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Short communication

Widespread resistance to anticoagulant rodenticides in *Mus musculus domesticus* in the city of Barcelona

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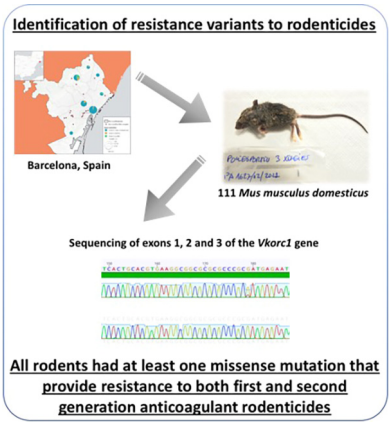
HIGHLIGHTS

- All *Mus musculus domesticus* carried missense mutations in at least one of the exons of the *Vkorc1* gene.
- 94.60 % of individuals carried mutations in exon1 associated with past introgression events.
- Only 13.51 % of analyzed individuals presented at least one mutation in exon 3.
- Detected mutations provide resistance to anticoagulant rodenticides.
- Resistance to rodenticides in Barcelona is driven by adaptive introgression and selective pressure.

GRAPHICAL ABSTRACT



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ABSTRACT

Control of rodent populations is a big challenge because of the rapid evolution of resistance to commonly used rodenticides and the collateral negative impacts that these products may have on biodiversity. Second-generation anticoagulants are very efficient but different single nucleotide polymorphisms (SNPs) in the *Vkorc1* gene may confer resistance in rodents. We sequenced exons 1, 2 and 3 of the *Vkorc1* gene from 111 mice (*Mus musculus domesticus*) captured across the city of Barcelona and found SNPs associated with resistance to first- and second-generation anticoagulants in all of them. Although most of the SNPs were associated with resistance to bromadiolone, we also found SNPs associated with resistance to brodifacoum. Out of all the individuals analyzed, 94.59 % carried mutations associated to introgression events with *Mus spretus*, a sympatric rodent species. Currently most of the chemical products for rodent control commercialized in the area are based on bromadiolone, although recent public control campaigns have already shifted to other products. Thus, the widespread occurrence of resistant mice to bromadiolone represents a challenge for rodent control in Barcelona and may increase the risk of secondary poisoning of animals preying on this species. Public health managers, pest control companies and citizens should be aware that the use of bromadiolone based products is ineffective and represents a risk for the environment, including human and animal health.

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1. Introduction

The emergence and spread of pesticide resistance are major challenges in the management and control of rodent populations. Rodents are responsible for a wide range of impacts on the environment, public health and economy, and over the centuries different biological, physical and chemical methods have been used to attempt to control rodent populations. Currently, anticoagulant rodenticides are the most commonly used pesticides worldwide (Buckle and Eason, 1994). Its use started in the early 1950s, with the development of warfarin, diphacione and coumatralyl (Buckle and Eason, 1994). However, a decade after its use began, the first cases of resistance in mice and rats started to be reported worldwide (Boyle, 1960; Buckle, 2013; Endepols et al., 2007; McGee et al., 2020). To overcome this resistance, a battery of second-generation anticoagulants including bromadiolone, difenacoum, flocoumafen, brodifacoum and diphetialone, among others, were developed. Second-generation anticoagulants are very efficient single-feeding compounds, and are extensively used to control rodent populations. However, there are several concerns regarding its use. First, some of these compounds are also affected by resistance (Greaves et al., 1982; Endepols et al., 2007; MacNicoll and Gill, 1987; Rowe et al., 1981). Second, they have longer half-life periods in individuals than the first-generation anticoagulants and can bioaccumulate leading to potential secondary toxicity when predators consume those rodents (Nakayama et al., 2019; Ray et al., 1989; Vein et al., 2013). Thus, evaluating the prevalence of resistance in rodent populations is critical to minimize the health, ecological and economic impact of rodent pest control.

One of the most studied resistance mechanisms involves the enzyme vitamin K epoxide reductase (VKOR) which is the target of anticoagulant rodenticides (Rost et al., 2004). When the anticoagulants block VKOR, the lack of bioavailable vitamin K leads to the absence of gamma-carboxilated clotting factors and compromises the coagulation process. As a result, anticoagulant rodenticides act causing internal hemorrhages and eventually death. Mutations in the gene *vkorc1* that codes for the complex subunit 1 of the VKOR enzyme (VKORC1) affect the efficiency of anticoagulant rodenticides (Pelz et al., 2005; Rost et al., 2004). The emergence of rodenticide resistances seems to have different origins. In general, pesticide resistance is the result of rapid evolution under a novel selective pressure (Hawkins et al., 2019) that acts on either de novo mutations or standing variation. In addition, pre-adaptation through pleiotropic effects of existing adaptations, and interspecific gene transfer, including interbreeding that leads to adaptive introgression, can play an important role in the rapid development of pesticide resistance (Hawkins et al., 2019). In rodents, the pattern of different *vkorc1* variants increasing in different areas suggests that de novo mutations could underlie adaptation at this locus (Pelz et al., 2005, 2012). However, the high speed of adaptation suggested that the different variants were already present in low frequency and that adaptation resulted from standing variation (Hedrick, 2013; Hermisson and Pennings, 2005). In addition, for mice, the origin of some of the resistance variants is the result of adaptive introgression between the western European mouse (*Mus musculus domesticus*) and the Algerian mouse (*Mus spretus*) (Liu et al., 2015). Song et al. (2011) identified a 20-megabase segment in the chromosome 7 of *M. m. domesticus* introgressed from *M. spretus* that contains anticoagulant resistance variants of gene *vkorc1*. This introgression has a selective advantage because the mice carrying the variants have higher survival when exposed to different rodenticides (Song et al., 2011), despite the sterility of the first generation hybrid males and the partial genetic incompatibilities between the genomes of the two species (Orth et al., 2002).

