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The Hampshire-Berkshire focus of L120Q anticoagulant resistance in the Norway rat (*Rattus norvegicus*) and field trials of bromadiolone, difenacoum and brodifacoum

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Abstract

Anticoagulant resistance has been present in Norway rats (*Rattus norvegicus*) in Hampshire and Berkshire for forty years. All first-generation anticoagulants and two of the second generation, bromadiolone and difenacoum, are resisted by rats carrying the L120Q single nucleotide polymorphism (SNP). A regulatory restriction on the use of resistance-breakers brodifacoum, difethialone and flocoumafen in the UK effectively prevented their use against Norway rats for more than 30 years. During this time, L120Q spread from original localised foci eventually to cover most of central-southern England; with other more dispersed foci elsewhere in the UK. We summarise research on L120Q Norway rats and the field performance of anticoagulant baits against them. Bromadiolone (50 ppm), difenacoum (50 ppm) and brodifacoum (23 ppm) baits were each applied on two farmsteads where it had been established that Norway rats carried the L120Q SNP. Preliminary DNA resistance tests conducted at the farms found only one of 107 rats to be susceptible and 86.9% to be homozygous resistant. The bromadiolone and difenacoum applications were either partially or wholly ineffective; brodifacoum treatments were fully effective. Quantities of active substances used varied between farms and substances; but more bromadiolone and difenacoum baits were applied than brodifacoum baits during the treatments. Results confirm the high incidence of resistance and support advice that bromadiolone and difenacoum should not be used against the L120Q SNP. Prolonged use of resisted anticoagulants has resulted in a high prevalence of homozygosity and resistance spread. Failed treatments result in prolonged feeding on anticoagulant bait and leave Norway rats alive carrying, presumably, high residues. It remains to be

seen whether the use of now-permitted effective substances, and the introduction of a rodenticide stewardship regime, will curtail the spread of resistance and reduce anticoagulant residues in wildlife.

KEY WORDS: Anticoagulant resistance, *Rattus norvegicus*, second-generation anticoagulant rodenticide, L120Q, rodent control, non-target wildlife

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1 INTRODUCTION

The Norway rat (*Rattus norvegicus*) anticoagulant resistance focus in central southern England was first identified in 1969 (Greaves and Rennison, 1973) and came to be known as Hampshire difenacoum resistance (Greaves and Cullen-Ayres, 1988). The focus was then considered to be 'relatively inconsequential' but was recognised probably to be caused by a 'different genotype' to those found in the better-known Scottish, Welsh and Kent foci. Fifty years later this focus has become one of the most extensive and severe anticoagulant resistance phenomena found anywhere in the world, due to the presence of the L120Q (leucine120glutamine) single nucleotide polymorphism (SNP) (Pelz and Prescott, 2015; Boitet *et al*., 2018). Presently, the focus covers almost all of central-southern England, and L120Q rats are increasingly found more widely across the UK (Jones *et al*., 2019)

Since their discovery, resistant rats from this part of the UK have been studied extensively, both in the laboratory and field, in attempts to resolve questions about the genetic nature of this resistance, the efficacy of anticoagulant rodenticides and behavioural traits that appear to co-exist with it. All

of these questions remain only partially answered. A laboratory feeding test for the identification of resistance to difenacoum was developed as a precautionary measure on the assumption that resistance to this, then, newly-introduced substance was likely to emerge among Norway rats sooner or later (Redfern and Gill, 1978). This was prescient because the failure of difenacoum to control Norway rats on farms on the north Hampshire-Berkshire border was soon reported. Rats taken from this area, and tested using the new resistance feeding test, were confirmed to be difenacoum-resistant, one female survived the consumption of 123.9 g of difenacoum bait (37.3 $mg.kg^{-1}$ of the active substance) (Redfern and Gill, 1978).

Two further studies were conducted; one to identify the geographical extent of the new resistance focus and another to test the efficacy of three newly-developed anticoagulants within it. The focus was found to be mainly within the boundaries of lines joining the Berkshire and Hampshire conurbations of Reading, Newbury, Andover, Winchester and Alton (Greaves *et al*., 1982a). Field trials showed that, while bait containing 50 ppm difenacoum was largely ineffective within the focus, the efficacy of 50 ppm bromadiolone was also substantially impaired (Greaves *et al*., 1982b). Only 20 ppm brodifacoum baits were fully effective but control operations using this active substance took longer than normal, suggesting an albeit lesser degree of resistance.

The results of these early studies were relatively unequivocal until resistance factors were derived from feeding tests for Norway rats from the 'Hampshire' focus as follows: difenacoum 3.9 (males) and 2.7 (females), bromadiolone 1.5 (males) and 2.9 (females), brodifacoum 2.0 (males and females) (Greaves and Cullen-Ayres, 1988). Resistance factors were somewhat higher for a laboratory-bred 'selected line' created by crossing and back-crossing to select for anticoagulant resistance. However, resistance factors of this magnitude would not normally be expected to result in the degree of practical treatment failure observed (Greaves *et al*., 1982b), and factors of a similar magnitude for the same compounds were reported in other foci, such as Scotland and Wales, but without any similar diminution of practical treatment efficacy (Greaves and Cullen-Ayres, 1988).

Several explanations are conceivable for these observations but none is wholly convincing. They include the presence of a behavioural trait that prevented feeding on rodenticide baits in Hampshire resulting in reduced intake of the active substances, a second additive resistance mechanism not related to the conventional resistance gene and the possible selection in field populations of an advanced form of resistance, caused by the repeated use of resisted rodenticides, which meant that the laboratory strains tested by Greaves and Cullen-Ayres had been superseded. It subsequently transpired that each of these explanations was at least partially relevant.

Following the development of the 'selected line', carrying the strongest resistance phenotype then known (Greaves and Cullen-Ayres, 1988), a resistance feeding test was developed for one of the most potent anticoagulants available, brodifacoum, involving bait containing 5 ppm of the active substance (Gill and MacNicoll, 1991). Animals that survived consumption of this bait for seven days were termed 'brodifacoum-resistant'. The somewhat illogical implication of this was that rats declared 'brodifacoum resistant' were likely to be fully susceptible during field operations because these employed commercial baits carrying 50 ppm of the active substance. Several hundred wild-caught rats from the difenacoum resistance area were tested using the brodifacoum resistance test and a single male survivor was used to found a selected 'brodifacoum-resistant' laboratory strain. The form of resistance it exhibited was termed 'low-grade' and, as the farms from which these animals were taken were in Berkshire, the strain came to be called "Berkshire-resistant" (Gill *et al*., 1992). However, breeding studies showed that this resistance was not genetically stable in the same way as other resistance mutations known at the time (Gill *et al*., *loc. cit.*).

Research also continued in the field, particularly to investigate the influence of rat behaviour on treatment efficacy within the difenacoum resistance focus. Differences were found between the responses of rats to both poisoned and unpoisoned baits in the Welsh and Hampshire resistance areas (Quy *et al*., 1992). Rats readily consumed novel foods such as rodenticide baits on Welsh farms, with consequent rapid control of entire infestations. While in Hampshire rats were often

reluctant to consume novel foods, resulting in control being delayed for many weeks. There was also frequent failure on these farms to achieve complete control. These differences were attributed to rat behavioural responses to farming practices and the structures of the farmsteads themselves. In Wales, mixed arable and livestock farms (mainly dairy and sheep) present highly disturbed environments, where food sources and harbourage change frequently. In such situations, rats quickly overcome their suspicion of novel objects and new foods to acquire the resources they need. There was little animal husbandry on many Hampshire farms and farmsteads presented habitats where food sources, often bulk grain flat-stores (i.e. large quantities of cereals held in metal-sided bins on concrete floors), were stable over long periods. Rats established feeding patterns undisturbed and were reluctant to divert their feeding onto novel food, such as rodenticide baits, and away from known, stable and abundant alternative food resources (Quy *et al.*, *loc. cit.*).

The UK regulatory framework throughout this period, and indeed until 2016, was one wherein only bromadiolone and difenacoum were effectively available for the control of anticoagulant-resistant Norway rats. Concerns about the potential environmental effects of the more potent resistance-breakers, brodifacoum, flocoumafen and, latterly, difethialone, had resulted in their restriction to use indoors only (HSE, 2012). This restriction virtually precludes their use against rat infestations because usually the greater part of these reside outdoors (Buckle, 2013). Finally, carefully monitored applications of bromadiolone bait on a farm in West Berkshire proved entirely ineffective, with 830 kg of bait consumed on the farm over an eight month period, resulting in no measurable reduction in the size of the infestation (Quy *et al.*, 1995). The use of bait markers revealed that one rat survived the consumption of at least 450 g of bromadiolone bait. Such a massive environmental emission of a second-generation anticoagulant, when replicated elsewhere in anticoagulant resistance foci, because the more potent resistance-breaking compounds could not be used, may go some way to explain both the large current extent of resistance foci in the UK and the prevalence of residues of bromadiolone and difenacoum in UK wildlife (Buckle, 2013; Shore *et al.*, 2015; Jones *et al.*, 2019).

