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Research

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Insecticide Resistance and Resistance Management

Synthetic pyrethroid resistance in *Haematobia irritans* subsp. *exigua* (Diptera: Muscidae): knockdown-recovery observed, a new putative resistance-linked mutation detected, and a complete voltage-gated sodium channel gene transcript sequenced

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Haematobia irritans subsp. *exigua* (Diptera: Muscidae), locally known as buffalo flies, are the costliest pest of cattle in Australia. Control of *H. i. exigua* is primarily through insecticides despite resistance to many control chemicals now confirmed. Elevated resistance to synthetic pyrethroids in Queensland *H. i. exigua* populations has been observed, which led to this study to investigate if new voltage-gated sodium channel (VGSC) mutations might be involved. The complete VGSC coding region of *H. i. exigua* has been sequenced. The gene consists of 25 putative exons plus 7 alternate-splice sites. Five genetic assays were developed that amplify *H. i. exigua* DNA and target 26 resistance-linked mutations characterized in other insects. Deltamethrin and α-cypermethrin tested flies from a field population of *H. i. exigua* were screened and a new putative resistance-linked mutation, *T929I* along with previously characterized *kdr* (*L1014F*) were found in 82% of the resistant flies. The *T929I* mutation has been shown to provide a synergistic enhancement of *kdr* in other insects and may be providing a synergistic enhancement of resistance to α-cypermethrin in *H. i. exigua*. A new class of resistance, knockdown-recovery, was observed in *H. i. exigua* populations. Knockdown-recovery in other insects has been linked to the elevated expression of detoxifying enzymes. This alternative pathway may explain how 18% of flies without VGSC resistance mutations survive high concentrations of insecticide.

Keywords: Insecticide resistance, genetics, behaviour, molecular biology, vertebrate pests

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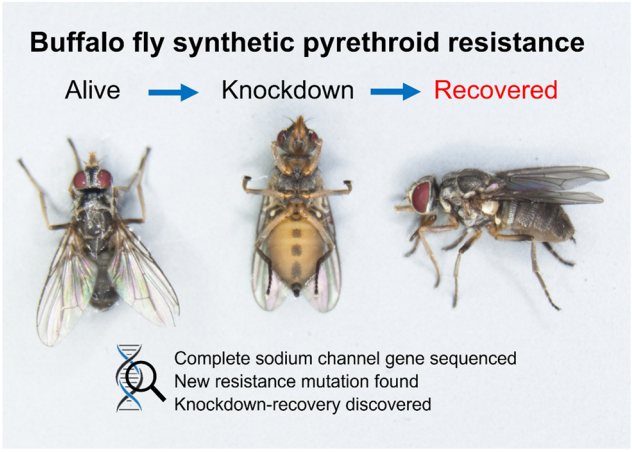
Graphical abstract



Introduction

Haematobia irritans exigua (Diptera: Muscidae) (de Meijere 1903), locally known as buffalo flies, are ranked as the number one economic pest of cattle in Australia costing the cattle industry $111.7M p.a. in production losses, and control measures contributing an additional $58.6M p.a. (Shephard et al. 2022). Due to its blood feeding (hematophagous) behavior, *H. i. exigua* causes significant irritation to cattle, leading to skin lesions and decreased milk and meat production, reducing profitability for cattle producers. Control of *H. i. exigua* is primarily through the application of insecticides. However, after many years of exposure to insecticides, resistance to many *H. i. exigua* control chemicals is now confirmed or suspected (Agrisearch Services Pty Ltd, 2000, Rothwell et al. 2011).

Insecticide resistance in insects is defined as a genetic adaptation that allows certain populations to survive exposure to insecticides (Hemingway et al. 2002). Insecticide resistance can occur through several mechanisms, including mutations that alter the target site of the insecticide, enhanced metabolism or detoxification of the insecticide, or behavioral adaptations that reduce exposure to the insecticide (Belinato and Martins 2016). The sodium channel, a transmembrane voltage-gated protein (VGSC) which is responsible for conduction of electrical signals in neurons, is the target site of pyrethroid insecticides. Pyrethroids bind to these proteins, extending the opening of channels causing repetitive nerve firing that leads to insect knockdown, a rapid state of intoxication and partial paralysis which usually precedes death (Dong 2007). Target site insensitivity to pyrethroids, mainly through knockdown resistance, has been associated with resistance in *H. i. exigua*, and closely related horn fly, *Haematobia irritans irritans* (Linnaeus, 1758) (Jamroz et al. 1998, Rothwell et al. 2011). Point mutations in the VGSC that confer resistance are named using a standard nomenclature developed in house fly, *Musca domestica* Linnaeus, 1758, that identifies the susceptible amino acid, then the amino acid position within the protein sequence, followed by the modified amino acid. Genetic assays have been developed to detect *kdr* (*L1014F*) and super *kdr* (*skdr, M918T*) mutations in horn fly (Guerrero et al. 1998, Domingues et al. 2019). Rothwell et al. (2011) demonstrated that the same assays are effective for screening *H. i. exigua*, and they identified the *kdr* mutation in *H. i. exigua* from south-east Queensland. They did not find any evidence of the *skdr* mutation (Rothwell et al. 2011).

Closely related species with fully sequenced VGSC coding regions (exons) are the stable fly, *Stomoxys calcitrans* (Linnaeus, 1758) (6,661 bp, GenBank accession XM_013245458) and *M. domestica* (6,327 bp, GenBank accession X96668). Complete DNA genomes for these model species are available. They show that the annotated VGSC gene is large, 170,938 bp in *S. calcitrans* (Genbank accession NC081555) and 176,312 bp in *M. domestica* (extracted from whole genome shotgun sequence strain M3 Contig6478 JAVQME010006552) and consists of around 30 exons separated by noncoding introns.

Although VGSC in insects is coded by a single gene, alternative splicing and RNA editing appear to be important in creating VGSC diversity in insects (Tan et al. 2002, Dong et al. 2014). In the fruit fly *Drosophila melanogaster* (Meigen 1869), 9 splice sites have been identified with 7 optional splice sites (a, b, i, j, e, f, and h), as well as 2 sites (c/d and l/k) where the exons are mutually exclusive and code for amino acids in the transmembrane spanning regions of the channel (Thompson et al. 2020). Alternative splicing has been shown to affect voltage sensitivity of the sodium channel in the German cockroach *Blattella germanica* Linnaeus, 1767 (Tan et al. 2002), *D. melanogaster* (Lin et al. 2009) and *M. domestica* (Thompson et al. 2020).