In this study we will focus on identifying resistance variants in the house mice (*M. m. domesticus*), which is considered one of the 100 most invasive species in the world (Global Invasive Species Database, 2022). House mice are also recognized to be one of the species with bigger economic and ecological impact (Capizzi et al., 2014). Until now, 15 different mutations in the *Vkorc1* gene of mouse have been associated with resistant phenotypes (Goulois et al., 2017; McGee et al., 2020). The distribution and frequency of these mutations vary between areas. For example, while some studies report a low frequency of mutations in the *vkorc1 gene* (3.3 % (Iannucci et al., 2019) others found percentages of resistance associated variants over 70 % (Goulois et al., 2017; Mooney et al., 2018; Song et al., 2011). Such differences are probably largely caused by the differences in management of rodent populations and the anticoagulants used for control. But there are other factors that could drive this pattern. For example, standing genetic variation present in the population and/or the natural history and distribution of different mouse species can largely determine how populations respond to the use of anticoagulants. In particular, individuals carrying the resistance variants derived from the adaptive introgression with *M. spretus* have been found in rural areas in Spain (where *M. musculus* and *M. spretus* can naturally hybridize because they are sympatric; Song et al., 2011), France (Goulois et al., 2017) and Germany (Rost et al., 2009), but they haven't been described in the United Kingdom (Pelz et al., 2005), Ireland (Mooney et al., 2018), Italy (Iannucci et al., 2019) or the Azores archipelago (Rost et al., 2009). When comparing different studies, it is also important to consider the sampling effort, which varies greatly between studies and in some regions might be biased towards resistant mice. In addition, to improve management strategies and have an accurate representation on resistance frequency, studies need not only to use larger sample sizes, but also to consider the history of rodenticide use. The goal of this study was to map for the first time *vkorc1* gene mutations in *M. musculus domesticus* in an urban area in Spain and analyze the diversity and frequency of single nucleotide polymorphisms (SNPs) and haplotypes, to evaluate how widespread these mutations are. Compared to previous studies, we significantly increased the analyzed sampled size to have a good representation of mutation frequencies at the city level (Song et al., 2011). Furthermore, to fully understand resistance mutations found we also considered the history of rodenticide use in the area. This study represents a prime example of the importance of monitoring the emergence and spread of pesticide resistance considering both the compounds used and natural history of populations.

2. Material and methods

2.1. Sampling

The sampling was carried out in 726 municipal facilities in the city of Barcelona within the framework of the urban pest surveillance and control programme conducted by the *Agència de Salut Pública de Barcelona* (Barcelona Public Health Agency). The attention to complaints is the core of the programme, including those related to mice. These complaints are communicated by those in charge of the centres. Within 24–48 h, an inspection is carried out by the urban surveillance and pest control services. In those cases where it is considered necessary, an action plan is initiated, which may include the communication of corrective measures, as well as the placement of safety boxes with biocide, and a follow-up is carried out until the problem is resolved. The use of biocides in the pest surveillance and control programme in municipal buildings follows a technical approach based on scientific evidence (https://rrac.info/). We quantified in kg the different rodenticides used since 2014 under the rodent control program.

During the sampling period from 29/10/2018 to 31/01/2020, traps were installed inside the municipal facilities where an active control programme was in place or with a recent complaint about mice presence (Fig. 1). The traps were checked every two days. If there was no indication of the presence of mice, the traps were removed within one week. If there were signs of activity, the traps were kept in place. In total we captured 111 mice in 24 facilities (civic centres (45 %), administrative (30 %), municipal markets (9 %), social service centres (6 %), sports (6 %) and security forces (3 %)) (Table 1). All individuals trapped were initially assigned to the species *Mus musculus domesticus* based on morphology (*Mus musculus*: head + body length: 73.0–101.5 mm. Tail: 68.0–98.5 mm). *Mus spretus* was ruled out based on the biometric measurements of the specimens (*Mus spretus*: head + body length: 69.0–91.0 mm. Tail: 52.0–73.0 mm.) (Gosàlbez, 1987). A tail fragment was taken and stored at −20 °C for subsequent molecular analysis and species confirmation.