The genetic basis of Norway rat anticoagulant resistance mutations was revealed to be a series of SNPs in the VKORC1 gene located on chromosome 1 (Rost *et al*., 2004). Pelz and his co-workers showed that rats from the Hampshire-Berkshire focus possess the L120Q SNP (Pelz *at al*., 2005; Pelz and Prescott, 2015). However, the research cited above appeared to show that there is more than one resistance phenotype at this focus, with Berkshire-resistant rats having a higher level of resistance to anticoagulants than Hampshire-resistant rats but each possessing the same resistance SNP. The presence of a second resistance mechanism was postulated to explain this (Buckle, 2013), and it was later found that elevated CYP450-oxidative metabolism, leading to an accelerated anticoagulant detoxification, is involved in the Berkshire phenotype (Boitet *et al*., 2018). A mechanism of enhanced metabolism and clearance of anticoagulants, involving CYP450 enzymes, was also found to explain some characteristics of bromadiolone resistance in Norway rats from Denmark (Markussen *et al*., 2008).

The purpose of the field trials described here was to test the efficacy of proprietary bromadiolone and difenacoum formulations against the anticoagulant-resistant Norway rat genotype present at the heart of the Hampshire/Berkshire resistance focus in 2008. No similar trials had been conducted since 1991 (Quy *et al*., 1995). For the first time, the treated populations were screened for the resistance mutation they carried using the (then) new DNA sequencing method (Pelz *at al*., 2005). After the difenacoum and bromadiolone trials had been completed, the intention was to test the effectiveness of a proprietary brodifacoum formulation in anticipation that this more potent active substance might offer both a practical solution to resistance in this focus and the potential to reduce wildlife exposure to second-generation rodenticides. However, since outdoor bait applications were needed, permission for the brodifacoum field trials was required from the UK biocides regulatory body, the Health and Safety Directorate (HSE). This permission was denied on the grounds that the risk to the environment of the two trials, acknowledged to be limited, was not justified by their anticipated scientific value. Therefore, the brodifacoum trials presented here could not be attempted until eight years later; after the removal in 2016 of the ‘indoor-only’ regulatory restriction

on brodifacoum (and consequently also on the other potent, resistance-breaking anticoagulants, difethialone and flocoumafen). In the interim period, the use of bromadiolone and difenacoum baits, shown in these trials to be largely ineffective, was the major anticoagulant intervention permitted to those whose premises were infested by resistant Norway rats. This likely had important consequences for resistance management, resistance spread and the exposure of wildlife to rodenticides (Daniells *et al*., 2011; Buckle, 2013).

Recent resistance surveys have revealed that the original Hampshire/Berkshire resistance focus has apparently spread widely from the areas where it was first discovered and now covers most of central southern England (Greaves and Rennison, 1973; Jones *et al*., 2019). There is also evidence of pockets of this resistance in many other parts of the UK, including East and West Sussex, Wiltshire, Somerset, Devon, Monmouthshire, Yorkshire and the counties of East Anglia (Rodenticide Resistance Action Committee http://www.rrac.info/). It is impossible to know whether these have become established either as a result of the transportation of resistant rats from original foci or from *de novo* mutation events. In any case, the lessons learned about the use of rodenticides against rats carrying the L120Q mutation during this study are now more widely relevant.

2. MATERIALS AND METHODS

2.1 Trial sites

General

The trial sites were working farms in West Berkshire and north-west Hampshire, within the L120Q resistance focus (Jones *et al*., 2019). Each site differed from the others in the composition of livestock and farming practices (see below), although they were typical of farm enterprises in the area that had been previously used for field trials of anticoagulant rodenticides (Greaves *et al*., 1992b; Quy *et al*., 1995; Cowan *et al*., 1995). All were infested with Norway rats, living predominantly outside the farm buildings, although in very close proximity to them. Another

common feature was that there were plentiful food sources and harbourage for rats, although few of these could be eliminated given conventional farm practices involving *ad libitum* feeding of livestock and the methods of storing cereal grains on-farm. All of the infestations were circumscribed with very little sign of movement of rats onto the farms from neighbouring areas, though in some places low-level infestations were to be found in neighbouring hedgerows, as is normal for the rural landscape of Hampshire and Berkshire. All who operated these enterprises had expended considerable resources in rodent pest management. These efforts mostly involved baiting with either the permitted difenacoum or bromadiolone baits, in conjunction with burrow fumigation and trapping. Two of the sites were under contract to a reputable commercial pest control company, but all had achieved only partially satisfactory control of rat infestations.

Site 1. Large dairy unit, nr. Stockbridge, Hampshire

The site comprised a large, modern milking parlour and four large open barns in which approximately 600 dairy cattle were housed in sheds set on a concrete apron. Other infested buildings included a feed store, concrete bunkers in which maize silage was stored, a vehicle shed and barns used for housing calves and storing straw bales. The moderate rat infestation was mainly feeding on the silage maize available from the cattle stalls and concrete bunkers. The site was very clean and well-kept, with limited availability of harbourage for rats. Rat burrows were mainly found on waste ground bordering the site, beneath the concrete foundations of buildings and areas of disturbed ground.

Site 2. Small mixed farm, nr. Welford, West Berkshire

The site involved a machinery barn, two smaller barns used for grain storage, a large barn used for vehicle parking and the storage of grain used in pheasant feeders and a Dutch barn with straw bales. In the centre of the site were two rows of pig-sties containing about 100 sows and piglets. The farmstead was in a poor state of maintenance, with large areas of overgrown vegetation around the

site and between the buildings. Food was continuously available for the heavy rat infestation in the pig stalls, at a flat store of whole wheat grains used for pheasant feeding and from spillage in a granary and milling barn. Rats had found harbourage in and around the buildings and barns across the site.

Site 3. Large free-range poultry unit, nr. Stockbridge, Hampshire

The site comprised thirteen large free-range poultry sheds, with raised floors above compacted earth bases. The sheds accommodated approximately 15,000 birds and applied high standards of animal welfare, cleanliness and biosecurity. However, the moderate rat infestation had continuous access to food from the chicken feed hoppers in each of the poultry sheds and, to a limited degree, from the spillage at augers and feed silos across the site. A long hedge ran down the entire north-east border of the site with rat burrows along its length and there were rat runs and burrows around and under the poultry sheds.

Site 4. Large mixed farm, nr. Wickham, West Berkshire

The site comprised three large barns for pigs, cattle and sheep and further barns for machinery and equipment, grain and straw bales. In addition there was a neighbouring hanger for light aircraft and several smaller buildings with varying uses. The farm was well-maintained although there were neglected areas and grain spillage in a storage barn and mill. There was evidence of rat infestation across the site; although mainly restricted to the farm buildings, there was evidence of low-level infestation in hedgerows leading away from the building in which pigs were housed.

Site 5. Small dairy unit, nr. Reading, West Berkshire

The site consisted of an office building, with a large shed for the intensive feeding and milking of cattle and a smaller shed for dairy cows. Pheasants were present throughout the site, particularly where there was woodland cover. There was evidence of rats being present, with runs and burrows

221 in several areas and live rats seen around the site. Food resources for rats were available at the site
222 at feeding troughs provided for sheep and cattle.

223 *Site 6. Small mixed farm, nr. Basingstoke, Hampshire*

224 The site comprised a residential house, an adjacent workshop, a large open pen for chickens and
225 geese, and four barns which housed cows, sheep, hay and straw bales. There were abundant signs
226 of a substantial rat infestation, with rat runs and rat burrows in all areas and with live rats frequently
227 seen. Feeding troughs for sheep, cattle and chickens gave rats access to feed and feed stored in
228 polymer bags in one of the barns was also accessible.

229 2.2 Laboratory resistance studies

230 Following preliminary site surveys, initial work was conducted on all of the farmsteads to obtain
231 information on the resistance status of their Norway rat infestations prior to the commencement of
232 the bait applications. Blood-clotting response (BCR) tests were conducted on rats from four of the
233 farms (sites 1-4) during 2009/2010 to assess the degree of resistance to anticoagulants they
234 exhibited and, in particular to compare current resistance factors with those published previously
235 based on feeding tests (Greaves and Cullen-Ayres, 1988). For this, a standard protocol was
236 employed which involved the administration by gavage of solutions containing various
237 concentrations of bromadiolone and difenacoum, subsequent removal of blood samples under
238 anaesthesia and assessment of blood clotting responses (BCR) (Prescott *et al*., 2007).

