Title: Gene editing in agricultural, health, and veterinary pest arthropods: recent advances

Authors: Madhusudan Manjunatha[1], Michael Pham[2], Monika Gulia-Nuss[2], Andrew Nuss[1, 2*]

[1]Department of Agriculture, Veterinary, and Rangeland Sciences, University of Nevada, Reno, USA

[2]Department of Biochemistry and Molecular Biology, University of Nevada, Reno, USA

*Corresponding author

Nussab@unr.edu

Highlights:

- Recent genome editing technological developments pioneered specifically in pest arthropods.
- Highlights of genome editing milestones in vector arthropods within the last two years.
- New genome editing updates in agricultural pests.

Abstract

Pest arthropods cause significant crop damage or are vectors of pathogens for both plants and animals. The current standard of pest management prevents against crop losses and protects human and animal health, but shortcomings exist, such as insecticide resistance and environmental damage to nontarget organisms. New management methods are therefore needed. The development of new tools, such as site-specific gene editing, has accelerated the study of gene function and phenotype in non-model arthropod species and may enable the development of new strategies for pathogen and arthropod control. Here, the most recent developments in gene editing in arthropod pests are briefly reviewed. Additionally, technological advances that could be applicable to new species or enhance the success rates of gene editing in species with already established protocols are highlighted.

Introduction

Arthropods are the most diverse animal phylum, making up over 80% of all known animal species. A small fraction of these have drastic impacts on public health and food production. Currently 40% of world crop production is lost to arthropod agricultural pests, either directly or through the pathogens they vector [1,2]. Additionally arthropod disease vectors (e.g., mosquitoes, sandflies, and ticks), are responsible for 17% of all infectious diseases and result in over 700,000 deaths globally per year [3]. These problems are predicted to increase, with climactic changes resulting in elevated arthropod population growth rates and range expansion. Together, arthropod pests represent significant global health and agricultural burdens despite current control methods.

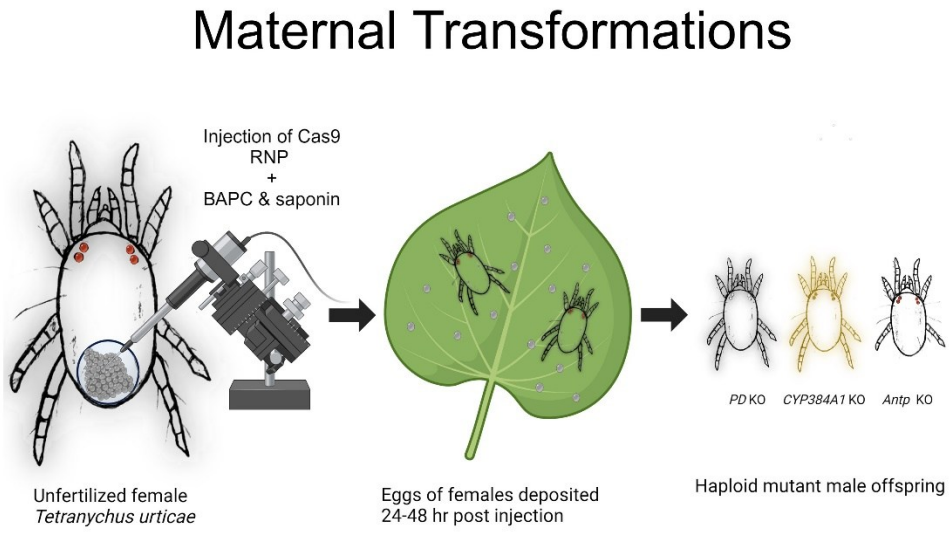
Numerous methods are currently employed to manage pest arthropod populations, most notably the use of chemical pesticides. However, chemical controls have drawbacks, including the development of pesticide resistance and environmental impacts on beneficial and other non-target species. The development of gene-editing tools, notably CRISPR/Cas9 and related techniques, has advanced the idea of more targeted approaches for pest population management, particularly those enabling gene drive and sterile insect strategies [4,5]. However, applying these tools to non-model organisms requires extensive research investments, such as establishing reliable lab rearing protocols and troubleshooting idiosyncratic life history details, as well as the development of functional tools necessary for genetic modification, including marker phenotypes, tissue- or developmental-dependent promoters, and embryo injection protocols, among others. In this mini-review, we highlight cutting-edge gene editing developments within the last two years in pest arthropods of medical, veterinary, and agricultural importance (Table 1). This brief snapshot provides insight into current arthropod genome-editing developments and what the future might hold.

