RNA editing: an overlooked source of fine-scale adaptation in insect vectors?

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Abstract

RNA editing is a source of molecular diversity that regulates the functional repertoire of animal transcriptomes. Multiple studies in *Drosophila* have revealed that conserved editing events can be a source of evolutionary adaptations, and there is a solid body of evidence linking editing and the fine-tuning of neural genes, which are often targeted by insecticides used in vector control. Yet, despite these suggestive connections, genome-wide analyses of editing in insect vectors are conspicuously lacking. Future advances will require complementing the growing wealth of vector genomes with targeted transcriptome analyses. Here, we review recent investigations of the genetic footprints of adaptive RNA editing in insects and provide an overview of new methodologies applicable to studies of RNA editing in insect vectors.

Highlights

- RNA editing introduces transcript-specific mutations that are not detected in genetic assays.
- The regulated edition of transcriptomes is conserved and globally adaptive across various *Drosophila* species, suggesting a general principle in insects.
- RNA editing fine-tunes the functions of neural channels that are involved in insecticide resistance. Yet, genome- and transcriptome-wide studies in insect vectors are still lacking.

Introduction

The synthesis of transcripts involves post-processing and chemical modifications of the RNA molecules, which can fine-tune their functions and create distinct isoforms from a single DNA template. RNA editing is a form of transcript post-processing that involves the chemical modification of single bases in immature RNA molecules, resulting in transcript-specific ribonucleoside mutations [1]. RNA editing is a source of molecular novelty that may fuel adaptive evolution [2,3], in common with other mechanisms of transcriptome diversification—with which it should not be confused—such as alternative splicing. RNA editing is regulated by conserved *cis*-encoded signals [1,3] that are subject to natural selection. Consequently, both the regulatory causes and the adaptive consequences of these transcriptomic mutations can be readily studied from a population genomic perspective.

Yet surprisingly, there have been very few studies of RNA editing in insect vectors, and none focusing on its population genetics. Here we review evidence of editing in disease vectors, in which it may generate functional changes in genes involved in adaptation to insecticide resistance. The emergence of resistance is an important public health issue, as it jeopardises the effectiveness of vector control programmes. Genetic monitoring programmes of insecticide resistance, however, do not routinely probe possible adaptations mediated by RNA editing. We also consider recent studies on the role of editing in environmental adaptations in model insects, primarily *Drosophila melanogaster*, and its regulation via population-specific polymorphisms. Finally, we provide examples of genome-wide approaches on the interaction between microevolutionary processes and RNA regulation that can inform future studies utilising vector genomic resources.

The molecular basis of RNA editing

Animals exhibit multiple types of RNA editing, each of them effected by a different family of enzymes that target specific nucleotides, and often show preference for certain types of transcripts and sub-regions within transcripts (coding and non-coding). The most common type of editing is the deamination of adenosine into inosine (A-to-I) by ADAR enzyme family [1,4], which is conserved in most animals [5]. Inosines are recognised as guanosines by the translational machinery and the reverse transcriptase used in RNA sequencing protocols [1,3], making A-to-I, effectively, a transcript-specific A-to-G substitution. Insects also undergo other, less common [3], types of editing: C-to-U deamination effected by the cytidine deaminase APOBEC-1 [6], and U-to-C or G-to-A trans-aminations [7].

RNA editing can have various effects at the molecular level (Figure 1) [1,3]. The most direct consequences are 'recoding' changes, which is relatively common in *Drosophila* [8], and can result in non-synonymous substitutions and possibly new protein isoforms (Figure 1a). Editing can also influence alternative splicing: it can disrupt or create new *cis*-regulatory signals that regulate splicing (e.g. the acceptor/donor splice sites) [9] (Figure 1b); alter the stability of the dsRNA structures formed during splicing [10,11]; and the editing molecular machinery can compete with splicing factors for physical access to the nascent RNA [10,11]. A-to-I changes also regulate microRNA activity (Figure 1c): editing of precursor mRNAs (3′ or 5′ untranslated regions) or the microRNA itself can reconfigure microRNA binding sites and influence transcript expression and degradation rates [1,12]. Finally, ADAR enzymes also act on clustered editing sites located in repetitive pre-mRNA regions, often rich in retroelements such as *Alu* that are prone to form dsRNA structures [3]. Intense editing of repetitive elements been linked to the regulation of the cytosolic immune response against dsRNA structures [3], and to the exonisation of retroelements via creation of new splicing sites [1].

