Warfarin resistance: a balanced polymorphism in the Norway rat

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SUMMARY

The frequency of monogenic resistance to anticoagulant rodenticides in *Rattus norvegicus* in an area straddling the England–Wales border was monitored from 1967 until 1975. Rats were trapped on farms and tested in the laboratory by administering a dose of warfarin lethal to susceptibles. The mean incidence of resistance was 44 % and did not change significantly, despite the extensive use of anticoagulants by farmers during the 9-year period. In 1975 more refined techniques showed that the frequencies of susceptible (SS) and resistant (RR) homozygotes were significantly below the Hardy–Weinberg expectations and simple estimates of the relative fitness ratios for the RR, RS and SS phenotypes were 0·37, 1·0 and 0·68 respectively. In two relatively isolated valleys, where selection with anticoagulants was minimal, the frequency of resistance decreased significantly from 57 % to 39 % during 1973–5. The results are consistent with the hypothesis that a balanced polymorphism is being maintained. Selection against susceptible homozygotes by the use of anticoagulant rodenticides, and against the resistant homozygote due to its high susceptibility to a primary deficiency of vitamin K gives the heterozygotes a selective advantage. A number of ecological factors that influence the incidence of the resistance are discussed briefly.

1. INTRODUCTION

The Norway rat, *Rattus norvegicus* (Berkenhout), is a common agricultural and urban pest in Britain. Since the early 1950s it has normally been controlled by baiting with anticoagulant rodenticides such as warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin). These rodenticides act as antagonists of vitamin K, inducing symptoms identical with severe deficiency of the vitamin, namely hypoprothrombinaemia leading to haemorrhage and death. In 1959 infestations of rats resistant to warfarin were discovered on two farms near Manafon, Powys, and by 1966 the resistant population was reported to have spread through a roughly circular area about 53 km in diameter, straddling the England–Wales border (Drummond, 1966).

Genetical studies have shown that the resistance is due to an autosomal gene with dominant effect (Greaves & Ayres, 1967, 1969*a*). At the biochemical level

anticoagulants are believed to act in the non-resistant rat by inhibiting the enzymic reduction of vitamin K oxide, a product of vitamin K metabolism; in warfarin-treated rats the accumulation of oxide decreases the physiological availability of the vitamin and prevents further synthesis of the vitamin K-dependent prothrombin-complex blood clotting factors (Bell, Sadowski & Matschiner, 1972). It has been proposed that the resistance is due to an alteration in the enzyme system that converts the oxide to the vitamin so that the enzyme system is less inhibited by warfarin; the altered enzyme appears however to be less effective in catalyzing the oxide-K conversion, with the result that the resistant animals are abnormally sensitive to vitamin K deficiency (Bell & Caldwell, 1973).

The high susceptibility of warfarin-resistant rats to vitamin K deficiency was first reported by Hermodson, Suttie & Link (1969), who noted that, as compared with susceptibles, heterozygotes needed 2–3 times as much vitamin K, while resistant homozygotes needed nearly 20 times as much to cure the hypoprothrombinaemia induced by keeping the rats on a vitamin K-deficient diet. Subsequently, spontaneous hypoprothrombinaemia was reported in resistant homozygotes by Bell & Caldwell (1973) and Greaves & Ayres (1973). Thus, the dominant character of warfarin resistance is also expressed as an almost recessive susceptibility to vitamin K deficiency.

Evidently, in a natural population where warfarin resistance is segregating, if there is selection against non-resistant homozygotes by farmers and others using warfarin, and against resistant homozygotes due to a shortage of vitamin K in the diet, then a balanced polymorphism with heterozygous advantage should develop. This possibility is of considerable genetical interest for there can be few, if any, situations in which the selective pressures maintaining a balanced polymorphism can be so clearly identified and laid open to further study. It is also of obvious practical interest to investigate the factors that control the frequency of a resistance gene in the pest population.

Since 1967 we have had several opportunities to ascertain the prevalence of resistance in the affected rat population in Wales and in this paper we present evidence gathered over a 9-year period to show that warfarin resistance has been maintained as a balanced polymorphism of considerable stability.

2. METHODS

The study was made in the Welsh 'resistance area' described by Drummond (1966), a tract of small mixed farms centred upon Welshpool, Powys. The Norway rat is essentially a commensal pest and its distribution is largely determined by the location of food sources provided by man; for much of the year, therefore, the majority of the rats in rural areas are to be found in or near farm buildings, particularly those in which grain or animal feeding-stuffs are stored. Enquiries made of the farmers indicated that during 1967–70 about half (44/86) were using an anticoagulant (i.e. a warfarin-type) rodenticide each year, with a further third using non-anticoagulant (and therefore non-selective) rodenticides such as

zinc phosphide. In comparison, in areas not affected by resistance, almost all rat destruction is done with anticoagulants.

