

How many genetic options for evolving insecticide resistance in heliothine and spodopteran pests?

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Abstract

The widely accepted paradigm for the development of insecticide resistance in field populations of insects is of selection for one or a very few genes of major effect. Limited genetic mapping data for organophosphate and pyrethroid resistance in heliothine and spodopteran pests generally agrees with this paradigm. However, other biochemical and transcriptomic data suggest a more complex set of changes in multiple P450 and esterase gene/enzyme systems in resistant strains of these species. We discuss possible explanations for this paradox, including the likely embedding of these genes in regulatory cascades and emerging evidence for their arrangement in large clusters of closely related genes. We conclude that there could indeed be an unusually large number of genetic options for evolving resistance in these species.

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1 INTRODUCTION: CONVENTIONAL WISDOMS ON THE GENETICS OF INSECTICIDE RESISTANCE

The dominant paradigms which emerged from several decades of classical genetic work on insecticide resistance were that major gene inheritance accounts for high level resistance to insecticides in the field and polygenic inheritance explains the generally lower level tolerance generated in selection experiments in the laboratory.¹ The rationale for the field results was that only genes conferring high level resistance will be useful against the high doses of insecticide to which field populations are exposed, but that the large sizes of field populations means that such genes can be recovered even if they are initially very rare, or even absent until new mutations occur. By contrast, laboratory selection experiments almost always use lower insecticide doses that can select for various genes of smaller effect because the much smaller sizes of laboratory populations means that only relatively common, pre-existing mutations are likely to be captured and higher insecticide doses could kill the whole population. Notably most of the data from which these paradigms emerged involve resistances/tolerances to the older organophosphate (OP) and organochlorine (OC) chemistries in various Diptera (both flies and mosquitoes).

Subsequent molecular work has generally borne out these paradigms and, at least for target site resistance in the field, revealed a marked tendency for the same or very similar mutations to be found in orthologous systems in different species. Evidence for the latter phenomenon now encompasses resistance to carbamates and pyrethroids, as well as the OPs and OCs.^{2–4} There is also one case of metabolic resistance to OPs where one or other of the same two amino acid

substitutions in orthologous carboxylesterases in several species confers significant OP hydrolase activity on the enzyme and resistance on the insect.^{5,6} Most of the molecular data involve Diptera again, as well as some Hemiptera (certain aphids and plant hoppers), but there is also mounting evidence that the parallelism of the resistance mutations extends to some other insect orders, and even into some non-insect groups such as Acari.⁷ One overall conclusion has therefore been that only a very finite number of genetic options are available through which field populations of insects can evolve insecticide resistance.

This conclusion has also been borne out by experiments involving laboratory mutagenesis and selection for high level resistance in *Drosophila*. Unlike the laboratory selection experiments that do not use mutagenesis above, these experiments are suited to the recovery of major gene effects. Work with several of the chemistries above plus the newer neonicotinoids and spinosyn has recurrently recovered the same resistance mutations in independent experiments, and these

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mutations are similar, and sometimes identical, to those found in field populations.⁴

2 EMERGING PATTERNS AND PARADOXES

At the same time, however, evidence is accumulating for certain species, particularly lepidopterans (but also including OC resistant houseflies⁸), suggesting that much more complex genetics may apply to some cases of field resistance. Large numbers of studies on several major pest lepidopterans such as the heliothines and spodopterans have correlated resistance with both simply inherited target site mutation and more complex changes in a range of putative detoxification enzymes [cytochrome P450s, esterases and glutathione S-transferases (GSTs)].^{9–11} Moreover, in cases such as the esterases, which are amenable to isozyme analysis, several different gene–enzyme systems have been implicated in the metabolic resistance.¹²