When this project commenced, one genome for horn fly was available (Hi v1.0, Konganti et al. 2018), which did not contain a complete VGSC gene. The small fragments sequenced covered around 1,000 base pairs (bp) of the VGSC gene surrounding the *kdr* (*L1014F*) and *skdr* (*M918T*) resistance mutation sites.

The aim of this project was to investigate field observations of elevated synthetic pyrethroid resistance in *H. i. exigua* populations. The complete VGSC coding region for the Queensland Department of Primary Industries (QDPI) laboratory colony of *H. i. exigua* was sequenced. DNA primers were designed in exons containing potential insect resistance-linked mutations

identified from the literature. These primers were then used to screen for new resistance mutations in field collected *H. i. exigua* that survived in bioassays against α-cypermethrin and deltamethrin. The bioassays were conducted at concentrations determined to reliably kill susceptible flies.

The benefit to industry of understanding synthetic pyrethroid resistance is better resistance management programs through the rotation of chemical groups. The practice of increasing chemical concentration to overcome resistance may be having undesirable effects on the long-term resistance profile of local fly populations.

Materials and Methods

Haematobia irritans exigua Samples

The Queensland DPI have maintained a colony of *H. i. exigua* since 2012. The colony originates from the University of Queensland Research Farm at Pinjarra Hills, Brisbane and has been in continual culture at QDPI's EcoSciences Precinct for 13 years (James 2013). Adult flies are fed daily with citrated cattle blood and are kept in cages made of an aluminum frame with insect mesh covering the top and sides, measuring 300 mm in height, 300 mm in width, and 450 mm in depth. These cages are maintained in a controlled environment room set to 28.0 °C with 70.0% relative humidity and a photoperiod of 14 h light and 10 h dark (14:10 L:D). The *H. i. exigua* colony was sampled for the component of this study focused on sequencing the VGSC coding region.

Field-collected flies were collected from a property in Millaa Millaa, north Queensland (animal ethics permit CA 2021/01/1457). Chemical test dishes were prepared (Farnsworth 1997) with the insecticides α-cypermethrin and deltamethrin. Flies were collected from cattle using a sweep net and transferred to a small insect cage. Flies were removed from the cage using a small vacuum, immobilized with carbon dioxide, and approximately 30 mixed sex flies were added to each treatment testing dish. Dishes were held closed with tape and stored at ambient temperature.

Each dish contained a filter paper impregnated with a set concentration of each chemical. The range of chemical concentrations chosen was based on previous testing to provide a range of low (3.4 µg/cm^2 α-cypermethrin and 0.2 µg/cm^2 deltamethrin) to high (1,433 µg/cm^2 α-cypermethrin and 500 µg/cm^2 or 2,000 µg/cm^2 deltamethrin) fly mortality. For all tests, flies were added on-site, with numbers of live, dead (permanent and complete cessation of all movement), and morbid (noticeably affected by chemical, in a state of severe sickness, inactivity or is near death but still exhibits some signs of life) assessed after 20 h. After chemical testing, flies identified for genetic testing were placed in pure molecular grade ethanol (Merck, Bayswater Victoria, Australia, Cat. E7023) or RNA*later* (Thermo Fisher Scientific, Seventeen Mile Rocks, Queensland, Australia, Cat. AM7020) followed by storage at –20 °C.

For genetic screening, *H. i. exigua* that died in bioassays at the lowest chemical concentration were categorized as Susceptible (dead after 2 h at α-cypermethrin concentration 3.4 µg/cm^2; dead at deltamethrin concentration 0.20 µg/cm^2). Flies that survived at the highest bioassay chemical concentration were categorized as Resistant (alive after 20 h at α-cypermethrin concentration 1,433 µg/cm^2; alive at deltamethrin concentration 2,000 µg/cm^2 or 500 µg/cm^2 due to low survivorship at the highest dose). An additional category, Knockdown-Recovered (flies appearing to be dead or morbid at α-cypermethrin concentration 1,433 µg/cm^2 but recovered) was created following observations of fly behavior during bioassay testing. The genetic study aimed for a sample size of 10 for each category; however, resistant fly numbers varied with bioassay survival.

RNA and DNA Extraction

RNA was extracted from individual RNA*later* stored *H. i. exigua* using an RNeasy Mini Kit (Qiagen, Chadstone, Victoria, Cat. 74104). Purified RNA was DNase-treated (TURBO DNase) (Ambion, Milton, Brisbane, Australia. Cat. AM2238) as per the manufacturer's instructions prior to first-strand cDNA synthesis (SuperScript III Reverse Transcriptase, Invitrogen, Waltham, Massachusetts, Cat. 18080-44).

DNA was extracted from individual *H. i. exigua* using a DNeasy Blood and Tissue Kit (Qiagen, Chadstone, Victoria, Cat. 69504). Ethanol-preserved samples were soaked in 1 ml of milli-Q water for 1 h prior to DNA extraction to remove the ethanol preservative. After the addition of lysis buffer, the flies were mechanically lysed with a plastic pestle against the inside wall of a 1.5 ml sample tube. They then underwent chemical lysis and DNA isolation following the manufacturer's instructions for "Purification of Total DNA from Animal Tissues."

VGSC Primer Design

Sodium channel gene sequences from *H. i. exigua* and related species (partial gene of *H. irritans irritans* and complete genes for *S. calcitrans* and *M. domestica*) were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/ accessions GU049672, GU049673, U83871, U83873, XM_013245458, X96668). The sequences were aligned using the pairwise MUSCLE alignment algorithm in Geneious Prime V11.1.2 (https://www.geneious.com). Resulting alignments were checked by eye to correct poorly aligned regions often associated with sequence insertions or deletions. Annotations were added to the alignment to mark the 4 structural domains, each consisting of 6 subunits, characterized for *M. domestica* (Williamson et al. 1996). The position of 36 arthropod resistance-linked mutation sites extracted from the literature (consolidated from Pasay et al. 2008, Dong et al. 2014, Chen et al. 2020, Jouraku et al. 2024) were also added to the alignment. Generic primers were designed within conserved regions of the alignment. As sequencing progressed, new generic primers were created, and new *H. i. exigua* specific primers were designed to span regions of the gene where the first-round generic primers failed to amplify a product. The successful primers used in this study are provided in Table 1.