Infested farm buildings were located by surveying 3×3 km squares that had been selected by means of random numbers, and the rats were collected by live trapping. In addition, during 1970–5 we obtained rats by systematic trapping from farmsteads in the relatively isolated valleys of the rivers Carno and Banwy. In these valleys we tried to ensure, by carrying out all rat control with our own pest control operators using traps and non-selective rodenticides, that selection with anticoagulant rodenticides was minimal.

The trapped rats were brought to the laboratory, caged singly and, after a settling-in period, were tested for resistance. The usual test was to inject the rats subcutaneously with 200 mg/kg of warfarin dissolved in dimethyl formamide, recording mortality during the next 14 days. An alternative technique was to feed the rats on a rodenticidal bait, medium oatmeal containing 0·005 % warfarin, for 6 days, again recording mortality during a 14-day period. These tests are generally lethal to susceptibles and therefore simply identified the rats as resistant or susceptible to warfarin. By 1975 we were able to identify the three phenotypic classes (RR, RS or SS) utilizing a two-stage procedure described by Martin, Steed, Redfern & Gill (in preparation). Briefly, each rat is injected intraperitoneally with 1 mg/kg of vitamin K oxide and 5 mg/kg of warfarin; 24 h after the injection the thrombotest time is found to be prolonged in susceptibles but not in resistants, due to the fact that, in the presence of warfarin, only the resistant rats can use the oxide as a source of vitamin K. After a suitable recovery period, usually a week, the resistant rats receive a topping-up injection of 0·36 mg/kg of vitamin K, and are placed on a vitamin K-deficient diet. After 4 days the thrombotest time is found to be distinctively prolonged in the resistant homozygotes owing to their high susceptibility to vitamin K deficiency.

The different tests gave very similar results and the results for animals tested by different techniques were pooled whenever appropriate.

3. RESULTS

The incidence of resistance in rats trapped in the main area is shown in Fig. 1(*a*). The annual changes are all insignificant, as is the largest difference, between 1969 and 1974. Pooling the annual samples, 364/836 ($44 \pm$ S.E. 2 %) of the rats are found to be resistant.

The samples from the valleys were pooled for each year as the difference between them was not significant ($t = 0·62$; $P > 0·5$). The annual incidence of resistance in the valleys, shown in Fig. 1(*b*), remained fairly high, around 50 % during 1970–2. A check prompted by this high level disclosed that during October 1972 to March 1973 about 20 % (16/75) of farmers were continuing to apply anticoagulant rodenticides; following intensive local publicity for the study, the proportion fell to 10 % (7/70) in April–October 1973, after which it remained low at 1 %, 4 %, 4 % and 0 % in successive 6-month periods. The percentage of resistant rats in the valley samples increased significantly from 49 % in 1972 to

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57% in 1973 ($P < 0.025$) but then decreased significantly in 1974 to 47% ($P < 0.01$) and again in 1975 to 39% ($P < 0.05$).

The numbers of rats of the three phenotypes trapped in 1975 are summarized in Table 1. The expected numbers are Hardy–Weinberg expectations calculated from the observed gene frequencies. The data show, for the first time, that resistant homozygotes of both sexes occur in the wild. Confirmation of this observation was obtained by test-crossing one male and one female, producing respectively 20 and 9 offspring, all of which were resistant. The observed numbers of the three
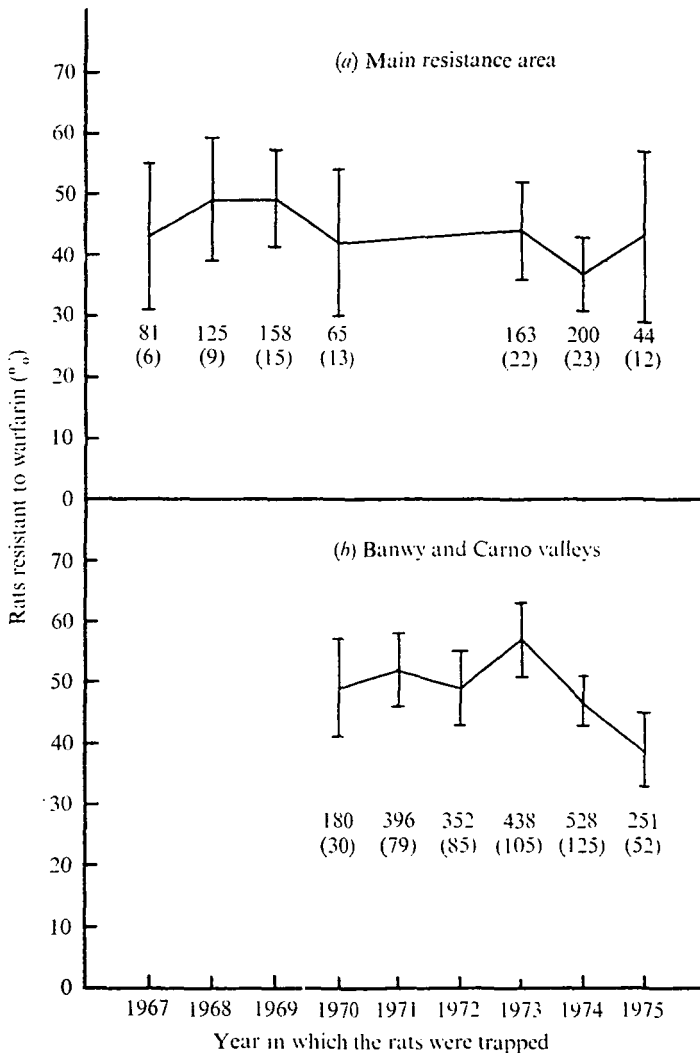

