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A.P. Buckle
Zeneca Public Health, Fernhurst, Haslemere, Surrey, GU27 3JE, United Kingdom

C.V. Prescott
Vertebrate Pests Unit, Department of Pure and Applied Zoology, University of Reading, United Kingdom

K.J. Ward
Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, Berkshire, RG12 6EY, United Kingdom

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A. P. BUCKLE, Zeneca Public Health, Fernhurst, Haslemere, Surrey, GU27 3JE, United Kingdom.

C. V. PRESCOTT, Vertebrate Pests Unit, Department of Pure and Applied Zoology, University of Reading, P.O. Box 228, Reading, RG6 2AJ, United Kingdom.

K. J. WARD, Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, Berkshire, RG12 6EY, United Kingdom.

ABSTRACT: Warfarin resistance was first discovered among Norway rat *(Rattus norvegicus)* populations in Scotland in 1958 and further reports of resistance, both in this species and in others, soon followed from other parts of Europe and the United States. Researchers quickly defined the practical impact of these resistance phenomena and developed robust methods by which to monitor their spread. These tasks were relatively simple because of the high degree of immunity to warfarin conferred by the resistance genes. Later, the second generation anticoagulants were introduced to control rodents resistant to the warfarin-like compounds, but resistance to difenacoum, bromadiolone and brodifacoum is now reported in certain localities in Europe and elsewhere. However, the adoption of test methods designed initially for use with the first generation compounds to identify resistance to compounds of the second generation has led to some practical difficulties in conducting tests and in establishing meaningful resistance baselines. In particular, the results of certain test methodologies are difficult to interpret in terms of the likely impact on practical control treatments of the resistance phenomena they seek to identify. This paper defines rodenticide resistance in the context of both first and second generation anticoagulants. It examines the advantages and disadvantages of existing laboratory and field methods used in the detection of rodent populations resistant to anticoagulants and proposes some improvements in the application of these techniques and in the interpretation of their results.

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INTRODUCTION

The development of resistance in rodents to the anticoagulant rodenticides threatened the great strides towards improved efficacy and safety that the introduction of these compounds had made possible. This phenomenon was first discovered among Norway rats *(Rattus norvegicus)* in Scotland in 1958 (Boyle 1960) but the initial outbreak was quickly followed by others occurring in the United Kingdom (UK) (Drummond 1966a), Denmark (Lund 1988), the United States (USA) (Jackson and Kaukeinen 1972) and elsewhere. Some of these resistance foci spread rapidly in spite of rigorously-applied and varied attempts to overcome them. Others were successfully managed, either so that they disappeared completely or their spread was substantially curtailed. Still other foci seemed to disappear without human intervention (see Smith and Greaves 1987 for a review). Meanwhile, the other important commensal rodent pest species, the House mouse *(Mus musculus/ domesticus),* never very susceptible to the early anticoagulant compounds such as warfarin, diphacinone and coumatetralyl, also developed resistant populations in several countries (Wallace and MacSwinney 1976, Ash ton and Jackson 1984).

The discovery of the second generation compounds (Hadler and Shadbolt 1972) redressed the balance for several years but, in a few localities, resistance to the first generation anticoagulants brought with it a measure of cross resistance to the second generation compounds and soon populations of rats and mice began to appear with reduced susceptibility to these more potent compounds (Greaves et al. 1982). However, resistance to the modern anticoagulants, such as brodifacoum, bromadiolone and difenacoum, has never become as widespread as that to the first generation compounds and nowhere is it impossible to control rodents with at least one of these materials (Buckle 1994). Nevertheless, great interest remains both among researchers and pest control practitioners in the resistance phenomenon; the former in their attempts to measure and record the development and spread of resistance in different rodent species and the latter in their desire to conduct effective rodent control programs.

