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Genetic factors potentially reducing fitness cost of organophosphate-insensitive acetylcholinesterase(s) in *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae)*

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Abstract

Acaricidal activity of organophosphate (OP) and carbamate acaricides is believed to result from inhibition of acetylcholinesterase (AChE). Previous studies in *Rhipicephalus* (*Boophilus*) *microplus* demonstrated the presence of three presumptive AChE genes (*BmAChE*s). Biochemical characterization of recombinant BmAChE proteins expressed in the baculovirus system demonstrated that each of the three *R.* (*B.*) *microplus* rBmAChEs have enzymatic properties consistent with designation as functional acetylcholinesterases. Complementary DNAs (cDNAs) for each of the three *BmAChE*s were cloned and sequenced from individual adult tick synganglia excised from OP-susceptible and OP-resistant strains. The data revealed the presence of multiple transcript sequences within individual ticks for each of the *BmAChE*s, suggesting alternative mRNA splicing or expression of multiple alleles for each of the *BmAChE* genes. Quantitative real-time PCR provided evidence of possible gene duplication or amplification for each of the *BmAChE* genes, and direct sequencing of genomic DNA provided evidence of structural *BmAChE* gene diversity with respect to presence or absence of introns, as well as the presence or absence of sequence polymorphisms. Baculovirus expression of rBmAChE1 and rBmAChE3 proteins containing some of the predicted amino acid sequence polymorphisms resulted in production of OP-insensitive AChE, demonstrating that at least some OP-resistant individuals contain mutations that reduce OP-inhibition for at least two of the three known BmAChEs. RNA interference was utilized to silence *in vivo* expression of the *BmAChE* genes in adult ticks, resulting in tick mortality if all three *BmAChEs* were silenced simultaneously, strongly suggesting that the BmAChE proteins functionally complement one another *in vivo*. Together, the results presented provide strong evidence that OP-resistance in *R. microplus* is at least partially mediated by a combination of the expression of multiple genes encoding acetylcholinesterase, mutations in *BmAChE*s resulting in OP-insensitivity, gene duplication, and maintenance of allelic diversity, including both OP-sensitive and OP-insensitive alleles within individual ticks. The authors propose that these factors may mitigate fitness costs that might otherwise result from *BmAChE* mutations, and demonstrate the extreme complexity of OP-resistance in *R.* (*B.*) *microplus*. It is hoped that elucidation of the complex interactions among the multiple BmAChEs and their physiological roles may enable development of new opportunities for tick control.

Key words: Acaricide, resistance, gene amplification, complementation, fitness.

Introduction

The Southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini), was discovered to vector bovine babesiosis and anaplasmosis in 1893, which led to an intensive effort over many decades culminating in its eradication from the United States (Graham & Hourrigan, 1977). Since

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1936, a permanent quarantine zone along the Texas-Mexico border together with treatment and inspection of all imported cattle has prevented the re-establishment of "cattle fever ticks" within the United States. The Veterinary Services Branch of the Animal and Plant Health Inspection Service of the United States Department of Agriculture manages the Cattle Fever Tick Eradication Program (CFTEP), including inspection and acaricide treatment of imported cattle, and eradication of "outbreaks" when *R.* (*B.*) *microplus* or *R.* (*B.*) *annulatus* ticks are discovered within the United States (summarized in Temeyer *et al*., 2004b). A key component of the CFTEP is the acaricide treatment of imported or infested cattle. The only acaricide approved and routinely utilized for dipping cattle in the U.S. since 1970 is the organophosphate, coumaphos (George, 1996), and OP-resistance is considered to be a major threat to continued success of the CFTEP (Davey & George, 1999; Davey *et al*., 2003; Temeyer *et al*., 2004b).

Organophosphate (OP) and carbamate pesticides kill arthropod pests by targeting the acetylcholinesterase (AChE) of the central nervous system. AChE hydrolyzes the neurotransmitter, acetylcholine, and is a key enzyme in the nervous systems of eukaryotic organisms. OP targeting of AChE results in quasi-irreversible inactivation of the enzyme, leading to collapse of the nervous system function (O'Brien, 1967; Fournier & Mutero, 1994). Resistance to OP pesticides has become widespread in arthropod pests throughout the world (Rosario-Cruz *et al*., 2009) and is frequently the result of one or more mutations leading to production of altered AChE that is insensitive to OP inhibition. Molecular structures of AChE from the electric ray *Torpedo californica* Ayres, the vinegar fly *Drosophila melanogaster* Meigen, and other organisms have been determined by x-ray crystallography, and OP-insensitivity in arthropod pests frequently results from alteration of key amino acid residues located at the catalytic gorge or from "second shell" allosteric effects on the catalytic gorge residues (Harel *et al*., 2000; Villatte *et al*., 2000).

