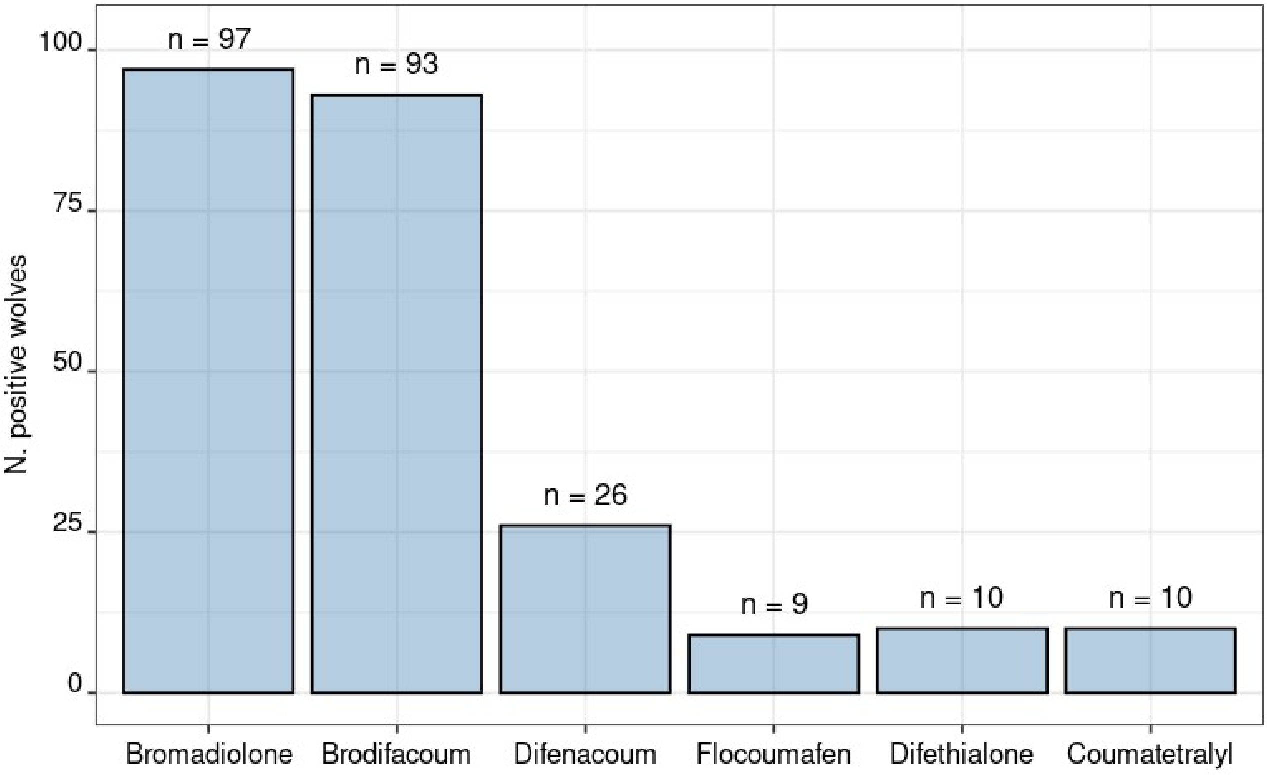

Fig. S3. Most common anticoagulant rodenticides (ARs) that were found in wolves. Total number of individuals that tested positive for each compound.

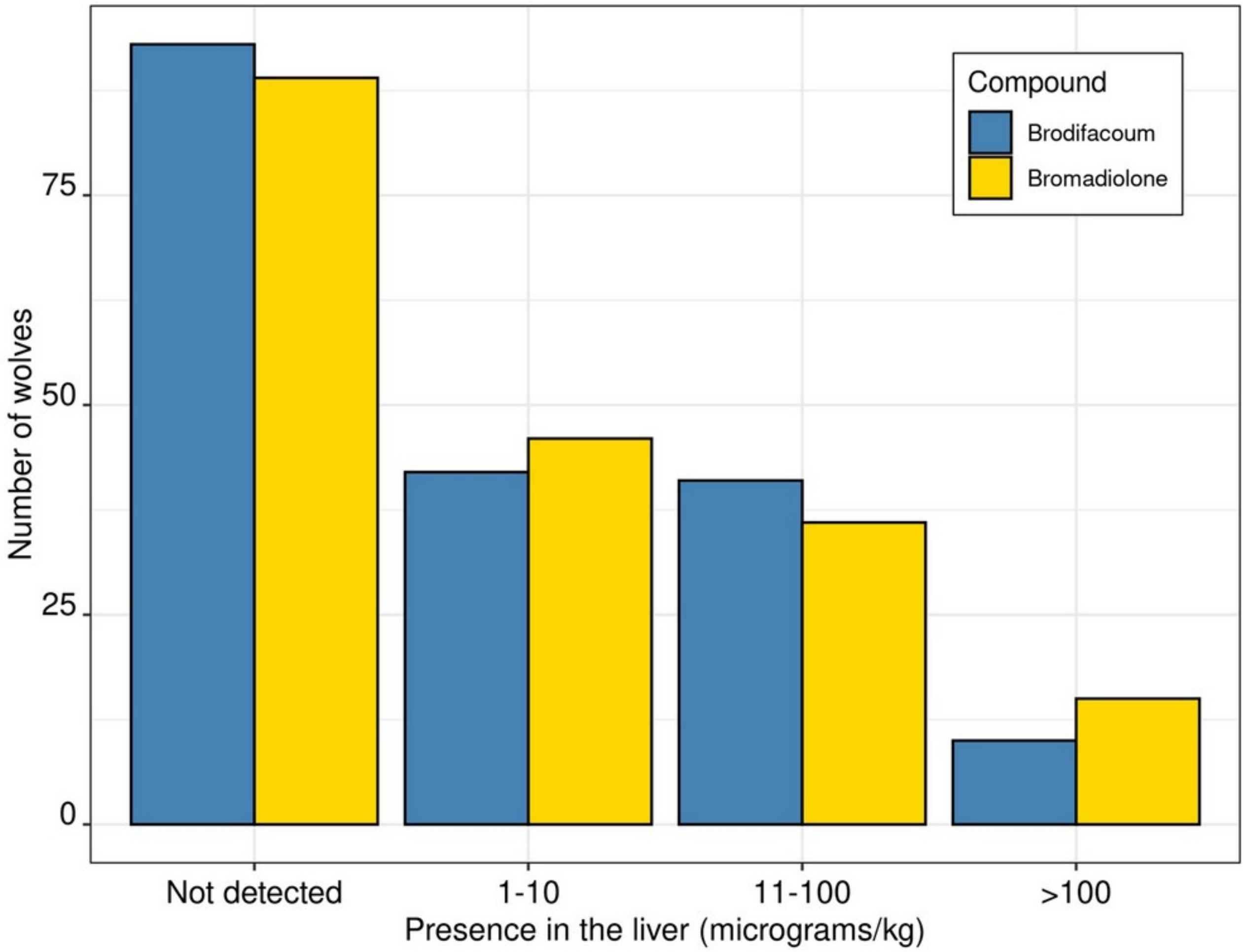

Fig. S4. Presence in the liver, as micrograms/kg, of Brodifacoum and Bromadiolone. Number of wolves where compounds were not detected, and where concentration was between 0 and 10, 11 and 100 and over 100 micrograms/kg.