DEFINITIONS OF RESISTANCE

The term resistance means different things to different people. Researchers commonly define it as "the development of an ability in a strain of a pest to survive doses of a toxicant which would prove lethal to the majority of individuals of a normal population of the same species." In other words, if a technique can be applied which distinguishes between the susceptibility of individuals belonging to two strains of a pest species, the less susceptible strain may be considered "resistant." Such a definition is important to those wishing to detect the early onset of resistance, perhaps to allow the implementation of measures to interrupt its spread. However, it gives no indication of the likely practical significance of the resistance phenomenon described. To the pest control practitioner, a statement that defines a species as resistant to an anticoagulant rodenticide implies that the compound will be ineffective when used against it. But it is evident from the foregoing that this is not necessarily the case.

Greaves (1994) recently conceived the following definition:

> Anticoagulant resistance is a major loss of efficacy in practical conditions where the anticoagulant has been applied correctly, the loss of efficacy being due to the presence of a strain of rodent with a heritable and commensurately reduced sensitivity to the anticoagulant.

This definition encompasses three important elements. Firstly, that the phenomenon described as resistance should involve a significant change in susceptibility that brings about a practical effect. Thus, where the term resistance is applied to a compound and pest species, practitioners should anticipate a real loss of efficacy at the resistance focus. Secondly, that the compound should have been applied correctly and, incidentally, be normally effective for the species involved. Often, when an anticoagulant treatment fails, the cause is attributed to resistance when the real reason is faulty application. Thirdly, that the resistance should have a genetic basis that makes it transmissible between rodent generations.

If the term resistance is to be reserved for cases satisfying these criteria, what term should be used when a heritable change of susceptibility is observed that falls short of this definition in terms of its practical effect? Gill et al. (1992) introduced the phrase "low grade resistance" to describe a situation of this type. However, unless genetical experiments are done, it is very difficult to distinguish between this phenomenon and differences in the response of rodent populations to anticoagulants that would be predicted on the basis of natural variability.

Rodenticide resistance in the sense defined by Greaves (1994) has been found only to the anticoagulant compounds. Therefore, in this paper we will restrict ourselves to dealing with these compounds, which are the basis of the majority of rodent control program worldwide. The main purpose of the paper is to present a critical review of the methods currently used in the detection of resistance to anticoagulants. Later we will look at some anticoagulant resistance phenomena detected in the UK and judge them against the definition of Greaves (1994).

RESISTANCE DETECTION METHODS

It quickly became apparent to those investigating early outbreaks of resistance to warfarin in Norway rats in the UK that methods were required by which to distinguish resistant rat infestations from normally susceptible ones. The two lines of research initially followed were described by Drummond (1966b). The first involved a method in which the effectiveness of field applications of warfarin against suspected warfarin-resistant Norway rats were compared with the results of treatments against anticoagulant-naive populations. This technique became known as the resistance "monitoring graph" method and was fully described by Drummond and Rennison (1973). A second method was developed following the same principle but based on laboratory testing techniques. This became known as the World Health Organization (WHO) "lethal feeding period" test because its result was a discriminating dose of the anticoagulant under investigation, expressed in terms of the number of days of consecutive feeding (the lethal feeding period or LFP), that would kill a defined percentile of normally susceptible animals (WHO 1982). A third line of research resulted in the development of another laboratory resistance detection technique base upon differences observed in the contrasting abilities of the blood of warfarin-susceptible and warfarin-resistant rats to clot after the administration of warfarin (Martin et al. 1979).

Resistance Monitoring Graph

As a susceptible rodent population feeds on poisoned bait in a practical rodent control treatment, the numbers of takes of bait from bait points and the quantity of bait eaten increases at first and then quickly decreases as the poison takes effect. Drummond and Rennison (1973) recorded this process in relation to three populations of warfarin-susceptible Norway rats exposed to either 0.005 or 0.025% warfarin. The data were pooled for the three treatments and plotted, as a regression equation, on a graph in which the abscissa was the day of the recording visit, expressed as a common logarithm, and the ordinate was the number of bait points with takes, expressed as a proportion of the number of takes recorded on the second day of the treatment. The 95 % confidence limits for the expected bait take at any given time were calculated and also plotted. The derived graph therefore illustrates the expected effect of the application of warfarin against susceptible Norway rats infesting UK farmsteads.