Fig. 1. The incidence of warfarin resistance in rats trapped on farmsteads during 1967–75. The vertical bars indicate the range of two standard errors above and below each point. Also shown are the numbers of rats tested and, in parentheses, the numbers of infestations from which they were drawn. Selection with anticoagulants was minimal in the Banwy and Carno valleys from 1973 onwards.

phenotypes differ significantly from the Hardy–Weinberg expectation in the main area ($\chi^2 = 5\cdot3$; $P < 0\cdot025$) with shortages of both types of homozygotes and an excess of heterozygotes. This incidates that selection was favouring the heterozygotes. In the valleys however the observed numbers do not differ significantly from the expected numbers ($\chi^2 = 0\cdot876$; $P > 0\cdot3$).

Table 1. *The frequencies of different warfarin-resistance phenotypes and their Hardy–Weinberg expectations in rats trapped during* 1975

(The relative fitness ratios were calculated for the main area, where the gene frequency was stable, but not for the valley population since here the frequency of the resistance gene was decreasing.)

Trapping area		Resistance phenotype				No. of infestations sampled
		RR	RS	SS	Total	
Main resistance area	Number observed	4	42	28	74	16
	Number expected	8·5	33·1	32·4	74	—
	Relative fitness	0·37	1·00	0·68	—	—
Banwy and Carno valleys	Number observed	4	47	80	131	26
	Number expected	5·8	43·5	81·7	131	—

4. DISCUSSION

The failure of the resistance to increase in the main area during 9 years of continued selection with anticoagulant rodenticides proves that a balance was being maintained by some opposing factor or factors. Since there is good evidence that the frequency of the resistance gene was stable it is valid to calculate the fitness ratios from the relative frequencies of the three phenotypes in the main area, as shown in Table 1. Besides confirming that there was substantial selection against susceptibles, the fitness ratios show that there was strong selection against the resistant homozygote, giving rise to a balanced polymorphism based upon heterozygous advantage. In practice our estimates of selection against the two homozygotes are almost certainly too low since if we had been able to allow for inbreeding the expected numbers of homozygotes in the sample would have been larger. Inbreeding in rat populations can arise from the isolation of infestations by distance, particularly as the average size of infestations is small; further, the social organization of rat colonies tends to limit successful breeding to a relatively small number of dominant, closely related individuals (Calhoun, 1962, p. 142) which would also tend to increase homozygosity. We note, however, contrary to our previous suggestion (in Ford, 1971), that resistant homozygotes do exist in the wild and therefore that the resistance gene does not act as a recessive lethal in natural populations.

In the valleys, the high level of resistance encountered during 1970–3 may be attributed to the extensive use of anticoagulants by farmers. The subsequent reduction in anticoagulant usage during 1974–5 probably caused the significant fall in resistance during this period. Since the numbers of the three phenotypes in

the valley sample (Table 1) are a good fit to the Hardy–Weinberg expectation they provide no evidence of selection. This may be partly due to the smaller proportion of resistant animals in the valley population. However, since the frequency of the resistance gene was demonstrably decreasing (precluding, in any event, the calculation of valid fitness ratios) the data are consistent with selection against the resistant homozygote only, or perhaps against both resistant pheno-types. It remains to be shown whether, in the absence of anticoagulant treatment the heterozygote is at some selective disadvantage compared with the susceptible homozygote, as may be suspected from the slightly greater vitamin K requirement of the heterozygote.