VGSC DNA Amplification, Sequencing and Annotation

PCR amplification reactions (20 µl total volume) contained 1.5 µl of DNA (10 to 50 ng), 0.4 µM of each primer pair (Table 1), 5× MyTaq PCR buffer containing 3 mM of magnesium and 1 mM of dNTPs, and 2 units of MyTaq DNA polymerase (MyTaq DNA Polymerase, Bioline, Eveleigh, NSW, Australia, Cat. BIO-21105). Amplification reactions were carried out at 96 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 60 s and 72 °C for 120 s with a final extension of 7 min at 72 °C. Amplified products were analyzed by 1.2% agarose Tris–borate–EDTA gel stained with GelRed (Biotium

Table 1. cDNA primers used to obtain the complete *H. i. exigua* VGSC gene

Primer pairs[a]	Primer direction	Primer sequence 5′-3′	Product size (bp)
F22 to	F	ATGACAGAAGATTCCGACTCGATATC	221
BR244	R	GTGGACCTTCATCTTCGTCCTC	
F135 to	F	AWAGAAAGAGAGCCGCCGAA	1,000
BR1164	R	CCAACCAAATGAATCGAAACTGGT	
BF721 to	F	AACATTTAGGGTACTGCGAGCTC	803
R1500	R	CGCCGCCAACAAACAGTTCA	
BF1455 to	F	GGGCCAATGTAGCAGCTCAA	560
R1985	R	ATGTGATGTATACGAGATTCTTGATTGATG	
F1937 to	F	CCAGCTTACTATTGTAATTTAGGTTCTAGAC	721
BR2668	R	TCATGGCCATGAGTTTCATGCT	
F2462 to	F	ACAATGTTYATGGCCATGGATCA	1,010
R3469	R	GTGGTTTATTGAGTTTCCAATCATCG	
BF3004 to	F	GGTTCATCTAGTTTATCAGCACCGAC	830
BR3801	R	GCTAAGCTACTCATTAAAATCATAGTGATAACTG	
BF3740 to	F	GGATGGGGCAATTTACGACTGA	877
R4951	R	TGAACATTTCTAATGATCCACCTGCT	
BF4277 to	F	ATTCTTCCTGGAGATGTTAATCAAATGG	698
R4951	R	TGAACATTTCTAATGATCCACCTGCT	
BF4514 to	F	ATTATATTTGGATCATTTTTCACACTCAATCTG	780
R5386	R	ACCGGCTGAGGTAGACATCT	
BF5300 to	F	CATGTCCTTCTTCATGCATGTCAA	745
BR5895	R	CCTTCTTCGGCCACATTGTCTTC	
BF5500 to	F	ACTGTGGCGCCAGCGTG	435
R5950	R	TCAGACATCTGCCGTCCTGGATG	

[a]Primer names beginning with B were designed from *H. i. exigua* sequence; those without a B were designed within conserved regions of a *S. calcitrans* and *M. domestica* sequence alignment.

distributed by Gene Target Solutions, Dural, New South Wales, Australia, Cat. 41003). PCR products were cleaned prior to sequencing with Exosap-it (USB Corporation, distributed by GE Healthcare Bio-Sciences, Rydalmere New South Wales, Australia) according to the manufacturer's instructions. PCR products were direct sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Seventeen Mile Rocks, Queensland, Australia, Cat. 4337455) according to the manufacturer's instructions and submitted to a commercial sequencing service (Genetic Research Services, The University of Queensland, Australia). Raw sequences were aligned and edited in Sequencher Vers. 5.4.6 (Gene Codes Corporation, Ann Arbor, Michigan, United States). The edited *H. i. exigua* VGSC gene was then added to the previously described alignment of other fly species for comparison using the pairwise MUSCLE alignment algorithm in Geneious Prime V11.1.2 (https://www.geneious.com). Putative exon boundaries were confirmed by aligning the sequences to the complete VGSC gene of *M. domestica* (mined from JAVQME010006552 whole genome shotgun sequence strain M3 Contig6478). Prior to publication, putative exon positions were further validated by sequence alignment to the complete VGSC gene of horn fly (mined from whole genome KBUSLIRL chromosome 3 CM113867.1). The annotation of putative alternative splice elements was based on those summarized in Lee et al. (2002) for *M. domestica*.

Screening Field Collected Flies for Potential Resistance Mutations

New DNA primers were designed to amplify regions containing at least 2 insect resistance-linked mutations in the alignment. The regions needed sufficient flanking sequence around the mutation sites to anchor primers (minimum of 18 bp) and needed to be large enough for Sanger sequencing (larger than 150 bp). Primers were designed by eye with the additional assistance of primer design software in Geneious Prime V11.1.2 (https://www.geneious.com) to calculate melt temperatures and to minimize hairpins and self-dimers (Table 2). Preparation of PCR amplification and DNA sequencing reactions using the new primers (Table 2), followed those detailed above under "VGSC DNA amplification and sequencing".

Statistics

Spearman's rank tests (www.socscistatistics.com/tests/spearman/default2.aspx) were used to determine if the resistance mutations found in *H. i. exigua* were correlated to survival in synthetic pyrethroid bioassays. For the 2 chemicals tested (α-cypermethrin or deltamethrin), each fly was assigned to a treatment rank (bioassay outcome) and genotype rank (*kdr* alone, *T929I* alone and *kdr* plus *T929I*) as shown in Table 3. A significant correlation between bioassay outcome and resistance mutation or mutations was determined using two-tailed *P*-values, with degrees of freedom calculated as d.f. = $n-2$, and the significance level set at $\alpha = 0.05$.

Results

Field Flies Selected for Molecular Screening

The exposure of *H. i. exigua* to α-cypermethrin and deltamethrin provided samples for genetic screening (Table 4). Flies that survived at the highest chemical doses were classed as either alive or morbid, and flies that died at lowest dose were selected for comparison.

