Article

Control of Common Vole (*Microtus arvalis*) in Alfalfa Crops Using Reduced Content of Anticoagulants

Goran Jokić * and Tanja Blažić

Laboratory of Applied Zoology, Institute of Pesticides and Environmental Protection, Banatska 31b, P.O. Box 163, 11080 Belgrade, Serbia; tanja.scepovic@pesting.org.rs
* Correspondence: jokicg@ptt.rs; Tel.: +381-113-076-133

Abstract: The common vole, *Microtus arvalis*, which is prone to cyclic overpopulation, poses a significant threat to sustainable alfalfa production by either chewing shoots periodically or gnawing and damaging roots permanently. In areas with established vole colonies, the density of alfalfa plants was shown to decrease 55.3–63.4%. Simultaneously, the number of alfalfa shoots decreased by 60.9–71.7%. These experiments were conducted in compliance with an EPPO standard method in alfalfa fields at three geographically remote sites. The experiment tested the efficacy of the most widely used acute rodenticide zinc phosphide (2%), and anticoagulants applied at significantly reduced doses of active ingredients, i.e., bromadiolone (25 ppm) and brodifacoum (25 ppm), as well as a combination of these active ingredients at a low concentration (10 + 10 ppm). Three weeks after treatment, zinc phosphide and brodifacoum achieved the highest average efficacy, at 98.5% and 92.05%, respectively, while the average efficacy of the anticoagulant combination and bromadiolone was 87.2% and 75.5%, respectively. The achieved efficacy of baits based on brodifacoum and the combination of brodifacoum and bromadiolone in controlling common voles indicates their possible utilization in the field. Baits with 25 ppm of brodifacoum and the combination of bromadiolone and brodifacoum (10 + 10 ppm) showed satisfactory results and their introduction could significantly improve pest management programs for rodent control. At the same time, the use of anticoagulant rodenticides with reduced contents of active ingredients would significantly reduce their exposure to non-target animals, especially predators and vultures. By further improving the palatability of tested baits for target rodent species, their efficacy and safety of application would be significantly improved.

Keywords: common vole; brodifacoum; bromadiolone; zinc phosphide; combination

1. Introduction

The common vole, *Microtus arvalis*, is the most widespread and most harmful Eurasian vole, and is especially frequent in habitats rich in green plant biomass such as alfalfa [1,2]. Common voles live in colonies and are highly adaptable to various conditions and types of soil, preferring especially fertile soils (black soil, loam, sandy loam) with low levels of underground water. Many entrances are made to lead down into tunnels by oblique angles, but rodents also make openings with nearly vertical entrances. According to Ryszkowski [3], alfalfa fields provide significantly better conditions than many other crops for the development of common vole colonies, as voles spend most of the day in their underground tunnels [4]. The common vole reacts clearly to the changing height of plant cover. The height of alfalfa does not exceed 15–20 cm after harvesting and, according to Jacob and Hempel [5], the home range of common voles is about 100–150 square meters. A system of tunnels, once established, may assume a permanent character and provide a place for developing several generations of voles. Colonies formed in this way may occupy areas of no more than several square meters [6,7]. Voles cause irreparable damage during winter vegetation standstill when they feed on underground alfalfa parts [7,8]. It is sometimes possible for common voles to form tens of colonies on a hectare of alfalfa field.

Cultivated alfalfa offers shelter and a rich source of food to herbivorous rodent species, primarily voles. In some seasons, the number of voles reaches the level of overpopulation. Even though unpredictable, such periods have a cyclic pattern. The year 2019 was observed in many European countries to be the latest season of vole overpopulation [9].