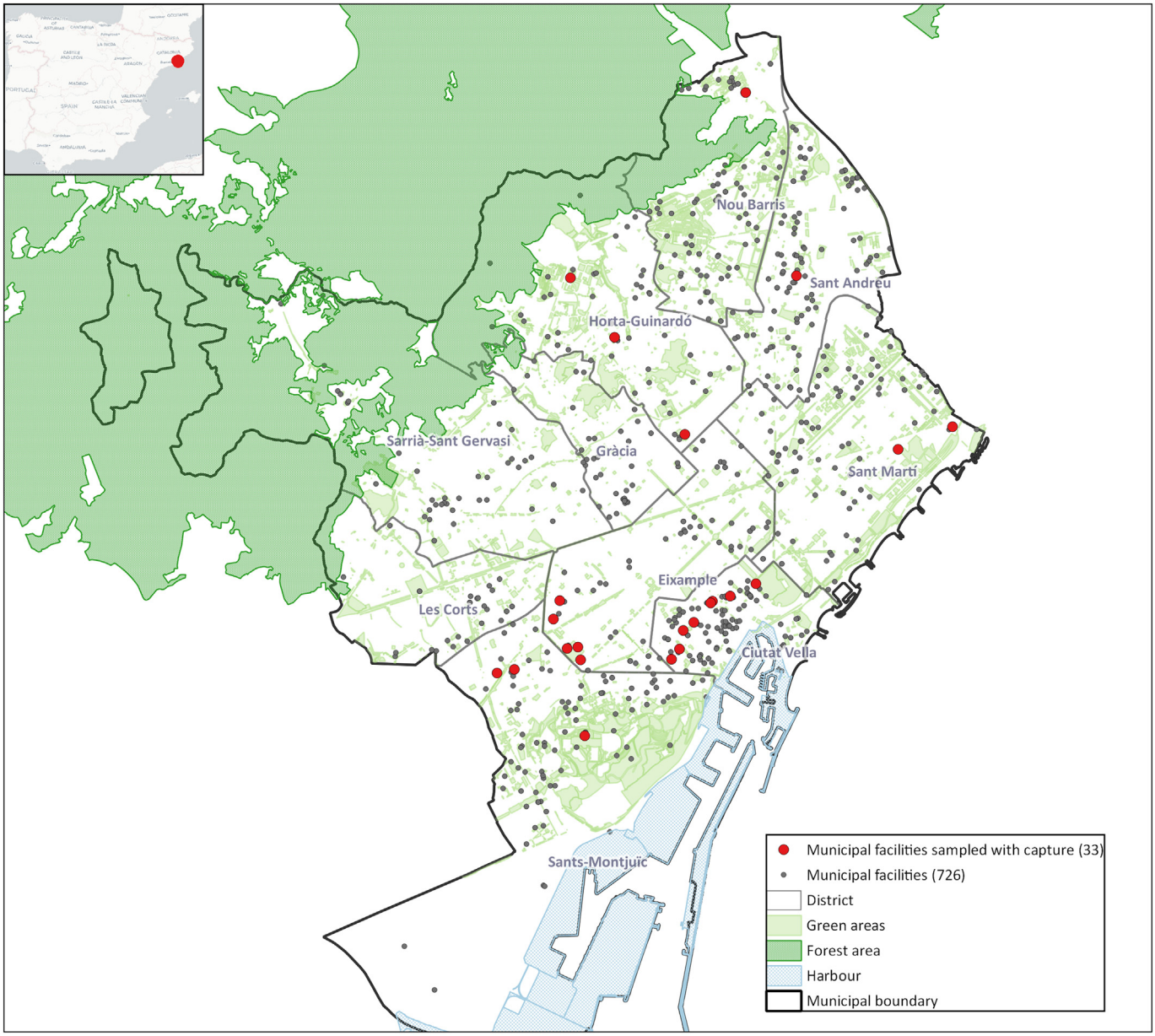

Fig. 1. Map showing the districts of Barcelona and the facilities sampled.

2.2. DNA extraction and molecular confirmation of the rodent species

Genomic DNA was extracted individually from 111 tail samples using the Maxwell®16 LEV system Research (Promega, Madison, WI) with the Maxwell®16 LEV Blood and Tissue DNA kit following the manufacturer's protocol. For each sample, a maximum of 50 mg of tissue with tail clippings shorter than 0.5 cm were used. We confirmed the species of rodent by the amplification and sequencing of a fragment of 748 bp of the cytochrome *c* Oxidase Subunit I (COI) mitochondrial gene following Alcaide et al., 2009. This procedure includes a first PCR using the primer pairs M13BC-FW (5′-TGT AAA ACG ACG GCC AGT-HAA YCA YAA RGA YAT YGG-3′) and BCV-RV1 (5′-GCY CAN ACY ATN CCY ATR (T)(A)-3′). The product of this PCR was subsequently used for a nested PCR using the primers M13-FW (GTA AAA CGA CGG CCA GTG) and BCV-RV2 (5′-ACY ATN CCY ATR TAN CCR AAN GG-3′). The amplified products were sequenced on both strands using Capillary Electrophoresis Sequencing by Macrogen (Madrid, Spain). Sequences were analyzed using Geneious v.2020.0.3 (Kearse et al., 2012). To identify the species two complementary steps were used: first COI sequences were mapped to the mitochondrial genomes of *Mus musculus* (GenBank accession number: AY172335.1) and *Mus spretus* (GenBank accession number: NC_025952.1) to identify mutations and haplotypes. Second, COI sequences were assigned to vertebrate species by comparison with those deposited in the Barcode of Life Data (BOLD) Systems platform (http://boldsystems.org/views/login.php). All 111 samples analyzed were identified to the species level as *Mus musculus*, matching the biometric determination and were used in subsequent analyses (GenBank accession number: ON453646-ON453655, see supplementary material for further details).