Some of the findings from the application of modern genomic technologies to insects from a variety of orders are also reinforcing this notion that many genes could potentially be involved in field resistance. Whole genome sequencing is revealing large numbers of genes in the P450, esterase and GST families that could be candidates for detoxification roles for the chemical insecticides; figures of ~50–130, ~20–70 and ~10–40, respectively, are commonly found for these families in the insect genomes so far characterised. Notably, it appears that clades within these families associated with dietary and detoxification functions may contain many more members in polyphagous pest species in which resistance has recurrently emerged than in more host-specialised species less prone to develop resistance.^{13–15} Several of the Lepidoptera for which the genetics of resistance appear to be most complex are highly polyphagous pests.^{10,11}

The emerging use of transcriptomics is also indicating higher levels of expression of several P450s, esterases and GSTs in insecticide resistant strains. Moreover, this pattern is evident even in several of the Diptera. In particular, an 'Aedes Detox Chip' containing over 200 P450, esterase and GST genes has been used in a series of microarray studies (validated with quantitative rtPCR work) that have implicated higher levels of expression of multiple P450 genes as well as several esterase and GST genes in OP and pyrethroid resistance in various field and laboratory-selected populations of *Ae. aegypti*.^{16–18} While these associations do not themselves establish causal connections between the elevated expression levels and detoxification/resistance, some aspects of the data, such as correlations between the expression levels and the levels of OP resistance during the course of selection and then the relaxation of that selection in the laboratory,¹⁶ certainly reinforce the idea. Interestingly also, the number of the detoxification genes implicated in the pyrethroid resistance appears to be inversely correlated with the frequency of target site resistance across different populations.¹⁷

'Next gen' genome resequencing technologies have not yet been deployed for genome-wide mapping of quantitative trait loci (QTL) contributing causally to insecticide resistance but microsatellites, amplified fragment length polymorphisms (AFLPs) and some other molecular markers have been used to this end. Results for pyrethroid resistance in three mosquito species are typical, with one to three loci explaining most of the variation, but still leaving significant genetic variation to be explained by other, as yet unidentified, factors.^{19–21} Furthermore, intensive analysis of two major loci contributing to pyrethroid resistance in the malaria vector *Anopheles funestris* has recently found that each in fact

includes at least two closely related P450 genes and each of these is over-expressed in resistant individuals.^{22,23}

So how do we reconcile the different sorts of data? Is the genetic basis for field resistance actually more complex than the limited power of classical genetics applied to non-model insects can detect? Just how limiting are the genetic options available to confer insecticide resistance? Are there real differences between laboratory and field populations in respect of the genetic basis of resistance? Are there qualitative differences in the genetic complexity of resistances between insect orders or between polyphagous pests and more specialised feeders? Herein we present some important insights into these issues emerging from recent classical genetic, biochemical and genomic studies of OP and pyrethroid resistance in some highly polyphagous lepidopteran pests, specifically heliothines, particularly *Helicoverpa armigera*, and *Spodoptera*.

3 THE CLASSICAL GENETICS AND BIOCHEMISTRY OF THE LEPIDOPTERAN CASES

Numerous studies have now reported OP and pyrethroid resistances in various African, Asian and Australian populations of *H. armigera*, with some reports indicating resistance factors exceeding 100-fold for the OPs and 1000-fold for the pyrethroids.^{10,24} Bioassays with diagnostic synergists implicate predominantly esterase- and P450-based metabolic resistance mechanisms in both cases, although the relative importance of the two mechanisms varies between populations. Broadly similar findings have also emerged from several studies of resistances to the two chemistries in *Helicoverpa punctigera*, *Heliothis virescens*, *Spodoptera littoralis* and *Spodoptera litura* (with more limited data to the same effect also for *Spodoptera exigua* and *Spodoptera frugiperda*), although not always for both sets of chemistries and other mechanisms are also influential in some cases.^{10,24}

Classical genetic analyses are difficult in these species and we have only found two relevant to our remit. The results of one back-crossing study on esterase-based pyrethroid resistance in an African *H. armigera* population were most consistent with the action of a single dominant autosomal gene, albeit minor effects of other genes or the combined effects of several closely linked genes could not be discounted by the data.²⁵ The other study, on an *S. litura* strain which bioassay data indicated had P450- and some esterase-based pyrethroid resistance, found major effects of at least two dominant autosomal genes,²⁶ although the precise number of genes could not be specified from the data and the caveats above about very limited power to detect genes of smaller effect or resolve the effects of closely linked genes again apply.