Rhipicephlus (*B.*) *microplus* expresses multiple genes encoding acetylcholinesterases

Strains of *R.* (*B.*) *microplus* resistant to OP have been biochemically characterized, demonstrating that resistance was due, at least in part, to AChE insensitivity to OP inhibition (summarized in Pruett, 2002; Li *et al*., 2003; Pruett & Pound, 2006). Unlike drosophilid flies that contain only a single AChE gene (Fournier *et al*., 1992), or mosquitoes and some other dipterans that contain two AChE genes (Villatte & Bachman, 2002; Xu *et al*., 2003; Ilg *et al*., 2010), *R.* (*B.*) *microplus* was reported to contain three different cDNAs presumptively encoding AChEs, *BmAChE1* (Baxter & Barker, 1998), *BmAChE2* (Hernandez *et al*., 1999), and *BmAChE3* (Temeyer *et al*., 2004a). In each case, initial surveys comparing cDNA sequences from OP-susceptible or OP-resistant strains failed to identify *BmAChE* mutations that correlated with resistance. In addition, identification of each of the *BmAChE*s as presumably encoding AChE, was initially based solely on nucleotide and amino acid sequence homology; however, the cholinesterase gene family contains a number of related enzymes and structural proteins (Cygler *et al*., 1993; Cousin *et al*., 1997; Oakeshott *et al*., 1999; Zimmerman & Soreq, 2006), so verification of the biochemical identity of the encoded gene products was needed.

The first *R.* (*B.*) *microplus* cDNA presumably encoding AChE to be expressed as a recombinant protein was *BmAChE3*. Biochemical characterization of recombinant BmAChE3 (rBmAChE3) produced in the baculovirus system demonstrated that it was an active enzyme exhibiting biochemical properties consistent with AChE (E.C. 3.1.1.7). It exhibited substrate preference for acetylthiocholine over butyrylthiocholine, was inhibited by the AChE-specific inhibitors eserine and BW284c51, and exhibited similar sensitivity to paraoxon as AChE prepared from OP-susceptible *R.* (*B.*) *microplus* adults, thereby verifying the identity as a functional ixodid

AChE (Temeyer *et al*., 2006). Recently, rBmAChE1, rBmAChE2 and rBmAChE3 were expressed in the baculovirus system, biochemically characterized (Table 1; Temeyer *et al*., 2010), and were each found to exhibit properties consistent with AChE (E.C. 3.1.1.7) as defined for insect AChE by Toutant (1989). Therefore, it is evident that *R*. (*B*.) *microplus* has at least three different genes, each encoding an enzymatically active AChE, but each with different properties (Table 1).

TABLE 1. Biochemical properties of rBmAChEs[1].

Expression Construct	K_m AcSCh[2] (μM)	K_m BuSCh[2] (μM)	I_{50} Eserine (nM)	I_{50} BW284c51 (nM)	Paraoxon Insensitivity Ratio
rBmAChE1 Deutch5	4.25a[3]	1.42	1	0.39	2.11
rBmAChE1 Tx-11	5.20a	320.28	-	-	40.29
rBmAChE2 SR12	52.7b	71.91	250	0.15	2.06
rBmAChE3 SR-BC26	90.19c	309.30	150	2	1.00
rBmAChE3 SR-R86Q	99.6c	-	-	-	19

[1]Data from Temeyer *et al*. (2007, 2010).
[2]AcSCh, acetylthiocholine; BuSCh, butyrylthiocholine.
[3]Letters denote significant differences $p \leq 0.05$.