2.3. Vkorc1 sequencing

We analyzed the mutations in the *Vkorc1* gene in exon 1, exon 2 and exon 3 that are 174 bp, 110 bp and 202 bp long, respectively, and encode in total 161 amino acids. Independent PCR reactions were used for each exon using the primer pairs detailed in Table 2, which included musVKORC1-ex1F and musVKORC1-ex1F for exon-1 (Rost et al., 2004, Rost et al., 2009, and personal communication), VKORC1_ex2_F and VKORC1_ex2_R for exon-2 and VKORC1_ex3_F and VKORC1_ex3_R for

Table 1
Facilities monitored in this study, number of samples obtained and number of visits at each facility.

Name of municipal facility	District	Category	Number of samples	Visits in the study period
BIBLIOTECA JOAN MIRÓ	Eixample	Civic centres	5	17
BIBLIOTECA SANTA CREU	Ciutat Vella	Civic centres	5	31
BOMBERS EIXAMPLE	Eixample	Security forces	5	31
CAMP DE FUTBOL MENORCA	Nou Barris	Sports	1	2
CASAL DE BARRI TORRE BARO	Nou Barris	Civic centres	5	7
CASAL DEL JUBILAT JOSEP TRUETA	Ciutat Vella	Civic centres	35	51
CASAL GENT GRAN VALL D'HEBRÓN	Horta-Guinardó	Civic centres	1	21
CASTELL 3 DRAGONS	Ciutat Vella	Administrative	1	26
CB PROSPERITAT	Nou Barris	Civic centres	0	19
CENTRE CÍVIC CAN DEU	Les Corts	Civic centres	0	22
CENTRE CIVIC CASA GROGA	Horta-Guinardó	Civic centres	0	17
CENTRE DE BARRI DIAGONAL MAR	Sant Martí	Civic centres	2	6
CENTRE PENITENCIARI LA MODEL	Eixample	Administrative	1	26
CSS BAIX GUINARDÓ- CAN BARÓ	Horta-Guinardó	Social service	6	17
CSS ROQUETES - TRINITAT NOVA - CANYELLES	Nou Barris	Social service	0	11
DIRECCIÓ DE PROGRAMES PER LA DONA	Ciutat Vella	Administrative	4	20
EDIF.C.CENTRE CIVIC TALL- CGG COTXERES DE SANTS	Sants-Montjuïc	Civic centres	0	18
EDIFICI D AUDITORI COTXERES DE SANTS	Sants-Montjuïc	Civic centres	1	28
ESPAI ARENES	Eixample	Administrative	12	40
ESPAI ASSOCIATIU LOLA ANGLADA	Eixample	Civic centres	5	32
ESPAI D'ENTITATS PLAÇA 8 DE MARÇ	Ciutat Vella	Civic centres	2	35
ESPAI JOVE BOCA NORD	Horta-Guinardó	Civic centres	0	14
CENTRE FABRA I COATS	Sant Andreu	Civic centres	1	17
MERCAT CARMEL	Horta-Guinardó	Municipal markets	8	22
MERCAT SANTA CATERINA	Ciutat Vella	Municipal markets	4	25
MERCAT SANTS	Sants-Montjuïc	Municipal markets	2	22
MUSEU BLAU	Sant Martí	Administrative	1	20
PALAU DE FORONDA	Eixample	Administrative	0	5
PALAU DE LA VIRREINA	Ciutat Vella	Administrative	1	3
PISCINES PICORNELL	Sants-Montjuïc	Administrative	1	14
PISTES ESPORTIVES SANT RAFAEL	Ciutat Vella	Sports	2	28
SEU DISTRICTE_RECURSOS INTERNS	Ciutat Vella	Administrative	0	22
LA CAPELLA DEL H. ST. PAU	Ciutat Vella	Administrative	0	14

exon-3 (Iannucci et al., 2019). Fragments amplified were 298 bp, 801 bp, and 308 bp long, respectively. Reactions for *vkorc1* exon1 amplifications were carried out following Rost et al. (2004) conditions. Amplifications of *vkorc1* exons 2 and 3 were carried out in 25ul reaction volumes containing around 50 ng of genomic DNA, 1× Buffer, 3 mM MgCl2, 2 mM dNTPs (Bioline), 0.16 mg/ml BSA (Roche Diagnostics), 0,5uM of each primer, and 1Unit of Taq polymerase (BIOTAQTM DNA polymerase, Bioline). The amplification was performed at 94 °C, for 5 min, followed by 35 cycles of 94 °C for 45 s, 63 °C for exon 2 and 64 °C for exon 3 for 45 s, and 72 °C for 90 s, and a final extension step at 72 °C for 10 min. The amplified products were sequenced on both strands using Capillary Electrophoresis Sequencing by Macrogen (Madrid, Spain).

The sequences of each exon were analyzed using Geneious v. 2020.0.3 (Kearse et al., 2012). To screen for mutations, all the sequences were mapped to the *vkorc1* gene of both *M. m. domesticus* (GenBank accession number: GQ905715.1) and *M. spretus* (GenBank accession number: GQ905711.1). Heterozygous mutations were confirmed by the presence of double peaks in the examination of the sequencing electropherograms. Individuals that carried heterozygous mutations for any of the exons or presented rare (low frequency/unique) genotypes ($n = 18$) were sequenced twice in independent PCRs to confirm the results. The repeatability of all independent PCR repetitions was 100 %.