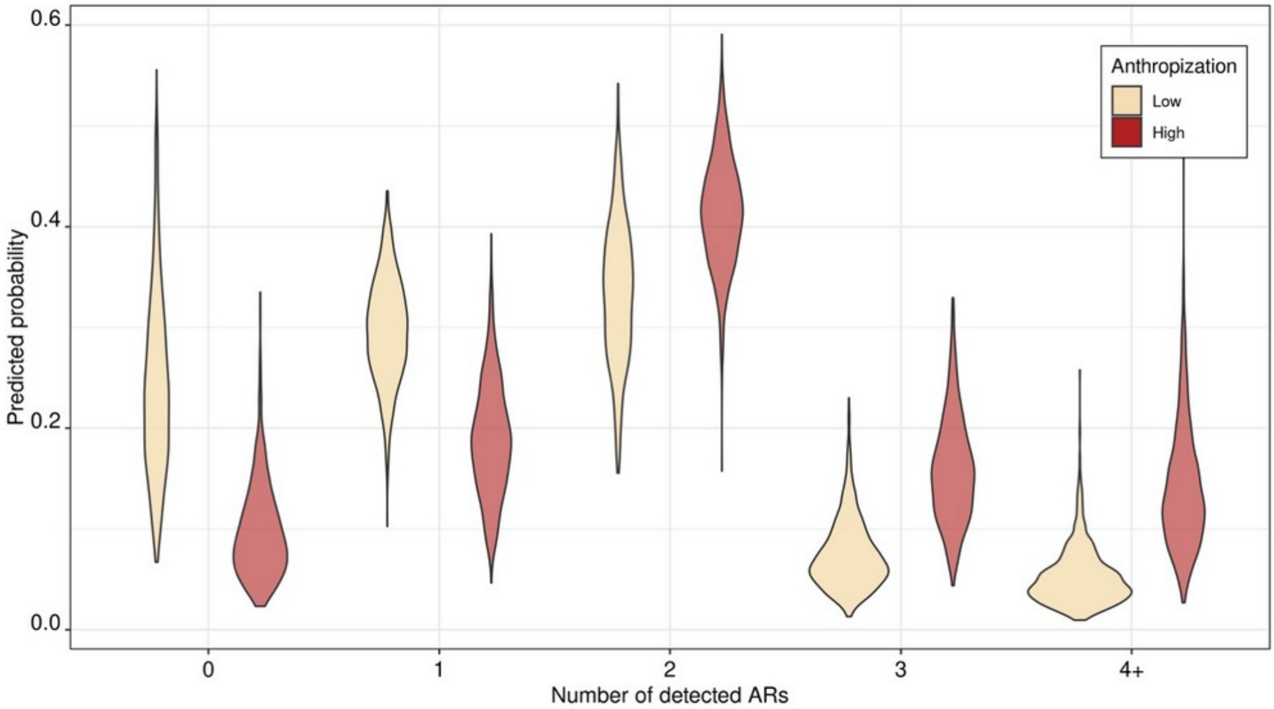

Fig. 2. Predicted probabilities that wolves tested positive for a certain number of anticoagulant rodenticides (ARs), between areas with different levels of anthropization. Conditional effect plot from the Bayesian ordered logit model, representing the posterior distribution: the largest section of the violin plot indicate values with the highest probability.

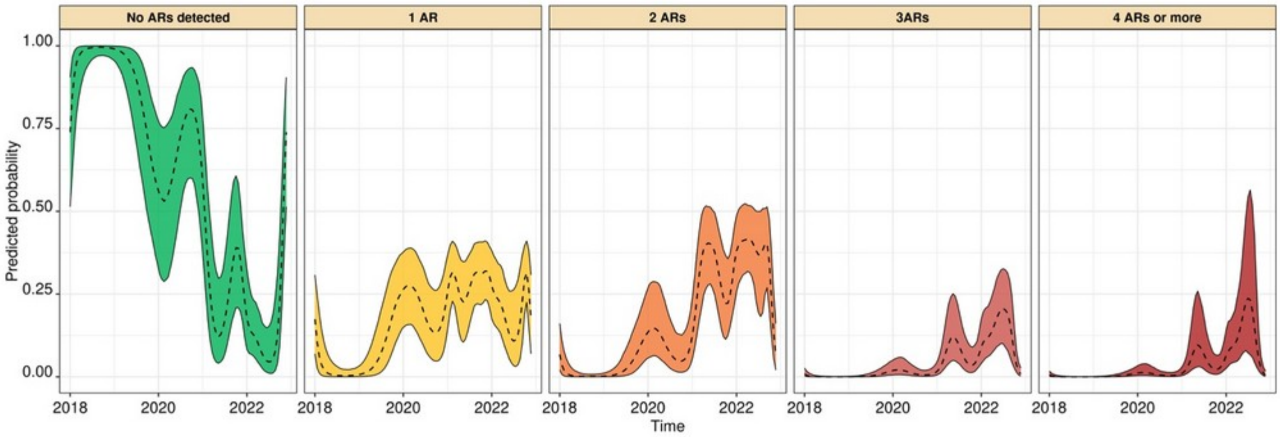

Fig. 3. Predicted probabilities that wolves tested positive for a certain number of anticoagulant rodenticides (ARs), through time. Conditional effect plot from the Bayesian ordered logit model.