Mutations in *BmAChE1* or *BmAChE3* result in organophosphate-insensitivity

Association of *BmAChE* mutations with OP-insensitivity has been evaluated; however, it remains far from complete. The R86Q substitution in BmAChE3 was the first mutation in ticks demonstrated to confer insensitivity to OP (Table 1), resulting in approximately 20-fold reduction in paraoxon sensitivity (Temeyer *et al*., 2007). In addition, Tx-11, a recombinant construct of BmAChE1 containing several amino acid substitutions (Table 2) was shown to result in an approximate 20-fold reduction (Table 1) in paraoxon sensitivity compared to the OP-sensitive Deutch5 construct (Temeyer *et al*., 2010). Transcripts expressing the OP-insensitive rBmAChE3 and rBmAChE1 constructs were each isolated from OP-resistant strains of *R*. (*B*.) *microplus*, suggesting that phenotypic resistance to OP may involve mutations in more than one of the three known *BmAChE* genes. Presence of *BmAChE* mutations encoding OP-insensitive AChE in susceptible strains further suggests that a single mutation producing OP-insensitivity in one of the *BmAChEs* may be insufficient to result in phenotypic resistance to OP (Temeyer *et al*., 2007, 2009). Genotyping surveys of OP-resistant and OP-susceptible strains utilizing allele-specific assays (Fig. 1) or cDNA sequencing has suggested additional mutations potentially associated with OP-insensitivity (Temeyer *et al*., 2009, unpub. results). Confirmation of the role of specific mutations in OP-insensitivity requires expression and biochemical characterization of rBmAChEs containing the specific mutations individually and in various combinations. Each of the three known BmAChEs is expressed in the adult tick synganglion (Temeyer *et al*., 2010), but the physiological role of each of the individual BmAChEs remains to be fully elucidated.

TABLE 2. Unmatched amino acid substitutions in rBmAChE1 Tx11 insensitive to paraoxon[1]

Substitution	Notes
D188G*	Conserved in BmAChE2, BmAChE3; position adjacent to catalytic gorge Tyr
E196G	Gly in BmAChE2, BmAChE3 & Drosophila AChE
V331A	Not conserved in BmAChE2, BmAChE3, or Drosophila AChE
F390S*	Conserved in BmAChE2, BmAChE3; 2nd position from catalytic gorge Tyr

[1]Data from Temeyer *et al*. (2010).
*Substitutions likely to be involved in OP-insensitivity.

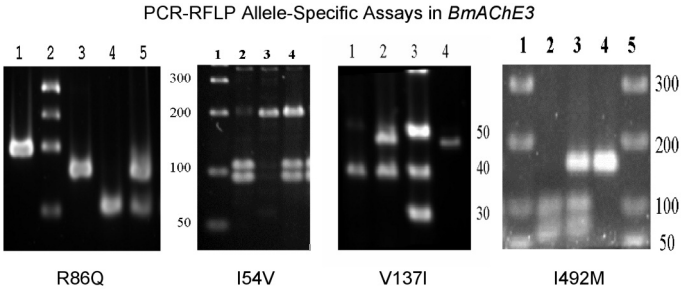

FIGURE 1. Allele-specific PCR-RFLP assays for *BmAChE3* mutations designated as R86Q, I54V, V137I, and I492M. In each case, a specific DNA fragment of *BmAChE3* is amplified by PCR, then incubated with a specific restriction endonuclease to test for the presence or absence of the mutation. **R86Q** (HaeIII): lane 1, undigested 306 bp PCR product; lane 2, DNA size standards; lane 3, homozygous R86Q mutant; lane 4, homozygous wild type; lane 5, heterozygous for R86Q locus (Temeyer *et al*., 2007). **I54V** (RsaI): lane 1, DNA size stds; lane 2, homozygous I54V mutant; lane 3, homozygous wild type; lane 4, heterozygous for I54V locus (Temeyer *et al*., 2009). **V137I** (BtsCI): lane 1, homozygous V137I mutant; lane 2, heterozygous for V137I locus; lane 3, DNA size stds; lane 4, homozygous V137I mutant (Temeyer *et al*., 2009). **I492M** (BccI): lanes 1& 5, DNA size stds; lane 2, homozygous I492 wild type; lane 3, heterozygous I492:I492M; lane 4, homozygous I492M mutant (Temeyer *et al*., 2009).