Frequencies of SNPs and haplotypes/genotypes were calculated considering all the individuals and plotted considering the division of the city of Barcelona by districts in a map using QGIS Geographic Information System open source.

Table 2
Sequences of primers used for the amplification of *Vkorc1* exon 1, exon 2 and exon 3.

	Primer name	Sequence 5′-3′	Annealing temperature
Exon 1	musVKORC1-ex1F	GACCAATCTTCCGGTAGGAG	57 °C
	musVKORC1-ex1R	CGACCCCAGACTCCAAAAT	57 °C
Exon 2	VKORC1_ex2F	CTGTGCTGAGGGGACAAAGT	63 °C
	VKORC1_ex2R	TTGCCATAAAACTGAGATTGTGA	63 °C
Exon 3	VKORC1_ex3F	TTTCACCAGAAGCACCTGCTGYC	64 °C
	VKORC1_ex3R	ACACTTGGGCAAGGSTCATGTG	64 °C

3. Results and discussion

Detection of resistance to anticoagulant rodenticides is crucial to set up efficient management strategies in rodent control. Here, molecular characterization of the *vkorc1* gene shows that the house mice population in Barcelona has a high frequency of SNPs that alters the amino acid sequence of the enzyme VKOR. In fact, all 111 samples analyzed presented at least one missense mutation in one of the three exons of the *Vkorc1* gene. The frequency of mutations varied by exon: 94.60 % of individuals carried at least one mutation in exon 1; 85.59 % of individuals carried at least one mutation in exon 2; and 13.51 % of individuals carried at least one mutation in exon 3. We found seven different mutations in the *Vkorc1* gene in the mice sampled (Table 3). Four mutations were identified in the exon 1, one in the exon 2 and two in the exon 3. Six of these mutations lead to amino acid changes and the only silent mutation found in exon 1 (Glu37Glu) was always accompanied by a missense mutation. These mutations have been previously described in different mice populations and have been shown to provide resistance to both first- and second-generation anticoagulant rodenticides (Blažić et al., 2018, Mooney et al., 2018, Pelz et al., 2005, Rost et al., 2009, Šćepović et al., 2016, Song et al., 2011). Eight different genotypes were observed in the individuals analyzed (Table 4). 80.18 % of individuals were homozygotes for all the five mutations in exon 1 and 2 (Arg12Trp/Ala26Ser/Glu37Glu/Ala48Thr/Arg61Leu) and did not carry any mutation in exon 3. This group of mutations are associated with the introgression of *Vkorc1* from *M. spretus* (from now on *vkorc1*^{spr}; Song et al., 2011, Liu et al., 2015). Individuals carrying *vkorc1*^{spr} in homozygosis show a

Table 3
Details of the seven SNP mutations found in mice in the city of Barcelona including the allelic frequencies of the mutations and number of homozygote and heterozygote individuals found. Complete genotypes and exon sequences can be found at http://hdl.handle.net/10261/273122.

Exon	Mutation	Codon Wild Type	Codon mutated	Nucleotide position	SNP Frequency	Homozygotes	Heterozygotes
Exon 1	Arg12Trp	CGG	TGG	34	0.865	89	14
	Ala26Ser	GCA	TCA	76	0.901	95	10
	Glu37Glu	GAA	GAG	111	0.901	95	10
	Ala48Thr	GCC	ACC	142	0.865	89	14
Exon 2	Arg61Leu	CGG	CTG	976	0.856	95	0
Exon 3	Leu128Ser	TTA	TCA	2190	0.050	4	3
	Tyr139Cys	TAT	TGT	2223	0.086	8	3

strongly resistant phenotype. Both in vitro and in vivo studies have shown that the introgressed SNPs confer resistance to both first-generation anticoagulants (coumetralyl and chlorophacinone) and second-generation anticoagulants (bromadiolone and difenacoum) (Goulois et al., 2017; Song et al., 2011). No significant resistance has been found to brodifacoum, difethialone and flocoumafen. Several of the SNPs associated with *vkorc1*^{spr} seem to confer limited resistance individually, but is the combined effect with other mutations present in the same individuals what appears to provide greater levels of resistance (Goulois et al., 2017). Previous studies have found some of these mutations in isolation (Goulois et al., 2017; Rost et al., 2004). This includes mutations Ala26Ser in exon 1 and the mutation Arg61Leu in exon 2 which has been suggested to be less tightly linked to the *M. spretus* genotype that the other four mutations because it is sometimes absent or alone (Goulois et al., 2017, Peltz et al., 2012, Song et al., 2011). In our population however, neither of those mutations were found alone. Ala26Ser was always found with Glu37Glu in exon 1 and Arg61Leu in exon 2. In addition, Arg61Leu in exon 2 seems to be highly linked to the other four mutations in exon 1. We never found the mutation alone, and although it was absent in 9 % of individuals with mutations in exon 1, it only occurred in individuals that were heterozygote for all the mutations in exon 1.