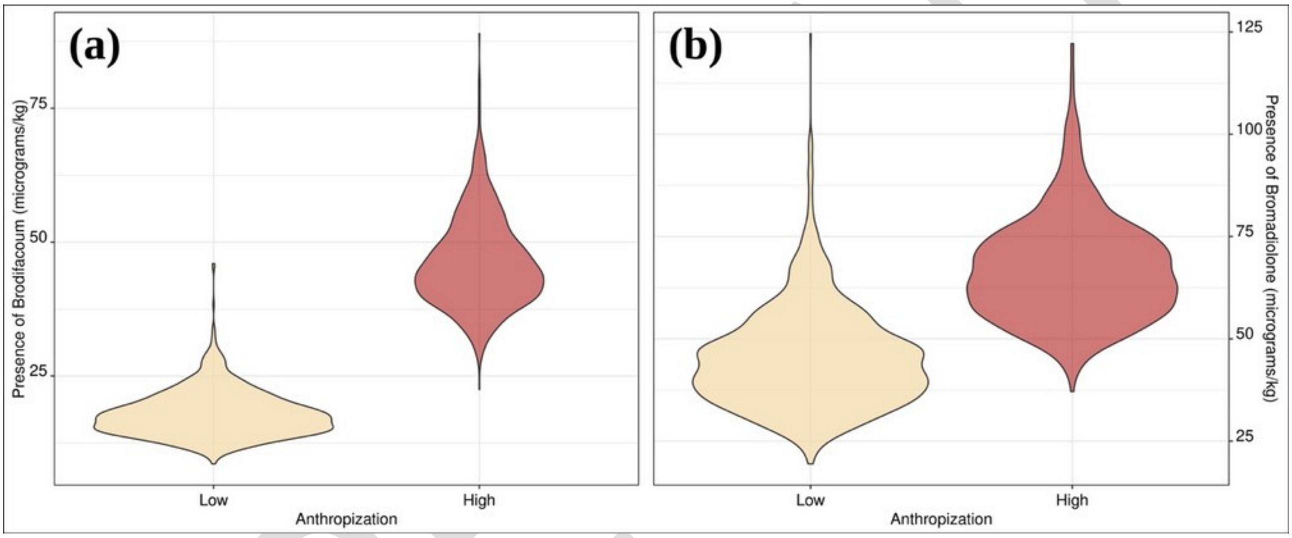

Fig. 4. Predicted concentrations of Brodifacoum and Bromadiolone, expressed as micrograms per kg, between areas with different levels of anthropization. Conditional effect plot from the Bayesian zero-altered Gamma model, representing the posterior distribution: the largest section of the violin plot indicates values with the highest probability.

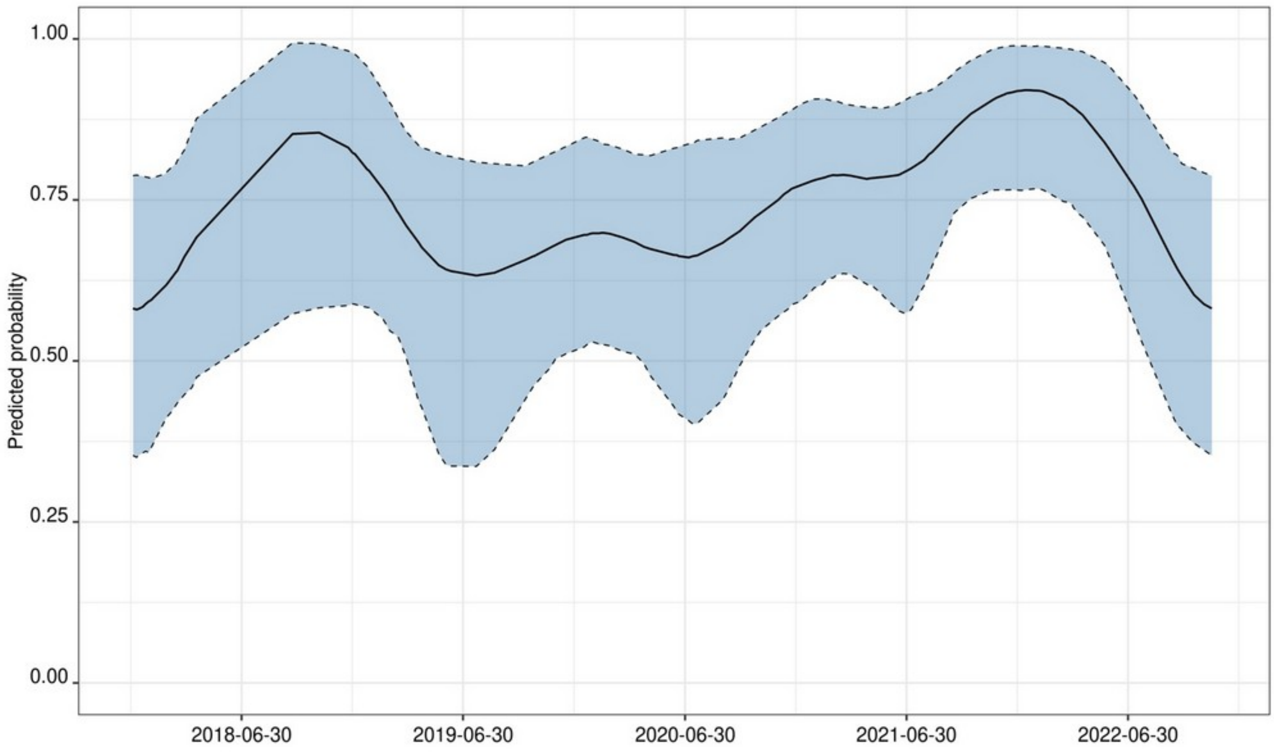

Fig. S5. Predicted probability that wildlife recovered in Emilia-Romagna region tested positive for anticoagulant rodenticides (ARs), through time. Conditional effect of the Bayesian Bernoulli regression. Mean value from the posterior distribution (dashed line) altogether with 95% Bayesian credibility intervals (highlighted area between solid lines.

Tables

Table 1. Number of anticoagulant rodenticide formulations available on the market in Italy, based on active ingredient (data updated to 2020). These include rodenticides falling under Product-Type (PT) 14, i.e., formulations intended for Trained Professionals, Professionals, General public. Source: Sinergitech, 2020.

Active ingredient	n	generation
Brodifacoum	121	2nd
Bromadiolone	98	2nd
Difenacoum	68	2nd
Chlorophacinone	4	1st
Coumatetralyl	4	1st
Difethialone	8	2nd
Flocoumafen	3	2nd
Bromadiolone+Difenacoum	3	2nd

Table S1. MS/MS transitions parameters and retention times for 11 ARs (the quantifier transitions are reported in bold)

Analyte	*TRANSITIONS (CE=COLLISION ENERGY)*	Retention Time (min)
COUMAFURYL	**297,1 → 161** (CE 12 V, Fragmentor 132 V) 297,1 → 240 (CE 12 V, Fragmentor 132 V)	1,58
WARFARIN	**307,1 → 161** (CE 12 V, Fragmentor 133 V) 307,1 → 250,1 (CE 16 V, Fragmentor 133 V)	2,67
COUMATETRALYL	**291,1 → 141** (CE 24 V, Fragmentor 158 V) 291,1 → 143 (CE 40 V, Fragmentor 158 V)	4,38
COUMACHLOR	**341,1 → 284** (CE 20 V, Fragmentor 148 V) 341,1 → 161 (CE 16 V, Fragmentor 148 V)	4,57
BROMADIOLONE	**525,1 → 250,1** (CE 36 V, Fragmentor 215 V) 525,1 → 93 (CE 40 V, Fragmentor 215 V)	6,86
DIPHACINONE	**339,1 → 167** (CE 20 V, Fragmentor 220 V) 339,1 → 116 (CE 40 V, Fragmentor 220 V)	7,21
DIFENACOUM	**443,2 → 135** (CE 36 V, Fragmentor 210 V) 443,2 → 293,1 (CE 32 V, Fragmentor 210 V)	7,70
CHLOROPHACINONE	**373,1 → 201** (CE 16 V, Fragmentor 220 V) 373,1 → 145 (CE 16 V, Fragmentor 220 V)	7,96
FLOCOUMAFEN	**541,2 → 161** (CE 36 V, Fragmentor 215 V) 541,2 → 382,1 (CE 24 V, Fragmentor 215 V)	8,17
BRODIFACOUM	**521,1 → 135** (CE 40 V, Fragmentor 210 V) 521,1 → 78,9 (CE 40 V, Fragmentor 210 V)	8,66
DIFETHIALONE	**537 → 79** (CE 40 V, Fragmentor 215 V)	9,58

	537 → 371,3 (CE 40 V, Fragmentor 215 V)	

Table S2. Most common co-occurrences of anticoagulant rodenticides in recovered wolves.