New technological improvements in gene editing in arthropods

Many techniques reported in this review are applications of existing insect gene-editing techniques applied to new species, yet several recent technological innovations have been applied for the first time in pest arthropods. For instance, a new embryo-screening method used shed pupal cases of *Bactrocera dorsalis* (oriental fruit fly) [6] or *Habrobracon hebetor* (an ectoparasitic wasp) [7] to screen for edited genes. This method may be useful in other species where a marker phenotype is not readily available, and prevents damage to edited individuals, in addition to reducing costs of maintaining non-edited animals.

Embryo injection is an acute challenge for several arthropods, as certain life history traits or egg morphology make embryo injections highly infeasible. In an innovation for CRISPR-mediated transgenesis not involving direct embryo injection, a method similar to Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control) was employed using cell-penetrating peptide (CPP) tags. This was used to deliver a fluorescent protein to several tissues and cell types (including bacteriocytes) in *Acyrthosiphon pisum,* the pea aphid [8]. Unlike ReMOT Control, this technique allows delivery of a Cas9-sgRNA ribonucleoprotein complex (RNP) to embryos in species that are non-vitellogenic. Another method used Branched Amphiphilic Peptide Capsules (BAPC) together with saponin in a synergistic interaction to deliver the RNP in the two-spotted spider mite, *Tetranychus urticae* (Fig. 1). This method, termed SYNCAS, was also used in the same study to successfully edit and achieve knock-in in the Western flower thrip, *Frankliniella occidentalis* [9]**. Yet another technique involved injecting high concentrations of RNP into the hemocoel of adult females before oviposition without using any type of carrier molecule and was used to edit the red flour beetle, *Tribolium casteneum*, and the German cockroach, *Blattella germanica* [10]**. This was termed direct parental (DIPA-) CRISPR by the authors, although this strategy has been used previously by other groups [11,12]. However, a novel development in the newer work was to achieve CRISPR knock-in events when co-injecting the RNP together with single stranded oligo DNA nucleotides (ssODNs) with homology arms [10]. However, DIPA-CRISPR may not work in every arthropod system [13]. Together, these advancements may facilitate gene-editing in species where embryo injection is difficult.

In an innovative departure from using CRISPR/Cas9, the CRISPR-Cas13 system (specifically Cas13d) was recently used for RNA editing in in the white-backed planthopper (WBPH), *Sogatella furcifera*, a migratory rice pest in Asia [14]* and injections were used to disrupt *SfTO* transcripts. Notably, Cas13d demonstrated a quicker phenotypic onset and decreased levels of SfTO transcript compared to RNA interference (RNAi). This suggests the CRISPR-Cas13d system has potential advantages over the current widespread usage of conventional RNAi techniques in terms of specificity and immediate outcomes. In addition, CRISPR-Cas13 was recently used to target chikungunya virus in *Ae. aegypti*, through a strategy known as REAPER (vRNA Expression Activates Poisonous Effector Ribonuclease) (Fig. 1). In this strategy, transgenic *Ae. aegypti*, upon taking up viral RNA in a blood meal, expressed CRISPR-13 targeted to specific arboviruses. This significantly diminished viral replication and even generated sufficient off-target collateral RNA cleavage to kill the majority of infected mosquitoes, preventing viral transmission to new hosts [15]**.