239 Tissue samples from rats on all of the farms were taken for DNA extraction and sequencing. On four
240 of the farms (sites 1-4) rats were trapped prior to the treatments specifically for this purpose. Dead
241 rats were collected both prior to and during the progress of the poison baiting treatments on the
242 other two farms (sites 5 and 6). The procedures used to detect the presence or absence of
243 anticoagulant resistance DNA mutations were similar to those described by Prescott *et al*. (2007)

244 and, although only the L120Q mutation was subsequently found, all other rat resistance mutations
245 known to be present in the UK would have been found had they been present.

246 2.3 Test substances

247 The rodenticide bait formulations tested in the trials are shown in Table 1. Two of the baits were
248 commercially available pellet formulations, one contained 50 ppm bromadiolone (Contrac®) and the
249 other 50 ppm difenacoum (Ratak®). The remaining two formulations both contained brodifacoum as
250 an active substance. One was a 20 g wax block experimental formulation, containing a nominal 23
251 ppm of the active substance, and the other a commercial pellet formulation containing the same
252 concentration of brodifacoum (Talon®). All the test baits, both proprietary and experimental,
253 contained 10 ppm of the human taste deterrent denatonium benzoate (Kaukeinen and Buckle,
254 1992).

Bait type	Active substance	Active substance conc. (ppm)	Trial site(s)	Quantity of bait applied per baiting point (g)	Replenishment frequency
Pellet (commercial)	difenacoum	50 ppm	1 and 2	100-300	Twice weekly
Pellet (commercial)	bromadiolone	50 ppm	3 and 4	150-300	Twice weekly
Wax block (20g) (experimental)	brodifacoum	23 ppm	5	20-60	Three times weekly
Pellet (commercial)	brodifacoum	23 ppm	6	50	Three times weekly

255 Table 1. Rodenticide baits used, the concentrations of the active substance, application rates and
256 the frequency of bait replenishment in the six field trials.

257 2.4 Field efficacy tests

258 The experimental programme was carried out in two phases for the reason given above. Trials 1-4
259 were conducted during the period February 2009 to January 2010. Trials 5 and 6 were conducted
260 from April to December 2016.

The field trial protocol closely followed that used in similar studies of anticoagulant rodenticide efficacy (Endepols *et al*., 2011; Buckle *et al*., 2012) and was in accordance with published guidelines from international agencies including the European and Mediterranean Plant Protection Organisation (EPPO, 1998), the European Commission (EC, 2011) and the European Chemicals Agency (ECHA, 2017a). The protocol was based on the application of poisoned baits according to their regulatory label requirements, or as otherwise advised by the manufacturers. Evaluations of efficacy relied on two different indirect assessments of the size of the rodent infestation applied in a similar manner before and after the treatment, namely census baiting and the measurement of tracking activity (Quy *et al*., 1993). In preparation for the field trials, the six sites were surveyed and scale maps were drawn. A thorough search of each site was conducted for the bodies of any Norway rats and non-target animals prior to bait applications and any bodies found were removed.

The field trial schedule comprised three main phases, each separated from the other by a lag period during which no activities were undertaken at the sites. The first phase involved a pre-treatment census, generally four-days in duration, in which census baiting and tracking were used to estimate the initial size of the rat infestations. The numbers of census bait points and tracking patches set out at the six sites are shown in Table 2. On the first day of the pre-treatment census, the wooden census bait trays were filled with 200g of whole dry wheat, and the sand tracking patches, each approximately 100 mm by 150 mm, were smoothed over. The census bait was weighed (± 1g) and replenished daily. Where complete takes of census bait occurred, the quantity of census bait put down at replenishment visits was doubled. This resulted in 800 g of bait on some bait points in heavily infested areas. The tracking patches were recorded and smoothed over daily for four consecutive days. The tracking patches were scored using the following scale: 0 = no signs of rodent tracks; 1 = 1-5 rodent tracks, 2 = >5 tracks and up to 25% of patch covered with footprints; 3 = 26 – 95% covered; 4 = >95% covered. A lag period of not less than four days was used to separate the pre-treatment census period from poisoned baiting to permit the rats to return to their usual foods and feeding places.

Site	Dates of trial	Active substance	No. tracking patches	No. census bait points	No. poisoned bait points
1	13.02.2009-08.05.2009	difenacoum	45	58	57
2	26.10.2009-24.01.2010	difenacoum	39	82	90
3	13.02.2009-21.04.2009	bromadiolone	54	78	77
4	26.10.2009-24.01.2010	bromadiolone	46	94	97
5	18.04.2016-29.07.2016	brodifacoum	35	50	55
6	05.05.2016-23.12.2016	brodifacoum	32	50	90*

Note: * 41 rat burrows were also baited for a 13-day period.

Table 2. The dates of trials, numbers of tracking, census bait and poisoned bait points used on the six farms.

The second phase was that of poisoned bait application. The difenacoum and bromadiolone baits were applied according to the regulatory labels found on the product packs. These permitted a range of bait quantities (100-300g) to be placed at each bait point. Initial bait point sizes were 100 g per point but this quantity was increased either when complete takes occurred or when the take of bait at a replenishment visit was found to have approached a complete take. Conversely, when takes of bait declined the quantity of bait set out was reduced to the minimum permitted. The applications of brodifacoum baits were of much smaller quantities, with either one to three 20 g blocks per bait placement (i.e. 20-60 g) or 50 g of pellet bait placed at each bait point, and replenishment visits three times per week.

The sites of the poisoned bait placements once again followed label instructions and were determined according to the intensity and distribution of signs of rat activity so that, so far as possible, all rats present had access to a nearby baiting point. Bait points were protected with materials available at the sites, such as pipes, bricks, wooden beams, corrugated metal sheeting and other materials. The use of locally-available materials for securing baiting points provides more rapid and greater uptake of bait than from purpose-made tamper-resistant bait boxes (Buckle and Prescott, 2011). However, specially-constructed wooden bait boxes (measuring approximately 31x22x14.5 cm, with two rectangular entrances of approximately 7.5x5 cm) were used where insufficient material for robust protection of baits was found at the sites. Plastic bait boxes were also used, having approximate dimensions of 27x18x12 cm, with two circular entrances with a

diameter of 9 cm. On some sites, when it became apparent that rats were present but were reluctant to feed from the covered bait stations, some baits were put directly into rat burrows. All bait points were inspected according to the treatment schedule and, at each visit, the remaining bait was weighed and replenished when necessary. An assessment of tracking activity continued throughout the poison baiting period, with recorded track scores being the aggregate score over the several days that separated baiting visits.

The trials ended with the completion of the third phase, the post-treatment census. This was conducted in a manner that was as similar as possible to that used in the pre-treatment census.

The basis for the calculations of the efficacy of treatments was a comparison between information obtained in the pre- and post- treatment census periods as follows (EPPO, 1998):

$$\% \text{ efficacy} = \frac{\text{pre-treatment activity} - \text{post-treatment activity}}{\text{pre-treatment activity}} * 100$$

3. RESULTS

3.1 Resistance tests

Resistance tests based on BCR methodology (Prescott *et al*., 2007) were conducted to estimate the magnitude of resistance to second-generation anticoagulants at four of the trial sites (Table 3). Female animals were given a dose eight times and six times greater than the susceptible ED_{50} for bromadiolone and difenacoum respectively, and male animals were given a dose three times the susceptible ED_{50} for both active ingredients and, 24 hours later, a high proportion of the animals were found to be non-responders. In almost all cases, the percentage of non-responders was greater than 50%, thus indicating female RFs of at least 8 and 6 for bromadiolone and difenacoum respectively, and male RFs of at least 3 for both active ingredients; a magnitude of resistance that is greater than that previously reported for both males and females for these substances using feeding tests (Greaves and Cullen-Ayres, 1998).

Trial site	Active substance	Sex and no. of animals tested	Doses administered	% non-responders
1	bromadiolone	Female (n=10)	5.00 $mg.kg^{-1}$ ($8xED_{50}$)	70.0
		Male (n=7)	1.41 $mg.kg^{-1}$ ($3xED_{50}$)	71.0
	difenacoum	Female (n=9)	4.74 $mg.kg^{-1}$ ($6xED_{50}$)	44.4
		Male (n=6)	1.95 $mg.kg^{-1}$ ($3xED_{50}$)	83.3
2	bromadiolone	Female (n=5)	5.00 $mg.kg^{-1}$ ($8xED_{50}$)	60.0
		Male (n=5)	1.41 $mg.kg^{-1}$ ($3xED_{50}$)	60.0
	difenacoum	Male (n=5)	1.95 $mg.kg^{-1}$ ($3xED_{50}$)	60.0
		Female (n=5)	4.74 $mg.kg^{-1}$ ($6xED_{50}$)	40.0
3	bromadiolone	Female (n=5)	5.00 $mg.kg^{-1}$ ($8xED_{50}$)	40.0
		Male (n=8)	1.41 $mg.kg^{-1}$ ($3xED_{50}$)	100.0
	difenacoum	Female (n=1)	4.74 $mg.kg^{-1}$ ($6xED_{50}$)	100.0
4	bromadiolone	Female (n=6)	5.00 $mg.kg^{-1}$ ($8xED_{50}$)	33.3
		Male (n=2)	1.41 $mg.kg^{-1}$ ($3xED_{50}$)	50.0
	difenacoum	Female (n=11)	4.74 $mg.kg^{-1}$ (6xED50)	63.3
		Male (n=13)	1.95 $mg.kg^{-1}$ ($3xED_{50}$)	53.4

Table 3. The results of the blood clotting response tests conducted on rats from four of the six treated farms. Doses administered are shown as both absolute values and as multiples of the ED50 for fully susceptible animals. Dates of the trials are given in Table 2.

