Experimental and Applied Acarology **23:** 897–905, 1999.

Monooxygenases play only a minor role in resistance to synthetic pyrethroids in the cattle tick, *Boophilus microplus*

A.L. CRAMPTON[a]*, P. GREEN[b], G.D. BAXTER[a] and S.C. BARKER[a]

[a]*Department of Microbiology and Parasitology, and Center for Cellular and Molecular Biology, The University of Queensland, Brisbane, 4072, Australia;* [b]*Animal Research Institute, Department of Primary Industries, Brisbane, 4072, Australia*

(Received 4 October 1998; accepted 9 July 1999)

Abstract. We investigated the role of monooxygenases in resistance to synthetic pyrethroids (SPs) in the cattle tick, *Boophilus microplus*. We found that monooxygenases play only a minor role in resistance to SPs in both resistant and susceptible strains of *B. microplus*. We blocked the monooxygenases with piperonyl butoxide (PBO) and simultaneously applied the SPs, flumethrin and cypermethrin to larval *B. microplus*. PBO increased the effect of flumethrin (synergism ratios 2.7–8.9) more than it increased the effect of cypermethrin (synergism ratios 1.9–3.1). Of the four strains tested, Parkhurst, which is resistant to SPs, was the least affected by the addition of PBO (synergism ratios after cypermethrin was applied 1.9; after flumethrin 2.7) whereas N.R.F.S., the strain susceptible to SPs, was the most affected by synergism between PBO and SPs (synergism ratio after cypermethrin was applied 3.1; after flumethrin 8.9). We hypothesize that *B. microplus* lacks monooxygenases capable of conferring resistance to SPs because it and its recent ancestors were blood-feeders rather than herbivores.

Key words: *Boophilus microplus*, tick, Ixodida, resistance, synthetic pyrethroid, monooxygenases

Introduction

Boophilus microplus, the cattle tick, is a haematophagous arachnid (suborder Ixodida, family Ixodidae). *B. microplus* is primarily a parasite of cattle and costs the Australian livestock industry more than $100 million a year (Angus, 1996). Although a vaccine against *B. microplus* is available (Willadsen *et al.*, 1995), acaricides, especially synthetic pyrethroids (SPs), remain the principal means of control of this tick. Some populations of *B. microplus* in Australia are resistant to all classes of commonly-used acaricides registered for their control (Kunz and Kemp, 1994).

Resistance to SPs in B. *microplus* is conferred by several different mechanisms (Schnitzerling *et al.*, 1989). Resistance to SPs in the DDT-R strain of *B. microplus*

* To whom correspondence should be addressed at: Department of Biochemistry, EngelHall 0308, Virginia Tech, Blacksburg VA 24061, USA; Tel: 0011 1 540 231 5729; fax: 0011 1 540 231 9070; e-mail: andreac@vt.edu

is due to a decrease in the sensitivity of an unknown target (Nolan *et al*., 1989), whereas resistance to the SPs cypermethrin and permethrin in another strain that is resistant to DDT, Malchi, is due to an increase in carboxylesterase activity (Schnitzerling *et al*., 1983; De Jersey *et al*., 1985). However, resistance of the Malchi strain to a different SP, flumethrin, involves neither the target insensitivity of the DDT-R strain nor an increase in carboxylesterase activity (Schnitzerling *et al*., 1989). Similarly, increased carboxylesterase activity does not apparently confer resistance to flumethrin (or cypermethrin) in two other highly resistant strains, Parkhurst and Lamington (Nolan *et al*., 1989). The lack of substantial cross-resistance to DDT in these SP-resistant strains indicates that the target-insensitivity mechanism, which confers resistance to SPs in the DDT-R strain, may not be the mechanism of resistance to SPs in the Parkhurst and Lamington strains (Nolan *et al*., 1989). Thus, the mechanisms of resistance to SPs in many extant strains of *B. microplus* are unknown.