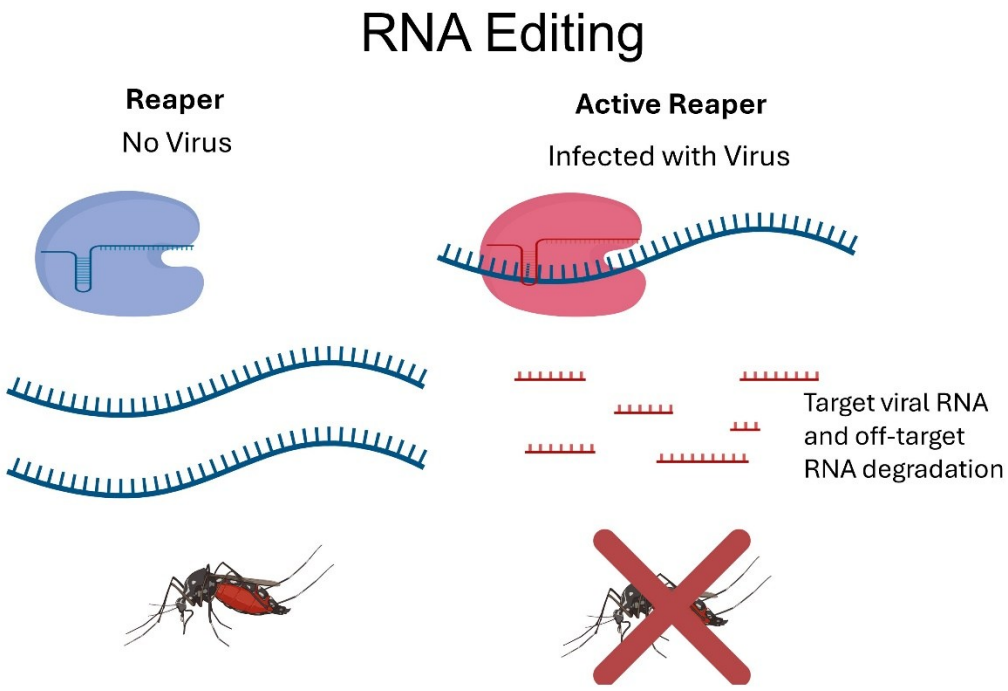


Figure 1: Highlight of recent, novel gene editing techniques in arthropods. Maternal transformations encompasses techniques in which gravid females are injected with a RNP for uptake into the oocytes and editing of the developing embryo, often with the help of a yolk peptide to facilitate oocyte uptake. SYNCAS is represented here in the two spotted spider mite, *Tetranychus urticae*. RNA-editing in arthropods has recently been achieved using Cas13. The example here depicts the action of REAPER, a system designed in *Aedes aegypti* for activation in response to Chikungunya virus in a blood meal, which then activates Cas13d that degrades viral specifically, and mosquito vector RNA nonspecifically, which results in mosquito mortality.

Gene editing in arthropods of medical and veterinary importance

Genome editing has been successfully demonstrated in several medically and veterinary important arthropod species, most notably mosquitoes. However, work has recently expanded to additional medically and veterinary important arthropod species. Of interest is New World Screwworm fly, *Cochliomyia hominvorax*, a myiasis causing parasite of warm-blooded animals. The eradication of this pest in North America last century was the first application of sterile insect technique (SIT) and is one of the greatest non-chemical pest management success stories. Although earlier methods relied on the release of irradiated sterile males, recent efforts are geared towards the development of genetically-controlled conditional lethal female strains to simplify rearing protocols and reduce costs. Recently, several U6 promoters were screened for use in *C. hominovorax* using the related pest, *Lucillia cuprina*, the Australian sheep blowfly [16]. The identification of optimal U6 promoters could be used in developing CRISPR/Cas9-based genetic lines such as sexing strains.

Gene editing in *Ixodes scapularis* ticks was reported for the first time, via two different methods: embryo injection and ReMOT Control [17]*. Among non-insect arthropods, *I. scapularis* is the most significant vector of public health concern in the United States due to its ability to transmit multiple pathogens, yet gene editing in this species was previously stymied by challenging egg morphology. Successful embryo injection required several technical advancements, including chemical treatments to soften the chorion and reduce the intraovular pressure as well as wax removal through draining of the wax gland of the mother tick. ReMOT Control involved injection of gravid females with a yolk protein or vitellogenin tagged Cas9 protein that directed the Cas9 into the ovaries. Edits to the homeobox gene, *Proboscipedia*, and a chitinase gene were demonstrated. This work represents a large step forward in a vector that was previously intractable to genome editing.

Another gene editing first was reported for the kissing bug, *Rhodnius prolixus,* an important vector of *Trypanosoma cruzi*, the causative agent of Chagas disease [18]. This was achieved using ReMOT Control. P2C (derived from *Drosophila melanogaster* Yolk Protein 1) and BtKV (derived from a vitellogenin binding sequence in *Bemisia tabaci*) were used for delivering RNPs to oocytes, with BtKV being slightly more effective in inducing edits. Potential phenotypic markers were targeted, with knockout of *Rp-scarlet* (*Rp-sca*) and *Rp-white* (*Rp-w*) resulting in red and white eyes, respectively. The *Rp-yellow* (*Rp-y*) gene was also edited, resulting in a yellow cuticle phenotype. DIPA-CRISPR was also attempted, but did not yield any visible phenotypes. This study opens the door for gene editing in triatomine bugs and potentially new Chagas disease control strategies.