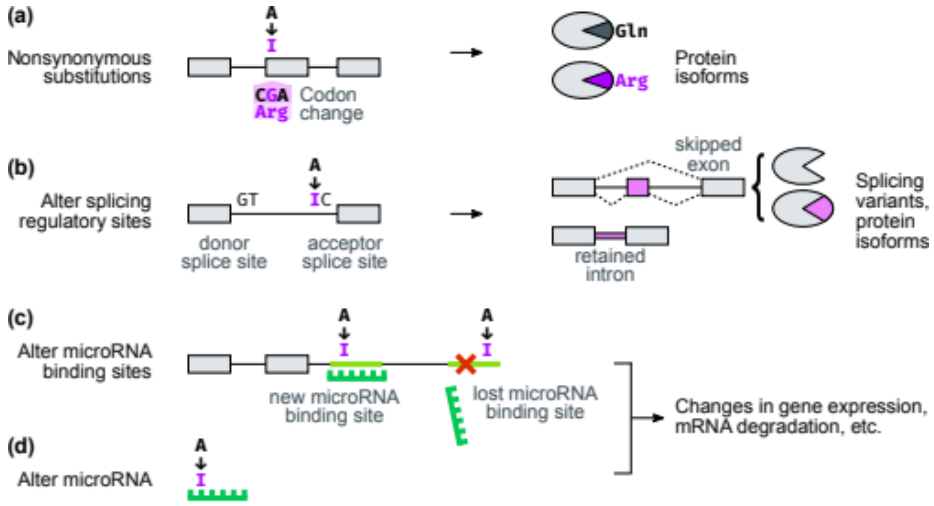

Figure 1. Molecular effects of RNA editing. **(a)** 'Recoding' events result in non-synonymous substitutions and the production of new protein isoforms. **(b)** Editing can modify conserved splicing regulatory signals present in precursor mRNA, such as donor (GT) or acceptor (AC) splice sites. **(c)** Editing can add/remove binding sites for microRNAs (often present in untranslated regions of the precursor mRNA), or **(d)** act on the microRNA molecule itself.

Genetic footprints of adaptive RNA editing

Transcript editing results in increased sequence diversity [3], potentially providing a source of evolutionary adaptations [2]. RNA variants enable the exploration of phenotypic space (e.g. novel protein isoforms) that is inaccessible by genomic mutations, which can carry fitness costs [2]. The incidence of editing can be regulated in a tissue- or stage-specific manner. For example, A-to-I

editing in *D. melanogaster* is enriched in brains and adult tissues [13,14], and it exhibits neuron type-specific profiles [15]. Editing is also responsive to environmental cues, e.g. the response to temperature acclimation in *D. melanogaster* [16,17].

If editing is linked to adaptive evolution, it should leave genetic footprints in the genome that can be detected by comparative analyses. Indeed, non-synonymous A-to-I sites in brain transcriptomes are frequently conserved and under positive selection across the *Drosophila* genus [18,19*,20*]. Interestingly, phylogenetic comparisons of editing in individual insect genes show that, as hypothesised [2], it expands phenotypic space by introducing sequence variation into highly conserved or invariant loci [21], or—more subtly—in variable regions within highly conserved genes, e.g. potassium voltage-gated channels [22]. These diversifying effects can be especially significant in neuronal genes that tend to evolve under strong functional constraints [20*], such as insect nicotinic acetylcholine receptors in which RNA editing provides substantial diversity [23].