The second group of mutations that we found were the two mutations located in exon 3, Leu128Ser and Tyr139Cys (Table 3). 5.4 % of individuals carried exclusively one or both of these mutations in exon 3 (Table 4). Mutations of exon 3 may confer significant resistance to first generation anticoagulants (warfarin, coumelatril and chlorophacinone). In addition, Tyr139Cys confers resistance to second generation rodenticide bromadiolone and Leu128Ser to second generation rodenticide bromadiolone, difethialone and brodifacoum (Blažić et al., 2018; Goulois et al., 2017; Mooney et al., 2018; Pelz et al., 2005; Šćepović et al., 2016). These mutations confer resistance both individually and combined, and the resistance is higher when they are in homozygosity (Baxter, 2019; Blažić et al., 2018) but they also confer some degree of resistance in heterozygosity (Rost et al., 2004). In addition, we found individuals that carried not only mutations in exon 3, but also mutations associated with *vkorc1*^{spr} (8.10 %). These individuals were heterozygotes for *vkorc1*^{spr} mutations in exon 1 and homozygotes for either Leu128Ser or Tyr139Cys. This is, to our knowledge, the first time that this combination of mutations is found and might lead to increased resistance profiles in the mice population.

Both the allelic and genotype frequencies differed between the districts sampled (Fig. 2, see also Table 1 in supplementary information). Overall in the studied population, *vkorc1*^{spr} mutations in exon 1 and 2 either in the complete form or partial were seven times more frequent than the mutations in exon 3. Although a high prevalence of the *vkorc1*^{spr} (93.1 %) was also observed in mice collected in 1991 in Spain (Song et al., 2011), a much smaller sample size (29 individuals) was analyzed and only rural localities in the province of Barcelona were included in the study. Our results thus indicate that *vkorc1*^{spr} is well stablished in the mice population in Barcelona and is not restricted to rural localities, where hybridization with *M. spretus* is more likely to occur. Although the city of Barcelona is within the distribution area of *M. spretus*, this species has not been detected during the sample collection at the municipal buildings, according both to the biometric measurements and the COI sequences. This result agrees with previous observations that indicate that *M. spretus* can be found in open habitat or different agrosystems that range from crops to orchards or forests (Palomo et al., 2009). The results of Song et al. (2011) indicated that hybridization might have occurred over 30 years ago and it is now segregating as polymorphisms in *M. m. domesticus*. In fact, other studies have found that this genotype is found in mice populations outside the sympatry area like Germany (Pelz et al., 2012) and France (Goulois et al., 2017). However, the frequency of *vkorc1*^{spr} in these countries is lower than those reported here, suggesting that frequency of *vkorc1*^{spr} might be higher in the sympatry area. In contrast, a recent study in Australia found evidence of the introgression in *M. musculus* but did not find the polymorphisms in *Vkorc1* associated with resistance (Duncan et al., 2020). This highlights the importance of carrying out similar studies in other areas in Spain, where *M. spretus* is present and consequently the potential for past hybridization events exists, to further understand the origin and evolution of rodenticide resistance.

3.1. Management implications

Results from this study show that the population of mice in Barcelona is resistant to bromadiolone as expected from the molecular characterization

Table 4
Frequencies of genotypes found, description of mutations and list of expected resistance to anticoagulant rodenticides. FGAR are first generation rodenticides, and SGAR are second generation rodenticides. Wild: wild type, Ho: homozygotes, He: heretozygotes.

Genotype	Frequency	Arg12Trp	Ala26Ser	Glu37Glu	Ala48Thr	Arg61Leu	Leu128Ser	Tyr139Cys	FGAR	SGAR
Vkorc^{spr}	0.802	Ho	Ho	Ho	Ho	Ho	Wild	Wild	Coumetralyl; Chlorophacinone	Bromadiolone; Difenacoum
Genotype 2	0.450	He	He	He	He	Wild	Wild	Ho	Warfarin; Coumetralyl; Chlorophacinone	Bromadiolone; Difenacoum
Genotype 3	0.090	He	He	He	He	Wild	Wild	Wild	Coumetralyl; Chlorophacinone	Bromadiolone; Difenacoum
Genotype 4	0.360	He	He	He	He	Wild	Ho	Wild	Coumetralyl; Chlorophacinone	Brodifacoum; Bromadiolone; Difenacoum; Difethialone
Genotype 5	0.270	Wild	Wild	Wild	Wild	Wild	Wild	Ho	Warfarin; Coumetralyl; Chlorophacinone	Bromadiolone; Difenacoum
Genotype 6	0.270	Wild	Wild	Wild	Wild	Wild	He	He	None	Bromadiolone
Genotype 7	0.180	Wild	Ho	Ho	Wild	Ho	Wild	Wild	Never tested	Never tested
Genotype 8	0.360	He	Ho	Ho	He	Ho	Wild	Wild	Coumetralyl; Chlorophacinone	Bromadiolone; Difenacoum