Mutations in the gene that produces the sodium channels of the nervous system and metabolism of pesticides by the enzymes of the monooxygenase system, are mechanisms of resistance to SPs in insects that might also occur in *B. microplus*. Oxidative detoxification by monooxygenases confers resistance to synthetic pyrethroids in other arthropods, like the house fly, *Musca domestica* (Wheelock and Scott, 1992; Tomita and Scott, 1995; Scott, 1996); cotton bollworm, *Helicoverpa armigera* (Xiao-ping and Hobbs, 1995); diamondback moth, *Plutella xylostella* (Sun *et al.*, 1992); cockroach, *Blattella germanica* (Scharf *et al.*, 1996; Valles and Yu, 1996); and the sheep blowfly, *Lucilia cuprina* (Kotze, 1993).

We tested whether or not monooxygenases confer resistance to flumethrin (supplied by Bayer, Australia) and cypermethrin (supplied by Cyanamid, Fort Dodge) in three SP resistant strains and one SP susceptible strain of *B. microplus*. We used a synergism approach, which has been used in studies of resistance to SPs in many other arthropod pests eg. *Amblyseius fallacis* (predator mite; Scott *et al.*, 1983); *H. armigera* (Daly and Fisk, 1992); *Bovicola ovis* (sheep body louse; Levot, 1994); and *Varroa jacobsoni* (mite ectoparasite of the honeybee; Hillesheim *et al.*, 1996). Synergism occurs when a compound like piperonyl butoxide (PBO) is mixed with another compound to produce an effect that is greater than the sum of their individual effects. PBO which blocks monooxygenases, is the most common synergist used to combat resistance to SPs in arthropods, and has been shown to block the monooxygenases of *B. microplus* (Knowles and Roulston, 1972).

Materials and methods

Ticks

We used four strains of *B. microplus*, three are resistant to SPs, and one is susceptible to these pesticides. Two of the resistant strains, Parkhurst and Ultimo, are laboratory

strains that originated from resistant ticks collected in the field. Parkhurst is resistant to all SPs used in commercial acaricides: cypermethrin, deltamethrin, cyhalothrin and flumethrin (Nolan *et al*., 1989). Ultimo has resistance to two different classes of acaricides, synthetic pyrethroids and amidines (Kunz and Kemp, 1994). The third resistant strain, R8372, is a field strain, whose larvae were bred from engorged females collected off cattle from Kingaroy in Queensland. The fourth strain is the non-resistant field strain, N.R.F.S., which is susceptible to all known acaricides and has been maintained in the laboratory for more than 20 years without exposure to acaricides.

Trial conditions

Concentrations of PBO (donated to P. Green by Dr G. Levot, NSW Department of Agriculture, Australia) used in experiments were chosen after preliminary trials of the toxicity of PBO in each strain. For all strains 2% PBO alone resulted in 7% or less mortality. The average mortalities due to 2% PBO, for each strain were Parkhurst, 6%; Ultimo, 2%; R8372, 7%; and N.R.F.S., 5%.

Concentrations of pesticides used against each strain were those routinely in use by one of us (P. Green) to test for resistance in *B. microplus* with the larval packet test (Stone and Haydock, 1962). Strains and final acaricide concentrations (% w/v) were: Parkhurst and Ultimo and flumethrin at 1, 0.5, 0.25, 0.125, 0.0625 with and without 2% PBO; Parkhurst and Ultimo and cypermethrin at 4, 2, 1, 0.5 with and without 2% PBO; field strain R8372 and flumethrin at 1, 0.5, 0.25, 0.125, 0.0625, 0.03125; field strains R8372 and flumethrin at 0.25, 0.125, 0.0625, 0.03125 with 2% PBO; N.R.F.S. and flumethrin at 0.002, 0.001, 0.0005, and 0.00025; N.R.F.S. and flumethrin at 0.00025, 0.000125, 0.00006 with 2% PBO; N.R.F.S. and cypermethrin at 0.06, 0.04, 0.02, 0.01, 0.005; N.R.F.S. and cypermethrin at 0.04, 0.02, 0.01, 0.005 with 2% PBO. Each trial had four controls, two with trichloroethylene solution only (the pesticides and PBO were diluted with trichloroethylene) and two with 2% PBO only. Concentrations of acaricides used in the PBO trials were lower than those in the non-PBO trials. This was to ensure that a wide range of mortalities occurred in each experiment so that the median lethal doses (LC_{50}) could be calculated.