The results of treatments, in which the susceptibility status of the infestation is unknown, may be plotted on the derived graph as a test for the presence of resistance. If the decline in bait takes with time follows the normal course, and the plotted line remains within the 95% confidence limits, the treated population is determined to be warfarin-susceptible. If the plotted line moves outside the upper 95% confidence limit for two successive baiting visits, the infestation is considered warfarin-resistant. However, the confidence limits chosen will give this result by chance alone on one occasion in forty and for this reason, and others, the authors recommended confirmatory laboratory test to be carried out on captured survivors of unsuccessful treatments. This method was widely used in the UK, not only for establishing the susceptibility status of suspected resistant populations but also as a method of determining the effectiveness of novel, resistance-breaking rodenticides against warfarin-resistant rat populations (e.g., Rennison and Dubock 1978).

The method has a number of flaws, both practical and statistical in nature. Rennison (1977) pointed out that complete cessation of feeding did not necessarily indicate that the rat infestation had been extinguished unless a careful examination of the site confirmed no other signs of continuing rat activity. The studies of Quy et al. (1992) have further emphasized the need for care in the interpretation of results when the effectiveness of treatments is assessed only by bait take data.

While experience has proven the original monitoring graph to have been robust for its purpose, there is considerable potential for improving its statistical basis. When data are pooled from several sites to obtain a single

regression line there must be confidence that the data employed are homogeneous. In this case, the three farm sites and the results Drummond and Rennison obtained from them show clear signs of heterogeneity (notwithstanding the obvious fact that, at one site, the concentration of warfarin in the bait differed from that used at the other two). This implies that the expected relationship between the decline in the number of bait takes and time is not fixed but may vary from one site to another. The authors also neglected the fact that the width of the 95% confidence limits would be expected to differ depending on the number of bait points monitored on the farms (i.e., sample size); the limits being wider for farms with smaller numbers of points. Furthermore, the regression equation applied assumed a linear relationship between log time and the numbers of bait takes. There is evidence that the relationship observed better fits a curve and, therefore, a suitable transformation should have been applied or a non-linear model fitted to the data.

In spite of these drawbacks, the monitoring graph offers the most easily interpreted positive indication of the existence of a resistant rat infestation. That is, when the rodenticide in question is used, as directed, against a natural pest infestation, it demonstrably fails to provide an acceptable level of control in comparison to its performance against a fully susceptible rat population. Of course, further laboratory work on the inheritance of the resistance trait is required before the definition of Greaves (1994) is fully satisfied.

World Health Organization (WHO) Lethal Feeding Period Test

This second methodology employs the same basic principles as the previous technique but the experimentation is laboratory-based. A susceptibility base-line is established using wild-captured animals from, preferably, anticoagulant-naive populations. Groups of these animals are fed, for differing numbers of days, a no-choice diet comprising a bait containing the poison under study at the strength normally used in control programmes. A dose-response curve is constructed in a similar way to that employed in tests to determine the acute oral LD^ of a compound but, in this case, feeding periods in days are used as the dose variable instead of differing quantities of active ingredient. Thus, lethal feeding period (LFP) percentiles are calculated rather than lethal dose (LD) percentiles.

This process is typified by the work of Buckle et al. (1980) to establish the susceptibility of the South-east Asian rice rat *(Rattus argentiventer)* to baits containing 0.025% warfarin. No-choice feeding periods of 2, 4, 6, 8, 10 and 12 days gave mortalities of 27.5, 57.5, 82.5, 87.5, 97.5 and 100% respectively. These data were used to construct a dose-response curve and, following WHO Guidelines (WHO 1982), the LFP,, was calculated to be 15.1 days, giving a 16-day no-choice feeding period on 0.025 % warfarin bait as the checking test for resistance in this species (Buckle 1983).