Effective methods of control are required to reduce the harmful impact of voles and ensure the economic sustainability of alfalfa production while simultaneously reducing the risk for environmental safety in accordance with the principles of good manufacturing practice. The use of rodenticides for alfalfa protection is the most effective and expeditious method of reducing harmful effects of rodent pests, which enables alfalfa cultivation to be profitable. To control common voles in alfalfa crops, baits based on the acute rodenticide zinc phosphide are often the most popular choice. In good weather without precipitation, significant reduction in common vole numbers is achieved in a matter of days after application [10]. An important weakness of zinc phosphide baits (inactive wheat grain supplemented with the active compound) is their sensitivity to high soil moisture or rainfall, which often results in delayed baiting or insufficient efficacy after bait application. Some improved bait formulations have significantly contributed to overcoming these shortcomings [11,12]. Slower-acting first and second generation anticoagulant rodenticides are used as an alternative to zinc phosphide. Due to their stability under conditions of precipitation or soil moisture, farmers have the advantage of greater flexibility regarding application. Owing to their mode of action, slow-activity anticoagulant rodenticides, such as bromadiolone and brodifacoum (a.i. content 50 ppm), enable reduction in rodent population size over a period of 7–10 days after application, i.e., bait ingestion. Also, a combination of sub-acute and anticoagulant rodenticides could be used as an alternative [13–15]. In response to European Commission regulations [16], rodenticides with reduced content of anticoagulants, bromadiolone, brodifacoum and others have played a dominant role on the market. In general, the concentration of active ingredients in anticoagulant rodenticides designed for amateur use for the control of commensal rodents must not exceed 30 ppm. Currently, there are no available data on the efficacy of baits with reduced concentrations of bromadiolone or brodifacoum in controlling common voles in alfalfa crops.

The present study tested the possibility of using reduced concentrations of active ingredients in bromadiolone and brodifacoum baits, as well as the efficacy of a combined bromadiolone and brodifacoum bait in controlling common voles in alfalfa crops. Using reduced concentration anticoagulant rodenticides for field applications should lower the exposure of non-target wildlife to them, ultimately increasing food safety and environmental protection.

2. Material and Methods

2.1. Study Area

Tests were performed in alfalfa crops in Serbia on three sites: Dolovo (44°87′ N; 20°81′ E), Stara Pazova (44°58′ N; 20°07′ E) and Štitar (44°45′ N; 19°37′ E). To test the susceptibilities of different populations of common vole, mutually remote sites were chosen that were separated by rivers as natural barriers. The sites were situated in agricultural areas suitable for alfalfa cultivation at least 1 km away from any human settlement. The alfalfa fields on which biological efficacy of rodenticides was tested were two years old (Štitar) or three years old (Dolovo and Stara Pazova sites). All fields were sown with the same amount of 10 kg of seed per 1 ha, which is sufficient to provide the required plant density under our agroecological conditions. The soil type in all sites was black soil. As zinc phosphide was used in the experiment according to the manufacturer's recommendations, the warmer and drier period of autumn was chosen for setting up the experiment. At Stara Pazova, the first rains in the amounts of 0.5 and 1.7 mm fell on the third and sixth day after the experiment began. At Dolovo, rainfall was recorded five and twelve days after the experiment's commencement, and the amounts were 1.3 mm and 5.8 mm, respectively. Over the initial two weeks of the experiment, the total precipitation at Dolovo was 8 mm. The lowest total precipitation of 3.6 mm for the two-week period was noted at Štitar and

the first rain, in the amount of 1.6 mm, occurred on the sixth day after the start of the experiment. Efficacy trials were conducted almost simultaneously after the last alfalfa cutting. No other method of plant protection or fertilization was applied during the course of the trials.

2.2. Experimental Design

In compliance with the relevant method of EPPO standards [17], trials were performed using a random block design with four replications and 0.25 ha plot size. The distance between the experimental plots and crop margins were at least 40–50 m in order to minimize the risk of plot-to-plot migration of rodents that could affect our efficacy trials. Bait efficacy was calculated based on the ratio of the number of open active burrows on treated and control plots, including the number of active burrows calculated from at the start of the efficacy trial. Rodenticide efficacy was evaluated 3, 7, 14 and 21 days after treatment.