	Brodifacoum	Bromadiolone	Difenacoum	Flocoumafen	Difethialone	Coumatetralyl
Brodifacoum	-	61	20	7		9
Bromadiolone	-	-	19	7		9
Difenacoum	-	-	-	3	1	5
Flocoumafen	-	-	-	-	3	0
Difethialone	-	-	-	-	-	1
Coumatetralyl	-	-	-	-	-	-

Appendix S1 – Overview of model selection and diagnostics

Ordered logit regression

Table S2. Model comparison from leave-one-out cross-validation, representing theoretical expected log pointwise predictive density (ELPD) and their standard error (SE). Leave-one-out cross retained the time when wolves were found and the level of anthropization of the site where they had been found. Splines follow the following nomenclature (Wood, 2017): “s” = thin plate spline, “cc” = cyclic cubic spline.

Model structure	ELPD ± S.E.
N. rodenticides ~ 1	-274.8 ± 7.2
N. rodenticides ~ anthropization	-268.2 ± 7.4
N. rodenticides ~ anthropization + sex	-268.9 ± 7.9
N. rodenticides ~ anthropization + sex + age class	-264.8 ± 7.8
N. rodenticides ~ anthropization + sex + s(time, bs = “cc”)	-216.7 ± 11.0
N. rodenticides ~ anthropization + sex + s(time, bs = “cc”) + s(lon, lat)	-216.8 ± 11.0

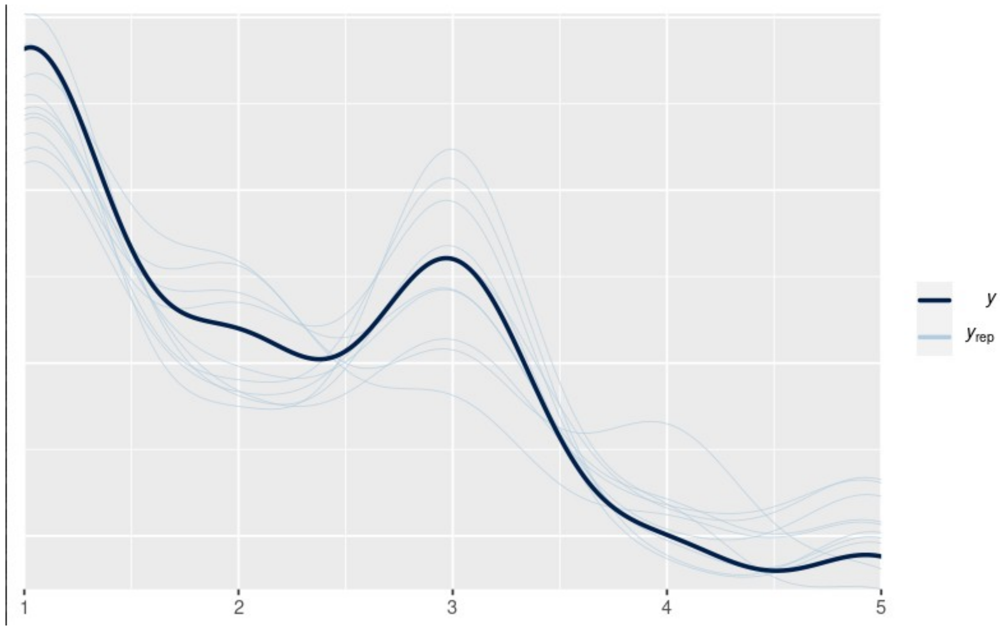

Fig. S6. Comparison between the empirical distribution of the data (*y*) with the distributions of simulated/replicated data from the posterior predictive distributions (y_{rep}). See: https://mc-stan.org/bayesplot/reference/PPC-distributions.html