Application of PBO and SPs to larval ticks

We used a form of the larval packet test (Stone and Haydock, 1962). Acaricides and PBO were made up to their final concentrations in trichloroethylene solution (per 600 ml: 400 ml trichloroethylene, 200 ml olive oil and 0.04 g ionol). For each combination of chemicals two sets of filter papers were impregnated with 650 μl of acaricide or acaricide- PBO. The filter papers were then dried at room temperature (about 25 °C) for one hour before being folded in half and clamped with bull-dog clamps to form a packet with an open top. 70–200 larvae were put in each packet which was

sealed with another bull-dog clip. The packets were then placed in a incubator at 27 °C and 85–95% relative humidity for 24 hours. Packets were then placed on a light box and the bull-dog clips removed. The number of live larvae (i.e. those that could walk forward) and dead larvae, were recorded. We breathed on the packets whilst counting to stimulate live but inactive larvae. Note that R8372 was tested with only one of the two SPs used on the other strains: flumethrin.

Analysis

Mortality and dose data were converted to probits (Finney, 1971) and analysed with a probit analysis program on a PC. Mortalities in the controls (PBO alone and trichlorethylene alone) were factored into the analysis. The program calculated LC_{50} levels, their fiducial limits, slope of the probit line and the standard error of the slope.

Results and discussion

PBO increased mortalities in all four strains, N.R.F.S., Ultimo, Parkhurst and R8372, i.e. there was a shift, left, in the probit lines (Figs. 1–3). However, PBO failed to increase mortality to the point where the resistant strains (Ultimo, Parkhurst and R8372) were as susceptible to these SPs as the susceptible strain, N.R.F.S. The width of the gaps between the probit lines of the N.R.F.S. strain and those of the resistant strains treated with PBO, plus the high resistance factors of the resistant strains when

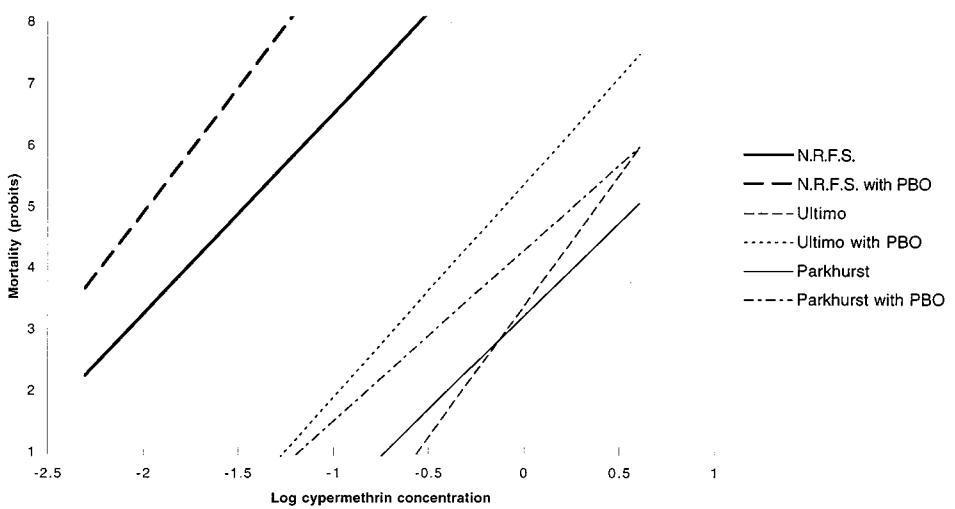

Figure 1. Regression lines of probit analysis of cypermethrin with and without PBO against N.R.F.S., Ultimo and Parkhurst strains of *B. microplus*. Probit of 5.01 = 50% mortality.