Gene duplication and expression of multiple transcripts for each *BmAChE*

More than two transcripts each for *BmAChE1*, *BmAChE2*, or *BmAChE3* were isolated and sequenced from synganglia of single individual adult ticks (Fig. 2), suggesting that each of the *BmAChE*s may be present in multiple copies or that they may be subject to alternative splicing. Quantitative-PCR also indicated the presence of multiple copies of each of the *BmAChE*s in *R*. (*B*.) *microplus*, as does PCR amplification of *BmAChE1* genomic DNA fragments encompassing the presence or absence of an intron (Temeyer, unpublished results).

	cDNA	Amino acid positions altered in coding sequence of cDNA transcript																	
Gene	Clone	A13S	H16Y	V29A	E60K	P78T	K89E	R96G	D98G	D109G	K116E	L148P	K149R	P157S	A194F	T219A	Y230C	M231V	Q237R	A241V
BmAChE1	a			X	X	X								X	X	X	X			
	b				X	X					X	X		X		X	X			
	c				X	X								X		X	X			X
	d	X			X	X								X		X	X	X		
	e				X	X		X	X	X			X	X		X				
	f		X		X	X	X							X		X			X	

	cDNA	Amino acid positions altered in coding sequence of cDNA transcript																		
Gene	Clone	A260T	L280P	D295G	K296E	V331A	A349V	S366P	V373A	W384R	F389L	L417P	F477S	W486R	Q488R	E514G	T547I	K549H	W571R	P593L
BmAChE1	a	X		X	X		X					X			X			X	X	X
	b	X					X					X			X			X	X	
	c	X	X				X						X		X	X	X	X	X	
	d	X					X			X					X			X	X	
	e	X				X	X								X			X	X	
	f	X					X	X	X		X			X	X			X	X	

	cDNA	Amino acid positions altered in coding sequence of cDNA transcript								
Gene	Clone	F9L	A26T	K46R	K56R	L196P	I210V	K271R	M406V	W808R	K555M
BmAChE2	a	X	X	X	X	X		X			
	b	X	X			X	X		X		X
	c	X	X			X				X	
	d	X	X			X					X

	cDNA	Amino acid positions altered in coding sequence of cDNA transcript								
Gene	Clone	I123M	Q157R	D158G	N165D	M256V	S372D	V509E	D551A	
BmAChE3	a					X				
	b	X			X					
	c		X							
	d	X			X					
	e			X						
	f						X	X	X	

FIGURE 2. Predicted amino acid substitutions encoded by transcripts of *BmAChE1*, *BmAChE2*, or *BmAChE3* isolated from single individual synganglia were determined by cDNA high fidelity cloning and sequencing. In each case, gene-specific cDNA prepared from individual synganglia resulted in more than two sequences, listed as the conceptual translation differences among sequenced cDNAs.

BmAChEs functionally complement one another *in vivo*

Post-transcriptional gene silencing by RNA interference (RNAi) was utilized to investigate the physiological role of the BmAChEs. Double stranded RNA (dsRNA) for each of the three *BmAChEs* was prepared (T7 RiboMAX™ Express RNAi System, Promega, Madison, WI) and microinjected in unfed female adult *R*. (*B*.) *microplus* ticks using drawn capillary glass needles. Microinjected female ticks were paired with uninjected males and placed in stockinette sleeves glued to the backs of cattle. Female ticks that had been microinjected with dsRNA were removed and dissected at various post-injection time intervals and synganglia were placed in RNA*later*® (Ambion, Life Technologies Corporation, Austin, TX) for subsequent quantitative RT-PCR. Engorged females were collected as they detached, and were weighed and allowed to oviposit in individual vials maintained under constant temperature and humidity conditions in an incubator. Egg mass and larval hatch were monitored. Similar experiments were conducted by microinjecting fully engorged female adult ticks with *BmAChE* dsRNAs and subsequent reproductive parameters monitored, as described above. Injection of *BmAChE* dsRNA resulted in specific silencing (≥80%) of the target gene, but did not produce significant mortality of injected females unless all three *BmAChE* genes were silenced simultaneously. Increasing the dose of microinjected dsRNA for individual *BmAChEs* alone did result in adult tick mortality, but appeared to be less than that from silencing all three *BmAChEs*.