Adaptive editing can also be studied from a population genetic perspective. For example, evolutionarily recent A-to-I sites in rhesus macaques are more common than expected in loci with recent G-to-A mutations (relative to humans) for both fixed and currently polymorphic loci, and these novel A-to-I sites are under positive selection across macaque populations [24]. These results suggest that A-to-I compensates the costs of recent G-to-A mutations, a view also supported by detailed analyses of editing conservation in insect nicotinic acetylcholine receptors [23]. In contrast, Popitsch *et al.* [25*] reported that the adaptiveness of A-to-I in human and *D. melanogaster* populations was due to higher relative fitness of G alleles in these sites, which A-to-I effectively mimics. These conflicting hypotheses, which can be tested with transcriptomic and population genetic methods, imply that different natural selection mechanisms could be acting on editing sites [25*].

RNA editing is a source of environmental adaptation in insects

A recent study by Yablonovitch *et al.* [26**] provides strong support for the relationship between editing, adaptation, and fine-scale population genetic diversity. Several editing events were associated with aridity tolerance in *D. melanogaster* from opposite slopes of the ‘Evolution Canyon’, near Israel’s Mount Carmel, which show dramatic microclimatic differences. The study combined whole-genome sequencing, RNA-seq, and microfluidics-based multiplex PCR (a high-throughput assay to measure allele-specific transcript frequencies [27*]) (Box 1) to investigate the role of DNA mutations in regulating gene expression and the frequency of A-to-I editing in flies

106 originating from opposite slopes of the canyon.

Fine-scale population structure in the 'Evolution Canyon' flies correlated with transcriptomic
108 regulation both at the editing and expression levels. Furthermore, differentially edited A-to-I sites were frequently associated with highly-differentiated DNA polymorphisms in their editing
110 complementary sequences (the region in dsRNA molecules that is required for ADAR-mediated A-to-I deamination); and the genomic regions surrounding differential editing sites had stronger inter-
112 population differentiation than those of constitutive editing sites (Figure 2). A CRISPR-Cas9 mutagenic assay was used to demonstrate the effect of DNA variation in editing rates for the
114 *prominin* gene, in which an intronic polymorphism exclusive to the north-facing population hindered dsRNA stability and resulted in lower editing rates.

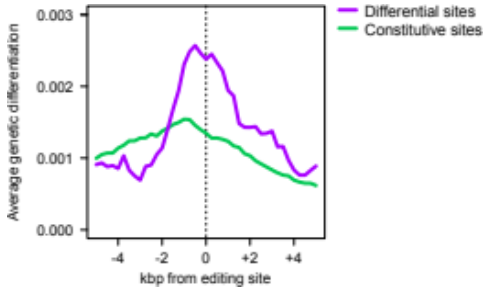

Figure 2. Genetic differentiation around A-to-I editing sites between *D. melanogaster* populations collected from south-facing (arid) and north-facing (humid) slopes in the 'Evolution Canyon'. Differentiation is higher in A-to-I sites that are differentially edited between the two populations (purple) than in constitutive sites (green), reflecting slope-specific regulatory polymorphisms. Figure adapted from Yablonovitch *et al.* 2017 [26**], with permission from the authors, and reproduced under a Creative Commons Attribution 4.0 International License (creativecommons.org/licenses/by/4.0).

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The link between genetic and editing variation is based on the assumption that ADAR activity is
118 regulated by genetically-encoded signals [20*]□. Current evidence suggests that multiple *cis*-regulatory factors influence editing, such as sequence motifs in A-to-I sites (depletion/enrichment of
120 guanosines upstream/downstream of adenosine sites [16,28]□) and their complementary sequences (e.g. cytosines opposite to the editing site increase dsRNA stability and facilitate ADAR activity
122 [21,29]□). A study of *D. melanogaster* polymorphisms with quantitative effects on A-to-I frequency showed that regulatory loci are located close to (but not overlapping) editing sites, and influence
124 editing frequency by altering dsRNA stability [30]□. Interspecific variation in editing frequency is also influenced by sequence conservation in the *D. melanogaster*/*D. sechellia* species pair [31]□;
126 and functionally relevant, conserved editing sites in *Drosophila* are often under positive selection [18,19]□.