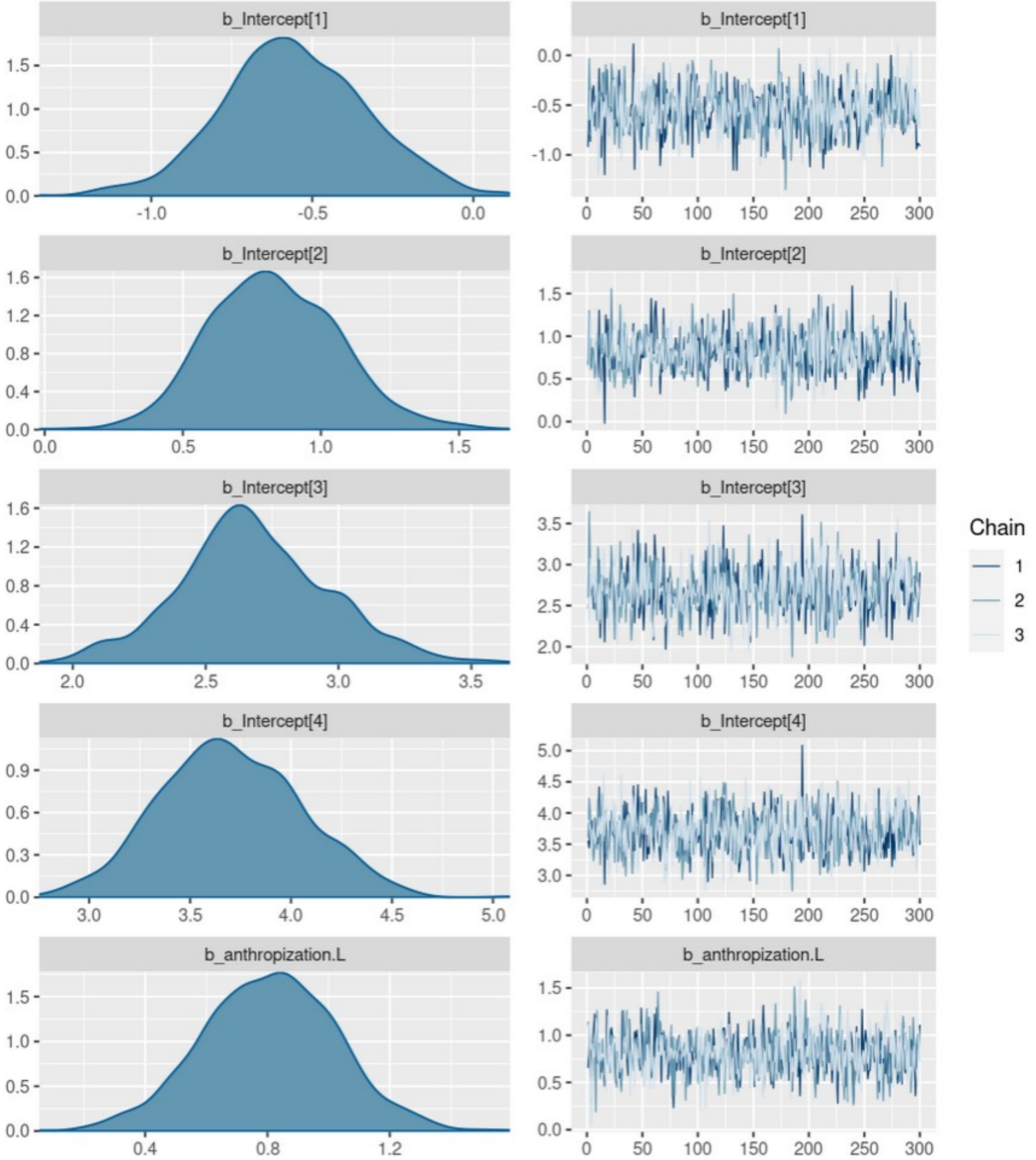

Fig. S7. Overview of the posterior distribution of model parameters (left) and MCMC (right).

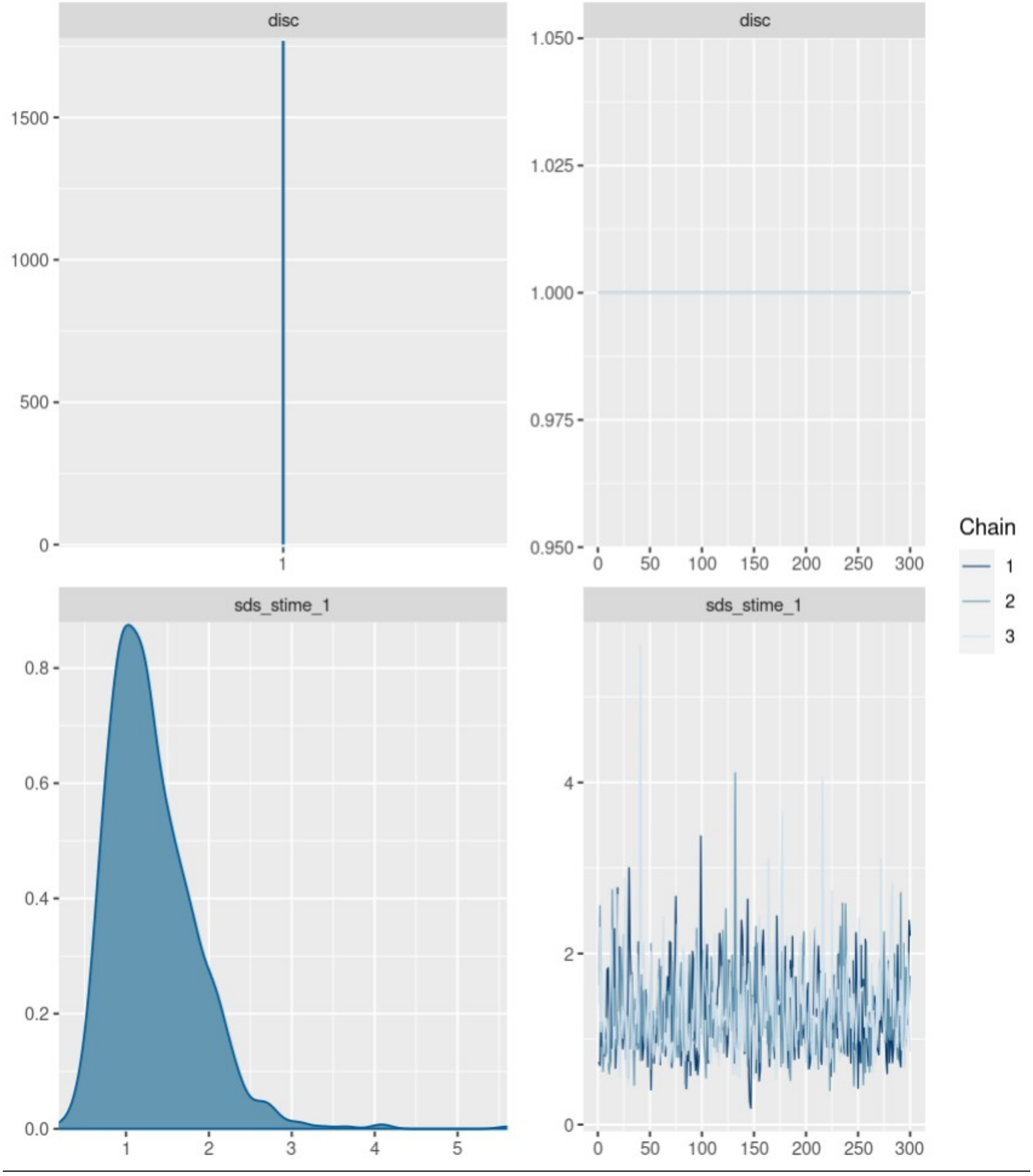

Fig. S8. Overview of the posterior distribution of model parameters (left) and MCMC (right).

Zero-altered gamma regression: Brodifacoum concentration

Table S3. Model comparison from leave-one-out cross-validation, representing theoretical expected log pointwise predictive density (ELPD) and their standard error (SE). Leave-one-out cross retained the level of anthropization of the site where they had been found. Splines follow the following nomenclature (Wood, 2017): “s” = thin plate spline, “cc” = cyclic cubic spline.