Baits containing 2% zinc phosphide (manufactured by EKOSAN a.d., Belgrade, Serbia) were used in the experiment. Baits containing the anticoagulants bromadiolone and brodifacoum were prepared in the laboratory by mixing appropriate amounts of their liquid concentrates (manufactured by EKOSAN a.d., Belgrade, Serbia) with a mix of broken wheat and maize grain. Baits based on bromadiolone (bromadiolone bait) and brodifacoum (brodifacoum bait) contained 0.0025% of respective active ingredients, while mixing 0.001% of each active ingredient produced their combination (combination bait). Concentrations of these active ingredients, i.e., zinc phosphide and anticoagulants, were checked in the Institute Chemical Laboratory. Deviation in active ingredient concentration was within the acceptable limit of 5%. One day before bait application, all active burrows were covered with soil. The next day, baits were applied around reopened burrows, and the burrows were then again covered with soil after application. The amount of zinc phosphide baits adhered to the manufacturer's recommendation, which is 5 g per active burrow, while anticoagulant baits were applied in doses determined for anticoagulant rodenticides under field conditions, i.e., 10 g of bait per active burrow.

Estimates of alfalfa density reduction and green plant biomass losses caused by voles were made in the period of the last cutting and before rodenticide application. Estimation of alfalfa density reduction was made by counting alfalfa plants in the central part of each of ten randomly selected vole colonies. As a control plot, we used a part of each alfalfa field which was free of voles. A stainless steel wire mesh (0.5 cm mesh size) fence (dimension 20×2 m) was set up after sowing to prevent rodent activity. The fence was 30–35 cm high above the ground and was equally buried under the ground. The counting of alfalfa plants on control plots was done in ten replicates. In order to clearly determine the detrimental effects of common voles and their feeding on green plant biomass, alfalfa shoots were counted on the same surface. Undamaged shoots at least 5 cm high were counted. The area of each plot for assessment of alfalfa density and green biomass reduction was one square meter. A wooden frame measuring 1×1 m was used to mark the surface at the colony center.

2.3. Data Analysis

Bait efficacy was computed comparing the number of opened active burrows on treated and control plots using the Henderson-Tilton formula [18]. The observed efficacy data were log (sqrt(x) + 1) transformed before analysis to normalize the variance [19]. A factorial analysis of variance (ANOVA) was performed using the sites, days after treatment and types of bait as fixed factors to detect significant influence between these parameters and bait efficacy as the dependent variable.

In addition, rodenticide efficacy data were submitted to one-way ANOVA and the means were separated by Tukey-Kramer's (HSD) test. Student's t-test was used to determine the effect of common vole on alfalfa density and the number of shoots in control enclosures and plots in which vole colonies had formed. In all analyses, the level of signifi-

cance was at least $p < 0.05$ [16]. All data were processed in StatSoft version 7.1 (StatSoft Inc., Tulsa, OK, USA).

3. Results

On all sites, alfalfa density at the center of vole colony and the number of shoots showed statistically significant differences, compared to surfaces in which no active burrows were present (Table 1). Alfalfa density at the colony center was the least reduced at Dolovo, at 55.3%, while the highest reduction of 63.4% was noted at Stara Pazova. The most significant reduction in shoot counts was found on the Štitar site, at 71.7%, and the lowest reduction was at Dolovo, at 60.9%.

Table 1. Average number of alfalfa plants and shoots at the center of common vole colonies and in control plots.

Site	Oasis †		Control Plot		Damage (%)	Student *t*-Test	
	MS	SE	MS	SE		*t*	*p*
Average Number of Plants							
Dolovo	24.0	1.8	53.7	2.4	55.3	9.9	0.0000 *
Stara Pazova	15.4	1.4	42.1	1.7	63.4	12.3	0.0000 *
Štitar	25.3	2.7	68.2	2.5	62.9	11.6	0.0000 *
Average Number of Shoots							
Dolovo	520.1	45.0	1331.6	20.6	60.9	16.4	0.0000 *
Stara Pazova	321.8	36.6	1044.9	116.4	69.2	5.9	0.0000 *
Štitar	488.6	89.2	1728	47.4	71.7	12.3	0.0000 *

† Oasis: center of common vole colony; * Within each row, an asterisk indicates significant differences; Students' *t*-test at 0.05; df = 18.

All main effects and days after treatment × bait associated interactions for rodenticide efficacy level at the end of the experiment were significant except for the interactions: site × days after treatment, site × bait, and site × days after treatment × bait, which were not significant at the $p < 0.05$ level (Table 2). Three days after the experiment began, the highest efficacy of 92.90% was noted for zinc phosphide on the Štitar site, while brodifacoum achieved the lowest efficacy level of <1% at Dolovo.