The recommended statistical method for the analysis of these data (WHO 1982) is that of Finney (1971), in which the dose variable (days) is expressed as a logarithm and the response variable (percent mortality) as a probit. However, other models for instance involving logit mortality and absolute time rather than log time are arguably equally appropriate. The effect of the model used is apparent when the data of Buckle et al. (1980) are examined using each of the four possible models (Table

There is no obvious biological reason why any one of the models used in Table 1 is more correct than any other. Nor does any of the four calculated dose-response relationships fit the observed data better than any other. Thus, at the LFP,, percentile recommended by the WHO, discriminating doses ranging from 11 days to 20 days are equally valid. In this unsatisfactory situation it is prudent, therefore, to adopt a less extreme LFP percentile at which the effect of the model used is unimportant. For example, Table 1 shows the discriminating dose to be eight days for all four models at the LFP^. The use of this percentile has the additional benefits that the feeding tests are of shorter duration and that the discriminating dose is derived by interpolation rather than by the less reliable method of extrapolation.

The WHO lethal feeding period test has been widely applied to first generation anticoagulants and to one of the second generation compounds (i.e., difenacoum, Redfern and Gill 1978). However, difficulties are encountered when using the technique to derive discriminating doses to detect resistance to the more potent compounds. This is because the steep slope of the dose-response curve does not allow sufficient data points for proper statistical analysis (Gill and MacNicoll 1991). For example, all susceptible Norway rats succumb to a single day of feeding on 0.005% brodifacoum and flocoumafen baits (Buckle 1994). In this case, the WHO guidelines recommend that the concentration of the anticoagulant in the bait should be reduced to produce survivors at a sufficient number of dosage (days) intervals. Gill and MacNicoll (1991) used 0.0005% brodifacoum bait (10% of the concentration of active ingredient normally used in rodenticide treatments) to establish a discriminating dose of brodifacoum and pointed out the difficulty of this approach. However, the result was the anomalous situation of rats being declared "resistant" to the compound in spite of the fact that they succumb to baits containing it at full field strength (i.e., 0.005%). The difficulty of determining the impact of this so-called resistance on the outcome of practical control treatments is readily apparent and certainly, in this case, the criteria for true resistance proposed by Greaves (1994) are not met.

Resistance Ratios

If the LFP test is inappropriate for use with the potent second generation anticoagulants, how then should resistance to them be monitored? The concept of the lethal feeding period was introduced in rodenticide resistance testing to overcome difficulties presented by the chronic nature of the first generation anticoagulants, such as warfarin, and the consequent requirement to administer them over several days for full effect. However, resistance to other pesticides (e.g., insecticides) is more normally established by the comparison of dose-response data from susceptible and resistant strains in which the dose variable is an administered quantity of the compound in question, rather than a period of time. It seems

Table 1. The data on the susceptibility of *R. argentiventer* to 0.25% warfarin (Buckle et al. 1980) are here used to demonstrate the effect of the model used to derive discriminating doses for use in checking tests for anticoagulant resistance using the WHO lethal feeding period method.

LFP percentile	Lethal feeding period (days)			
	Probit/days	Logit/days	Probit/log days*	Logit/log days
LFP_{50}	3.6	3.6	3.2	3.2
LFP_{90}	7.7	7.5	7.5	7.6
LFP_{95}	8.8	8.9	9.6	10.1
LFP_{98}	10.1	10.6	12.6	14.7
LFP_{99}	11.0	11.8	15.1	19.3

* method of Finney (1971) recommended by WHO.

sensible then to revert to this methodology with the second-generation anticoagulants which are active after single doses.