The biochemical phenotypes associated with the two resistance mechanisms in these species have almost always been elevations in the levels of esterase and P450 activities against artificial substrates in larval homogenates.^{10,24} Many of the individual studies reporting these associations could be criticised because of the limited number of strains involved, the different genetic backgrounds of the resistant and susceptible strains and, in some cases, the co-occurrence of resistances to other chemistries in the resistant strains. However, the combined data across a large number of studies (approaching 30 for *H. armigera* alone) make a strong case. Moreover a few studies have shown a quantitative correlation between resistance levels and the esterase and/or P450 activities across strains, or over the course of laboratory selection for increased resistance, and subsequent relaxation

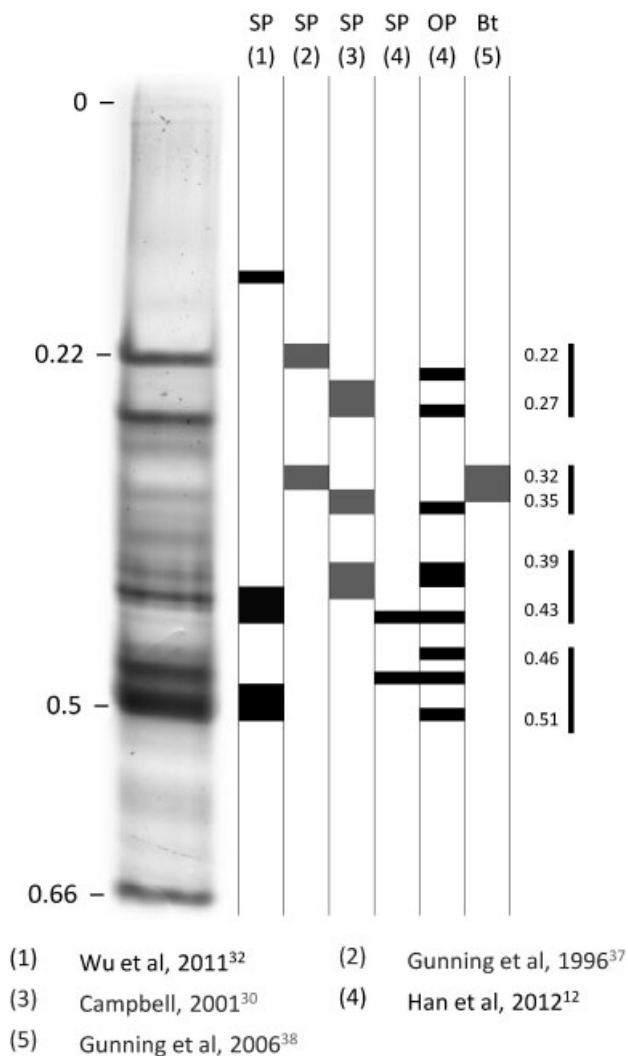


Figure 1. Isozyme regions associated with organophosphorus (OP), pyrethroid (SP) and *Bacillus thuringiensis* resistance in *Helicoverpa armigera* in five studies using comparable electrophoretic methods. The reference isozyme lane shown is from fifth instar larvae of the Australian GR strain and the schematic lanes shown for the five studies are coded black for Chinese and grey for Australian strains. The numbers in the margins refer to relative mobility (Rm) values.

of selection.^{27–29} Such patterns support the idea of a direct connection between the resistance and the biochemistry.