Gene editing in arthropods of agricultural importance

The promise of gene-editing technologies for management of agriculturally relevant arthropods is keenly recognized. Recently, a massive increase in research has emerged for a variety of agricultural pests, focused mainly on various stages of genome editing tool development including identification of phenotype markers, promoters, fertility disruption, and facilitation of CRISPR-Cas9 delivery to germline cells. A brief sample of new developments in genetically edited agricultural pests is included below and in Table 1.

As noted above, SYNCAS was recently used for gene editing in the highly polyphagous and widespread two-spotted spider mite, *T. urticae*. This approach improved knockout efficiency over previous methods to edit this mite. Two genes were targeted for knockout as a proof of concept, *CYP384A1* and *Antennapedia* (*Antp*), which incurred “lemon” phenotypes (a yellow body color due to the absence of keto-carotenoids) and severe leg deformities, respectively. Further, knock-ins were also achieved by SYNCAS coupled with enhancer V2 (an HDR stimulating agent) to induce an I1017F mutation to the *chitin synthase 1* gene (*CHS1*) which conferred etoxazole resistance [9]. These advancements facilitate gene editing in an arthropod which is challenging to manipulate due to its small size.

In Hemiptera, advancements have recently been reported for *Lygus hesperus*, the western tarnished plant bug, an agricultural pest in Mexico and the western United States. RNAi screening of the eye pigments *cardinal* (*LhCd*), *cinnabar* (*LhCn*), and *white* (*LhW*) demonstrated that *LhW* knockdown was fatal to developing embryos in *L. hesperus*, but knockdowns of *LhCd* and *LhCn* produced viable insects with bright red eye phenotypes [19]. Subsequent CRISPR/Cas9 editing created heritable germline knockouts of *LhCd* (Card) and *LhCn* (Cinn), with a stable red eye phenotype, particularly in the Cinn strain. Further, RNAi screening and subsequent CRISPR/Cas9 knockout of *β-tubulin2* in *L. hesperus* resulted in males with non-viable sperm that could mate but were effectively sterile, an important step in developing genetically tractable sterile-male strategies in this pest species [20].

Similar to the work in *L. hesperus*, advancements in phenotype markers and successful CRISPR/Cas9 gene editing were recently made for *Homalodisca vitripennis*, the glassy-winged sharpshooter (GWSS), a leafhopper vector in several crops, particularly grapes. A conserved FAD-binding domain region of *cinnabar* (*cn*) and the region of the ABC-transporter-like domain (conserved AAA domain) of *white* (*w*) were targeted for editing and resulted in white eye phenotypes. This was aided by *in situ* embryo microinjections of embryos on host plant tissue (in contrast to removing and positioning the eggs), to reduce disruptions to the eggs and enhance survival [21].

Huanglongbing (HLB), a devastating citrus disease caused by *Candidatus Liberibacter asiaticus*, is primarily spread by the insect vector *Diaphorina citri* (Asian citrus psyllid). Knockout of two eye pigmentation genes, *white* (*w*) and *kynurenine hydroxylase* (*kh*), in this insect each produced observable phenotypic variations in eye color and may serve as phenotypic markers of gene editing. Notable innovations in the embryo injection protocol include techniques to prevent needle blockage and, similar to the GWSS work noted above, injecting eggs attached to plant tissue, both of which improved embryo survival and facilitated the first successful use of CRISPR-Cas9 gene editing in *D. citri*. In addition, the ReMOT Control technique was used to inject adult females and successfully edit offspring, resulting in noticeable eye mutants with somatic mosaicism [22].

The eye pigment gene *tryptophan 2,3-dioxygenase* (*SfTO*) was targeted in *S. furcifera* [23]. Mutation of this gene resulted in a white eye phenotype. Instead of embryo injections, this group used DIPA-CRISPR [10], as described above, and injected RNPs into the hemocoel of adult females before oviposition to edit offspring.

Advancements have also been made to curb reproduction in the brown planthopper (BHP), *Nilaparvata lugens*, a major rice pest. CRISPR-Cas9 facilitated knockout of *NlugGr23a*, a putative fecundity-related gustatory receptor, which resulted in sterility in homozygous males [24]. As with strategies to disrupt reproduction in other insects, this target may have utility in sterile insect approaches.