Table 2. Genomic DNA primers to amplify *H. i. exigua* DNA surrounding resistance-linked mutation sites characterized in other insects

Primer[a]	Sequence 5′ to 3′	Tm[b] °C	Size bp	Insect resistance-linked mutation site captured
Region 1: Spans complete Domain I P-loop and Domain I S6 within Exon 8				
BF1135	GTCCAAATCCCAATTATGATTATACCAGT	55	276	*V410L/M, E435K*
BR1388	GCTTCTCGTATCGCCTCCT	56		
Region 2: Spans complete Domain II S1 and partial Domain II S2 within Exon 15				
BF2401	CATTTAAGGACATAGCCCTCGAGTA	56	222	*C785R, M827I*
BR2516	CACTTTTGAGAACCTTCTCCAATTCTG	56		
Region 3: Spans partial Domain II S4 and complete Domain II S5 within Exon 17				
FG235[c]	CTTCGTGTATTCAAATTGGCA	55	168	*M918T (skdr), L925I,*
skdrR[d]	GTGATCAATATAGTTCTTTCCG	60		*T929I, L932F, I936V*
Region 4: Spans complete Domain II P-loop and complete Domain II S6 within Exon 18, Intron 18 and Exon 19				
kdrF[d]	AATTCAAAGATCATGAATTACC	56	444	321 bp exons with variable sized intron
FG138[c]	CAATATTACGTTTCACCCAG	56		*F979S, S989P, V1010L/A, I1011M, N1013S, L1014F (kdr), V1016G, F1020S, L1024V*
Region 5: Spans complete Domain III S6 within Exon 27				
BF4827	GGACAAGCAGCCGATTCGAG	58	234	*M1524I, F1528L*
BR5014	GGAATGGCTTTTAATGGTTTTTTAGAGC	56		*F1534C, F1538I, D1549V, E1553G, N1575Y*

[a]Primer names commencing with B were designed from *H. i. exigua* sequence by the authors.
[b]Predicted primer melt temperature °C.
[c]Primer from Guerrero et al. (1998).
[d]Primer from Rothwell et al. (2011).

Table 3. Treatment and genotype rankings used for Spearman's rank testing to determine if a correlation could be found between the presence of sodium channel mutations and fly survival following exposure to insecticide

Treatment ranking	α-Cypermethrin	Deltamethrin
1	Susceptible 3.4 (μg/cm²)	Susceptible 0.2 (μg/cm²)
2	Resistant 1433 (μg/cm²)	Resistant 500 (μg/cm²)
3	Morbid 1433 (μg/cm²)	Resistant 2000 (μg/cm²)
Genotype ranking	***kdr* alone (amino acids)**	***T929I* alone (amino acids)**
1	Homozygous susceptible (L)	Homozygous susceptible (T)
2	Heterozygous (LF)	Heterozygous (TI)
3	Homozygous resistant (F)	Homozygous resistant (I)
Genotype ranking	***kdr* plus T929I (amino acids)**	
1	Homozygous susceptible (L) + Homozygous susceptible (T)	
2	Homozygous susceptible (L) + Heterozygous (TI)	
3	Heterozygous (LF) + Homozygous susceptible (T)	
4	Homozygous resistant (F) + Homozygous susceptible (T)	
5	Heterozygous (LF) + Heterozygous (TI)	
6	Homozygous resistant (F) + Heterozygous (TI)	

Table 4. Field *H. i. exigua* samples selected for genetic screening following exposure to insecticide treatments

Treatment	Dose (μg/cm²)	Number of flies	Status	Resistance category
α-Cypermethrin	3.4	11	Dead	Susceptible
	1,433	8	Alive	Resistant
	1,433	4	Morbid	Knockdown-recovered
Deltamethrin	0.2	10	Dead	Susceptible
	500	9	Alive	Resistant
	2,000	1	Alive	Resistant

VGSC Gene

Complete VGSC DNA sequences, including introns were extracted from genome sequences of *S. calcitrans* (GenBank accession NC081555, 170,938 bp) and *M. domestica* (extracted from GenBank accession JAVQME010006552, 176,335 bp). The VGSC DNA alignment of these 2 species is 176,889 bp and consists of around 36 characterized exons including up to 11 alternative splice sites. This alignment was used to identify likely intron positions in the *H. i. exigua* coding sequence.

Based on the comparison, the complete *H. i. exigua* VGSC gene (including 5′ and 3′ terminus primers) is 6,348 to 6,393 bp long. The QDPI *H. i. exigua* laboratory colony mRNA expressed the VGSC gene with and without optional alternate splice sites b and j (see Lee et al. 2002, for splice site sequences). By sequence alignment inference, the *H. i. exigua* VGSC gene contains 32 putative exons including 7 expressed alternate-splice sites a, b, d, h, i, j and l (Fig. 1 and Supplementary Table S1 for exon, structural domain and resistance mutation positions within the sequence). Sequence information for alternate-splice sites c, e, f, and k found in *M. domestica* and *S. calcitrans* likely exist but were not obtained as their expression was not detected in the QDPI *H. i. exigua* laboratory colony.

The *H. i. exigua* sodium channel gene sequence aligns with 95.2% similarity to *S. calcitrans* transcript variant X16 (XM_059367561.1, 100% cover) and with 93.8% similarity