The biochemical work on the esterases has gone a step further because of the facility of separating and staining individual esterase enzymes on native PAGE gels. The esterase isozyme profiles of the lepidopterans under discussion here are found to be considerably more complex than those of the dipteran and hemipteran precedents above. For example, Campbell³⁰ found over 30 distinct isozymes in fourth instar *H. armigera* larvae alone, with several others also evident in other life stages of this species. Moreover greater staining intensities of several of the isozymes (6–9 in some cases) have been associated with OP or SP resistances in *H. armigera*,^{12,31,32} *H. punctigera*,³³ *H. virescens*,²⁷ *S. littoralis*,³⁴ *S. litura*³⁵ and *S. exigua*.³⁶ Figure 1 shows that at least four regions of the esterase zymogram, each containing multiple isozymes, have been associated with OP and pyrethroid resistance in *H. armigera*.^{12,30,32,37,38}

The biochemical mechanisms linking the more intensely staining isozymes to the resistances are not yet known but a mix of enhanced sequestration and metabolism of the insecticides is widely assumed to apply.^{10,39} In fact there is as yet little direct empirical evidence that the enzymes can actually bind or metabolise the insecticides. A couple of studies have shown esteratic cleavage of OPs and pyrethroids in whole larval extracts and there is also evidence for binding/inhibition of several of the relevant isozymes by the pesticides.^{30–36,39,40} Wu *et al.*³² also showed that an excised extract of one of the major isozymes associated with pyrethroid resistance in *H. armigera* was able to hydrolyse a pyrethroid, and purified esterases from other insects have been shown to have good activity for several pyrethroids, and much lower but possibly still protective activities against the activated, oxon forms of OPs.⁴¹ However, the outstanding need is for biochemical and physiological work testing directly for the sequestration and/or metabolic capabilities of the relevant heliothine and spodopteran isozymes.

Intriguingly, some level of OP/pyrethroid cross-resistance which is synergised by esterase inhibitors has also been noted in some of the work on these species.^{35,42,43} It is not found in all such studies but, in some cases involving correlated responses in laboratory selection experiments, the evidence is quite strong and encompasses carbamates as well.⁴⁰ Synergistic effects among the three classes of chemistry have been well documented and are commonly explained in terms of the sensitivity of the relevant detoxifying esterases to inhibition by OPs and carbamates, as per the sensitivity of the closely related acetylcholinesterase that is the primary target of OP and carbamate insecticides. It is assumed that some cross-resistance eventuates because over-expression of the relevant enzymes produces higher levels of sequestration/degradative activities for the different sets of chemistries. The overlap in isozyme bands associated with OP and pyrethroid resistances in *H. armigera* noted above is consistent with this idea.

4 INSIGHTS INTO THE LEPIDOPTERAN RESISTANCES FROM THE 'OMICS'

Projects to sequence the genomes of *H. armigera* and *S. littoralis* are under way but not yet completed. Meanwhile, however, tissue-specific transcriptome data are providing early insights into an unusual diversity of genes for P450s and esterases in heliothine and spodopteran pests. Sequencing of over 1600 paralogous midgut transcripts in *H. virescens* identified 20 different P450s and nine esterases,⁴⁴ while a similar project on about 3000 unique midgut transcripts from *H. armigera* identified 40 paralogous esterases alone.^{32,45} Transcriptomes of adult antennae (which also appear to include many detoxification genes) likewise reveal large numbers of P450s and esterases, 37 and 30, respectively, in *S. littoralis*.^{46,47} Notably the CYP3 and –4 clans predominate among the antennal P450 transcripts, and the –4, –6, –9 and –12 clans among the midgut RNAs, with esterase clades CCE1, in particular, and –16/–17 found in the transcriptomes of both tissues. CYP-4, –6, –9 and –12 P450s are commonly associated with insecticide resistances in other organisms⁴⁸ and the esterase clades –1 and –16/–17 have been variously associated with resistance in *H. armigera*.¹² The phylogeny in Fig. 2 shows that the relevant section of esterase clade –1 indeed contains several more genes in *H. armigera* than in another lepidopteran which is highly host-specific, the silkworm *Bombyx mori*.^{45,49,50}