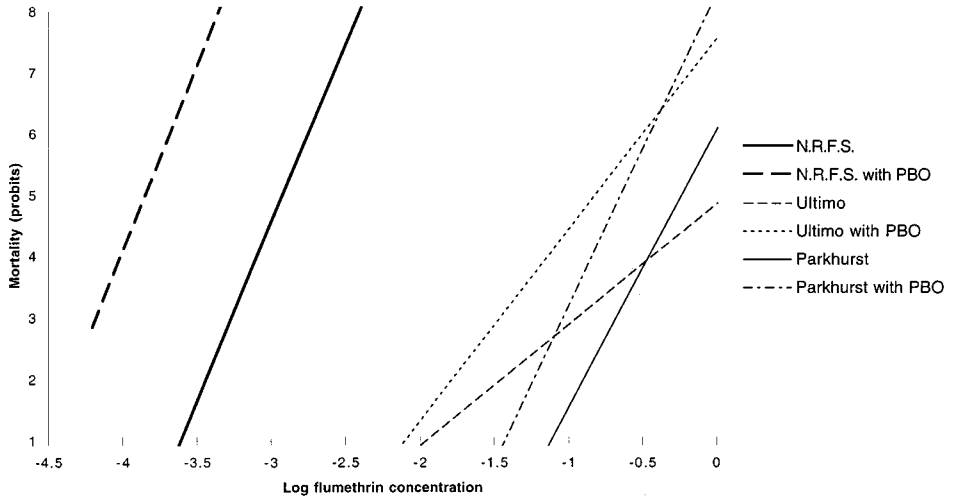

Figure 2. Regression lines of probit analysis of flumethrin with and without PBO against N.R.F.S., Ultimo and Parkhurst strains of *B. microplus.* Probit of 5.01 = 50% mortality.

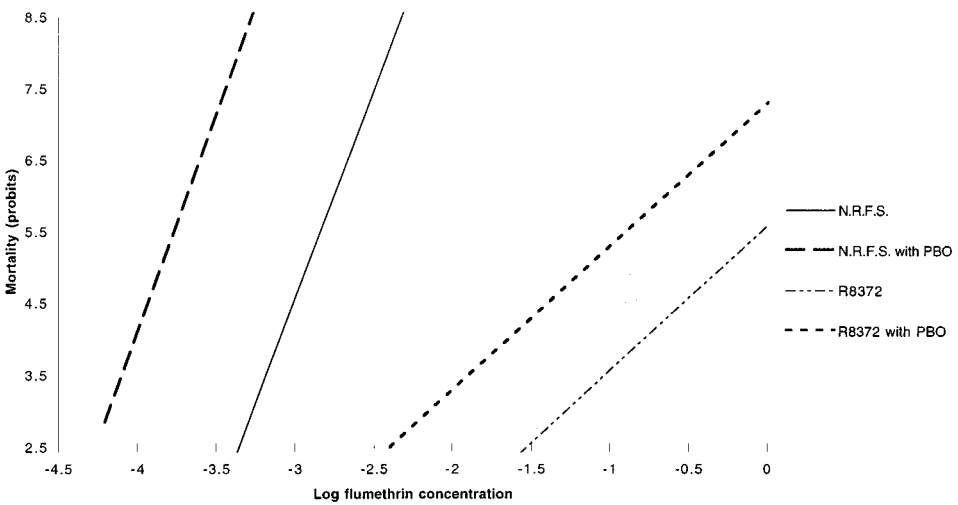

Figure 3. Regression lines of probit analysis of flumethrin with and without PBO against N.R.F.S. and R8372 strains of *B. microplus.* Probit of 5.01 = 50% mortality.

PBO was added to the SP (Table 1), indicate that a mechanism of resistance to SPs that is unaffected by PBO is present in the resistant ticks. In all trials the median lethal dose (LC_{50}) calculated from the packets treated with PBO was lower then that calculated from the packets without PBO (Table 1). Whilst decreases in LC_{50} after treatment with PBO and a SP have been recorded for a variety of other organisms, like cockroaches (Scharf *et al.*, 1996; Valles and Yu, 1996), house flies (Scott and

Table 1. Median lethal dose (LC_{50}), slope of the probit lines and resistance factors (RF), with and without 2% piperonyl butoxide (PBO). Resistance factor = LC_{50} trial/LC_{50} of susceptible strain (i.e. N.R.F.S.). Synergism ratio (SR) = LC_{50} without PBO/LC_{50} with PBO