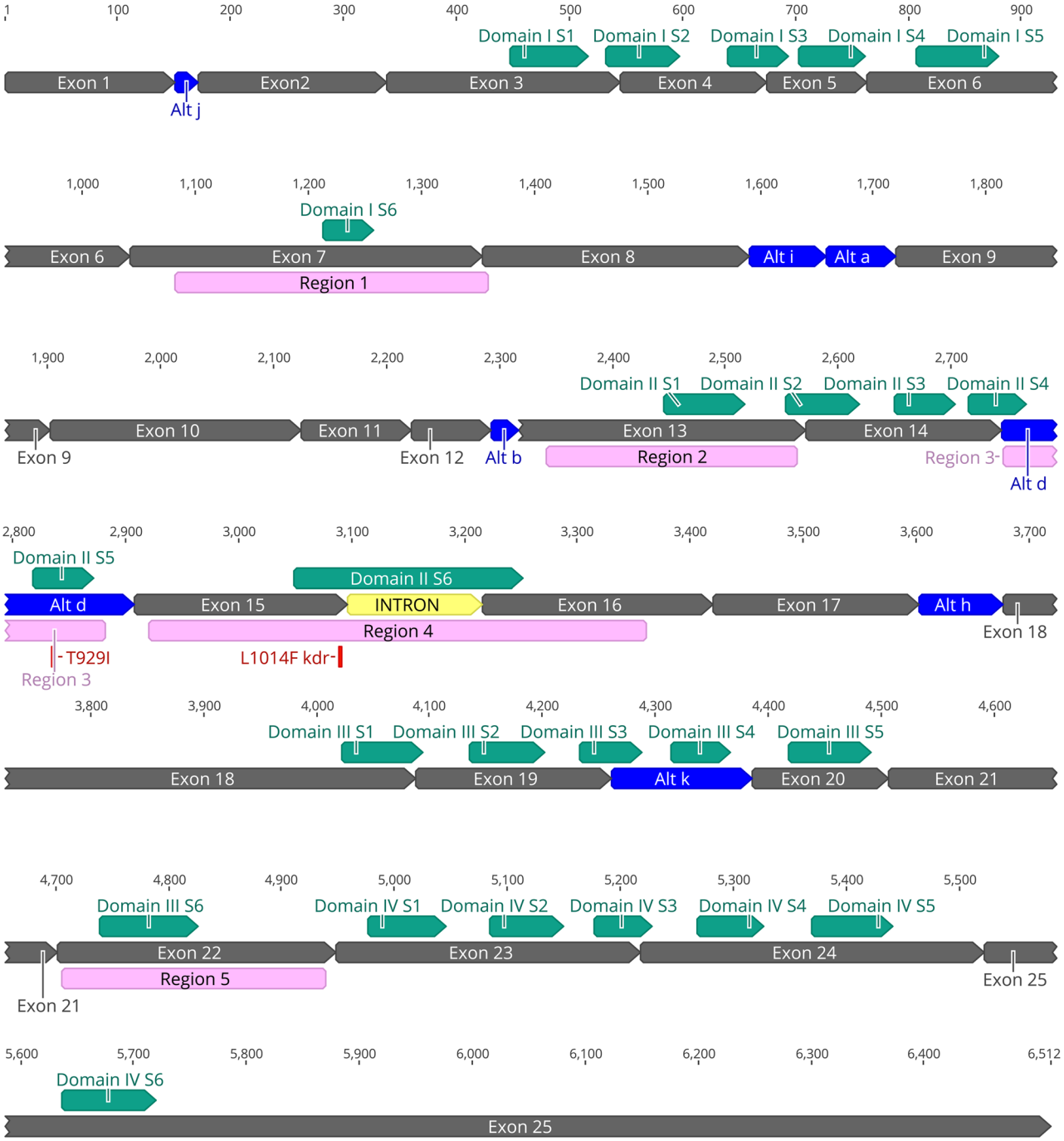


Fig. 1. *H. i. exigua* sodium channel gene sequence structure.

to *M. domestica* transcript variant X10 (XM_059122211.1, 96% cover). As with other insects (Soderlund and Knipple, 2003), the gene likely consists of 4 structural domains (I to IV), each consisting of 6 subunits (S1 to S6). When compared to *S. calcitrans* and *M. domestica* mRNA, the highest sequence and amino acid divergence occurs in the last, and largest exon of the gene. While preparing this manuscript, a new horn fly genome was submitted to GenBank (Luecke et al. 2025). Although not yet annotated, the new genome assembly does contain the complete 151,358 bp VGSC gene (isolate KBUSLIRL chromosome 3 CM113867.1 position 3,860,413 to 4,011,771). The *H. i. exigua* transcript has 99.47% similarity to horn fly differing at 38 sites in the 6,393 bp sequence resulting in 8 amino acid changes and 3 amino acid deletions in horn fly.

The *H. i. exigua* VGSC sequence has been submitted to GenBank (Accession PV871995).

Screen of Field-collected Flies for Resistance Mutations

A total of 43 field-collected *H. i. exigua* were screened for 14 possible resistance-linked mutations found in other insect

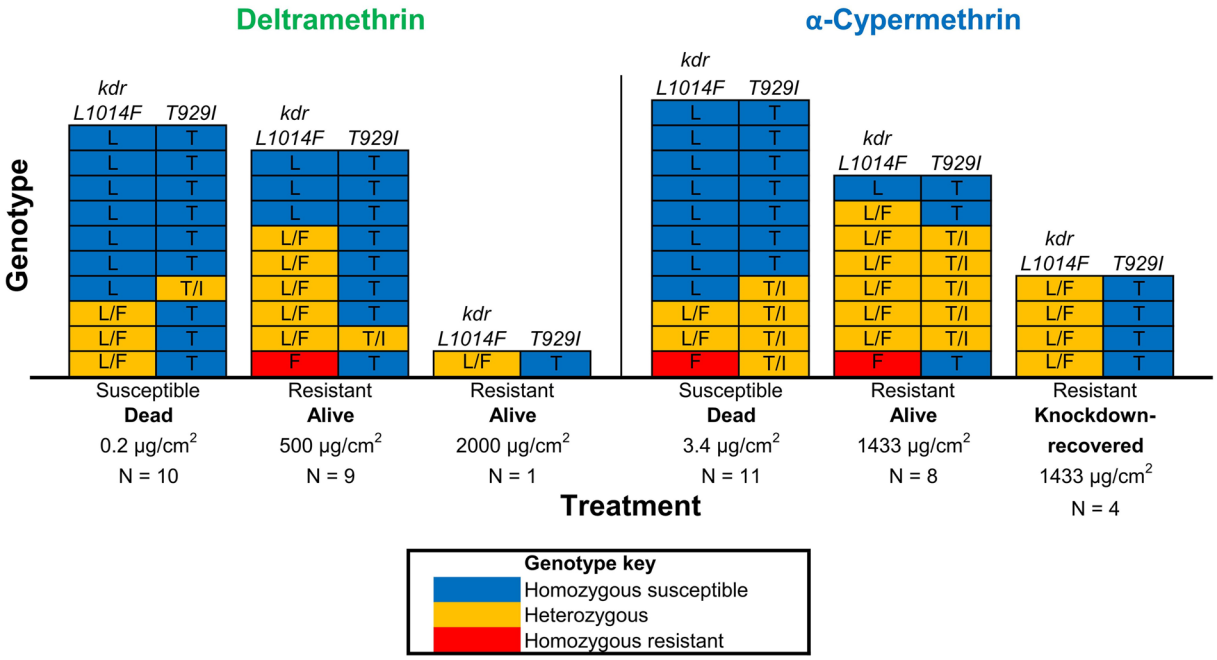


Fig. 2. Genetic screen of synthetic pyrethroid susceptible and resistant *H. i. exigua* from a field population with paired cells above each treatment displaying single fly amino acid genotypes for kdr and T929I loci.

species (regions 3 and 4 in Table 2). Among these flies, 21 were classed as susceptible, dead at lowest chemical concentration, and 22 as resistant, alive at high concentrations of deltamethrin or α-cypermethrin. The analysis detected the mutations *kdr* and *T929I*, with the first report of the putative resistant mutation *T929I* in *H. i. exigua*.