Conclusions and new hypotheses

It is clear from results of these investigations that AChE participation in *R*. (*B*.) *microplus* phenotypic resistance to OP acaricides is highly complex, involving multiple BmAChEs. These results strongly suggest that the three known BmAChEs functionally complement one another *in vivo*. Results also strongly suggest that investigated strains of *R*. (*B*.) *microplus* possess and express multiple copies of each of the three BmAChEs in the tick synganglion. It may be hypothesized that deleterious effects of *BmAChE* mutations are mitigated by gene duplication and maintenance of allelic diversity, including both OP-sensitive and -insensitive alleles. It is possible that the presence of multiple copies of *BmAChE1*, *BmAChE2* and *BmAChE3*, expressed in the central nervous system of *R*. (*B*.) *microplus*, may provide a significant selective advantage by simultaneous maintenance and expression of multiple alleles and functional complements. In the presence of OP acaricide, expression of OP-insensitive alleles may provide sufficient AChE activity to maintain nervous system function. In the absence of OP acaricide, expression of OP-susceptible alleles may reduce the fitness cost potentially associated with mutations conferring OP-insensitivity. In addition, maintenance of multiple alleles for each of the three known BmAChEs may also account for previous failures to identify *BmAChE* mutations associated with OP-resistance in *R*. (*B*.) *microplus*. It is hoped that elucidation of the complex interactions among the multiple BmAChEs and the physiological roles they play *in vivo* may enable development of new opportunities for tick control.

References

Baxter, G.D. & Barker, S.C. (1998) Acetylcholinesterase cDNA of the cattle tick, *Boophilus microplus*: characterization and role in organophosphate resistance. *Insect Biochemistry and Molecular Biology*, 28, 581–589.

Cousin, X., Hotelier, T., Giles, K., Lievin, P., Toutant, J.-P. & Chatonnet, A. (1997) The α/β fold family of proteins database and the cholinesterase gene server ESTHER. *Nucleic Acids Research*, 25, 143–146.

Cygler, M., Schrag, J.D., Sussman, J.L., Harel, M., Silman, I., Gentry, M.K. & Doctor, B.P. (1993) Relationship between sequence conservation and three-dimensional structure in a large family of esterases, lipases, and related proteins. *Protein Science*, 2, 366–382.

Davey, R.B. & George, J.E. (1999) Efficacy of coumaphos applied as a dip for control of an organophosphate-resistant strain of *Boophilus microplus* (Acari: Ixodidae) on cattle. *Journal of Economic Entomology*, 92, 1384–1391.

Davey, R.B., George, J.E. & Miller, R.J. (2003) Efficacy of various concentrations of coumaphos to control adult, nymphal, and larval stages of an organophosphate-resistant strain of *Boophilus microplus* on infested cattle. *American Journal of Veterinary Research*, 64, 684–689.

Fournier, D. & Mutero, A. (1994) Modification of acetylcholinesterase as a mechanism of resistance to insecticides. *Comparative Biochemistry and Physiology*, 108C, 19–31.

Fournier, D., Mutero, A. & Rungger, D. (1992) *Drosophila* acetylcholinesterase: expression of a functional precursor in *Xenopus* oocytes. *European Journal of Biochemistry*, 203, 513–519.

George, J.E. (1996) The campaign to keep *Boophilus* ticks out of the United States: technical problems and solutions. In: Wagner, G.G. & Buisch, W.W. (eds) *Proceedings of the 100th Annual Meeting of the US Animal Health Association*. Spectrum Press, Richmond, pp. 196–206.

Graham, O.H. & Hourrigan, J.L. (1977) Eradication programs for the arthropod parasites of livestock. *Journal of Medical Entomology*, 13, 629–658.

Harel, M., Kryger, G., Rosenberry, T.L., Mallender, W.D., Lewis, T., Fletcher, R.J., Guss, J.M., Silman, I. & Sussman, J.L. (2000) Three-dimensional structures of *Drosophila melanogaster* acetylcholinesterase and of its complexes with two potent inhibitors. *Protein Science*, 9, 1063–1072.

Hernandez, R., He, H., Chen, A.C., Ivie, G.W., George, J.E. & Wagner, G.G. (1999) Cloning and sequencing of a putative acetylcholinesterase cDNA from *Boophilus microplus* (Acari: Ixodidae). *Journal of Medical Entomology*, 36, 764–770.

Ilg, T., Schmalz, S., Werr, M. & Cramer, J. (2010) Acetylcholinesterase of the cat flea *Ctenocephalides felis*: identification of two distinct genes and biochemical characterization of recombinant and in vivo enzyme activities. *Insect Biochemistry and Molecular Biology*, 40, 153–164.

Li, A.Y., Davey, R.B., Miller, R.J. & George, J.E. (2003) Resistance to coumaphos and diazinon in *Boophilus microplus* (Acari: Ixodidae) and evidence for the involvement of an oxidative detoxification mechanism. *Journal of Medical Entomology*, 40, 482–490.