Strains of *B. microplus*	LC_{50} %w/v (fiducial limit)	Slope ± SE	RF	With 2% piperonyl butoxide: LC_{50} %w/v (fiducial limit)	Slope ± SE	RF
				Cypermethrin		
N.R.F.S.	**0.0358** (0.03750–0.0341)	3.701 ± 0.346	1	**0.0114** (0.0120–0.0109)	3.715 ± 0.324	1
Parkhurst	**2.926** (3.146–2.722)	3.740 ± 0.747	64	**1.535** (1.638–1.438)	3.860 ± 0.194	43
Ultimo	**2.683** (2.753–2.616)	9.444 ± 1.168	75	**0.793** (0.820–0.766)	3.990 ± 0.194	22
				Flumethrin		
N.R.F.S.	**0.00122** (0.00127–0.00172)	5.810 ± 1.212	1	**0.0001344** (0.000141–0.000128)	5.561 ± 0.738	1
R8372	**0.608** (0.695–0.532)	2.155 ± 0.584	498	**0.073** (0.0808–0.0652)	2.006 ± 0.170	60
Parkhurst	**0.609** (0.644–0.575)	4.999 ± 0.895	499	**0.227** (0.238–0.216)	5.081 ± 0.433	186
Ultimo	**0.949** (1.079–0.350)	2.124 ± 0.350	778	**0.151** (0.159–0.143)	3.173 ± 0.512	124

Georghiou, 1986; Liu and Scott, 1995), tobacco budworms (McCaffery *et al.*, 1991; Sparks *et al.*, 1993; Zhao *et al.*, 1996), cotton moths (Daly and Fisk, 1992; Forrester *et al.*, 1993) and sheep body lice (Kotze, 1994; Levot, 1994), ours is the first report of a reduction in LC_{50} in a species of tick. Nolan (1985, unpub. data cited in Schnitzerling *et al.*, 1989) found that the addition of PBO to flumethrin had no synergistic affect on strains of *B. microplus.* Nolan's work was done before the Ultimo and Parkhurst strains had developed, thus the difference in, results between his and our study apparently reflect the different strains of *B. microplus.*

For both cypermethrin and flumethrin, synergism ratios (1.9–8.3) were much lower than would be expected if monooxygenases were a major mechanism of resistance in the Ultimo, Parkhurst and R8372 strains. In situations where the monooxygenase system has been identified as the major mechanism of resistance, the synergism ratios have been 30–100 times greater than our observations, e.g. the PEG87 strain of *H. vierescens* in which increased metabolism by monooxygenases is a mechanism of resistance to SPs, has a synergism ratio of 288 when treated with cypermethrin and cypermethrin plus PBO (McCaffery *et al.*, 1991).

PBO increased the mortality caused by flumethrin more than it increased mortality caused by cypermethrin. This indicates that the monooxygenases of *B. microplus* metabolize flumethrin more efficiently than they metabolize cypermethrin. Intriguingly, Schnitzerling *et al.* (1989) also reported that *B. microplus* (Biarra, DDT-R, Malchi and Y strains) was better able to resist cypermethrin than flumethrin. Further they found that the carboxylesterases of *B. microplus* metabolize cypermethrin more efficiently than they metabolize flumethrin (Schnitzerling *et al.*, 1989). Forrester *et al.* (1993) suggested that the extra phenol-ring in flumethrin provides more sites for oxidation by monooxygenases than are available in cypermethrin.

Our choice of strains and acaricides for this study were directly related to the real problems currently faced by the livestock industry of Australia. Parkhurst, Ultimo and field strains that are resistant to SPs are prevalent in many areas of Northern Australia, yet despite several studies, the mechanisms used by these strains to resist the actions of SPs are unknown. The two SPs used in this study, cypermethrin and flumethrin, were chosen because not only are they two of the most commonly used SPs but they also represent two different structural classes of SPs. PBO was chosen as the synergist because it is the only chemical that has previously been shown to block the monooxygenases of *B. microplus (*Knowles and Roulston, 1972).