All possible *kdr* genotypes were found in the population but no *T929I* homozygous resistant flies were found. As sampling was nonrandom, allele frequencies were not tested for Hardy–Weinberg equilibrium.

Of the 21 dead flies in the 2 susceptible treatments, 8 carried resistance alleles (38%) (Fig. 2). The death of flies carrying resistance alleles in the low concentration chemical treatments may be explained by stress, age or starvation induced death in the test. In contrast, 70% of the deltamethrin resistant flies and 92% of the α-cypermethrin-resistant flies carried at least one resistant allele.

The most abundant resistance mutation was *kdr* with 82% of resistant flies carrying either homozygous resistant (F/F) or more commonly heterozygous (L/F) genotypes at this locus (Fig. 2). The *T929I* mutation was found in only 10% of deltamethrin resistant flies, no different to the proportion found in the deltamethrin susceptible treatment, but it had a higher prevalence of 62.5% in the α-cypermethrin-resistant treatment that displayed no knockdown. Interestingly, *T929I* was not found in any of the high-concentration α-cypermethrin knockdown-recovered flies.

Resistance mutations were not detected in 18% of flies that survived high concentration levels (3 flies in deltamethrin treatments, one in α-cypermethrin treatments).

Further screening of the complete 26 resistance SNP panel (Regions 1 to 5 in Table 2) was conducted on 12 of the field collected flies (6 resistant and 6 susceptible) including 2 flies with homozygous susceptible *kdr* and *T929I* genotypes that survived high concentration α-cypermethrin or deltamethrin treatments. No other sodium channel gene mutations were identified.

New field collected *H. i. exigua* VGSC DNA sequence variants have been submitted to GenBank (Accessions PV987407 and PV987408).

Cypermethrin Resistance

A significant correlation exists between the presence of the *kdr* resistant allele alone, and survivorship at high α-cypermethrin concentration (Spearman's Rho = 0.54, d.f. = 21, $P = 0.008$). Spearman's Rho ranges from −1 to +1. The closer Rho is to 1, the stronger the positive or negative relationship. The putative *T929I* resistance allele displayed a slightly higher prevalence in the α-cypermethrin resistant treatment but was completely absent from the α-cypermethrin treated Knockdown-Recovered group of flies ($n = 4$) (Fig. 2). Although the trend is visible (Fig. 2), the resistance correlation with *T929I* alone was not as strong as *kdr* and was not significant, even when the Knockdown-Recovered category was excluded (Spearman's Rho = 0.26, d.f. = 17, $P = 0.29$).

Flies with combined *kdr* and *T929I* mutations were most abundant in the α-cypermethrin treatment with active flies (Fig. 2). Results of the Spearman's rank correlation including ranks for the combined alleles, and excluding the resistant Knockdown-Recovered category, suggests a positive association does exist (Spearman's Rho = 0.47, d.f. = 17, $P = 0.044$, Fig. 3).

Deltamethrin Resistance

Although the trend of increasing *kdr* resistance alleles with increasing deltamethrin concentration is apparent in Fig. 2, the correlation was not significant (Spearman's Rho = 0.43, d.f. = 18, $P = 0.056$). The analysis would probably benefit from larger sample sizes to increase the statistical power to detect a correlation.

In contrast to α-cypermethrin, no evidence of a correlation exists with the *T929I* resistant allele and survivorship at increasing deltamethrin concentrations (Spearman's Rho = 0.016, d.f. = 18, $P = 0.95$).

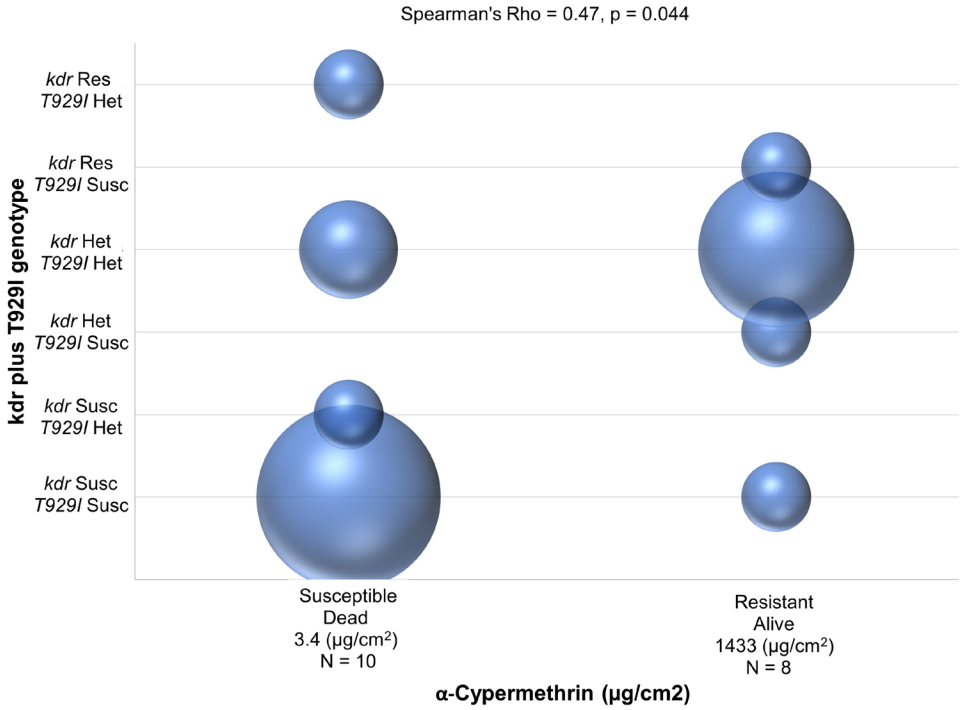


FIG. 3. Frequency bubble plot showing the different sodium channel genotype profiles of susceptible and resistant *H. i. exigua* exposed to α-cypermethrin.