Table 4 provides a summary of the DNA resistance tests that were conducted on a total of 111 rats taken from the farms. Tissue samples from four rats could not be sequenced. Among the others, only one individual did not carry the L120Q SNP; thus the frequency of resistance on the six farms was 99.1%. The incidence of homozygosity was also very high (86.9%). Such results are likely to be obtained where there has been a prolonged selection for the resistance trait caused by the frequent use of resisted anticoagulants.

Site	Number of animals	Wild type (susceptible)	Heterozygous	Homozygous	Sequencing failed	% resistant
1	32	0	1	30	1	100.0
2	27	0	3	24	0	100.0
3	13	0	1	12	0	100.0
4	20	0	2	18	0	100.0
5	9	1	1	5	2	85.7
6	10	0	5	4	1	100.0
totals	111	1	13	93	4	

Table 4. Results of DNA sequencing for the L120Q resistance mutation among rats taken from the trial sites. Dates of the trials are given in Table 2.

3.2 Efficacy of rodenticide treatments

Site 1. Large dairy unit, nr. Stockbridge, Hampshire

The survey of the farm had revealed a widespread infestation across the site, with a particular centre of activity around the barn that housed the calf stalls, stacks of straw bales and farm machinery. Therefore, the very low take of census bait of just 174 g on the first day of the trial was unexpected and indicated that the rats on the site were reluctant to switch their feeding to novel alternative foods. Census bait takes increased rapidly (Fig. 1), however, but it was considered by the fourth day, when the census would normally have been terminated, that bait take (1.37 kg) still did not provide a realistic assessment of the size of the population. Therefore, census baiting was conducted for a further two days at which time the daily consumption of wheat bait had increased to 2.20 kg. It seems likely that the take of census bait would have continued to increase had the census been continued for longer and, therefore, the size of the initial infestation may have been underestimated. It was also noticeable throughout the census that almost all bait consumption was from bait stations constructed from natural materials and very few takes were recorded from bait boxes, either of the wooden or plastic designs. The highest daily pre-treatment consumption of census bait was 2.20 kg and the highest daily track score was 55 (Table 5).

Site	Total pre-treatment census bait take (kg^{-1})	Highest daily pre-treatment census bait take (kg^{-1})	Total pre-treatment track score	Highest daily pre-treatment track score	Total post-treatment census bait take (kg^{-1})	Highest daily post-treatment census bait take (kg^{-1})	Total post-treatment track score	Highest daily post-treatment track score

1*	6.87	2.20	242	55	7.89	1.86	286	58
2	33.36	10.57	418	115	13.20	4.20	169	45
3	9.20	2.73	154	43	2.83	0.92	94	27
4	15.07	5.49	285	84	17.80	5.57	323	87
5	4.88	1.37	172	50	0.02	0.01	0	0
6	9.78	3.18	336	96	0.05	0.03	4	3

Note: * pre- and post-treatment census periods of six days at site 1.

Table 5. Results of pre- and post-treatment census baiting and tracking at the six experimental sites.

If certain assumptions are made about the rat infestation, it is possible to estimate the number of individuals present at the site from the quantity of census bait consumed. These assumptions are: 1) rats consume only census bait during the baiting period, 2) rats have a mean weight of 200 g, 3) Norway rats consume 10% of their body weight in dry food each day. These assumptions, and the maximum daily census bait take of 2.20 kg, result in an estimated population size of 110 rats. Such estimates are conservative as it is very unlikely, given the known neophobia of Norway rats and the presence of abundant alternative food sources at trial sites, the rats fed exclusively from the census bait (Barnett, 1958; Inglis *et al*., 1996).

The 50 ppm difenacoum pellet poisoned bait was applied according to the manufacturer's label instructions, following a lag period. Once again, initial takes of this bait were poor, and certainly less than might have been expected from the apparent size of the infestation, although they showed a steady increase over the first 20 days of the treatment. Thereafter, and until the treatment was terminated after 48 days of baiting, takes of poisoned bait fluctuated between replenishment visits from approximately 2 kg to 3 kg (Fig. 1). During the treatment period, track scores fluctuated between 37 and 61, but it should be noted that these scores are not directly comparable with the daily track scores recorded during the pre-treatment period because they are the accumulate track score for either three or four days.

The post-treatment was conducted after a lag period of six days and the maximum daily take of census bait (1.86 kg) was recorded on the last of the six days of census baiting. The maximum daily track score, also recorded on the sixth day, was 58 (Table 5).

During the treatment the estimated total quantity of poisoned bait consumed by the rat infestation was 28.57 kg (Table 6). The maximum daily quantities of census bait consumed during the pre- and post-treatment census periods were used in the equation shown above to estimate that treatment efficacy at 15.5% mortality. The maximum daily track scores were similarly used and provided an estimate that the rat infestation at the site had grown by 5.5% during the treatment period.

Site	Active substance (conc. ppm)	Maximum daily take of pre-treatment census bait (kg) [§]	Total quantity of rodenticide used (kg)	Total quantity of active substance applied (g)	Estimated mortality (%)*	
					Census baiting	Track Score
1	difenacoum (50ppm)	2.20 (110)	28.57	1.43	15.5	+5.5
2	difenacoum (50ppm)	10.57 (529)	59.24	2.96	60.3	60.9
3	bromadiolone (50 ppm)	2.73 (136)	17.67	0.88	66.3	37.2
4	bromadiolone (50 ppm)	5.49 (275)	43.19	2.16	+1.5	+3.6
5	brodifacoum (23 ppm)	1.37 (69)	4.38	0.10	99.3	100.0
6	brodifacoum (23 ppm)	3.18 (159)	9.68†	0.22	98.4	96.9

Notes: [§] values in brackets are the estimated numbers of rats present at the start of the treatments (assumptions are given in the text)
* mortality estimates preceded by + indicate estimated population growth during treatment
† includes burrow bait that was applied but some of which was probably not consumed

Table 6. The results of efficacy assessments conducted at the six trial sites. The maximum daily pre-treatment census bait take is shown as an indirect measure of the sizes of the initial infestations.

Site 2. Small mixed farm, nr. Welford, West Berkshire

Initial takes of census bait at site 2 were high, indicating that the rat infestation exhibited little or none of the bait shyness that had been seen at site 1 (Fig. 1). This was in spite of abundant alternative food at the site (section 2.1). Similarly, the rats readily entered the wooden bait boxes used from the first day of the census. These observations demonstrated that differences in the behaviour of the field infestations may influence both treatment durations and treatment outcomes. A total of 33.36 kg of census bait was consumed over four days of the pre-treatment census baiting, with a maximum daily take of 10.57 kg. The total track score for the four days was 169, with a maximum of 45 recorded on the last day of the census (Table 5). Using the same assumptions described above, an estimated population size of 529 Norway rats infested the site.

The takes by rats of difenacoum pellets at site 2 were also initially high, as might be expected from a large infestation showing little or no reluctance to take apparently highly palatable bait. For example 9.96 kg of bait were eaten over the first five days of baiting. Bait takes declined over the following 14 days (Fig. 1) and thereafter continued at a fairly constant rate for a period of about three weeks. This pattern of bait take may occur when a resistant infestation is treated with a resisted anticoagulant. The more susceptible animals succumb to the bait initially, particularly when bait takes are good, leaving only the more resistant animals remaining to continue feeding. It is this process which increases the incidence of resistance mutations within resistant populations, as well as the severity of resistance. After 35 days of baiting, it became apparent both from the quantities of poisoned bait taken and the track score (Fig. 1) that little further progress would be made and the treatment was discontinued.

The post-treatment census began, following a four-day lag period, but a very heavy fall of snow overnight prevented access to the farm on the third day of the census and for seven days thereafter. Eventually, the census bait was removed and a second, five-day lag period was applied before the census bait procedure began again. Overall, 19 days separated the removal of the poisoned bait and the commencement of the second census period. Given the extremely cold weather and the deep-laying snow, it seems unlikely that the rat infestation remaining on the farm was significantly supplemented by either immigration or breeding.