The pre-adaptation hypothesis has been proposed for the evolution of resistance to pesticides that is conferred by monooxygenases in some insects and mites (Gordon, 1961). The pre-adaptation hypothesis states that herbivores that are able to detoxify toxic plant compounds (allelochemicals) use the same mechanism to detoxify pesticides extracted from or modeled on compounds from plants, i.e. they are pre-adapted for resistance to these pesticides. Allelochemicals are primarily detoxified by monooxygenases in arthropods (Mullin *et al.*, 1982). The pre-adaptation hypothesis explains the ability of several herbivorous insects and mites to resist pesticides

(Rosenheim *et al.*, 1996; Snyder and Glendinning, 1996). Since obligative blood-feeders like *B. microplus* do not normally ingest chemicals from plants presumably they have not developed monooxygenases able to detoxify these compounds: thus they are not preadapted to detoxify SPs. This may explain why the monooxygenases of *B. microplus*, in contrast to those of herbivorous insects like *H. vierescens*, have not become a mechanism of resistance to SPs.

Acknowledgements

We thank Ralph Stutchbury and Chris McCarthy from the Animal Research Institute for technical assistance.

References

Angus, B.A. 1996. The history of the cattle tick *Boophilus microplus* in Australia and achievements in its control. Int. J. Parasitol. 26: 1341–1355.

Daly, C.D. and Fisk, J.H. 1992. Inheritance of metabolic resistance to the synthetic pyrethroids in Australian *Helicoverpa armigera* (Lepidoptera: Noctuidae). Bull. Ent. Res. 82: 5–12.

De Jersey, J., Nolan, J., Davey, P.A. and Riddles, P.W. 1985. Separation and characterization of the pyrethroid-hydrolyzing esterases of the cattle tick, *Boophilus microplus*. Pestic. Biochem. Physiol. 23: 349–357.

Finney, D.J. 1971. *Probit analysis*. Cambridge University Press, Cambridge.

Forrester, N.W., Cahill, M., Bird, L.J. and Layland, L.K. 1993. Management of pyrethroid and endosulfan resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Australia. Bull. Ent. Res. Suppl. 1: 54–96.

Gordon, H.T. 1961. Nutritional factors in insect resistance to chemicals. Ann. Rev. Entomol. 6: 27–49.

Hillesheim, E., Ritter, W. and Bassand, D. 1996. First data on resistance mechanisms of *Varroa jacobsoni* (OUD.) against tau-fluvalinate. Exp. Appl. Acarol. 20: 283–296.

Knowles, C.O. and Roulston, W.J. 1972. Antagonism of chlorphenamidine toxicity to the cattle tick *Boophilus microplus* by piperonyl butoxide. J. Aust. Ent. Soc. 11: 349–350.

Kotze, A.C. 1993. Cytochrome P450 monooxygenases in larvae in insecticide-susceptible and resistant strains of the Australian sheep blowfly, *Lucilia cuprina*. Pestic. Biochem. Physiol. 46: 65–72.

Kotze, A.C. 1994. Enhanced monooxygenase activity in pyrethroid-resistant strains of *Bovicola ovis* (Schrank) (Phthiraptera: trichodectidae). J. Aust. Ent. Soc. 33: 275–278.

Kunz, S.E. and Kemp, D.H. 1994. Insecticides and acaricides: resistance and environmental impact. Rev. Sci. Tech. Off. Int. Epiz. 13: 1249–1286.

Levot, G.W. 1994. Pyrethroid synergism by piperonyl butoxide in *Bovicola ovis* (Schrank) (Phthiraptera: Trichodectidae). J. Aust. Ent. Soc. 33: 123–126.

Liu, N. and Scott, J.G. 1995. Genetics of resistance to pyrethroid insecticides in the house fly, *Musca domestica*. Pestic. Biochem. Physiol. 52: 116–124.

McCaffery, A.R., Gladwell, R.T., El-Nayir, H., Walker, C.H., Perry, J.N. and Miles, M.J. 1991. Mechanisms of resistance to pyrethroids in laboratory and field strains of *Heliothis vivescens*. Southwestern Entomologist Suppl. 15: 143–158.

Mullin, C.A., Croft, B.A., Strickler, K., Matsumura, F. and Miller, J.R. 1982. Detoxification enzyme differences between a herbivorous and predatory mite. Science 217: 120–1271.

Nolan, J., Wilson, J.T., Green, P.E. and Bird, P.E. 1989. Synthetic pyrethroid resistance in field samples in the cattle tick (*Boophilus microplus*). Aust. Vet. J. 66: 179–182.