Discussion

A complete VGSC gene transcript has been sequenced for *H. i. exigua*. The gene has 99.47% similarity to horn fly and over 90% similarity to *S. calcitrans* and *M. domestica* transcripts. The genus *Haematobia* likely share the same structural domains and subunits as characterized in *M. domestica*.

Results of the α-cypermethrin and deltamethrin testing on a field-collected population of *H. i. exigua* from far north Queensland found flies surviving high concentrations of synthetic pyrethroids. Two types of resistant flies were observed, the first type displayed no effect when exposed to very high concentrations of synthetic pyrethroids; they remained active, and the second type appeared to be knocked down by high concentrations of the chemical, but surprisingly many of these flies made a full recovery back to active status. This is the first time knockdown-recovery has been observed in *H. i. exigua*. This discovery is important because in chemical bioassays, dead and morbid flies are typically pooled together to calculate average mortality (Farnsworth, 1997). It also flags that a proportion of flies believed to be knocked down during treatment, may instead be temporarily paralysed causing them to fall off the cattle and away from chemical exposure, where they have the capacity to rapidly recover. Sample sizes were too small to confidently test for genetic differences between the 2 α-cypermethrin resistant categories. The absence of *T929I* in the knockdown-recovered flies suggests that further study is warranted to determine if the 2 distinct resistance behaviors observed in *H. i. exigua* represent separate resistance mechanisms or simply variations in response within a single resistant population.

H. i. exigua resistance to α-cypermethrin in far north Queensland is increasing. Surveys show that average survival at the 3.4 µg discriminating concentration in 1995 was 63.2%, and in 2000 it was 87.6% (Agrisearch Services Pty Ltd, 2000). This 2023 study of a single property found live flies after 20 h exposure to 421 times the discriminating concentration (91.5% survival).

The *H. i. exigua* population also showed very high resistance to deltamethrin, with some flies surviving 1,000 times the discriminating concentration.

Primer sets to amplify 5 regions of *H. i. exigua* VGSC DNA have been designed to enable screening of 26 mutation sites associated with insecticide resistance in other insects. In addition to the known *kdr* (*L1014F*) mutation in *H. i. exigua*, a new putative resistant-linked mutation, *T929I*, was detected in a field population of flies. The *T929I* mutation has been identified in a pyrethroid-resistant strain of diamondback moth, where it is believed to work in combination with *kdr* (Schuler et al. 1998, Sonoda 2010). Schuler et al. (1998) have suggested that mutation *T929I* may produce additive or synergistic enhancement of pyrethroid resistance when present with the *kdr* mutation.

At least one resistance allele (*kdr* or *T929I*) was found in 82% of flies that were alive in treatments at high concentration levels of α-cypermethrin and deltamethrin. The prevalence of *kdr* resistance, 56%, was higher than *T929I*, 26%, but due to the selective sampling of this study (ie non-random), the proportion of resistant flies in the field population could not be determined.

The *kdr* resistance mutation is correlated with α-cypermethrin resistance in *H. i. exigua* ($P = 0.008$) and is approaching significance with deltamethrin ($P = 0.056$). No correlation was detected for the *T929I* mutation alone with either chemical; however, the combined *kdr* + *T929I* genotype is significant ($P = 0.044$) for active α-cypermethrin resistance only suggesting it may be enhancing the *kdr* mutation. The *T929I* allele does not appear to be playing a role in deltamethrin resistance. Sun et al. (2017) found that the enhancement of *kdr* resistance by the addition of *T929I* was not the same across all pyrethroids. Enhancement of different pyrethroid groups varied from none

to ~1,000-fold increased resistance. Deltamethrin and α-cypermethrin belong to the same type II pyrethroid group, they are α-cyano-3-pheoxybenzyl pyrethroid esters with similar chemical structures. Possibly the 2 bromines in deltamethrin interact slightly differently with the *T929I* mutation in the sodium channel pore than the 2 chlorines in α-cypermethrin. This difference in the chemicals may explain why deltamethrin is more potent than α-cypermethrin (ie a lower discriminating concentration is needed).

The *kdr* locus is an incomplete recessive; the heterozygote SR (amino acids L/F) has a resistance level somewhere in between the dominant susceptible SS (L) and recessive resistant RR (F) phenotypes (Roca-Acevedo et al. 2023). The observation that the *kdr* resistant allele does not appear to drive to fixation in insect populations under heavy synthetic pyrethroid insecticide pressure suggests that the mutation induces some form of fitness cost (Chandre et al. 2000). The *kdr* mutation has been found to have a heterozygote SR advantage in some insects. In the mosquito *Anopheles gambiae* Giles, 1902 *sensu stricto*, for instance, the SR genotype has a fitness advantage over the homozygous SS, which are susceptible to insecticide, and the homozygous RR, which is associated with a loss of fitness in females (Chandre et al. 2000). The *H. i. exigua* samples screened in this study displayed all 3 *kdr* genotypes: 22 SS, 24 SR, and 5 RR, indicating that the homozygous fitness cost of RR is not lethal in this species. This is further confirmed by the QDPI *H. i. exigua* colony, which has, to date, always tested homozygous resistant RR at the *kdr* locus (bioassay results for the colony show 86% survival at 1,433 ug/cm^2 α-cypermethrin).