Oakeshott, J.G., Claudianos, C., Russell, R.J. & Robin, G.C. (1999) Carboxyl/cholinesterases: a case study of the evolution of a successful multigene family. *BioEssays*, 21, 1031–1042.

O'Brien, R.D. (1967) *Insecticides: action and metabolism*. Academic Press, New York.

Pruett, J.H. (2002) Comparative inhibition kinetics for acetylcholinesterases extracted from organophosphate resistant and susceptible strains of *Boophilus microplus* (Acari: Ixodidae). *Journal of Economic Entomology*, 95, 1239–1244.

Pruett, J.H. & Pound, J.M. (2006) Biochemical diagnosis of organophosphate-insensitivity with neural acetylcholinesterase extracted by sonication from the adult tick synganglion. *Veterinary Parasitology*, 135, 355–363.

Rosario-Cruz, R., Almazan, C., Miller, R.J., Dominguez-Garcia, D.I., Hernandez-Ortiz, R. & de la Fuente, J. (2009) Genetic basis and impact of tick acaricide resistance. *Frontiers in Bioscience*, 14, 2657–2665.

Temeyer, K.B., Davey, R.B. & Chen, A.C. (2004a) Identification of a third *Boophilus microplus* (Acari: Ixodidae) cDNA presumptively encoding an acetylcholinesterase. *Journal of Medical Entomology*, 41, 259–268.

Temeyer, K.B., Olafson, P.U. & Miller, R.J. (2009) Genotyping mutations in *BmAChE3*: a survey of organophosphate-resistant and -susceptible strains of *Rhipicephalus* (*Boophilus*) *microplus*. *Journal of Medical Entomology*, 46, 1355–1360.

Temeyer, K.B., Pound, J.M., Miller, J.A., Chen, A.C., Pruett, J.H., Guerrero, F.D., Davey, R.B., Untalan, P.M., Lohmeyer, K.H., Li, A.Y., Miller, R.J. & George, J.E. (2004b) Organophosphate resistance in Mexican strains of *Boophilus microplus*: a major threat to the U.S. cattle industry. *SAAS Bulletin: Biochemistry and Biotechnology*, 17, 43–51.

Temeyer, K.B., Pruett, J.H. & Olafson, P.U. (2010) Baculovirus expression, biochemical characterization and organophosphate sensitivity of rBmAChE1, rBmAChE2, and rBmAChE3 of *Rhipicephalus* (*Boophilus*) *microplus*. *Veterinary Parasitology*, 172, 114–121.

Temeyer, K.B., Pruett, J.H., Olafson, P.U. & Chen, A.C. (2007) R86Q, a mutation in *BmAChE3* yielding a *Rhipicephalus microplus* organophosphate-insensitive acetylcholinesterase. *Journal of Medical Entomology*, 44, 1013–1018.

Temeyer, K.B., Pruett, J.H., Untalan, P.M. & Chen, A.C. (2006) Baculovirus expression of *Bm*AChE3, a cDNA encoding an acetylcholinesterase of *Boophilus microplus* (Acari: Ixodidae). *Journal of Medical Entomology*, 43, 707–712.

Toutant, J.-P. (1989) Insect acetylcholinesterase: catalytic properties, tissue distribution and molecular forms. *Progress in Neurobiology*, 32, 423–446.
Villatte, F. & Bachman, T.T. (2002) How many genes encode cholinesterase in arthropods? *Pesticide Biochemistry and Physiology*, 73, 122–129.
Villatte, F., Ziliani, P., Marcel, V., Menozzi, P. & Fournier, D. (2000) A high number of mutations in insect acetylcholinesterase may provide insecticide resistance. *Pesticide Biochemistry and Physiology*, 67, 95–102.
Xu, G., Fang, Q.Q., Keirans, J.E. & Durden, L.A. (2003) Cloning and sequencing of putative acetylcholinesterase cDNAs from the American dog tick, *Dermacentor variabilis*, and the brown dog tick, *Rhipicephalus sanguineus* (Acari: Ixodidae). *Journal of Medical Entomology*, 40, 890–896.
Zimmerman, G. & Soreq, H. (2006) Termination and beyond: acetylcholinesterase as a modulator of synaptic transmission. *Cell and Tissue Research*, 326, 655–669.