The impressive stability of the polymorphism tends to obscure its ecological complexity. As Bishop & Hartley (1976) have pointed out, different dynamic processes can occur in different local populations. Rodenticide treatment, and therefore selection for resistance is normally confined to farm buildings and rarely affects nearby field populations. However, if selection against resistance affects mainly homozygotes then it also must occur mainly in populations in farm buildings, for it is only here that significant numbers of resistant homozygotes could be expected to build up. Environmental heterogeneity in factors controlling the incidence of resistance is probably considerable, though little studied. For example, on some farms the rats have access to vitamin K-reinforced animal feed, which must minimize selection against resistance. Habitats also vary seasonally. In the winter, fields and hedgerows become very marginal as rat habitat, a factor which may bear more heavily against the resistant segment of the population. Farm buildings, however, become very favourable on account of the extensive storage of winter feed for livestock and, unless already fully colonized, they may become a haven for migrant rats.

Local infestations vary greatly in their degree of resistance (though almost all have some resistant rats) according to whether, and how recently the farmer has applied an anticoagulant rodenticide. Since anticoagulants are relatively easy and safe to handle many farmers persist in their use, not turning to alternative, non-selective rodenticides until the degree of control achieved becomes unsatisfactory. At this stage usually all of the rats in an infestation are resistant. Thus selection is often related to the degree of resistance in an infestation, and this may be an important factor tending to stabilize the frequency of the resistance gene. To sum up, it is likely that the balance is maintained in a complex manner, involving migration, selective mating and great variation in selection for and against resistance at different times and between one section of the population and another.

A potentially important change in conditions that occurred during the study was the introduction of the anticoagulant coumatetralyl (4-hydroxy-3-(1,2,3,4-tetrahydro-1-naphthyl)coumarin), a compound which, though not completely non-selective, is relatively toxic to resistant rats (Greaves & Ayres, 1969b). From 1969 onwards this rodenticide was used by many farmers in the main area, owing to its effectiveness against resistant infestations. Interestingly, the use of couma-tetralyl seems not to have affected the incidence of resistance, probably because

the ordinary user lacks the skill to eradicate an infestation completely and, as with warfarin, the residual population is usually composed entirely of resistant individuals. More recently analogues of coumatetralyl have been developed that are still less selective and are generally believed to have overcome warfarin resistance as a practical problem in the Norway rat (Hadler & Shadbolt, 1975; Hadler, Redfern & Rowe, 1975; Redfern, Gill & Hadler, 1976). It will be interesting to discover whether the use of these new compounds will lead to a decrease in the frequency of the resistance gene or whether its prevalence will be maintained by the small remaining degree of selective toxicity that these compounds possess.

REFERENCES

BELL, R. G. & CALDWELL, P. T. (1973). Mechanism of warfarin resistance. Warfarin and the metabolism of vitamin K_1. *Biochemistry* **12**, 1759–1762.

BELL, R. G., SADOWKSI, J. A. & MATSCHINER, J. T. (1972). Mechanism of action of warfarin. Warfarin and metabolism of vitamin K_1. *Biochemistry* **11**, 1959–1961.

BISHOP, J. A. & HARTLEY, D. J. (1976). The size and age structure of rural populations of *Rattus norvegicus* containing individuals resistant to the anticoagulant poison warfarin. *Journal of Animal Ecology* **45**, 623–646.

CALHOUN, J. B. (1962). *The Ecology and Sociology of the Norway Rat*. U.S. Public Health Service Publication no. 1008. Washington, D.C.

DRUMMOND, D. (1966). Rats resistant to warfarin. *New Scientist* **30**, 771–772.

FORD, E. B. (1971). *Ecological Genetics*, 3rd ed., p. 350. London: Chapman and Hall.

GREAVES, J. H. & AYRES, P. (1967). Heritable resistance to warfarin in the rat. *Nature* **215**, 877–878.

GREAVES, J. H. & AYRES, P. (1969a). Linkages between genes for coat colour and resistance to warfarin in *Rattus norvegicus*. *Nature* **224**, 284–285.

GREAVES, J. H. & AYRES, P. (1969b). Some rodenticidal properties of coumatetralyl. *Journal of Hygiene* **67**, 311–315.

GREAVES, J. H. & AYRES, P. (1973). Warfarin resistance and vitamin K requirement in the rat. *Laboratory Animals* **7**, 141–148.

HADLER, M. R., REDFERN, R. & ROWE, F. P. (1975). Laboratory evaluation of difenacoum as a rodenticide. *Journal of Hygiene* **74**, 441–448.

HADLER, M. R. & SHADBOLT, R. S. (1975). Novel 4-hydroxycoumarin anticoagulants active against resistant rats. *Nature* **253**, 272–277.

HERMODSON, M. A., SUTTIE, J. W. & LINK, K. P. (1969). Warfarin metabolism and vitamin K requirement in the warfarin resistant rat. *American Journal of Physiology* **217**, 1316–1319.

REDFERN, R., GILL, J. E. & HADLER, M. R. (1976). Laboratory evaluation of WBA 8119 as a rodenticide for use against warfarin-resistant and non-resistant rats and mice. *Journal of Hygiene* **77**, 419–426.