Lethal dose (LD) percentiles can be determined in base-line experiments in which graded doses of the anticoagulant are given to groups of susceptible animals. To assess the resistance status of rodent populations, equivalent tests can be conducted with groups of suspected resistant animals and the relationship, at a certain LD percentile, between the potency of the active ingredient in susceptible and resistant animals expressed as a conventional "resistance ratio." Reference to established Norway rat resistance ratios for the second generation anticoagulants in existing resistance foci (Greaves 1994) would assist in determining the likely practical impact of any resistance detected. For tests on individual rodents, a certain LD percentile dose, say the LD99 or a multiple of it, can be used in checking tests. Further work is required, however, to establish the potential of these proposals to provide effective and practicable resistance tests. Particularly, it is important to recognize that this technique would be expected to reflect accurately the resistance status of populations only when the frequency of the resistant phenotype is high. If the frequency is low, even a very high level of resistance in resistant individuals would not result in a significantly increased resistance ratio (Ward, personal observations).

Blood Clotting Response (BCR) Tests

As warfarin resistance (and cross-resistance to many of the other first generation anticoagulants) took hold in Norway rat populations in the UK, work began towards the development of more cost-effective tests to determine the resistance status of these rodents. This was because the tests described in the preceding paragraphs required either extensive field investigations or laboratory experiments of many weeks duration. Work on the biochemistry of resistance (Bell and Caldwell 1973) led to the observation that, in the Vitamin K cycle, the reduction of Vitamin K, epoxide to the quinone was less liable to inhibition by warfarin in resistant than in susceptible rats. Martin et al. (1979) used this effect in the development of a blood clotting response (BCR) test to distinguish warfarin resistant from warfarin susceptible Norway rats.

As in the previous tests, base-line observations were first required. In BCR tests the response variable is the reduction in blood clotting activity. This is measured as a clotting time and expressed as a percentage (of normal) coagulation activity (PCA). Coagulation times are determined using commercially available test methods (e.g., "Thrombotest") and converted to PCAs by reference to calibration curves. These curves are generated by determining coagulation times for a blood (or plasma) dilution series prepared form pooled blood samples from specific numbers of male and female animals. Thus, a blood sample with a PCA of 50% has a coagulation time equivalent to that of a 50% dilution of the pooled normal blood sample.

The next step in the development of the warfarin BCR test was to derive a discriminating dose of warfarin required to produce a specific effect on the blood coagulation of susceptible rats. (The effect chosen by Martin et al. (1979) was a reduction of blood clotting activity to less than 17% of that seen in normal animals.) This was done by administering graduated doses of warfarin to groups of animals and observing, 24 hours later, their effect in preventing blood coagulation in different proportions of the animals tested. Vitamin K, epoxide was co-administered with the anticoagulant to reduce the tendency of resistant animals to become Vitamin K deficient.

Martin et al. (1979) proposed a discriminating dose of 0.5 mg of warfarin per 100 g of animal body weight. This was administered in saline solution with 0.1 mg per 100 g of Vitamin K, epoxide. When this dosage was given to 212 Norway rats, all warfarin resistant animals had PCAs that were greater than 17% and all warfarin susceptible animals had a PCAs less than 17 %. Thus, the chosen dose was effective in discriminating resistant and susceptible Norway rats. The same authors went on to develop the test method further so that homozygous and heterozygous resistant animals could be distinguished.

MacNicoll and Gill (1993a) revised this method, replacing Vitamin K, epoxide with a water-soluble form of Vitamin K_3 (menadione sodium bisulphite or MSB) and also using a water-soluble form of the anticoagulant, sodium warfarin. In the new test, both anticoagulant and vitamin are administered by oral intubation rather than by

intraperitoneal injection. Subsequently, Gill et al. (1993) proposed a BCR test for difenacoum resistance and a test for resistance to bromadiolone is also available (Gill et al. 1994).

The doses of active ingredient and Vitamin K administered, the time from the administration of anticoagulant to measurement of PCA and the threshold PCA selected for each of these tests are summarized in Table 2.