Table 2. Analysis of variance for bait efficacy comparing site, days after treatment and type of bait (error df = 144).

Source of Variation	Df *	*F*-Value	*p*-Value
Site	2	3.38	**<0.05**
Days after treatment	3	228.97	**<0.05**
Bait	3	54.48	**<0.05**
Site × days after treatment	6	0.91	0.49
Site × bait	6	0.53	0.76
Days after treatment × bait	9	23.75	**<0.05**
Site × days after treatment × bait	18	0.38	0.98
Error	144		

* df: degree of freedom; significant values are given in bold.

At Dolovo, the lowest average efficacy levels of brodifacoum and the combination of anticoagulants 21 days after application were 91.19% and 84.94%, respectively. On the Stara Pazova site, the combination of anticoagulants increased its efficacy between days 14 and 21 by 13 and reached 86.4%. At the end of the experiment, efficacy levels of the test baits were statistically significantly different on all sites, i.e., Dolovo ($F_{3,12} = 11.0$; $p < 0.05$), Stara Pazova ($F_{3,12} = 33$; $p < 0.05$) and Štitar ($F_{3,12} = 10.55$; $p < 0.05$). On the Štitar site, zinc phosphide achieved the highest efficacy level of 100% at the end of the experiment, while

the lowest efficacy was observed for bromadiolone, at 72.94%. The highest average efficacy levels of baits based on brodifacoum and the combination of anticoagulants were 93.23% and 90.22%, respectively (Table 3).

Table 3. Average number of active burrows of common vole in alfalfa crop and efficacy of rodenticides 3, 7, 14 and 21 days after the beginning of trial at Dolovo, Stara Pazova and Štitar (for all analysis, df: 3, 12).

Rodenticide	A *	A + 3		A + 7		A + 14		A + 21		F	p
	MS ± SE	MS ± SE	Ef (%)	MS ± SE	Ef (%)	MS ± SE	Ef (%)	MS ± SE	Ef (%)		
Dolovo											
Zinc phosphide	51.75 ± 3.56	14.75 ± 1.31	70.71 a A †	2.00 ± 0.91	95.71 a B	1.50 ± 0.64	97.16 a B	1.75 ± 0.85	96.83 a B	23.50	<0.05
Bromadiolone	58.50 ± 2.90	57.25 ± 2.75	2.10 b A	53.25 ± 1.44	9.77 c B	31.25 ± 6.41	51.61 b C	13.25 ± 1.93	79.76 b C	24.72	<0.05
Brodifacoum	48.25 ± 2.46	48.00 ± 2.74	0.57 b A	27.75 ± 2.87	43.28 b B	10.25 ± 2.05	79.87 a B	4.75 ± 0.48	91.19 ab B	35.53	<0.05
Combination	51.00 ± 1.35	50.00 ± 1.68	2.01 b A	27.25 ± 4.62	47.01 b B	12.75 ± 3.33	77.02 ab B	8.50 ± 2.53	84.94 ab B	25.45	<0.05
Control	53.75 ± 5.17	53.75 ± 5.17		54.50 ± 5.42		58.25 ± 5.67		59.75 ± 6.13			
F			11.19		28.16		7.34		7.00		
p			<0.05		<0.05		0.004		<0.05		
Stara Pazova											
Zinc phosphide	51.00 ± 5.71	11.00 ± 2.12	78.94 a A	0.75 ± 0.48	98.56 a B	0.75 ± 0.48	98.58 a B	0.75 ± 0.48	98.63 a B	32.50	<0.05
Bromadiolone	57.25 ± 5.02	54.50 ± 2.75	5.16 b A	39.50 ± 3.96	32.20 d B	24.25 ± 1.97	58.06 c B	16.00 ± 2.27	73.66 c B	13.74	<0.05
Brodifacoum	64.50 ± 4.73	63.25 ± 4.71	1.97 b A	19.50 ± 1.19	69.35 b B	11.00 ± 1.78	83.39 b B	5.75 ± 1.18	91.73 ab B	42.21	<0.05
Combination	65.00 ± 2.27	64.25 ± 2.25	1.15 b A	29.25 ± 1.70	55.29 c B	17.50 ± 0.96	73.24 b B	9.25 ± 1.03	86.39 b B	64.28	<0.05
Control	56.00 ± 2.97	56.00 ± 2.97		56.75 ± 2.62		57.00 ± 2.55		59.00 ± 3.11			
F			11.69		86.03		47.01		33.00		
p			<0.05		<0.05		<0.05		<0.05		
Štitar											
Zinc phosphide	34.75 ± 2.98	2.50 ± 1.89	92.90 a A	0	100 a A	0	100 a A	0	100 a A	2.0	0.172
Bromadiolone	33.00 ± 4.95	32.25 ± 4.58	3.00 b A	22.25 ± 3.94	34.44 c B	14.75 ± 3.47	58.32 b B	9.50 ± 2.33	72.94 b B	21.65	<0.05
Brodifacoum	28.75 ± 2.78	28.00 ± 2.48	3.41 b A	8.25 ± 0.63	70.68 b B	5.50 ± 0.65	81.15 b B	2.00 ± 0.41	93.23 b B	20.39	<0.05
Combination	26.25 ± 2.87	25.25 ± 3.47	6.11 b A	9.75 ± 1.03	62.66 b B	3.75 ± 1.25	85.97 b B	2.75 ± 1.55	90.22 b B	15.28	<0.05
Control	39.00 ± 1.58	39.50 ± 1.94		39.50 ± 1.94		41.00 ± 2.67		43.25 ± 2.28			
F			7.61		53.55		15.50		10.55		
p			<0.05		<0.05		<0.05		<0.05		

