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THE ROYAL SOCIETY

Predicting insecticide resistance: mutagenesis, selection and response

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Strategies to manage resistance to a particular insecticide have usually been devised after resistance has evolved. If it were possible to predict likely resistance mechanisms to novel insecticides before they evolved in the field, it might be feasible to have programmes that manage susceptibility. With this approach in mind, single-gene variants of the Australian sheep blowfly, *Lucilia cuprina*, resistant to dieldrin, diazinon and malathion, were selected in the laboratory after mutagenesis of susceptible strains. The genetic and molecular bases of resistance in these variants were identical to those that had previously evolved in natural populations. Given this predictive capacity for known resistances, the approach was extended to anticipate possible mechanisms of resistance to cyromazine, an insecticide to which *L. cuprina* populations remain susceptible after almost 20 years of exposure. Analysis of the laboratory-generated resistant variants provides an explanation for this observation. The variants show low levels of resistance and a selective advantage over susceptibles for only a limited concentration range. These results are discussed in the context of the choice of insecticides for control purposes and of delivery strategies to minimize the evolution of resistance.

Keywords: genetic response; insecticide resistance; *Lucilia cuprina*; mutagenesis; selection

1. INTRODUCTION

Effective pesticides play a key role in the management of agricultural ecosystems. For example, in sheep the control of ectoparasites is currently dependent on the use of chemicals. This situation will continue to apply in, at least, the intermediate term (Levot 1993). Pesticide usage leads to chemical residues, resulting in issues of occupational health and safety and, particularly after processing of the fleece, potential environmental degradation. Similar difficulties arise in other agricultural systems.

The residue problem is exacerbated by the evolution of resistance to the pesticide. To maintain control, the response is commonly more frequent application of higher concentrations of pesticide (Daly & McKenzie 1986; Roush & Tabashnik 1990; Denholm & Rowland 1992; McKenzie 1996) with resistance-management strategies usually based on general models influenced by the anticipated genetic basis of resistance (Georghiou & Taylor 1977*a*,*b*; Roush 1989; Tabashnik 1990; Rosenheim & Tabashnik 1990; Gressel 1995). Specific strategies are generally put in place only after resistance has already evolved (Daly & McKenzie 1986; Forrester *et al.* 1993).

There would be many advantages if a likely resistance mechanism could be predicted before a new insecticide was introduced into the field. The availability of genetic, toxicological, biochemical, cross-resistance and relative fitness data would maximize the chance of devising strategies to minimize the evolution of resistance, that is, enable the management of susceptibility (Leeper *et al.* 1986; Daly & McKenzie 1986; Firko & Hayes 1990; Tabashnik 1990; McKenzie 1996). To attempt to make a meaningful prediction, it is essential to have an understanding of the genetic basis underlying the observed phenotypic basis of resistance. It is on this variation that evolutionary processes will act (Roush & McKenzie 1987).

2. THE GENETIC BASIS OF RESISTANCE

Monogenic and polygenic control of resistance is observed in natural populations (Roush & McKenzie 1987; McKenzie 1996). There is considerable debate about the relative importance of these mechanisms (Roush & McKenzie 1987; Mallet 1989; Macnair 1991; McKenzie & Batterham 1994, 1995; Groeters 1995; Tabashnik 1995; McKenzie 1996). Such discussions are a subset of a more general evolutionary debate concerning monogenic and polygenic responses during adaptation (Lande 1983; Macnair 1991; Orr & Coyne 1992).