Model structure	ELPD ± S.E.
N. rodenticides ~ 1	-414.3 ± 15.9
N. rodenticides ~ anthropization	-410.5 ± 15.3
N. rodenticides ~ anthropization + sex	-410.3 ± 15.2
N. rodenticides ~ anthropization + sex + age class	-412.0 ± 15.5
N. rodenticides ~ anthropization + sex + s(lon, lat)	-411.6 ± 15.3
N. rodenticides ~ anthropization + sex + s(time, bs = “cc”) + s(lon, lat)	-411.9 ± 15.5

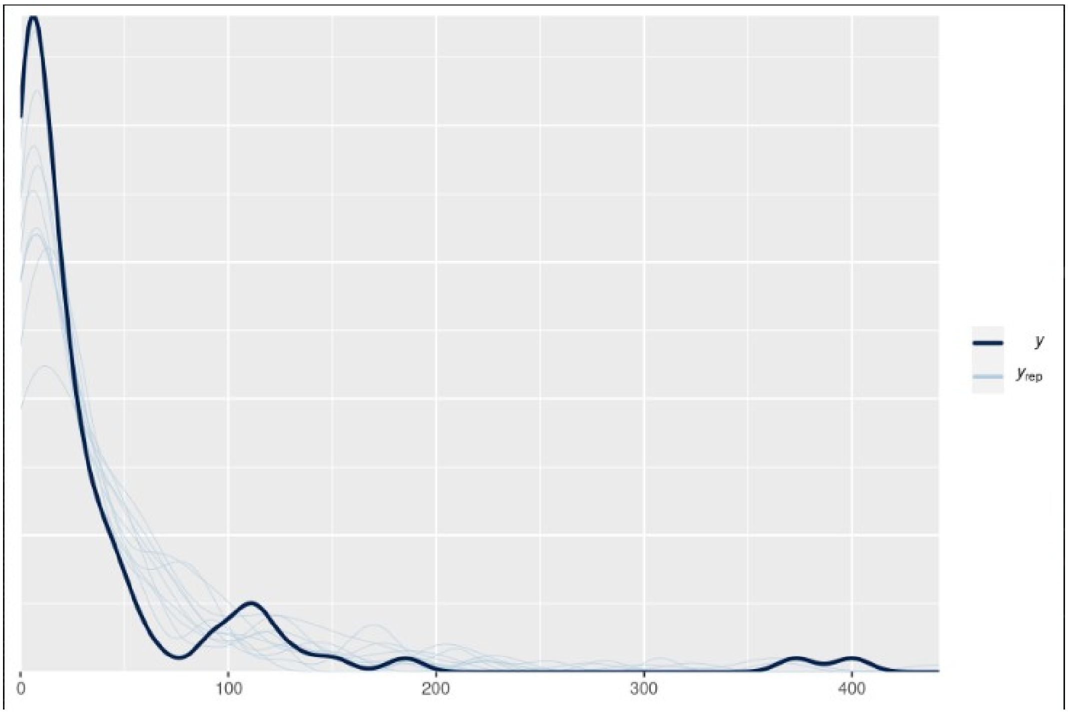

Fig. S9. Comparison between the empirical distribution of the data (*y*) with the distributions of simulated/replicated data from the posterior predictive distributions (y_{rep}). See: https://mc-stan.org/bayesplot/reference/PPC-distributions.html

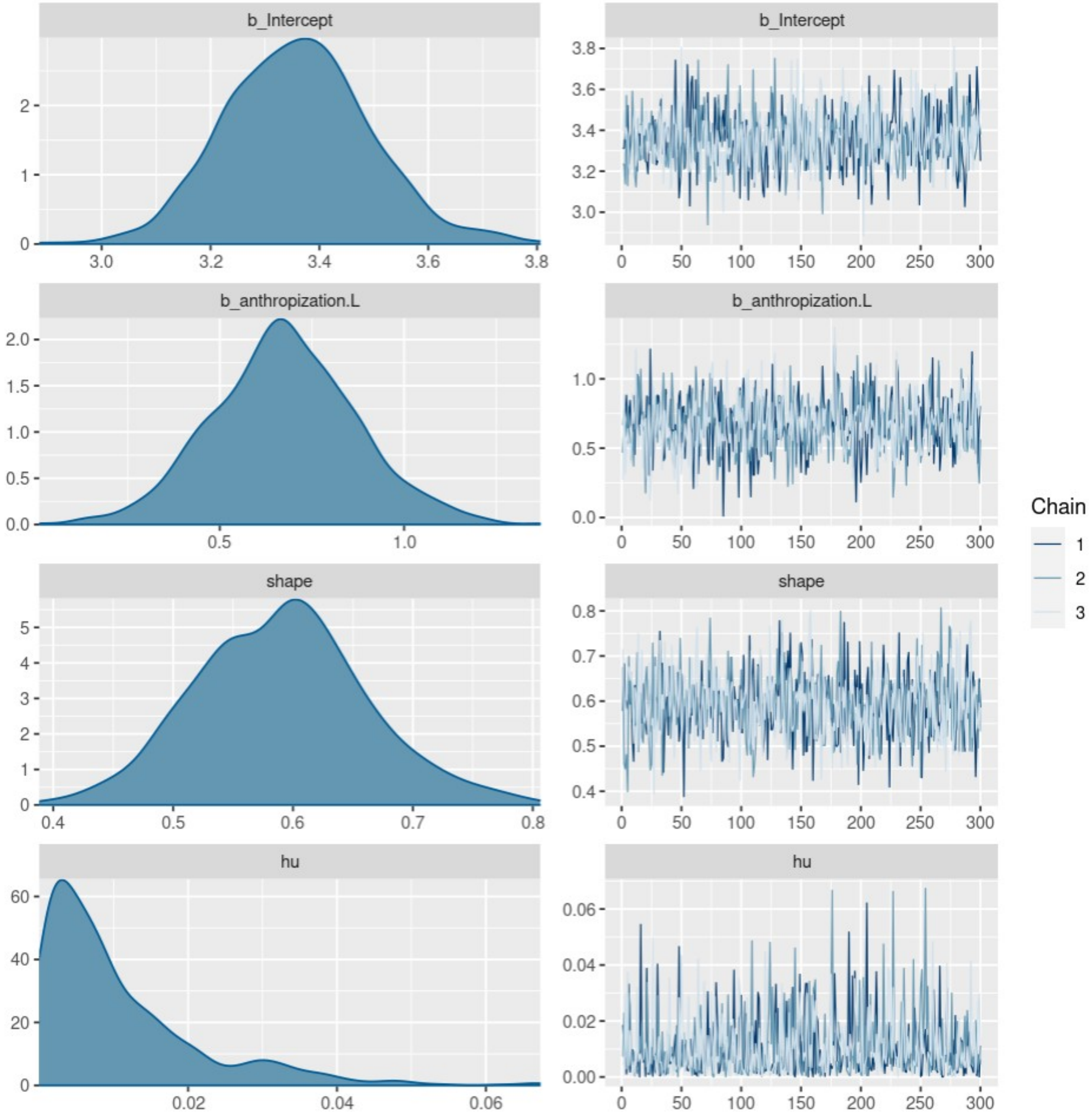

Fig. S10. Overview of the posterior distribution of model parameters (left) and MCMC (right).

Zero-altered gamma regression: Bromadiolone concentration

Table S4. Model comparison from leave-one-out cross-validation, representing theoretical expected log pointwise predictive density (ELPD) and their standard error (SE). Leave-one-out cross retained the time when wolves were found and the level of anthropization of the site where they had been found. Splines follow the following nomenclature (Wood, 2017): “s” = thin plate spline, “cc” = cyclic cubic spline.