A recent review by Jouraku et al. (2024) on resistance in onion thrips, *Thrips tabaci* Lindeman (1889), found that adult females of one strain with *T929I* exhibited shorter longevity and produced fewer eggs than those of the same strain without *T929I*. Larger fitness costs were observed when strains were also carrying *kdr* and *skdr* mutations. No *T929I* homozygous resistant RR flies were found in the *H. i. exigua* population, only heterozygous SR (29%), or homozygous susceptible SS (71%) genotypes. The absence of RR genotypes may reflect the lower abundance of flies found with the *T929I* allele (15 flies) or could indicate that there is a homozygous resistance fitness cost at this locus. When present on the same gene copy as *kdr*, *T929I* is thought to produce additive or synergistic enhancement of pyrethroid resistance (Schuler et al. 1998, Soderlund and Knipple, 2003, Vais et al. 2003, Sun et al. 2017). In this study, of the 15 flies found carrying the *T929I* allele, 12 also carried the *kdr* allele. Most active flies in the highest α-cypermethrin treatment (1433 µg/cm^2), were carrying the *T929I* + *kdr* genotype. This suggests that the *T929I* mutation may be providing a synergistic enhancement with *kdr* to resist α-cypermethrin. Interestingly, none of the flies in the knockdown-recovered α-cypermethrin treatment carried the combined *T929I* + *kdr* genotype. Further research is needed to better understand how *kdr* and *T929I* resistance operates within *H. i. exigua*. It is possible that, like thrips, only populations under high synthetic pyrethroid regimes can maintain the high fitness cost of the combined mutations.

Synthetic pyrethroid resistance in 82% of field collected *H. i. exigua* appears to be explained by 2 DNA mutations in the VGSC gene. The correlation is significant, but further investigation is needed to understand if the relationship is causal. The remaining 18% of bioassay survivors must be using a different mechanism to avoid the insecticide. Ten resistance-linked mutation sites were not captured in this study because they were either positioned too close to exon-intron boundaries to design DNA primers, or they were lone mutations reported from a single distant taxon. Targeted regions 3, 4, and 5 span the important S5 and S6 subunits in domains DII and DIII, which contain most characterized mutations linked to synthetic pyrethroid resistance in insects. It is possible that other VGSC mutations are still to be discovered in *H. i. exigua*.

Alternative mechanisms used by insects to modify their VGSC genes without direct DNA mutations include alternative splicing and RNA editing (Dong et al. 2014). Seven alternate-splice sites have been sequenced in *H. i. exigua* including transcript variants with and without alternate splice sites b and j. Research expressing *D. melanogaster* VGSC in *Xenopus laevis* Daudin 1802 oocytes has shown that optional exons may be regulating neuronal excitability (Lin et al. 2009). Insect k/l splice variants differ in their pyrethroid sensitivity with the l variant being more susceptible (Tan et al. 2002, Lin et al. 2009, Huahua Sun et al. 2020). The QDPI *H. i. exigua* colony expressed the l splice variant. Increased VGSC expression in *D. melanogaster* has been observed in transcripts lacking splice variant b. The QDPI *H. i. exigua* colony expressed VGSC with and without splice variant b. This might contribute to variability in bioassay responses in the inbred laboratory strain. Further research is needed to determine if and how splice variants contribute to *H. i. exigua* resistance to synthetic pyrethroids.

Insects can also use RNA editing to modify transcripts by converting one base to another or by inserting and deleting nucleotides. Research on the VGSC of *Drosophila suzukii* (Matsumura, 1931) found 33 RNA editing events (Yuan et al. 2024). Amino acid changes through RNA editing have been linked to changes in VGSC currents; however, their role in increasing insecticide resistance is unknown (Yuan et al. 2024).

Knockdown-recovery has been observed in other insects and research into this pyrethroid avoidance mechanism suggests that metabolic detoxification enzymes are involved (Lu et al. 2021). The enhanced detoxification pathway does not appear to be driven by single DNA mutations (Freeman and Scott, 2024). Instead, a superfamily of cytochrome P450 (CYP) enzymes is overexpressed in pyrethroid-resistant insects (Saavedra-Rodriguez et al. 2021, Freeman and Scott, 2024). Synergists such as piperonyl butoxide (PBO) are often added to insecticide formulations to assist in blocking detoxifying enzymes. In 1993, an American study demonstrated that synergists could be combined with pyrethroids in ear tag formulations for improved control of horn fly, however, doing so increased the frequency of pyrethroid-resistant individuals in the population substantially after 14 wk (Cilek and Knapp, 1993). Cattle ear tags registered for *H. i. exigua* control in Australia that include pyrethroids and the synergist PBO in their formulations (APVMA PubCRIS 30/4/25), may be assisting in blocking detoxifying enzymes in flies that would otherwise survive knockdown. It would be valuable to determine the importance of detoxifying enzymes in *H. i. exigua* resistance.

Knockdown-recovery was not observed in the deltamethrin bioassays and putative resistance mutation *T929I* does not appear to improve survivorship at higher deltamethrin concentrations. The cause of this substantial difference between the toxicity of α-cypermethrin and deltamethrin is not known. Studies on other fly species suggest that the difference may be linked to repeated or continued exposure to α-cypermethrin (Meijer et al. 2024).

Smith et al. (2021) compared VGSC mutations and metabolic detoxification resistance in the mosquito *Aedes aegypti* (Linnaeus, 1762) and found both resistance pathways had fitness costs for the insect. Understanding the fitness costs of resistance pathways in *H. i. exigua* would assist in predicting how quickly resistance would be lost if pesticide application were ceased.

The first complete *H. i. exigua* VGSC gene transcript has been sequenced. This sequence provides a foundation for future research on synthetic pyrethroid resistance in the species. A new putative resistance-linked mutation, *T929I*, has been found in *H. i. exigua* that in combination with the *kdr* mutation, appears to be providing a synergistic enhancement of α-cypermethrin resistance. A total of 82% of the resistant flies tested carried at least one of these 2 mutations. The fitness costs associated with carrying these mutations are still to be determined for *H. i. exigua*. A new class of resistance, knockdown-recovery, has been observed in a wild fly population suggesting the efficacy of synthetic pyrethroids may be significantly lower than current estimates. Future resistance surveys involving bioassays should take this new class of resistance into consideration. If detoxifying enzymes are involved in knockdown-recovery, improved efficacy might be achieved by using synthetic pyrethroid products containing synergists that block these enzymes. The importance of alternate splice elements and RNA-editing modifications of the VGSC gene in conferring resistance to synthetic pyrethroids requires further investigation in *H. i. exigua*. Surveys of *H. i. exigua* populations are needed to determine the prevalence of resistance in the field.

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Supplementary Material

Supplementary material is available at *Journal of Economic Entomology* online.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability

DNA and mRNA sequences obtained in this study are publicly available on GenBank (accessions PV871995, PV987407, and PV987408).

Animal Ethics

Flies collected from field populations were sourced under animal ethics permit CA 2021/01/1457.

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