For the purpose of this paper, let us assume that when an insecticide is first introduced for pest control the population consists of susceptible phenotypes. Within the susceptibles we also assume that viability is normally distributed with differences between individuals of that continuous distribution being under polygenic control (McKenzie & Batterham 1994). If selection acts within the distribution, the differences between selectively advantaged and disadvantaged individuals will be polygenically based and therefore a polygenic response will be expected. If a rare resistant mutation, outside the range of the original phenotypic distribution, confers a selective advantage over susceptibles, at concentrations that cannot

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be accommodated by a polygenic response, a monogenic response is expected (Whitten & McKenzie 1982; Macnair 1991; McKenzie & Batterham 1994; McKenzie 1996). Comparative laboratory studies in the Australian sheep blowfly, *Lucilia cuprina*, support these propositions (McKenzie *et al.* 1980, 1992). In natural populations of *L. cuprina*, resistances to dieldrin, diazinon and malathion are primarily due to allelic substitutions at single genetic loci, an observation consistent with the way in which natural selection for resistance acts in this species (McKenzie 1993, 1996).

In the light of the above arguments and observations we have attempted to mimic, and predict, field resistance by selecting for monogenic responses in the laboratory.

3. PREDICTING RESISTANCE MECHANISMS

To have any confidence that variants, selected in the laboratory for resistance to a novel insecticide, will be predictive of mechanisms that may evolve in the field after the insecticide is introduced, it is first necessary to conduct the appropriate control experiments. That is, it is necessary to demonstrate that variants selected in the laboratory for resistance to previously used insecticides are equivalent to the resistant variants that evolved in natural populations. This has been done in *L. cuprina*.

(a) *Control experiments: resistance to dieldrin, diazinon and malathion*

Dieldrin, a cyclodiene, was used to control sheep blowfly between 1955 and 1958 (Hughes & McKenzie 1987). Resistance to this insecticide evolved in natural populations of *L. cuprina* within two years as a result of allelic substitution at the *Rdl* locus on chromosome V (McKenzie 1987). Resistance is due to less effective blocking of insect neuronal GABA receptors by the insecticide in resistant strains (Smyth *et al.* 1992). The resistance gene is the orthologue of the dieldrin resistance gene, *Rdl*, of *Drosophila melanogaster*. The same serine-to-alanine substitution is responsible for resistance in both species (P. Batterham, Z. Chen, R. H. ffrench-Constant, K. Freebairn, R. D. Newcomb and J. A. McKenzie, unpublished data; ffrench-Constant 1994).

The organophosphorus insecticide diazinon replaced dieldrin for the control of blowfly in 1958. Resistance, essentially controlled by allelic substitution of the *Rop-1* locus on chromosome IV (McKenzie 1993), was observed in the field in 1965 (Hughes & McKenzie 1987). The susceptible allele of the *Rop-1* gene encodes a carboxylesterase, E3 (Hughes & Devonshire 1982). The resistance substitution (glycine^{137} to aspartic acid) leads to a gain of organophosphorus hydrolase activity at the expense of the carboxylesterase activity (Newcomb *et al.* 1997; Campbell *et al.* 1998*a*). A second gene, *Rop-2*, on chromosome VI, also influences resistance to diazinon, but occurs very rarely in natural populations (Arnold & Whitten 1976). *Rop-2* codes for a mixed-function oxidase (Hughes & Devonshire 1982; Terras *et al.* 1983).

Malathion, also an organophosphorus insecticide, was not used specifically for blowfly control. However, resistance, of *L. cuprina* to this chemical was detected in 1968 and mapped to a gene on chromosome IV (Hughes *et al.* 1984). This gene, *Rmal*, was believed to be tightly linked to the *Rop-1* locus (Raftos & Hughes 1986; Smyth *et al.* 1994), but recent molecular data suggest that malathion resistance is, in fact, controlled by an allele of the *Rop-1* locus (Campbell *et al.* 1998*a*). For convenience the *Rmal* notation is maintained. As a result of a single amino-acid substitution (tryptophan^{251} to leucine), the resistant form of the E3 enzyme has both organophosphorus hydrolase and malathion carboxylesterase activities (Hughes *et al.* 1984; Campbell *et al.* 1998*a*,*b*). Hence, *Rmal* genotypes provide some resistance to diazinon but *Rop-1* genotypes do not provide no resistance to malathion (Campbell *et al.* 1998*b*).