The results of this census are shown in Fig. 1. The maximum census bait take (4.20 kg) was recorded on the fourth night of the census and the maximum track score of 45 was recorded on the third night. These data were used to obtain estimates of rat mortality achieved by the treatment of 60.3%, using census baiting, and 60.9% using track scores.

Site 3. Large free-range poultry unit, nr. Stockbridge, Hampshire

The Norway rats at this site apparently took the census bait without reluctance and there were good takes across the entire area. The maximum daily census bait consumption, 2.73 kg, was recorded on the fourth night of the census but the takes for the two preceding nights were not much less (Fig. 1). The maximum track score was 43 on the second night of the census. The census bait take was used to estimate the rat infestation at 136 rats, making as before the somewhat unlikely assumption that the rats only ate census bait. Takes of poisoned bait were initially good and a total of 6.80 kg of bromadiolone bait was consumed during days four to seven of the application. However, bait takes fell rapidly thereafter and continued at a regular, but considerably, lower level for the remaining period of the treatment. After 35 days of poisoned baiting it became apparent, from the continuing low level takes of poisoned bait and track score, that little further reduction of the infestation would be achieved and the treatment was therefore terminated.

The post-treatment census monitoring was initiated and was conducted in the same way as the pre-treatment census, using the same tracking patch and bait point locations. The four-day post-treatment tracking patch census was assessed daily, and produced a total census score of 94 and a maximum on day four of the census of 27 (Fig. 1). The post-treatment census bait consumption was measured daily for four days, and resulted in a total bait take of 2.83 kg, with a maximum take of 0.92 kg on the third day of the census (Table 5). These records provided estimates of the percentage mortality of rats at the site caused by the bromadiolone treatment of 66.3% using census baiting and 37.2% using the track score values. There was no obvious explanation for the difference between these two values but it is usually considered that mortality estimates derived from census bait data are more reliable than those obtained from track scores.

Site 4. Large mixed farm, nr. Wickham, West Berkshire

The consumption of census bait on site 4 followed a usual pattern in which takes increase during the four-day census period. Consumption was good on the first day of baiting, when the rats consumed 2.10 kg of bait overnight, but increased steadily until 5.49 kg were consumed on the fourth night of

446 the census (Table 5). It seems likely that census bait take would have continued to rise had the
447 census continued for a longer period. The number of rats present at the site was conservatively
448 estimated, from census bait consumption, to be 275 individuals. The total track score during the
449 four-day census was 285, with a maximum of 84 on the fourth day of the census.

450 Takes of bromadiolone bait were initially high, with a total of 7.67 kg of bait consumed during the
451 first four days of the treatment (Fig. 1). The average daily consumption of 1.92 kg was, however, less
452 than the average daily consumption of census bait of 3.75 kg. During the period of poisoned baiting
453 a pattern was observed in which consumption of bait remained high but showed a steady decline.
454 Given the high quantities of bait consumed, it is likely that some rats would have died during this
455 period, resulting in the observed decrease in both bait take and track score (Fig. 1). However, total
456 bait takes were 7.57 kg and 5.12 kg respectively in the fourth and fifth weeks of the treatment and
457 track scores showed an increase. The decision was therefore made to terminate the treatment after
458 35 days of poisoned baiting and a total consumption by the rats at the site of 43.19 kg of
459 bromadiolone bait (Table 6).

460 The work at site 4 was carried out synchronously with that nearby on site 2 and the post-treatment
461 census period was similarly disrupted by a very heavy fall of snow. When the post-treatment census
462 was carried out it resulted in a maximum daily take of census bait of 5.57 kg and a maximum daily
463 track score of 87 (Table 5). When these values were used to assess the effectiveness of the
464 bromadiolone treatment, both resulted in an estimated growth of the infestation, of 1.5% and 3.6%
465 for census baiting and track score respectively (Table 6).

466 *Site 5. Small dairy unit, nr. Reading, West Berkshire*

467 The infestation at this site was apparently the smallest of those present among the six trial sites. A
468 total of 4.88 kg of census was consumed over four days, with the maximum 1.37 kg taken on the
469 fourth day. This value provides a conservative estimate of 69 rats at the site. The nature of the site,

the fact that the activity of the infestation was mainly focussed outdoors and the frequent rain that occurred during the trial, made it difficult to establish tracking patch points that remained dry and capable of recording rat foot-prints throughout the census. However, a total track score of 172 was recorded, with a maximum daily score of 50 on the fourth day of the census.

The consumption of the brodifacoum wax blocks was initially poor, with only 210 g of bait eaten in the first two days of baiting. This may have been due either to the initial inability of the rats to recognise the blocks as food or, if they were recognised as food, a reluctance to consume them. However, bait takes then increased and reached a maximum at the end of the first week of baiting, when more than a kilogramme of bait was consumed over three days (Fig. 1). Thereafter, bait takes declined quickly, as did the tracking score, indicating that the rat infestation was being quickly extinguished. After three weeks of baiting, both bait takes and track score had reached very low levels. Unfortunately, at this time field signs at some bait stations indicated that a portion of the continuing activity observed may have been due to grey squirrels (*Sciurus carolinensis*). Camera traps were deployed and confirmed that this was the case. Grey squirrels are, of course, non-target animals in the context of outdoor baiting with anticoagulants and, therefore, bait was immediately removed from the site and the trial paused. Five squirrels were subsequently humanely trapped and removed from the site and the trial resumed. The duration of the pause was 24 days, but baiting continued thereafter for a further 18 days, until both track scores and bait takes were reduced to zero. Bait takes from stations that are considered likely to have been visited by squirrels were subtracted from the estimated total consumption of poisoned bait.

As in previous trials, the post-treatment census was carried out in the same way as the pre-treatment census. No activity was detected on any tracking patches, giving an estimate of mortality by that method of 100%. However, there were several very small takes of census bait, amounting to a total of 21 g over the four-day census period. Although it was likely that these takes were by either wood mice (*Apodemus sylvaticus*) or bank voles (*Myodes glareolus*), it was not possible from

the field signs entirely to dismiss the likelihood that they were small rat takes. Therefore, the estimate of mortality brought about by the application of 23 ppm brodifacoum wax blocks at site 5, using census baiting, was estimated to be 99.3%.

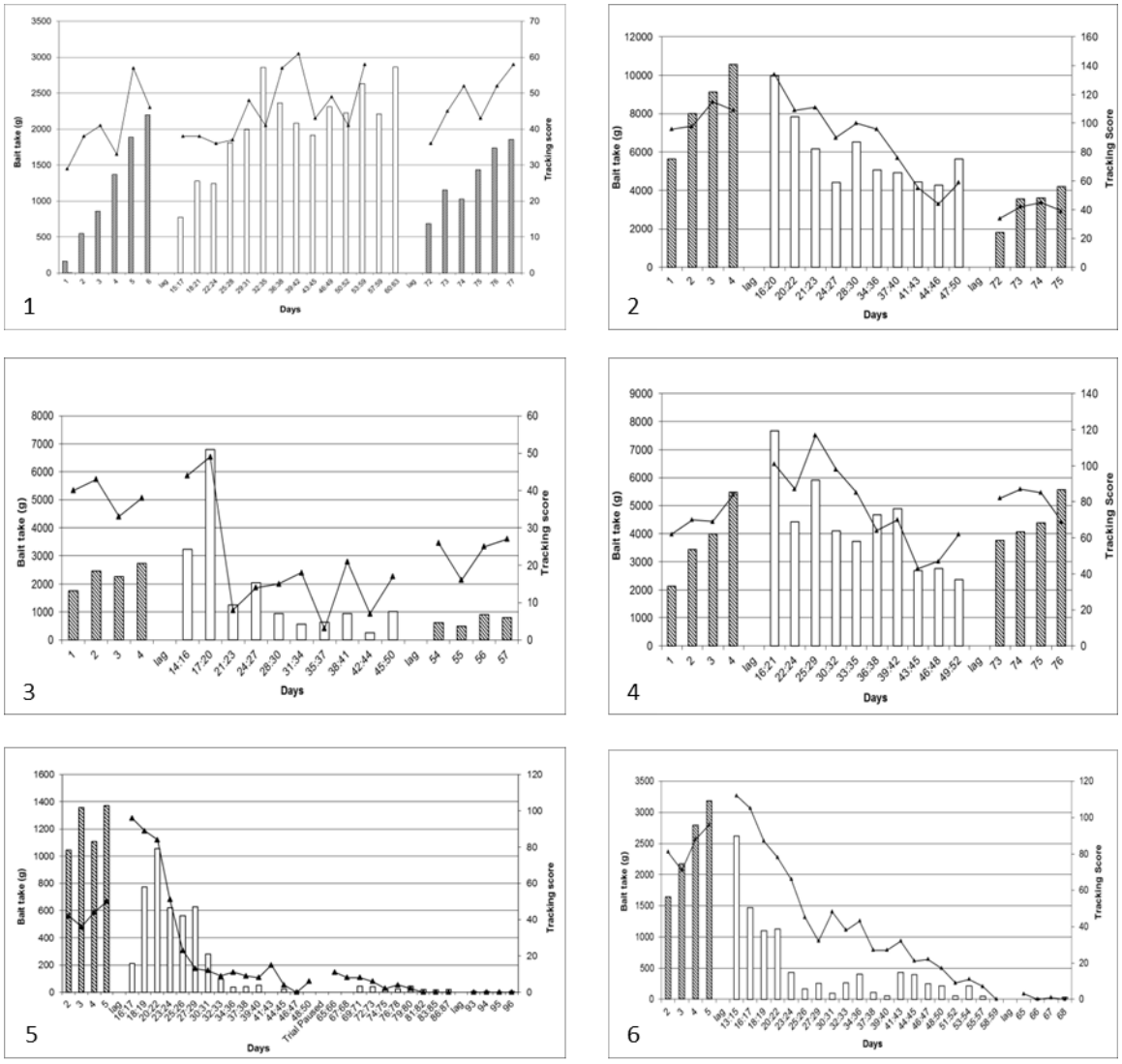
Site 6. Small mixed farm, nr. Basingstoke, Hampshire