Model structure	ELPD ± S.E.
N. rodenticides ~ 1	-467.9 ± 16.7
N. rodenticides ~ anthropization	-469.1 ± 17.5
N. rodenticides ~ anthropization + sex	-471.0 ± 17.2
N. rodenticides ~ anthropization + sex + age class	-471.8 ± 18.2
N. rodenticides ~ anthropization + s(lon, lat)	-468.4 ± 17.0
N. rodenticides ~ anthropization + s(time, bs = “cc”)	-464.8 ± 17.2

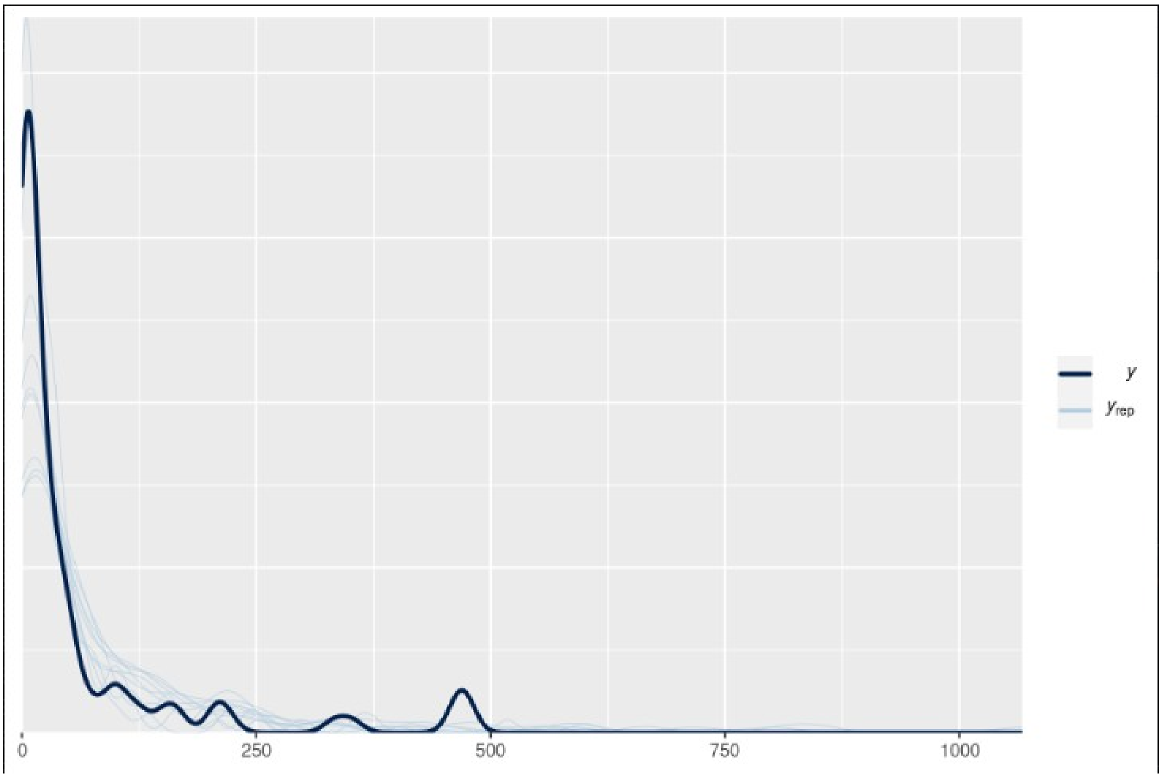

Fig. S11. Comparison between the empirical distribution of the data (*y*) with the distributions of simulated/replicated data from the posterior predictive distributions (y_{rep}). See: https://mc-stan.org/bayesplot/reference/PPC-distributions.html

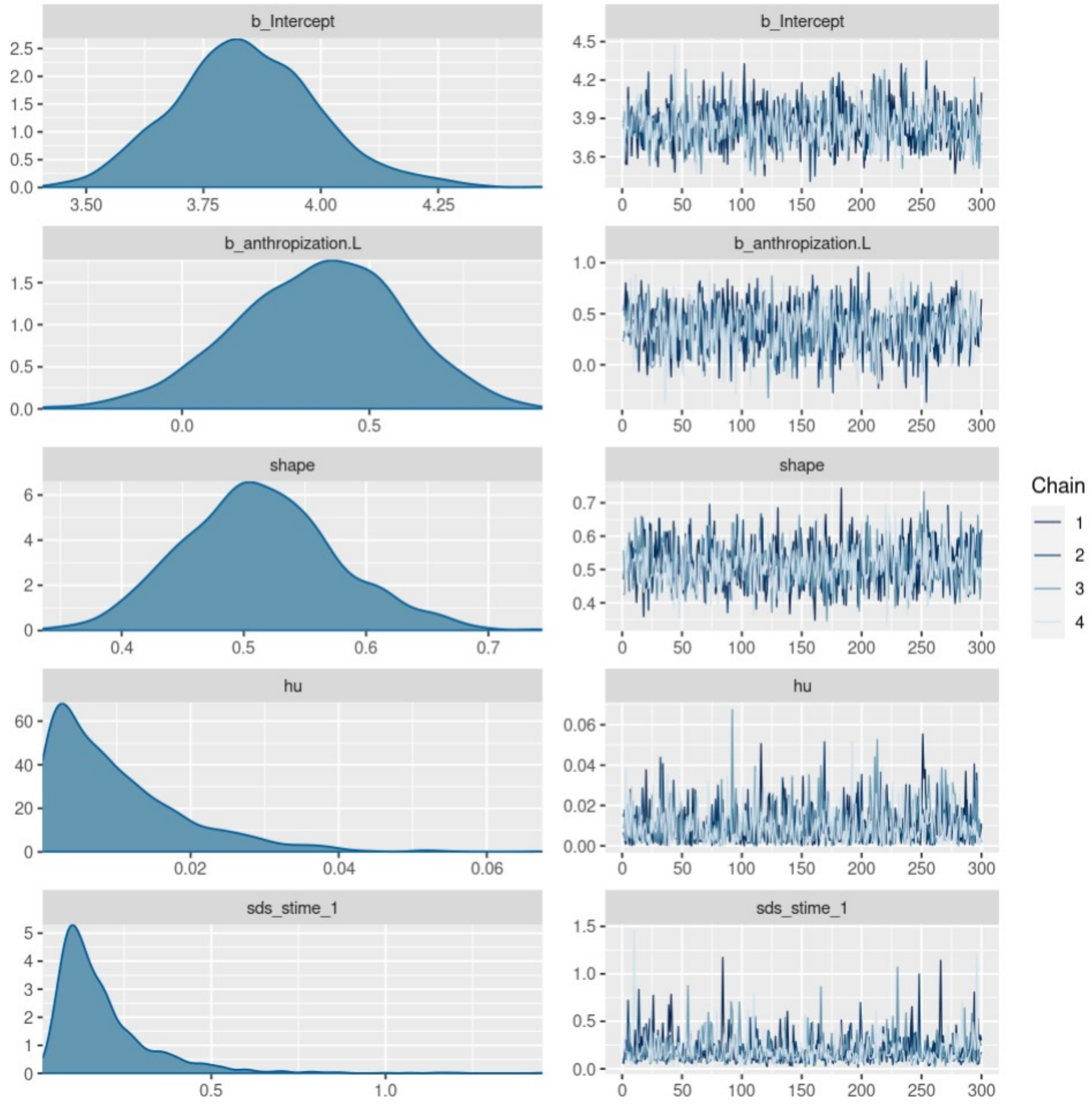

Fig. S12. Overview of the posterior distribution of model parameters (left) and MCMC (right).