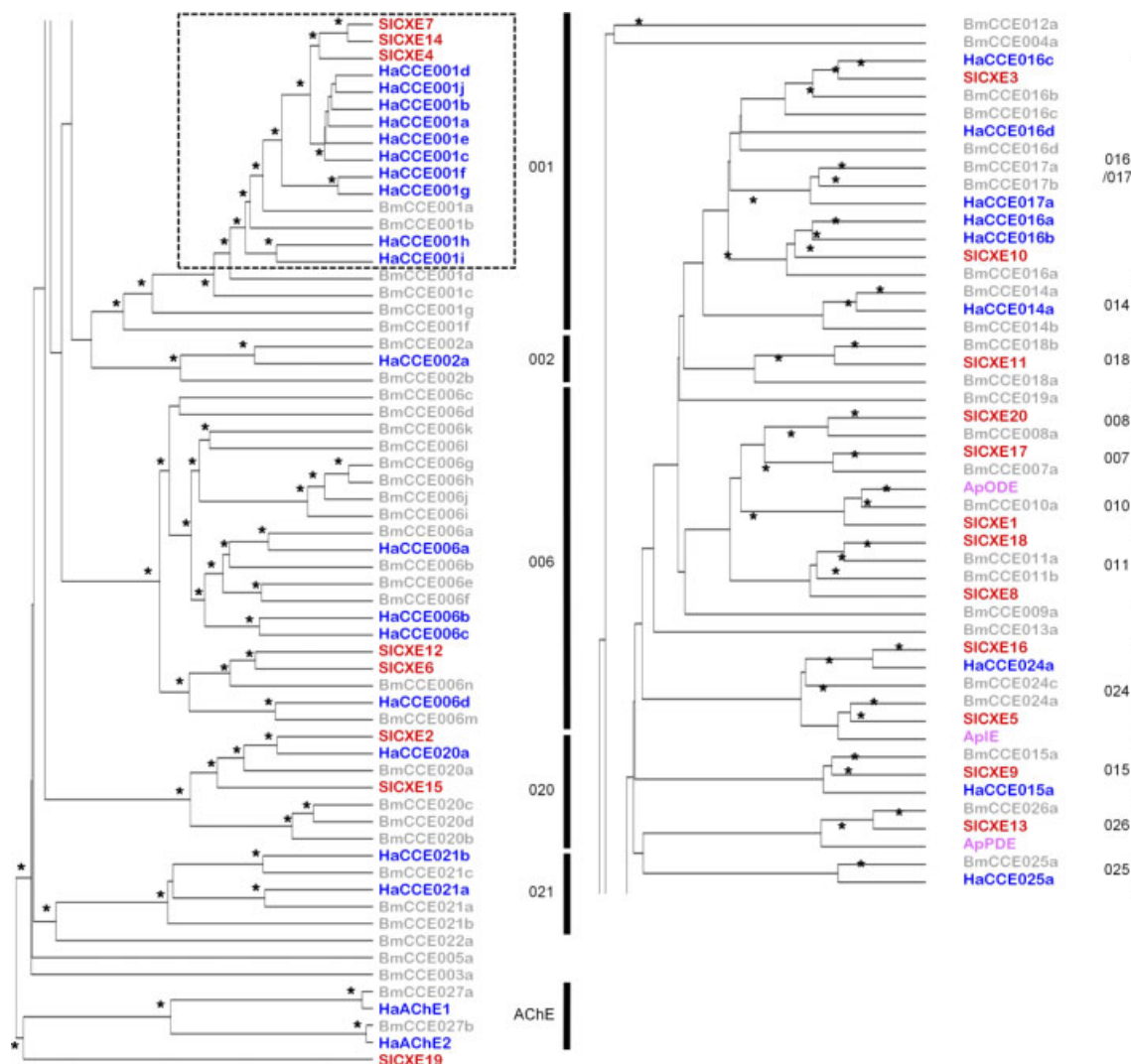


Figure 2. Phylogeny of the esterase genes recovered from the midgut transcriptome of *Helicoverpa armigera*,^{32,45} the antennal transcriptome of *Spodoptera littoralis*^{46,47} and the full genome sequence of *Bombyx mori*.^{45,49,50} Nomenclature is according to Teese *et al.*⁴⁵ and Durand *et al.*⁴⁷ The cladogram is based on an alignment of amino acid sequences trimmed as previously described (Claudianos *et al.*¹³) using default parameters of the sequence alignment and UGPMMA tree construction algorithms of CLC Main Workbench 6 software, Version 6.5.5 (CLC-Bio). Asterisks indicate nodes supported by at least 50% of bootstrap replicates ($n = 1000$). Major clades are indicated by their number and a solid vertical bar and the section of clade 1 containing enzymes implicated in xenobiotic metabolism is boxed. The sequences BmCCE001e and BmCCE024b of Teese *et al.*⁴⁵ subsequently identified as allelic variants of BmCCE001d and BmCCE024a, respectively (Tsubota and Shiotsuki⁵⁰) were omitted from this analysis. The partial sequence BmCCE023a was excluded from the alignment, but the additional *H. armigera* clade 1 sequence HaCCE001j of Wu *et al.*,³² not available to Teese *et al.*,⁴⁵ is included.

Several studies have now compared the expression levels of various *CYP* genes between pyrethroid susceptible and resistant strains of *H. armigera* (Table 1).^{51–57} A total of 30 different *CYP* genes have been compared in this way, several in more than one study, and 12 have been found to be more highly expressed in the resistant material in at least one study. While some (e.g. *CYP6AE11*, *-6B7*, *-9A12*, *-9A14*, *-332A1*, *-337B1*) show this pattern in multiple studies, others (e.g. *-4G8*, *-4L5*, *-4L11*, *-4M6*, *-4M7*) show more study-/population-specific effects. Individual resistant populations are reported in which as many as five *CYP* genes have been found to be over-expressed.