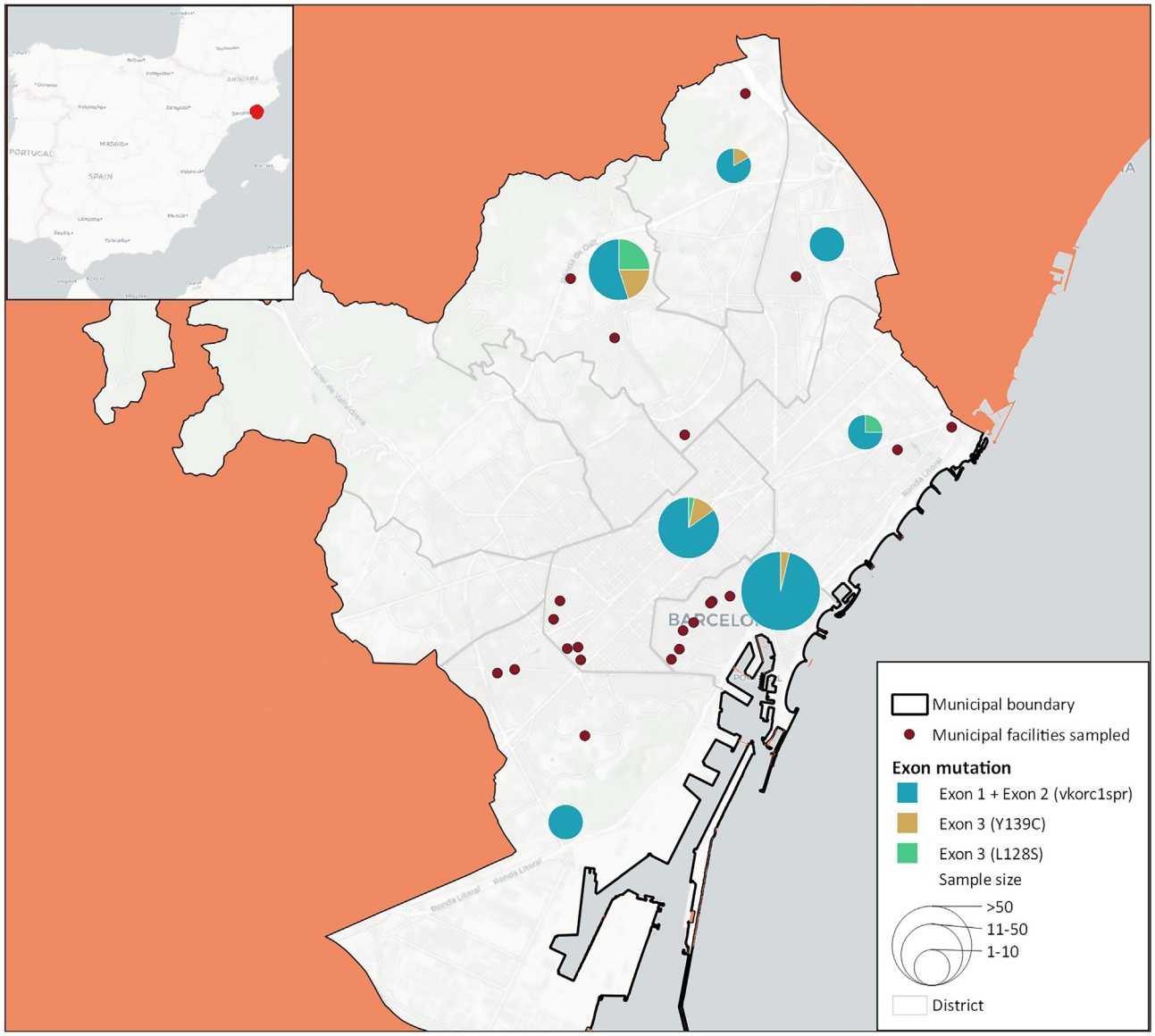

Fig. 2. Distribution of resistance by district, indicating mutations in exon 1 and 2 associated with the *vkorc1*^{spr} genotype, and both of the mutations in exon 3 (Leu128Ser and Tyr139Cys).

of the *vkorc1* gene, and consequently that brodifacoum is probably more effective than bromadiolone. These results support the management carried out by the *Agència de Salut Pública de Barcelona*, which since 2015 has implemented the preferential use of brodifacoum to avoid resistance (Fig. 3). However, in 2018, the regulation included a new classification of places of use. Because brodifacoum had restrictions on outdoor use, bromadiolone was used until the registration of brodifacoum allowed all uses (Reglamento (CE) n° 1272/2008, Reglamento (CE) n° 528/2012).

Currently, there are 366 rodenticide products authorised for use in Spain, 70 % of these have as principal component bromadiolone (Registro Oficial de Biocidas, 2022). Contrary to the case of the public agency, in the private sector control the use is based on bromadiolone. This is favoured by the availability of bromadiolone in large retail outlets (e.g. Centros Comerciales Carrefour S.A., Mercadona S.A., Alcampo S.A., Eroski S. Coop.). In addition, more than 87 companies provide pest control services in Barcelona (Registro oficial de Biocidas, 2022), where a large part of the control services they perform are against rodents and a fraction of the rodenticides used are bromadiolone-based. Under the current regulation in Spain only professionals with a specific permission are able to use baits of 50 ppm (Reglamento (CE) n° 528/2012). Such a regulation intents to avoid the use of high quantities of rodenticides by non-professionals. Nevertheless, the extensive use of bromadiolone probably explains why the resistance to this product in Barcelona is extremely high. In fact, Bishop et al. (1977) showed that when the selective pressure from the anticoagulant is removed the frequency of the resistance SNPs can decrease. However, our results are similar to that found in mice sampled in 1991 in rural areas (Song et al., 2011), even when the *Agència de Salut Pública de Barcelona* has greatly reduced bromadiolone use over the years. Thus, it seems that the high use of bromadiolone for mouse control in the home whether for domestic or professional use over many years has continued favouring the survival of individuals carrying the *vkorc1*^{spr} introgression in Barcelona. This case is a prime example of how anticoagulant resistance in an area can be widespread due to selection acting on standing variation (in this case an introgression).