Bernoulli regression

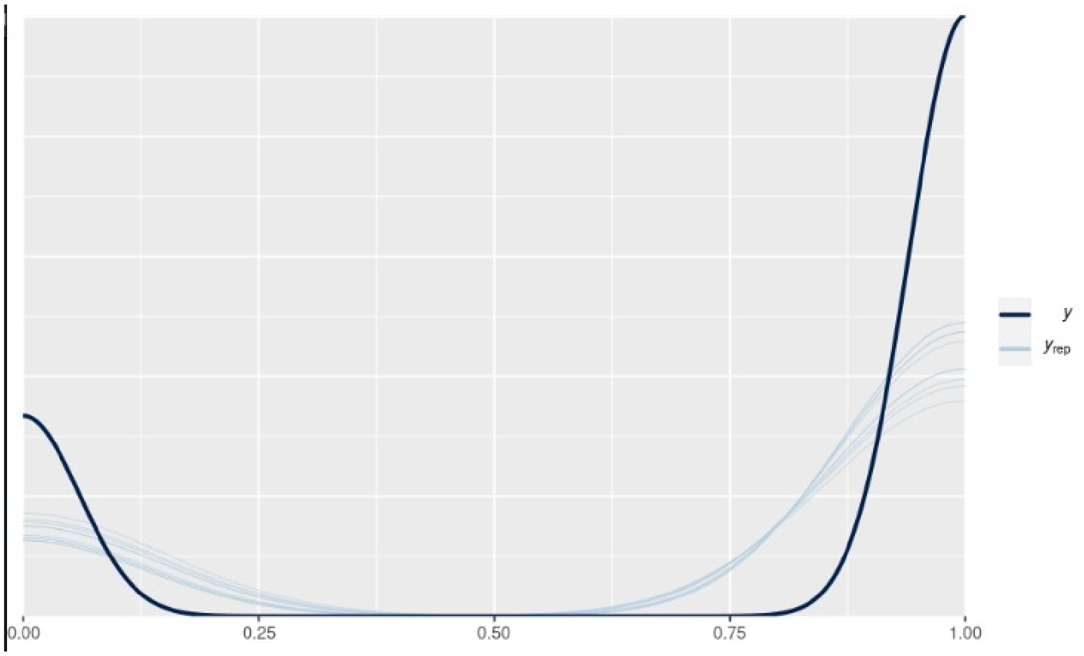

Fig. S13. Comparison between the empirical distribution of the data (*y*) with the distributions of simulated/replicated data from the posterior predictive distributions (y_{rep}). See: https://mc-stan.org/bayesplot/reference/PPC-distributions.html

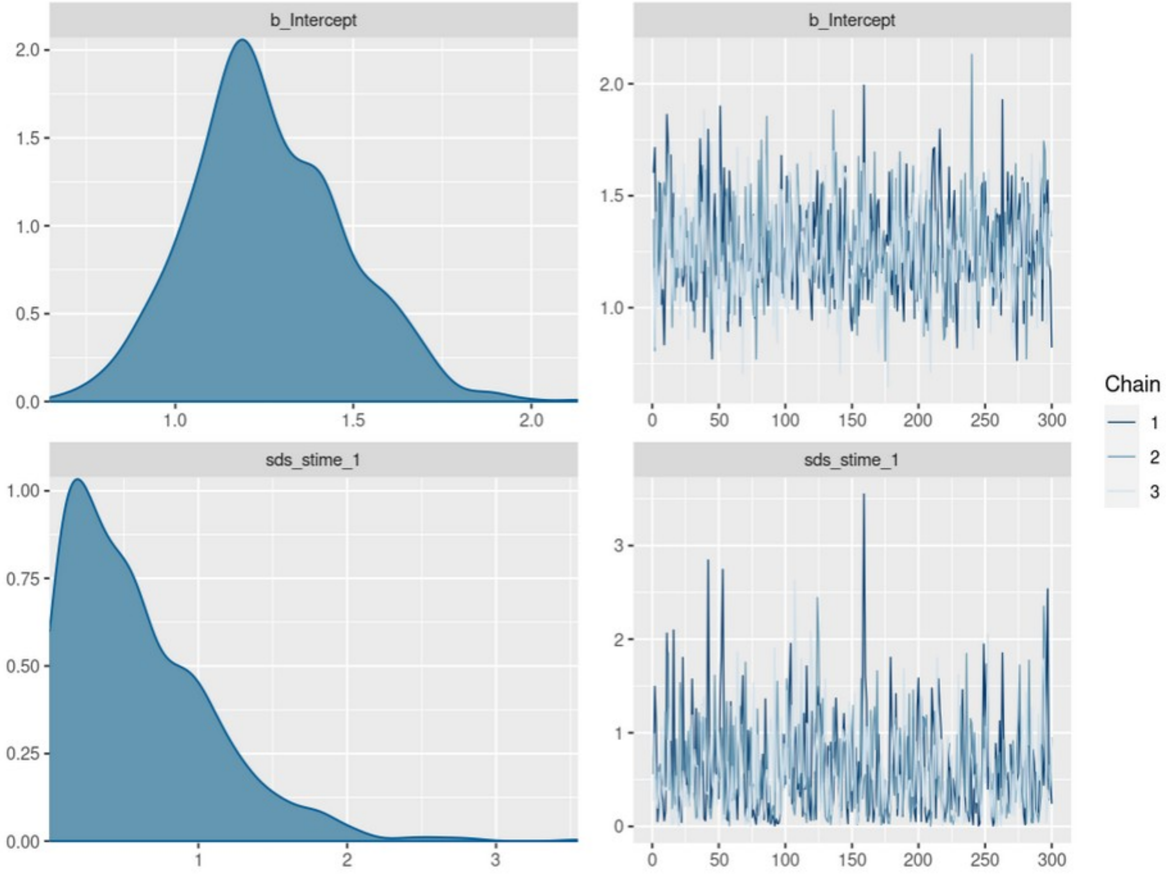

Fig. S14. Overview of the posterior distribution of model parameters (left) and MCMC (right).