⁵⁷ Given that only perhaps a third of the total complement of P450s in *H. armigera* have been included in any of these studies,⁵⁷ these transcriptomic experiments suggest considerable scope for many *CYP* genes to be involved in pyrethroid resistance in this species, in either population-specific or more general ways.

However, QTL mapping of pyrethroid resistance in *H. armigera* appears to tell a different story, with a single major locus found to account for most (~80%) of the ~50-fold P450-based metabolic resistance in an Australian population.⁵⁸ This locus was found to encompass the *CYP337B1* gene which was over-expressed in some of the pyrethroid resistant strains above.⁵⁹ *CYP337B1* is a hybrid of the closely linked *CYP337B2* and *CYP337B3* genes and produces an enzyme with an ability to metabolise pyrethroids that both the parental genes/enzymes lack.⁵⁹ These findings are significant in several respects. On the one hand they caution against the widely made assumption that the greater P450 activities measured with artificial substrates reported in many of the biochemical studies outlined above are necessarily relevant to resistance. And they also caution against the assumption that resistance based on greater P450 activity would generally be achieved by over-expression of existing P450s rather than the creation of novel P450s (and there

Table 1. Relative expression levels of *CYP* genes in pyrethroid susceptible (S) and resistant (R) strains of *Helicoverpa armigera*