Table 1. *The genetic and molecular bases of resistance to dieldrin, diazinon and malathion in laboratory- and field-selected variants of* L. cuprina

(Derived from: Hughes & McKenzie 1987; McKenzie *et al.* 1992; Smyth *et al.* 1992; Newcomb *et al.* 1997; Campbell *et al.* 1998*a*; P. Batterham, Z. Chen, R. H. ffrench-Constant, K. Freebairn, R. D. Newcomb and J. A. McKenzie, unpublished data.)

resistance	genetic locus		amino-acid change susceptible⟶resistant	
	field	laboratory	field	laboratory
dieldrin	*Rdl*	*Rdl*	ser^{302}⟶ala	ser^{302}⟶ala
diazinon	*Rop-1*	*Rop-1*	gly^{137}⟶asp	gly^{137}⟶asp
malathion	*Rmal*	*Rmal*	trp^{251}⟶leu	trp^{251}⟶leu

In the laboratory, susceptible strains were mutagenized by using ethyl methanesulphonate (EMS) and progeny selected for resistance to dieldrin or diazinon by screening above the lethal concentration (LC_{100}) of susceptibles. The dieldrin- (Smyth *et al.* 1992) and diazinon- (McKenzie *et al.* 1992) resistant strains generated showed similar toxicological, biochemical, genetic and molecular bases similar to those observed in natural populations (table 1). It can therefore be concluded that if these experiments had been conducted before the insecticides were introduced for blowfly control, the laboratory results would have been highly predictive of the mechanisms that evolved in natural populations. Two comments should be made to place this statement in context.

First, selection in the laboratory for resistance to diazinon generated variants which are resistant to just this chemical (*Rop-1*) and variants resistant to malathion, with some resistance to diazinon (*Rmal*). Second, *Rop-2* variants have not been generated, thus far, in the laboratory mutagenesis–selection regime.

The first observation is consistent with resistance to malathion having been selected in the field by exposure to diazinon. Although other explanations are possible (Campbell *et al.* 1998*b*), the laboratory result supports the initial hypothesis of Hughes *et al.* (1984) of such a selective process.

The absence of *Rop-2* variants from the laboratory screen may help provide an explanation for its rarity in field populations; however, a larger sample of resistant mutants is necessary for a definitive comment. This work is in progress.

In summary, taking these comments into account, the control experiments provide sufficient encouragement to

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Table 2. *Comparison of strains of* L. cuprina *resistant or susceptible to cyromazine*

(Derived from Yen *et al.* (1996).)

strain	resistance ratio*	viability	concentration range (% (W/V) $\times 10^{-5}$) of selective advantage**
susceptible	1	viable	0–4
Cyr 4 (1)	4.1	recessive lethal	6–30
Cyr 4 (2)	1.5	viable	6–20
Cyr 5 (1)	2.8	recessive subvital	4–25
Cyr 5 (2)	2.2	recessive subvital	8–25

*LC_{90} of heterozygote/LC_{90} of susceptible.
**Susceptible compared with resistant heterozygote.

extend the laboratory programme to the prediction of possible resistance mechanisms before they have evolved to a particular insecticide in natural populations. An attempt to select for resistance to cyromazine was therefore instigated.

(b) *Predicting resistance to cyromazine*

Cyromazine acts as an insect growth regulator (Binnington 1985) and was introduced during the late 1970s to control a number of pests (Hart *et al.* 1982; Iseki & Georghiou 1986; Hughes *et al.* 1989; Sirota & Grafius 1994). Moderate levels of resistance have been observed in natural populations of *Musca domestica* (Bloomcamp *et al.* 1987; Shen & Plapp 1990; Keiding *et al.* 1992), but resistance is yet to evolve in the field in *L. cuprina* (Levot 1993; Yen *et al.* 1996).