Yet, editing can also be influenced by environmental factors such as temperature. In *D. melanogaster*, A-to-I editing is more common at lower temperatures because ADAR enzymes are more active [14,17]□, recognise dsRNA motifs with higher specificity [16]□, and dsRNAs are more stable [16,17]□. The relative importance of *cis*-regulatory and environmental factors was investigated by Yablonovitch *et al.* [26**]□, who found that genetic effects were site-specific and stronger than environmental factors; whereas temperature increases had broad, unspecific effects by virtue of globally reduced editing rates.

RNA editing regulates the activity of insecticide target site proteins

Whilst genome- and transcriptome-wide analyses of RNA editing remain restricted to few taxa, there have been several studies focusing on individual genes and species, with a particular focus on neural ion channels whose kinetics can be fine-tuned by editing-mediated substitutions [32]□. Crucially, many ion channels where functional editing has been described are also target sites of insecticides [33,34]□ – for example, γ-aminobutyric acid receptors (GABA) [15,35]□, subunits of the nicotinic acetylcholine receptors (nAChR) [15,36,37]□, or voltage-gated sodium channels (VGSC) [15,38]□. Given that mutations in target site genes are a major cause of rising insecticide insensitivity, editing is well-suited to have similar adaptive effects [33,34]□.

γ-aminobutyric acid receptors (GABA receptors)

GABA receptors are targeted by the insecticides dieldrin, fipronil, and ivermectin [39–41]□, an anti-parasitic and insecticidal drug that shows considerable promise for vector control [41]□. Es-Salah *et al.* [42]□ characterised an editing event near the GABA binding site in *Drosophila* (*R122G*) that decreased its sensitivity to the GABA neurotransmitter and fipronil. Rather than creating a resistant phenotype, this modification enhanced survival in flies carrying resistance alleles (*A301S*/*A301G* and/or *T350M*, suggesting compensation of fitness costs [39,43]□).

A recent study in the mosquito vectors *Anopheles gambiae, Culex pipiens* and *Aedes aegypti* [44**]□ identified new editing sites with effects on insecticide resistance. Specifically, the combination of six non-synonymous editing sites in the *A. gambiae* receptor (*R119G, I162V, I176V, N183G, I278V, N289D*) altered the activating and inhibiting potencies of the receptor in presence of GABA and ivermectin. Interestingly, functional editing sites in mosquito vectors were located near, but not overlapping, described *D. melanogaster* sites [44**]□. This suggests that, unlike the conserved effects of known resistance mutations (codon 301 or 296 mutations in *D. melanogaster* or *A. gambiae*, respectively [43]□), the location of editing sites in GABA receptors could more

species-specific.

Nicotinic acetylcholine receptors (nAChRs)

The subunits of the nicotinic acetylcholine receptor (nAChR) assemble in heteromeric channels involved in cholinergic synaptic transmission, and are targeted by spinosad [45] and neonicotinoid insecticides [46]. Multiple conserved editing sites have been identified in the α5, α6 and α7 subunits of *D. melanogaster* nAChRs [15,36,37], some of which are differentially edited across neuron types [15], and located near functionally significant protein domains [47]. Editing has been linked to reduced sensitivity to the neonicotinoid imidacloprid in the major pest species, the brown planthopper *Nilaparvata lugens* (*N133D* and *N73D* in the nAChR β1 subunit) [48]. Concordantly, ADAR-defective *D. melanogaster* have increased susceptibility to imidacloprid and spinosad [49*], which suggests that A-to-I editing contributes to an unrecognised resistance mechanism to these insecticides.