Authors and Reference	Country	Genes in which there is no difference	Genes in which R>S
Xiao-Ping and Hobbs ⁵¹	Australia	6B2	
Pittendrigh <i>et al.</i> ⁵²	Australia	4S1, 4S2, 4G9, 4G10, 4M4	4G8
		6B2	
		9A3	
		43L	
Ranasinghe and Hobbs ⁵³	Australia	6B6	6B7
Yang <i>et al.</i> ⁵⁴	China	4G8	6B7
			9A12, 9A14
Wee <i>et al.</i> ⁵⁵	Australia	?	4S1
			337B1
Zhang <i>et al.</i> ⁵⁶	China		6B7
Brun-Barale <i>et al.</i> ⁵⁷	Burkina-Faso, Spain	4G8, 4M6, 4M7, 4M10	4L5, 4L11
		6AB9, 6AE12, 6AE15, 6AE16, 6B6, 6B7	6AE11
		9A12, 9A16, 9A17, 9A23	9A14
		321A1, 337B1	332A1
	Benin, Burkina-Faso, Mali	4G8, 4L5, 4L11, 4M10	4M6, 4M7
		6AB9, 6AE12, 6AE15, 6AE16, 6B6, 6B7	6AE11
		9A14, 9A16, 9A17, 9A23	9A12
		321A	332A1, 337B1

R>S indicates that expression was significantly higher in the resistant than in the susceptible strain.

are other examples of resistance due to structural mutations creating enzymes with new activities, both among the P450s and among the esterases⁵). Nevertheless, we also note that many of the biochemical and transcriptomic studies above involved significantly higher P450-based resistance factors (e.g. ~400-fold in Yang *et al.*⁵⁴) and some did not involve any over-expression of *CYP337B1* (e.g. Brun-Barale *et al.*⁵⁷). Thus *CYP337B1* only explains a proportion of the P450-based resistance so far described for this species.

There is no comparable QTL analysis of esterase-based resistances in the heliothines or *Spodoptera* but there are some intriguing data showing elevated expression of multiple esterases in resistant material, in this case involving both pyrethroids and OPs. Wu *et al.*³² and Han *et al.*¹² studied two pyrethroid- and one OP-resistant Chinese strains of *H. armigera* which each produced isozyme profiles with between three and nine bands that were more intensely staining than in a control susceptible strain. Two of these bands were more intense in all three resistant strains but otherwise there were considerable differences between them. Proteomic analyses on the isozymes from one of the pyrethroid resistant strains and the OP resistant strain yielded matches to nine of the 30 esterase genes in the transcriptomic databases above, with six of the nine (*CCE001a*, *-1c*, *-1d*, *-1g*, *-1i* and *-1j*) all being closely related to one another in a subclade that is considerably larger in *H. armigera* than in *B. mori* (8 cf 2 genes, respectively; see Fig. 2). The latter difference is also likely to be an underestimate because the *B. mori* figure is based on full genome sequence whereas the current transcriptome databases for *H. armigera* (and *S. littoralis*) may only contain about a third of its esterases.^{45,49,50} Native western analysis with a polyclonal antibody to the clade 1 esterases confirmed that the greater intensities of the corresponding isozyme bands in the resistant strain were due to greater amounts of the corresponding proteins. These esterase data thus bear out the findings from the P450 transcriptomics to date that a large number of often very closely related genes can be upregulated in resistant material, but that the specific genes upregulated can vary widely between strains.

5 CONCLUSIONS

A strong case emerges from a large number of studies for a direct link between OP and pyrethroid resistances and elevated levels of P450 and esterase activities in the heliothine and spodopteran species considered herein. Whilst some of the studies involve laboratory selection experiments, the husbandry and breeding issues with laboratory colonies of the species mean that the associations seen will generally reflect the effect of genes present in their wild-caught progenitors. The data already available also make a strong case for a direct link between the pyrethroid resistances in particular and elevated expression of several *CYP* and *CCE* genes, albeit some of the genes implicated in individual studies may well prove to be strain differences in genetic backgrounds rather than directly linked to the phenotype. It seems likely from studies such as those by Brun-Barale *et al.*⁵⁷ and Han *et al.*¹² that there are also genuine differences between populations in some of the specific genes that are upregulated in this way. Whilst the very limited number of classical genetic and QTL studies on the issue clearly point to some individual genes of major effect, it therefore seems likely that several members of the two families of detoxification genes could be involved in the resistances.