* A; A + 3; A + 7; A + 14 and A + 21: The number of active burrows at the beginning of experiment and after 3, 7, 14 and 21 days. † For each site separately, means within rows followed by the same uppercase letter and means within columns followed by the same lower-case letter are not significantly different, Tuckey's HSD test at $p > 0.05$.

4. Discussion

Crop density, i.e., the number of plants per square meter and number of healthy shoots, are the main factors determining the total yield, quality and durability of alfalfa crop [20,21]. Alfalfa crop density estimates based on the number of plants per square meter is changeable over the years of exploitation [22] and is dependent on a variety of agricultural practices and activities of pest organisms, especially rodents. Damage caused by common voles through cutting spouts for feeding during the vegetation period is instantaneous and affects fresh and dry matter yields of alfalfa. Estimated reduction in alfalfa shoot counts at the center of colony may indicate the high detriment that common voles are able to cause to alfalfa crops. Alfalfa tufts with many sprouts cut off also form young and weak shoots, causing the additional physiological feebleness of plants and their probable deterioration at a faster pace. Even though this phenomenon was not at the focus of attention of this study, we believe that damage caused in this way may add a negative impact immediately prior to the crop overwintering period, when additional soil moisture and wounds on plants enable a variety of pathogens to penetrate plants.

During vegetation, some alfalfa tufts remain undamaged and are covered in soil partially or fully in the midst of colony as a results of vole activity (digging of active burrows and tunnels). Based on our experience of field work, evaluation of alfalfa density reduction at colony center will be easier for farmers at the beginning of vegetation, when they are able to recognize a clear difference between damaged and intact tufts.

The number of common voles and the number of their active burrows, i.e., colony size, are positively and linearly dependent [23]. In this research, the reduction in the number of tufts at the colony center was up to 63.4%, which indicates a high potential of common vole as a pest and a need to control its presence in alfalfa crops. Tertil [24] and Sterner et al. [25] reported that the damage caused by common voles in the years of their overpopulation may rise up to 90%. During the last common vole overpopulation throughout Serbia in 2014, over 50,000 burrows per ha were found and they caused significant damage to several agricultural crops [26]. At the second cutting, damage from chewing shoots reached 100% on some sites. The last common vole overpopulation in Europe was reported in autumn 2019, when more than 6600 burrows per hectare were detected [27].