Blood clotting response tests have a number of significant advantages. The tests are conducted in the laboratory and provide rapid assessments of the resistance status of individual rodents. They are not necessarily lethal to the animals tested because an effective dose of antidote can be administered after PCA assessment. This has benefits in the welfare of the animals used and permits the resistance status of individuals to be determined sequentially to a number of different compounds. It also allows animals to be used in later breeding studies in which the genetical basis of any resistance observed can be studied. The tests are very sensitive and can be used to detect small differences in the susceptibility of individual rodents to anticoagulants.

However, some difficulties are associated with the use of the method. It relies on comparative measurements of the physiological effects of anticoagulants in susceptible and resistant rodents. Such observations provide no direct indication of the practical impact of the resistance observed and further work is required for this to be to determined. Because of the sensitivity of the tests, relatively small differences in the susceptibility of rodent populations to anticoagulants can be demonstrated and there is a risk that naturally-occurring intraspecific variation can be classified as "resistance." There is evidence that Vitamin K_3 is antidotal to the effects of anticoagulants in resistant, but not in susceptible, rodents (MacNicoll and Gill 1993b). Therefore, care is required when using MSB to prevent resistant animals from becoming vitamin K deficient during BCR tests to avoid producing a confounding antidote effect.

PRACTICAL USE OF RESISTANCE TESTS

The most thoroughly researched anticoagulant resistance focus in the UK is the area in which warfarin-resistant Norway rats infest farmsteads on the Anglo-Welsh border. In this locality, the resistance monitoring graph has been used successfully in the assessment of novel, resistance-breaking anticoagulants (e.g., Rennison and Dubock 1978) and both LFP and BCR tests have been developed for resistance detection (Drummond and Wilson 1968, Martin et al. 1979). These tests accurately reflect a resistance situation in which warfarin is almost useless for the control of Norway rat populations containing a high percentage of resistant animals.

Resistance in *R. norvegicus* to the second-generation compound, difenacoum, was reported on farms in central southern England soon after the introduction of the compound (Greaves et al. 1982). A WHO lethal feeding period test was established for the detection of difenacoum resistance (Redfern and Gill 1978) and, subsequently, a BCR test was also developed (Gill et al. 1993). Rats were trapped from farms in the resistance area and found to be resistant to difenacoum using both detection methods. However, Greaves and Cullen-Ayres (1988) speculated that the level of resistance to difenacoum observed at the focus (the resistance ratio is four-fold) was insufficient to account for the severity of the practical problem. Later, extensive field studies revealed that control difficulties were primarily due to behavioral factors, principally the constant availability of alternative food sources that made the rat infestations very difficult to bait (Quy et al. 1992, Quy et al. these proceedings).

A further focus of resistance to the second-generation anticoagulants was found to occur in Berkshire. At this site, rats are fully resistant, in practical terms, to bromadiolone (Prescott, personal observations) and work is in progress to determine a resistance ratio for this compound by the method proposed above. These infestations also contain individuals that survive the brodifacoum LFP test of Gill and MacNicoll (1991),

Table 2. Some parameters used in blood clotting response tests for detecting resistance to anticoagulants in Norway rats.

Active ingredient	Discriminating dose* of active ingredient	Type of vitamin K used	Dose* of vitamin K	Time (hrs) to checking PCA	Threshold PCA
Warfarin	0.5	K_1	0.1	24	17
Sodium warfarin	0.54	K_3	0.1	24	17
Difenacoum	0.5	K_3	0.2	96	10
Bromadiolone (males)	0.1	K_3	1.0	96	10
Bromadiolone (females)	0.24	K_3	1.0	96	10

* mg/kg per 100 g rat body weight

which involves feeding for seven days on 0.0005% brodifacoum. However, none would be expected to survive feeding on full strength (i.e., 0.005%) brodifacoum baits and the efficacy of brodifacoum for the control of these infestations remains to be determined. These observations serve to illustrate the difficulties facing those who try to interpret, in terms of likely practical effect on rodent control, the results of laboratory experiments conducted to determine the resistance status of rodent populations. The foregoing also suggests that more research is required before statistically valid and fully reliable resistance detection methods are available.

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