The transcriptome databases currently available for the heliothine and spodopteran species in question likewise suggest that there may be relatively large numbers of P450s and esterases that could provide options for the development of resistance if upregulated. These data include evidence for several more genes in *CYP* clans and *CCE* clades implicated in detoxification functions in *H. armigera* and *S. littoralis* than occur in, for example, *B. mori*.^{45,49,50}

How then to resolve the paradox that relatively few major genes for resistance are identified in the classical genetic and QTL mapping but large numbers are implicated as upregulated in the biochemistry and transcriptomics?

One explanation may be that at least some of the latter are incidentally over-expressed as part of the upregulation of a more general stress response controlled by genes operating upstream

in a regulatory cascade.¹⁰ Such P450s and esterases may have little or no degradative or binding activity against the insecticides or their metabolic products. Even assuming that several P450s and esterases do have physiologically important pyrethroid or OP degradative or sequestration activities, their titres may still be controlled by an upstream gene(s) in a regulatory cascade. In this case only the upstream regulatory gene(s) would contribute to the heritability of resistance and register in a classical genetic or QTL mapping experiment. The only case where a specific gene encoding a physiologically relevant (pyrethroid) detoxification activity is yet known to make a major contribution to the heritability of resistance is the *H. armigera* CYP337B1 above.⁵⁹ As noted, biochemical work is now required to test the metabolic activities of more of the upregulated P450s and esterases against the insecticides, and high density QTL mapping is required on more highly resistant strains to determine how the genes contributing to the heritability of resistances relate to the genes that actually encode the detoxifying functions. The application of new gene silencing technologies⁶⁰ and improved abilities to express P450 genes *in vitro*⁶¹ should facilitate the biochemical work required.

As well as the possibility of mutation in an upstream regulatory gene, there is another major genetic reason why the number of genetic options available for the development of various resistances might be under-represented by even a quite powerful QTL analysis. This involves the common observation of large clusters of closely related esterase and P450 genes in the genome. Such clusters have been found in all the insect species for which full genome sequence data have so far been produced. For example, over half the *B. mori* esterase genes sit in clusters of up to seven tandemly arranged members,⁴⁹ while a blowfly esterase implicated in major gene control of OP resistance sits in a cluster of at least eight members.⁶² Tightly linked clusters such as these generally behave as single loci in QTL experiments²² (and even genome-wide association studies⁶³), even though it is quite possible that several of their component genes could be independently upregulated in resistant strains. Similarly, two such tightly linked genes could contribute to resistance to different chemistries but co-segregate in both genetic mapping and laboratory selection experiments, giving an appearance of a shared cross-resistance phenotype.

Additionally we suggest that, while several esterases and P450s may indeed be selected for by OP or pyrethroid exposure, their relative contributions to resistance might be expected to vary substantially, depending on their biochemistry and physiology. A small number may exert major effects while polygenic effects result from smaller contributions from several others. This is clearly the case in the best characterised mosquito case (the pyrethroid resistance in *A. funestris* outlined above).^{22,23} The findings from the very extensive insecticide toxicology literature for mammals are also worth noting in this respect; the general findings are that the metabolism of most insecticides involves several enzymes which vary in their contributions to overall detoxification.⁶⁴

Finally, we note an emerging and still contentious literature reporting an association in *H. armigera* between midgut esterase activities and resistance to the Cry1Ac toxin of the biopesticide *Bacillus thuringiensis* which is now widely used in transgenic strategies for conferring endogenous insect protection on crops.^{38,45,65,66} The esterases involved migrate to the same zymogram region as one of those implicated in OP and pyrethroid resistance (see Figure 1). The mechanism underlying the association with Cry1Ac resistance is unclear but its existence is supported by the finding of a similar

association in the diamondback moth *Plutella xylostella*.⁶⁷ Further work is required to determine the generality and the basis of the association, and how it impacts on the associations of the esterases with the OP and pyrethroid resistances outlined herein.

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