Rats at site 6 ate more than 1.5 kg of wheat bait on the first day of census baiting, indicating that they were not reluctant to take a novel foodstuff. Bait takes increase daily until, on the last day of census baiting, the take of bait reached 3.18 kg, with a total consumption over the four days of baiting of 9.78 kg. The daily bait take provides, using the same assumptions as before, an estimate of 159 rats. The maximum track score during the pre-treatment census was 96.

Takes of the 23 ppm brodifacoum pellet bait were initially very good, with a total take of more than 2.5 kg over the first three days of baiting. Thereafter, takes of bait declined quickly during the following two weeks (Fig. 1). However, although the track score also showed a decline, its degree did not match that seen in the poisoned bait take data. About three weeks into the treatment it became apparent that rats remained active at the site but they were not taking the poisoned bait. Any open rat burrows seen were blocked and it was found that a considerable number of rat burrows remained active around an outdoor pen where poultry was kept and fed *ad libitum*. A normal procedure in this case during a conventional treatment would be to employ burrow baiting, in an attempt to induce the rats to take bait. However, although it is possible to record the quantity of bait put out in burrow baiting, it is not possible to estimate bait consumption, as is desirable in a monitored rodenticide trial. Therefore, conventional baiting continued for another week in the hope that the infestation could be extinguished. When this did not occur, burrow baiting was first employed on day 30 of the application. A total of 41 burrows were treated with 50 g of pellets each and burrows which remained active between treatment visits were baited until day 43 of the treatment. These burrow baits were included in the calculations of the quantities of bait applied but it seems likely that at least some of this bait was not consumed by rats.

The post-treatment census began after a five-day lag period. Census bait take was observed on two of the four census days, with a maximum of 32 g of bait eaten on the fourth and final day. Similarly tracking activity was observed on two days, with a maximum score of 3 on the first day of the census. Once again, it was impossible from the field signs to preclude the possibility that these takes were by rats and, therefore, these data provide estimates of mortality of 98.4% and 96.9%, using census bait takes and track scores respectively.

It is generally considered that effective anticoagulants should control a rat infestation that is susceptible to it within about 35 days (EC, 2011). The treatment at site six continued for a week longer than this but it is probable that the treatment would have ended earlier had burrow baiting been applied more expeditiously.



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531 Figure 1. Summary of the progression of six anticoagulant field trials conducted at farmsteads in
532 Berkshire and Hampshire, UK. Sites 1 and 2, 50 ppm difenacoum commercial pellet bait; sites 3 and
533 4, 50 ppm bromadiolone commercial pellet bait; site 5, 23 ppm brodifacoum experimental wax block
534 formulation; site 6, 23 ppm commercial brodifacoum pellet bait. Hatched bars, census bait takes;
535 open bars, poisoned bait takes; line, tracking score. Note: track scores are for a 24-hour period
536 during the pre- and post-treatment census, during the poisoned baiting they are aggregate scores
537 for two- to three-day periods. A six-day census period was employed at site 1 because of the initial
538 reluctance of rats to take census bait. The post-treatment lag period was prolonged at sites 2 and 4
539 because of heavy snowfall.

540 **4. DISCUSSION**

The application of anticoagulant rodenticides is the principle management intervention applied in almost all rodent pest scenarios worldwide, in both crop protection and public health (Jacob and Buckle, 2018). No alternatives exist that are sufficiently effective (ECHA, 2017b) and possess similar safety features, including a specific antidote (vitamin K_1) and a slow mode of action which provides time for its administration (Buckle and Eason, 2015). There is little evidence of technology capable of fully replacing anticoagulants becoming available in the foreseeable future (Buckle and Smith, 2015). However, anticoagulant resistance is a significant threat to the continued effective use of these active substances (Berny *et al*., 2018)and resistance management strategies are required to extend the useful lives of these essential products (RRAC, 2020).

Research in the Hampshire and Berkshire area, over many years and using different resistance monitoring techniques, has shown the prevalent nature of the L120Q resistance SNP in the area of this study (Buckle, 2013; Jones *et al*., 2019). The interactive resistance maps provided by the Rodenticide Resistance Action Committee of CropLife International (available here: http://guide.rrac.info/mapas-de-resistencia/reino-unido/?L=3%27A), based on DNA testing conducted at the University of Reading, demonstrate the known scale and geographical scope of the resistance focus. Therefore, it was unsurprising that the rats on six Hampshire/Berkshire farms were found to carry the L120Q SNP. What was surprising, however, was the very high incidence of resistance; with only one susceptible animal found in a sample of 107 rats from the trial sites, and the high incidence of homozygosity (86.9%). These results indicate prolonged selection pressure towards resistance exerted by the application, over a period of more than 30 years, of only partially-effective rodenticides. The late J.H. Greaves wrote, “the unabated use of anticoagulants to which resistance has developed will unremittingly cause resistance to these compounds to spread” (Greaves, 1994). Apparently this lesson was not learned and the consequences for resistance of a regulatory policy that, effectively, enforced the prolonged use of resisted active substances, is apparent (Jones *et al*., 2019). Also, the relatively high initial numbers of rats on the trial farms (Table 6) were not unusual at this resistance focus, in spite of almost continual but ineffectual attempts to

567 control them with bromadiolone and difenacoum baits (Quy *et al*., 1995), and by other physical
568 means such as trapping, dogs and shooting.

569 It is also clear from the foregoing that none of the six field trials described here progressed in an
570 entirely predicted manner. Rats appeared to be unusually reluctant to take bait at one site. This
571 may have resulted in an underestimate of the initial size of the infestation during census baiting, in
572 turn leading to an over-estimate of treatment efficacy. However, poor poisoned bait uptake and its
573 consequential detrimental effect on treatment efficacy would have exerted possible bias in the
574 opposite direction. Heavy snowfall caused the work of the post-treatment census to be unavoidably
575 abandoned and resumed later at two trial sites. There is a possibility that this prolonged,
576 unscheduled lag period may have permitted some rats to repopulate the sites, but the extreme
577 winter weather conditions, very low temperatures and deep-laying snow, would have militated
578 against this to a considerable extent. On another site the treatment was interrupted when it was
579 found, almost at the end of the treatment, that non-target grey squirrels were entering bait boxes
580 and taking poisoned bait. The trial could have been terminated at that point because very few rats
581 apparently remained. However, it was decided to pause the work, remove the squirrels, and then
582 continue in an attempt to extinguish the infestation. This resulted in a necessarily prolonged trial.
583 Finally, poor bait takes, probably due to the proximity of palatable alternative food, and justifiable
584 reluctance on the part of the researchers to employ burrow baiting because of the difficulty this
585 presented in quantifying bait takes, resulted in another prolonged treatment at site 6; although
586 virtually complete control was eventually achieved.