By planning areas for alfalfa fields and cover crops that would be away from natural vole habitats (pastures and meadows), and by applying deep tillage and other similar agricultural practices, it is possible to reduce the initial density of voles in agricultural fields [28]. The high reproductive potential of common voles, and their tendency to overpopulate, especially in crops such as alfalfa, quickly becomes the basic and greatest threat to economic interests of sustainable plant production. The average common vole abundance of 158–184 animals per ha caused up to 9.6% alfalfa green biomass yield decrease [29,30], while the average abundance of 250–285/ha caused 15.6–21.2% alfalfa green biomass yield decreases. Babinska-Werka [31] found that an average vole population of 145–220/ha caused 8.7% damage to alfalfa crop. A significant level of crop protection is achieved by stimulating the presence of predators in the field [32]. Although acceptable protection is possible by applying other nonchemical measures [33–35], rodenticide treatments are still the most widespread and most frequent measure for controlling common voles in alfalfa crops, particularly in situations of great urgency to reduce vole numbers, i.e., in periods of their high abundance or seasons of overpopulation. Different anticoagulant active ingredients are used in plant protection products or biocides in total amounts not exceeding 50 ppm. A European Union Regulation [16] has lowered the maximum content of anticoagulant active ingredient to 30 ppm. There is very little data on some possible applications of anticoagulants against rodents, i.e., the efficacy of rodenticide baits with reduced anticoagulant concentrations in the field. Frankova et al., [36] reported 95.7–99.8% efficacy of brodifacoum (25 ppm) baits in controlling house mice (*Mus musculus*) under field conditions. Difethialone (25 ppm) was found in the laboratory to cause 100% mortality of *Bandicota bengalensis* after 4–15 days [37]. Brodifacoum (23 ppm) was found to control a bromadiolone- and difenacoum-resistant brown rat population on a farm [38]. In our earlier research [29,30], the efficacy of bromadiolone (50 ppm) and brodifacoum (50 ppm) in controlling common voles in alfalfa crops was 81–85% and 95%, respectively. In this study, the average efficacy of brodifacoum three weeks after treatment was 92.05%, while the average efficacy of the anticoagulant combination and bromadiolone was 87.2% and 75.5%, respectively.

The pesticide market offers products with a variety of concentrations of zinc phosphide intended for field applications. The concentration of zinc phosphide used in this research was 2%, which is consistent with recommendations by Jacob et al. [39], who reported that zinc phosphide may affect bait palatability and should therefore not exceed 2.1%. High efficacy of zinc phosphide in controlling common voles in alfalfa crop, which reached 70–90%, was achieved only three days after the experiment started. Seven days after treatment its high efficacy was noted on all sites, reaching 95–100%. The results are not consistent with findings reported by Aria et al. [40], who found the average efficacy of zinc phosphide in controlling mixed populations of common vole and short-tailed bandicoot rat *Nesokia indica* in alfalfa crop to be 30–62%. The achieved swiftness of activity of zinc phosphide is consistent with its mode of action. According to Timm [41], mortality in rodents is achieved within 30 h after zinc phosphide ingestion. Different formulations and additives are used to preserve and stabilize baits, but bait susceptibility to moisture remains the most important deficiency of zinc phosphide. Anticoagulant rodenticides are significantly more resistant to surface soil moisture and rainfall, and have therefore attracted

a wider use by farmers because they provide greater freedom in timing the application. The persistence of antidotes for anticoagulants further facilitates the choice of farmers. Even though rodenticides ensure the most expedient effect of control, consequences of their application persist over the ensuing period, affecting non-target organisms, especially predators and vultures. The main flaw of anticoagulant rodenticides is possible secondary poisoning and bioaccumulation [42–46].

In this study, the rate of action of baits with reduced anticoagulant concentration was satisfactory 14 days after the start of the experiment and it did not differ from the rate of action of individually tested anticoagulants. The efficacy of combined anticoagulants on the Dolovo site was at the same level as zinc phosphide, which indicates that the tested combination of bromadiolone and brodifacoum could serve as an adequate replacement of baits based on zinc phosphide, as well as baits containing higher individual doses of either bromadiolone or brodifacoum. As the test baits were formulated without any attractants, further research might focus on testing improved baits with reduced concentrations of anticoagulants.

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