Our results also showed there is a SNP mutation, at low frequencies in the population, that confers resistance to brodifacoum (Leu128Ser). Goulois et al., (2017) showed that the combination of Ale26Ser and Leu128Ser also leads to brodifacoum resistance. Although none of the studied mice in the Barcelona population carried those 2 SNPs alone, some carried the combination of *vkorc1*^{spr} (or a derived genotype) and

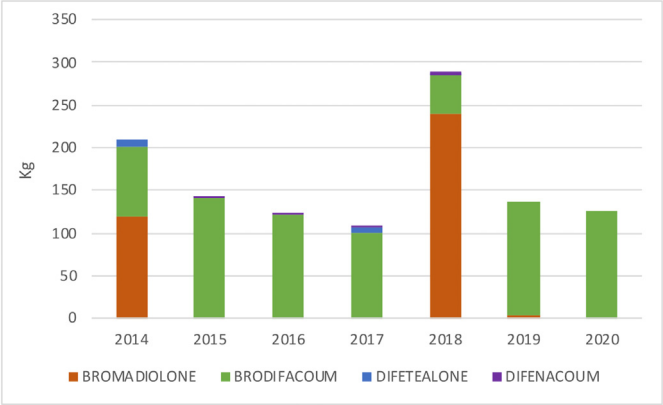

Fig. 3. Amount (in kg) of different rodenticides used by the Barcelona Public Health Agency from 2014 to 2020.

Leu128Ser, which may lead to a similar effect. This means that the presence of mice resistant to brodifacoum needs to be evaluated periodically. Although at the moment the presence of these mutations is restricted to some areas (Fig. 2), they might rapidly spread in the population if management is not adequate. In addition, Blažić et al. (2018) showed that 50 ppm of brodifacoum was able to kill over 90 % of mice population carrying these mutations. But changes in Regulation (EC) No 1272/2008 on classification, labelling and packaging of chemical substances and mixtures (CLP) lead to the use of concentrations below 30 ppm, which may lead to a longer control time and resistance problems because mice might be able to tolerate lower quantities better. The adequate use of these compounds is thus key. Second generation rodenticides are very efficient single feeding compounds with a delayed action and mortality occurring several days after consumption, so rodents do not avoid them. But they are very lipophilic and tend to bioaccumulate and lead to secondary toxicity in non-target species (Breckenridge et al., 1985; Duvall et al., 1989; Ray et al., 1989; Eason et al., 2001; Fernandez-de-Simon et al., 2022; Fournier-Chambrillon et al., 2004; Laakso et al., 2010; Vein et al., 2013). An increase in resistance and tolerance of mice to these compounds can therefore cause environmental damage through secondary toxicity. Our results also clearly highlight that using bromadiolone in the next years in Barcelona is senseless because of the extremely high presence of resistant individuals. This means that no benefit can be expected from its use, while the risks of secondary intoxication may be increased due to the high levels that can accumulate in the resistant and tolerant mice. In order to make a greener use of rodent control, it is important to use integrated control strategies that include non-chemical methods and incorporate structural, mechanical and physical measures, as well as behavioral measures to prevent food availability and shelter for mice (RRAC, 2016; Bonnefoy et al., 2008). The use of other recently marketed compounds such as cholecalciferol should be considered. These problems of high rodenticide resistance probably also occur in other cities and localities of the region, where populations of *M. spretus* and *M. m. domesticus* occur in sympatry.

4. Conclusion

Resistance to anticoagulant rodenticides is widespread in Barcelona, and it is mainly driven by adaptive introgression and the strong selective pressure of bromadiolone use. These results highlight the importance of using molecular tools to better understand the prevalence of resistance in mice populations. The high incidence of resistance found in mice in Barcelona has important consequences for public health authorities, pest control companies, and citizens in general because most widely available products are ineffective against mice. It is also necessary to consider the possible risk for predators of these resistant mice, due to the levels of rodenticides that they can accumulate. If the use of rodenticides for the control of rodents is needed, it is recommendable to use substances that are effective for mice, and not cause adverse effects on health, biodiversity and environment. It is also important to bring these results to the attention of pest control companies, manufacturers and distributors to make rational use of active substances and achieve effective control of mice in urban ecosystems.

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CRediT authorship contribution statement

María José Ruiz-López: Methodology, Formal analyis, Writing original draft, review and editing.

Laura Barahona: Conceptualization, validation, methodology, writing-review and editing.

Josué Martínez-de la Puente: Methodology, validation, writing-review and editing.

Marta Pepió: Data curation/Sampling, Methodology, validation.

Andrea Valsecchi: Data curation/Sampling, Methodology, validation.

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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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