587 In spite of these experimental difficulties, a consistent pattern emerges from this study on the field
588 efficacy of anticoagulants against Hampshire/Berkshire L120Q rats. The four 50 ppm difenacoum
589 and bromadiolone treatments were substantially ineffective and the two applications of baits
590 carrying 23 ppm brodifacoum were both highly effective. The current regulatory authorisation
591 requirement for proof of efficacy of a rodenticide is 90% mortality in field trials (ECHA, 2017a), and it

592 is clear that all the difenacoum and bromadiolone treatments failed this criterion by wide margins.
593 Previously, bromadiolone had performed consistently better than difenacoum against Norway rats
594 in the Hampshire resistance focus (Greaves *et al*., 1982b) but this was not seen in the present study.
595 The resistance factors calculated here, using blood-clotting response tests conducted in 2009-10,
596 confirmed a shift towards a higher degree of resistance than those observed previously (Greaves and
597 Cullen-Ayres, 1988). However, if the advanced 'Berkshire' phenotype had entirely replaced the
598 once-prevalent, and more susceptible, 'Hampshire' phenotype at all the trial sites, we might have
599 expected both compounds to perform consistently poorly where they were applied. This was not
600 the case, as at one site where each substance was used they provided a similar, partial degree of
601 control; difenacoum, 60.3-60.9% mortality on site 2 and bromadiolone 37.2-66.3% mortality on site
602 3. However, at the other sites where bromadiolone and difenacoum were used the outcomes were
603 similar in their degree of complete failure to that seen for bromadiolone in a field trial conducted
604 many years earlier (Quy *et al*., 1995). It seems likely, therefore, that at some sites other factors, in
605 addition to anticoagulant resistance, may have influenced the outcomes of treatments, possibly the
606 unwillingness of the rat infestations to take the commercial baits (Cowan *et al*., 1995). Whatever
607 the causes of the variable effectiveness of the two less-potent anticoagulants, it is clearly apparent
608 that they should not be recommended for Norway rat control where the L120Q resistance mutation
609 is prevalent (RRAG, 2018).

610 Evidence has been growing for the past thirty years of widespread contamination of UK wildlife with
611 SGARs (Shore *et al*., 2015). The list of exposed species is extensive and includes, among others, barn
612 owl (*Tyto alba*), red kite (*Milvus milvus*), buzzard (*Buteo buteo*), kestrel (*Falco tinnunculus*), stoat
613 (*Mustela erminea*), weasel (*Mustela nivalis*), polecat (*Mustela putorius*), fox (*Vulpes vulpes*) and
614 hedgehog (*Erinaceus europaeus*). One of the few positive notes to emerge from this, however, is
615 that there is currently no evidence that any exposed species has declined as a result of this exposure
616 (Smith and Shore, 2015). Indeed, some of the most exposed populations of birds (i.e. red kite, barn

617 owl and buzzard) are rapidly increasing, both in numbers and in their geographical distribution
618 across the UK (Harris *et al*., 2019).

619 In simple terms, the risk of non-target effects of rodenticide applications is a function of the intrinsic
620 toxicity of the active substance to exposed species, the quantity applied and the duration of its
621 availability in the environment (Newton, 2018). In turn, the quantities applied are influenced by
622 concentration of the active substance in the bait, the application rates (determined by the product
623 label) and the size and scope of the treated rodent infestation. Actual impacts are affected by the
624 degree of risk of any non-target species' exposure and the effectiveness of the risk mitigation
625 measures employed when using these substances (Buckle and Prescott, 2018; López-Perea and
626 Mateo, 2018; Shore and Coerdassier, 2018). Table 6 shows the quantities of active substance
627 released to the environment during SGAR applications reported here. These ranged from 0.10 g to
628 2.96 g of active substance per treatment; one almost thirty times the other. Unsurprisingly, the
629 unsuccessful bromadiolone and difenacoum treatments resulted in much larger emissions than the
630 more successful brodifacoum treatments. This was because smaller quantities of brodifacoum baits
631 were applied (i.e. pulsed baiting, see Buckle and Eason, 2015), the baits contained lower
632 concentrations of the active substance (23 ppm instead of 50 ppm) and the duration of feeding on
633 brodifacoum baits by the treated rat infestations was shorter (Fig. 1). The most significant period of
634 risk of secondary exposure of predators during anticoagulant treatments is likely to be during the
635 'latency period'; that is after consumption of bait by target animals and before their deaths (Buckle
636 and Prescott, 2018). During this time the animals remain active in the environment, continue to
637 consume rodenticide and exhibit normal behaviour until, latterly, showing some reduced sensory
638 and motor function, and abnormal behaviours, due to poisoning (Cox and Smith, 1992). When a
639 lethal dose of an anticoagulant is taken, this latency period may be quite short, usually only 3-5 days
640 and rarely longer than ten (Buckle and Eason, 2015). However, in the unsuccessful difenacoum and
641 bromadiolone treatments recorded here, the latency period is very much longer, in effect the entire
642 remaining natural life of surviving rodents (Atterby *et al*., 2005) because of the long biological half-

643 lives of these substances (Horak *et al*., 2018). A scenario therefore emerges in this part of the UK
644 where the L120Q SNP is widespread, and where difenacoum and bromadiolone continue to be used
645 extensively, that a high proportion of individuals within extant Norway rat infestations carry SGAR
646 residues that are virtually continuously available for ingestion by non-target animals through
647 predation and scavenging. This occurrence in Hampshire and Berkshire, and probably to a lesser
648 extent in other resistance foci (Jones *et al*., 2019), may go some way to explain the widespread
649 nature of anticoagulant residues in predatory birds and mammals, particularly of bromadiolone and
650 difenacoum (Shore *et al*., 2019). In the brodifacoum treatments, the relatively smaller quantities of
651 active substance emitted resulted in the virtual extirpation of the rat infestations (Table 6) and,
652 therefore, both to a lower overall SGAR emission and shorter duration of potential secondary
653 exposure of predators.

654 Important regulatory changes have been introduced recently in the UK concerning the permitted
655 uses of the second-generation anticoagulants. Firstly, as mentioned above, the more potent
656 anticoagulants, brodifacoum, difethialone and flocoumafen, have been authorised for use outdoors.
657 This has made available, for the first time, potentially highly-effective resistance-breaking active
658 substances for use in resistance foci, such as that of Norway rat L120Q resistance studied here.
659 However, to accompany this change, a programme of rodenticide stewardship has been introduced
660 which has brought about important changes in whom can purchase SGARs and how they can be
661 used (Buckle *et al*., 2017). UK Government assessment of the stewardship regime will determine
662 future rodenticide regulation and permitted use practices and, in so doing, will continue to affect the
663 development and spread of anticoagulant resistance and the prevalence of anticoagulant residues in
664 UK wildlife (GOG, 2019). However, so far no significant increase in total SGAR residues has been
665 detected in the chosen sentinel species, barn owl, as a result of the regulatory change which
666 occurred in 2016 to permit the use outdoors of the resistance-breaking SGARs (Shore *et al*., 2019).

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Table(s)

Bait type	Active substance	Active substance conc. (ppm)	Trial site(s)	Quantity of bait applied per baiting point (g)	Replenishment frequency
Pellet (commercial)	difenacoum	50 ppm	1 and 2	100-300	Twice weekly
Pellet (commercial)	bromadiolone	50 ppm	3 and 4	150-300	Twice weekly
Wax block (20g) (experimental)	brodifacoum	23 ppm	5	20-60	Three times weekly
Pellet (commercial)	brodifacoum	23 ppm	6	50	Three times weekly

Table 1. Rodenticide baits used, the concentrations of the active substance, application rates and the frequency of bait replenishment in the six field trials.

Site	Dates of trial	Active substance	No. tracking patches	No. census bait points	No. poisoned bait points
1	13.02.2009-08.05.2009	difenacoum	45	58	57
2	26.10.2009-24.01.2010	difenacoum	39	82	90
3	13.02.2009-21.04.2009	bromadiolone	54	78	77
4	26.10.2009-24.01.2010	bromadiolone	46	94	97
5	18.04.2016-29.07.2016	brodifacoum	35	50	55
6	05.05.2016-23.12.2016	brodifacoum	32	50	90*

Note: * 41 rat burrows were also baited for a 13-day period.

Table 2. The dates of trials, numbers of tracking, census bait and poisoned bait points used on the six farms.