The laboratory mutagenesis and selection regime described above generated four resistant strains (Yen *et al.* 1996). Resistance in each strain was controlled by a single gene. Loci (Cyr4 and Cyr5) mapped to chromosomes IV and V, respectively. Two alleles ((1) and (2)) have been identified at each locus. The resistance-ratio, genetic and viability characterizations of these strains (table 2) help to explain why cyromazine-resistance has been slow to evolve in natural populations of *L. cuprina*. If the laboratory variants are typical of those that arise in the field, the evolutionary window to select for resistance is only just open (Yen *et al.* 1996).

It should be noted that a number of other genes may control resistance to cyromazine. For example, in *Drosophila melanogaster*, Adcock *et al.* (1993) have selected genes different from, and similar to, the ones in *L. cuprina*. The conservation of genetic maps between these species (Weller & Foster 1993) allows this conclusion. More novel variants are expected as the mutagenesis and selection programmes have yet to achieve saturation in either species. However, thus far, all field- and laboratory-selected cyromazine-resistant variants appear to share one characteristic, low resistance ratios (Bloomcamp *et al.* 1987; Shen & Plapp 1990; Keiding *et al.* 1992; Adcock *et al.* 1993; Yen *et al.* 1996). Therefore, there is cause for optimism in the predictive nature of the laboratory experiments in *L. cuprina*. The relatively low LC_{100} of heterozygotes (table 2) and the toxicological properties of cyromazine also allow the possibility of delivery systems to minimize the probability of resistance evolving.

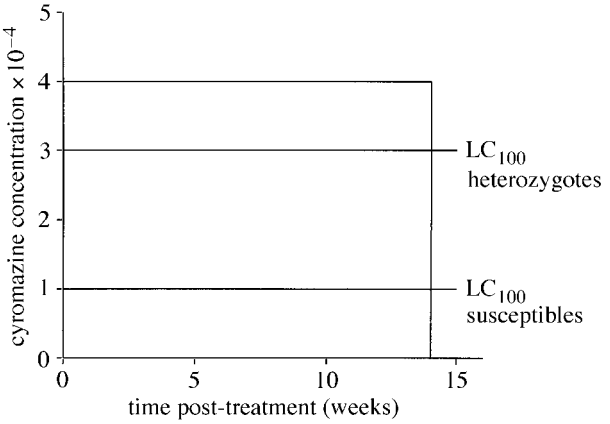

Figure 1. Idealized square-wave decay curve concentration to minimize the evolution of resistance to cyromazine in natural populations of *L. cuprina*. The concentrations for LC_{100} of susceptibles and heterozygotes are derived from Yen *et al.* (1996).

4. INSECTICIDE DELIVERY SYSTEMS

Selection for resistance is minimized when the concentration range over which resistant phenotypes are advantaged, relative to susceptibles, is restricted. When resistance at a single locus first evolves in natural populations, selection is for heterozygotes (Whitten & McKenzie 1982). Therefore, if an insecticide can be delivered at a concentration above the LC_{100} of heterozygotes, with a square-wave decay curve, the probability of resistance evolving can be theoretically reduced to zero (McKenzie 1987, 1996).

Cyromazine is an extremely safe compound to mammals and is taken into the serum after oral delivery in sheep. Through the use of intraruminal capsules, a square-wave decay curve can be generated (Anderson *et al.* 1989). The results from the laboratory variants allow the LC_{100} of susceptibles and heterozygotes to be identified. If these data were available before the release of the chemical for protection from blowfly, use of the intraruminal system, at the appropriate concentration, would allow control with little chance of resistance evolving (figure 1).

5. DISCUSSION

The laboratory experiments have demonstrated that it is possible to select for single-gene resistance by screening above the LC_{100} of susceptibles. Mutagens have been commonly used to enhance mutation rates but their use in insecticide-resistance studies has been restricted since the initial studies of Kikkawa (1964; McKenzie 1996).

The spontaneous rate of mutation from susceptible to resistant alleles is not known. Theoretical arguments can be made for rates in the range 10^{-3} to 10^{-13} (Whitten & McKenzie 1982). In the mutagenesis and selection experiments described, the average rate, after mutagenesis, was of the order of 4×10^{-6}. No resistant mutants arose spontaneously in unmutagenized cultures (McKenzie *et al.* 1992; Smyth *et al.* 1992; Yen *et al.* 1996) and therefore, EMS mutagenesis has increased the variation available to be screened by selection.