Voltage-gated sodium channels (VGSC)

VGSCs are the target site of pyrethroids and DDT [50]. Many base substitutions that reduce the channel sensitivity (knock-down resistance mutations, *kdr*) have been identified in insects, including disease vectors [50]. Initial reports of links between editing-mediated *kdr* substitutions and pyrethroid resistance in the mosquitoes *Culex quinquefasciatus*, *Aedes albopictus*, the house fly *Musca domestica*, and the cockroach *Blatella germanica* [51–53] were later attributed to methodological errors [54], which we speculate may have discouraged further investigations into RNA editing in vectors. Nevertheless, there is independent evidence of non-synonymous editing effecting changes in voltage dependence of activation/inactivation in *B. germanica* (A-to-I: *K184R* and *I1663M;* C-to-U: *L1285P* and *V1685A*) [55] and *D. melanogaster* (A-to-I: *I260V*) [38].

Conclusions

Genome-wide investigations of RNA editing in insect vectors have been, to date, noticeably lacking, preventing informed assessment of heir aggregate importance in generating phenotypic diversity. However, evidence from *D. melanogaster* suggests that this is a fertile line of inquiry for at least two medically-relevant phenotypes: environmental adaptations, and insecticide resistance.

There are multiple paths leading from RNA editing to adaptive evolution, each of them with distinct phylogenetic [19*,20*] and population genetic footprints [25*] that can be detected in *cis*-regulatory motifs governing editing rates [20*,26**,30,31]. Yablonovitch *et al.* [26**] provide a

blueprint for joint surveys of fine-scale genomic and transcriptomic variation in insects, a path to validate causal links between both, and valuable evidence of overlooked adaptive *cis*-regulatory changes.

Future investigations in vectors should go beyond single-gene approaches [35,44**] and leverage existing population and comparative genomic resources [56,57] to elucidate the dominant mechanisms of evolution of RNA editing in a wider selection of species, and identify regulatory polymorphisms involved in adaptive evolution in natural vector populations. Transcriptome-wide analyses can also expand the range of editing candidate genes to include, for example, enzymes involved in metabolic insecticide resistance [58], which have not been usually covered by target-gene approaches. Furthermore, it has recently become possible to investigate the cell type specificity of RNA editing using full-transcript single cell transcriptomic approaches [59,60], which can provide fine-grained insights on its functional effects—including resistance adaptations—and possibly inform the development of novel insecticides. Insect disease vectors have remarkable capacity to rapidly evolve and evade control, and going beyond focus on DNA substitutions to understand the range of contributory mechanisms is a key step for the vector genomics community.

Box 1 – Methods for genome-wide identification of RNA editing sites

Genome-wide scans of RNA editing sites can be performed using high-throughput sequencing approaches, often based on the fact that inosine bases are incorporated as guanosines by the reverse transcriptases used in RNA-sequencing protocols [1,3].

RNA editing detection methods based on RNA-seq (see [61] for a detailed review) require two steps: (i) RNA-to-genome mapping to identify transcript variants, and (ii) a series of filters aimed at discriminating between editing sites and other sources of polymorphism, such as genomically-encoded variants (SNPs) and sequencing errors [62,63] (Figure 3). A common solution to filter out genomic variants is the use of paired WGS and RNA-seq experiments from the same sample, under the assumption that variants present in RNA but not in the DNA reads will result from editing (suitable tools include *JACUSA* [62], *RES-Scanner* [64], or *reditools* [65]).

Less costly procedures based on RNA-seq alone can discriminate editing sites from SNPs by filtering out genomic variants from pre-compiled databases, complete [66] or partial (*GIREMI* [67]). Other tools discriminate between editing sites and SNPs by taking advantage of the tendency of editing to occur in hyper-editing clusters ([68], *SPRINT* [69]).

Any analysis of RNA editing might also benefit from a comparative perspective – i.e., differential editing between insect populations, tissues, or biological conditions, etc. In that respect, all the above-mentioned methods provide per-site editing frequencies that can be compared ad-hoc, and some are able to perform explicit differential analyses (*JACUSA* [62]).