Trial site	Active substance	Sex and no. of animals tested	Doses administered	% non-responders
1	bromadiolone	Female (n=10)	5.00 $mg.kg^{-1}$ ($8xED_{50}$)	70.0
		Male (n=7)	1.41 $mg.kg^{-1}$ ($3xED_{50}$)	71.0
	difenacoum	Female (n=9)	4.74 $mg.kg^{-1}$ ($6xED_{50}$)	44.4
		Male (n=6)	1.95 $mg.kg^{-1}$ ($3xED_{50}$)	83.3
2	bromadiolone	Female (n=5)	5.00 $mg.kg^{-1}$ ($8xED_{50}$)	60.0
		Male (n=5)	1.41 $mg.kg^{-1}$ ($3xED_{50}$)	60.0
	difenacoum	Male (n=5)	1.95 $mg.kg^{-1}$ ($3xED_{50}$)	60.0
		Female (n=5)	4.74 $mg.kg^{-1}$ ($6xED_{50}$)	40.0
3	bromadiolone	Female (n=5)	5.00 $mg.kg^{-1}$ ($8xED_{50}$)	40.0
		Male (n=8)	1.41 $mg.kg^{-1}$ ($3xED_{50}$)	100.0
	difenacoum	Female (n=1)	4.74 $mg.kg^{-1}$ ($6xED_{50}$)	100.0
4	bromadiolone	Female (n=6)	5.00 $mg.kg^{-1}$ ($8xED_{50}$)	33.3
		Male (n=2)	1.41 $mg.kg^{-1}$ ($3xED_{50}$)	50.0
	difenacoum	Female (n=11)	4.74 $mg.kg^{-1}$ (6xED50)	63.3
		Male (n=13)	1.95 $mg.kg^{-1}$ ($3xED_{50}$)	53.4

Table 3. The results of the blood clotting response tests conducted on rats from four of the six treated farms. Doses administered are shown as both absolute values and as multiples of the ED50 for fully susceptible animals. Dates of the trials are given in Table 2.

Site	Number of animals	Wild type (susceptible)	Heterozygous	Homozygous	Sequencing failed	% resistant
1	32	0	1	30	1	100.0
2	27	0	3	24	0	100.0
3	13	0	1	12	0	100.0
4	20	0	2	18	0	100.0
5	9	1	1	5	2	85.7
6	10	0	5	4	1	100.0
totals	111	1	13	93	4	

Table 4. Results of DNA sequencing for the L120Q resistance mutation among rats taken from the trial sites. Dates of the trials are given in Table 2.

Site	Total pre-treatment census bait take (kg^{-1})	Highest daily pre-treatment census bait take (kg^{-1})	Total pre-treatment track score	Highest daily pre-treatment track score	Total post-treatment census bait take (kg^{-1})	Highest daily post-treatment census bait take (kg^{-1})	Total post-treatment track score	Highest daily post-treatment track score
1*	6.87	2.20	242	55	7.89	1.86	286	58
2	33.36	10.57	418	115	13.20	4.20	169	45
3	9.20	2.73	154	43	2.83	0.92	94	27
4	15.07	5.49	285	84	17.80	5.57	323	87
5	4.88	1.37	172	50	0.02	0.01	0	0
6	9.78	3.18	336	96	0.05	0.03	4	3

Note: * pre- and post-treatment census periods of six days at site 1.

Table 5. Results of pre- and post-treatment census baiting and tracking at the six experimental sites.

Site	Active substance (conc. ppm)	Maximum daily take of pre-treatment census bait (kg) [§]	Total quantity of rodenticide used (kg)	Total quantity of active substance applied (g)	Estimated mortality (%)*	
					Census baiting	Track Score
1	difenacoum (50ppm)	2.20 (110)	28.57	1.43	15.5	+5.5
2	difenacoum (50ppm)	10.57 (529)	59.24	2.96	60.3	60.9
3	bromadiolone (50 ppm)	2.73 (136)	17.67	0.88	66.3	37.2
4	bromadiolone (50 ppm)	5.49 (275)	43.19	2.16	+1.5	+3.6
5	brodifacoum (23 ppm)	1.37 (69)	4.38	0.10	99.3	100.0
6	brodifacoum (23 ppm)	3.18 (159)	9.68†	0.22	98.4	96.9

Notes: [§] values in brackets are the estimated numbers of rats present at the start of the treatments (assumptions are given in the text)

* mortality estimates preceded by + indicate estimated population growth during treatment

† includes burrow bait that was applied but some of which was probably not consumed

Table 6. The results of efficacy assessments conducted at the six trial sites. The maximum daily pre-treatment census bait take is shown as an indirect measure of the sizes of the initial infestations.

Figure(s)

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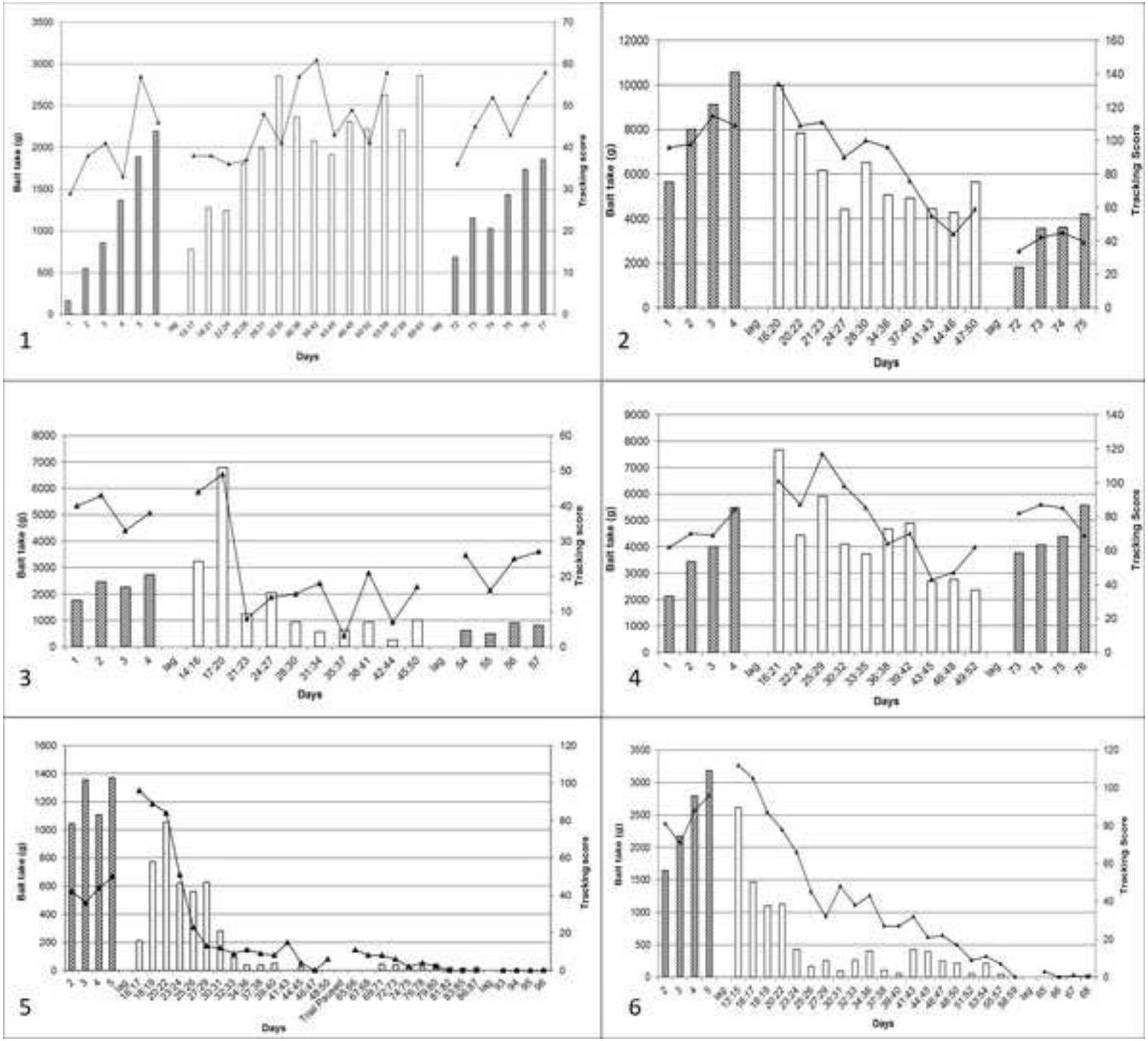

***Highlights (for review)**

Highlights

- Field trials were conducted with three anticoagulants in Hampshire/Berkshire UK
- Six farm sites were used where 87% of Norway rats were L120Q homozygous resistant
- Bromadiolone and difenacoum were ineffective and should not be used against L120Q
- Brodifacoum was fully effective, with lower anticoagulant environmental emissions
- Rodenticide stewardship is in place, with resistance and environmental monitoring

***Declaration of Interest Statement**

Declaration of interests

☐ 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.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The study was funded by Syngenta AG, Basel, Switzerland. Dr Alex Cornish, of Syngenta, provided comment on some aspects of the study including application rates for the brodifacoum formulations. The funding company and Dr Cornish, however, played no part in collection, analysis and interpretation of data, in writing the report and in the decision to submit the article for publication.

***Credit Author Statement**

Alan Buckle: Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Writing - original draft; Writing - review & editing. **Clare Jones**: Data curation; Formal analysis; Investigation; Writing - review & editing. **David Rymer**: Data curation; Formal analysis; Investigation. **Emily Coan**: Data curation; Formal analysis; Investigation. **Colin Prescott**: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Writing - review.