Rosenheim, J.A., Johnson, M.W., Mau, R.F.L., Welter, S.C. and Tabashnik, B.E. 1996. Biochemical pre-adaptions, founder events and the evolution of resistance in arthropods. J. Econ. Entomol. 89: 263–273.

Scharf, M.E., Hemingway, J., Reid, B.J., Small, G.J. and Bennett, G.W. 1996. Toxicological and biochemistry characterization of insecticide resistance in a field-collected strain of *Blattella germanica* (Dictyoptera: Blattellidae). J. Econ. Entomol. 89: 322–331.

Schnitzerling, H.J., Nolan, J. and Hughes, S. 1983. Toxicology and metabolism of some synthetic pyrethroids in larvae of susceptible and resistant strains of the cattle tick *Boophilus microplus* (can.) Pestic. Sci. 14: 64–72.

Schnitzerling, H.J., Nolan, J. and Hughes, S. 1989. Toxicology and metabolism of isomers of flumethrin in larvae of pyrethroid-susceptible and resistant strains of the cattle tick *Boophilus microplus* (Acari: Ixodidae). Exp. Appl. Acarol. 6: 47–54.

Scott, J.G. 1996. Cytochrome P450 monooxygenase-mediated resistance to insecticides. J. Pestic. Sci. 21: 241–245.

Scott, J.G., Croft, B.A. and Wagner, S.W. 1983. Studies on the mechanism of permethrin resistance in *Amblyseius fallacis* (Acarina: Phytoseiidae) relative to previous insecticide use on apple. J. Econ. Entomol. 76: 6–10.

Scott, J.G. and Georghiou, G.P. 1986. Mechanisms responsible for high levels of permethrin resistance in the house fly. Pestic. Sci. 17: 195–206.

Snyder, M.J. and Glendinning, J.I. 1996. Causal connection between detoxification enzyme activity and consumption of a toxic plant compound. J. Comp. Physiol. A 179: 255–261.

Sparks, T.C., Graves, J.B. and Leonard, B.R. 1993. Insecticide resistance and the tobacco budworm: past, present and future. Rev. Pestic. Toxicol. 2: 149–183.

Stone, B.F. and Haydock, K.P. 1962. A method for measuring the acaricide-susceptibility of the cattle tick *Boophilus microplus* (can.). Bull. Ent. Res. 53: 563–578.

Sun, C.N., Tsai, Y.C. and Chiang, F.M. 1992. Resistance in the Diamondback moth to pyrethroids and benzoylphenlureas. In: Molecular mechanisms of insecticide resistance: diversity among insects, C.A. Mullin and J.G. Scott (eds), pp. 149–167. American Chemical Society, Washington, D.C.

Tomita, T. and Scott, J.G. 1995. cDNA and deduced protein sequence of *CYP6D1*: the putative gene of a cytochrome P450 responsible for pyrethroid resistance in the house fly. Insect Biochem. Molec. Biol. 25: 275–283.

Valles, S.M. and Yu, S.J. 1996. Detection and biochemical characterization of insecticide resistance in the german cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. 89: 21–26.

Wheelock, G.D. and Scott, J.G. 1992. The role of cytochrome $P450_{ipr}$ in deltamethrin metabolism by pyrethroid resistant and susceptible strains of house flies. Pestic. Biochem. Physiol. 43: 67–77.

Willadsen, P., Bird, P., Cobon, G.S. and Hungerford J. 1995. Commercialisation of a recombinant vaccine against *Boophilus microplus.* Parasitol. Suppl. 110: S43–S50.

Xiao-ping, W. and Hobbs, A.A. 1995. Isolation and sequence analysis of a cDNA clone for a pyrethroid inducible cytochrome P450 from *Helicoverpa armigera.* Insect Biochem. Mol. Biol. 25: 1001–1009.

Zhao, G., Rose, R.L., Hodgson, E. and Roe, R.M. 1996. Biochemical mechanisms and diagnostic micro-assays for pyrethroid, carbamate, and organophosphate insecticide resistance/cross resistance in the tobacco budworm *Heliothis virescens.* Pestic. Biochem. Physiol. 54: 183–195.