Finally, the microfluidics-based multiplex PCR (mmPCR-seq) is a general approach to measure transcript allelic ratios, including editing events [27*]. This high-throughput method requires prior knowledge of the sites, but it enables the estimation of editing rates at higher accuracy than RNA-seq. It has been used to investigate population- [26**] and tissue-specific [15,20] editing profiles in *D. melanogaster*.

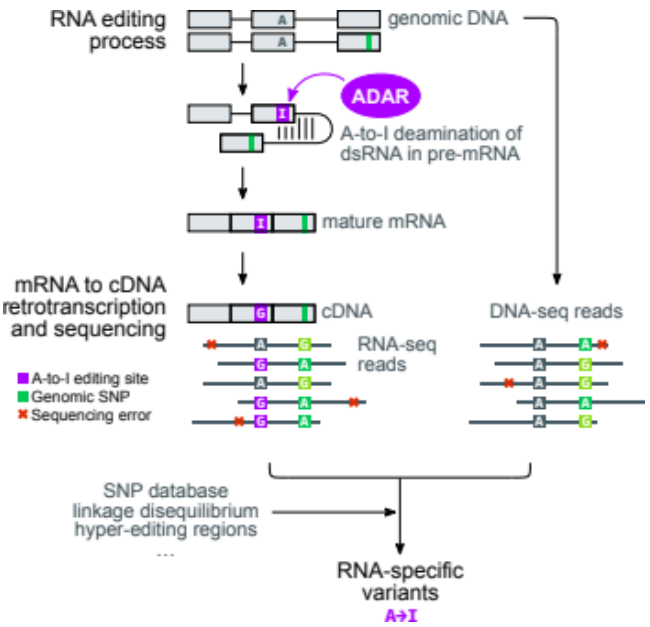

Figure 3. Summary of a high-throughput approach to detect RNA editing events, based on paired RNA-seq and DNA-seq experiments. A-to-I editing is used as an example.

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Key references

Duan *et al.* 2017 [19*]□. Investigation of A-to-I editing in neural tissues in closely-related *Drosophila* species. The authors demonstrate that editing is enriched in neural tissues and affects functionally constrained genes, and highlight the adaptive value of conserved editing sites in insects.

Zhang *et al.* 2017 [20*]□. Using comparative transcriptomic and genomic analyses of multiple *Drosophila* species, the authors demonstrate the importance of the *cis*-regulatory landscape in regulating editing variation. The authors also trace gains and losses of editing sites across species, and show that widely-conserved sites are enriched in slow-evolving neural genes.

Popitsch *et al.* 2017 [25*]□. Investigation of the population-genetic footprints underpinning the evolution of adaptive editing. The authors provide a comprehensive list of hypotheses with testable predictions. They find support for an adaptive role of A-to-I editing as a transcriptomic 'mimicry' of adaptive A-to-G mutations in both *D. melanogaster* and humans.

Yablonovitch *et al.* 2017 [26**]□. The authors use a combination of WGS, RNA-seq and targeted assays to unravel the role of A-to-I editing in two closely related populations of *D. melanogaster* with divergent climatic adaptations. They are able to link population genetic divergence to regulatory variation in editing, and they identify candidate genes for validation.

Zhang *et al.* 2014 [27*]□. The authors propose a new high-throughput assay to measure allelic ratios in transcripts at high precision, which can be coupled with genomic and transcriptomic analyses to RNA editing variants.

Taylor-Wells *et al.* 2018 [44**]□. This ground-breaking study demonstrates that multiple editing events in the GABA receptor of *A. gambiae* can change the electrophysical properties of the channel, and result in reistane to ivermectin. The authors also study the evolutionary conservation of the mutations in other vectors and *D. melanogaster*.

Rinkevich *et al.* 2012 [49*]□. The authors demonstrate that ADAR-defective *D. melanogaster* are more susceptible to insecticides that target the heavily edited nicotinic acetylcholine receptors.

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