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In the control experiments, the resistance to dieldrin, diazinon and malathion generated in the laboratory was identical to that that evolved in the field (table 1). This is perhaps not surprising in the case of resistance to dieldrin, as the molecular basis of resistance has been due to parallel mutations over a number of species (ffrench-Constant 1994). However, for resistance to the organophosphorus insecticides mechanisms involving acetylcholinesterases, carboxylesterases, mixed-function oxidases and glutathione-S-transferases have been recorded in the literature (Russell *et al.* 1990; McKenzie 1996). Resistances based on carboxylesterases and, rarely, on mixed-function oxidases have been recorded in natural populations of *L. cuprina* (Hughes & Devonshire 1982; Terras *et al.* 1983) but, only the former have been generated in the laboratory (McKenzie *et al.* 1992). It remains to be seen whether further laboratory selection results in other variants but, in the context of this paper, the control experiments have been successful. They provide a foundation for the use of the mutagenesis and selection approach to predict, and allow analysis of, resistance mechanisms to novel insecticides before the chemicals are introduced into the field.

The results for the cyromazine-resistant variants (table 2) are also encouraging in this regard. The low-resistance ratios generated in the laboratory strains of *L. cuprina* (Yen *et al.* 1996) and *D. melanogaster* (Adcock *et al.* 1993) match those observed in natural populations of *M. domestica* (Bloomcamp *et al.* 1987; Shen & Plapp 1990; Keiding *et al.* 1992). The availability of resistant variants also allows the current lack of knowledge about the actual physiological and molecular foundations of resistance to cyromazine (Binnington 1985; Friedel & McDonell 1985; Kotze & Reynolds 1990) to be addressed. Molecular and genetic conservation across species provide considerable flexibility of the analysis of resistance systems (Oakeshott & Whitten 1992; ffrench-Constant 1994; Severson *et al.* 1997). Therefore, model systems may have an important role in predicting resistance mechanisms. *D. melanogaster* is already commonly used in resistance studies (McKenzie 1996) and there are compelling genetic and molecular arguments for its use as a model organism (Wilson 1988). This species has already proved useful in studies of laboratory mutagenesis and selection for resistance studies (Kikkawa 1964; Wilson & Fabian 1986; Adcock *et al.* 1993). Its role is likely to expand.

Information about the LC_{100} of resistant heterozygotes allows key delivery concentrations to be defined (figure 1) before a chemical is released. Relative fitness estimates can be made before resistance evolves in the field (Yen *et al.* 1996) and the selective concentration range determined (table 2). Such data have helped explain the relative rates of development of resistance to different insecticides (Roush & McKenzie 1987; Roush & Tabashnik 1990; McKenzie 1996) after resistance has evolved in the field. They will be equally important in determining the application strategies for a new insecticide if possible resistant mechanisms are predicted.

By way of example, if the laboratory experiments for dieldrin, diazinon and cyromazine had been performed before the chemicals were used for blowfly control, it would have been predicted that dieldrin-resistance would develop most rapidly. Furthermore, the level of resistance would render the insecticide ineffective for blowfly control.

Diazinon resistance would have been predicted to evolve more slowly and it would have been predicted that, even in the face of high *Rop-1* allelic frequencies, the insecticide would provide adequate protection against blowfly until a replacement insecticide was available. Cyromazine resistance would have been predicted to have relatively little chance of evolving. So far each of these predictions would have proved to be accurate (McKenzie 1996).

The strategy of selecting for resistance in the laboratory before a chemical is released, therefore, appears promising if our aim is to anticipate likely mechanisms before they evolve in natural populations. This information is important if we are to maximize the probability of managing susceptibility, an option preferable option to attempting to manage resistance (Daly & McKenzie 1986).

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