In Lepidoptera, phenotype markers were explored in *Plodia interpunctella,* the Indianmeal moth, a stored products pest, by mutating the *white* gene using CRISPR/Cas9. Knockout of *white* resulted in white-eye mutants, and subsequent rescue with homology directed repair (HDR) restored the brown-pigmented eye phenotype [25]. Subsequent work demonstrated that, in contrast to some other moth species, the white eye phenotype did not cause embryonic lethality [26]. Similarly, the *Tacinnabar* gene was mutated in *Tuta absoluta*, the tomato leafminer, using CRISPR/Cas9. This may serve as a phenotypic marker of editing, with knockouts displaying red eye coloration in adults, and is a first for genome editing in this species [27]. Also, several studies in recent years have reported editing in *Spodoptera frugiperda*, the fall armyworm, and an additional knockout screen of pigment related genes (*Sfebony, Sfyellow-y, Sflaccase2*, and *Sfscarlet*) resulted in mutants with distinct eye and cuticle phenotypes [28].

CRISPR/Cas9-mediated mutagenesis is also being used to manipulate reproduction in lepidopterans. In *Ostrinia furnacalis*, a pest of maize in Asia and beyond, knockout of ovary serine protease (*OfOsp*) induced female-specific sterility [29]. Knockout of ovary serine protease was also performed in *Plutella xylostella*, the diamondback moth, a pest of crucifers, particularly cabbage, and this also induced

female sterility with shorter ovarioles, reduced egg development and fragile eggs [30]. These may be of use in genetic pest control.

Although gene knockout through CRISPR/Cas9-induced mutagenesis is becoming more frequent, gene knock-ins are more complex. However, recent progress has been made using CRISPR/Cas9 to develop knock-in gene drive constructs in *P. xylostella* [31]*. This particular approach targeted the *Pxyellow* gene, previously demonstrated as a phenotypic marker in this species. A plasmid construct was then inserted via HDR. This construct contained a Cas9 gene (under the *Pxnanos* promoter) and an EGFP marker gene (HR5IE1 promoter). In addition, dsRNA of *Pxku70* was co-injected to suppress non-homologous end-joining (NHEJ) to increase the probability of HDR.

Among Diptera, development of marker genes to easily screen for phenotypes was recently explored in the melon fly (*Zeugodacus cucurbitae*) and oriental fruit fly (*Bactrocera dorsalis*) [32]. As with many other insects noted above, the *white eye* (*we*) gene was targeted, and heritable knockout lines of *we* were established. In addition, knockout of *sex peptide receptor (Bdspr*) disrupted fertility in female *B. dorsalis*, making it a potential target for SIT [33].

In Coleoptera, a Cas9-expressing line of the model beetle *Tribolium castaneum* (red flour beetle) was recently developed to simplify CRISPR/Cas9-editing [34]*. Lines generated from these experiments were effective at generating germline mutants after injections of sgRNAs, not containing Cas9, suggesting they will be a useful tool for further manipulation of the *T. castaneum* genome. As noted above, knock-in by DIPA-CRISPR was also achieved in this species in a separate study, although knock-in efficiency was low at 0.36% overall [10].

Parasitoids are an important biocontrol component for agricultural pests and genetic manipulation of these beneficials could confer useful traits, such as insecticide resistance. However, embryo injection is particularly challenging for many parasitoids because eggs are laid directly on or inside of hosts. Despite this challenge, successful CRISPR/Cas9-mediated mutagenesis was recently reported in the hymenopteran *H. hebetor,* an ectoparasitic wasp that parasitizes several caterpillars including *P. interpunctella* [7].

Conclusions

Overall, pest arthropod genome editing, a long-desired dream in the entomological community, has become a reality for the study and genetic manipulation of arthropods, largely aided by the development of CRISPR-Cas9. This has ushered in a new era, enabling the investigation of gene function and the development of new, targeted methods for controlling pest populations and vector-borne diseases that have advantages over the current standard of pest management. Although many obstacles remain, the future is bright for the use of genome modification in developing new

management tools to prevent disease transmission and crop damage by pest arthropods.

Author Contributions:

Conceptualization: MM, MP, ABN, MG-N; Funding acquisition: ABN, MG-N, MP; Writing: original draft: MM, MP, ABN; review & editing: MM, MP, ABN, MG-N.

Funding:

This publication was made possible by grants from the National Science Foundation (NSF) under Grant No. 2236698 to ABN and National Institutes of Health-National Institute of Allergy and Infectious Diseases (NIH-NIAID) grants R21AI176352 and R01AI172943 to MG-N, ABN, and MP.

Conflicts of Interest:

The authors declare no conflict of interest.

Table 1: CRISPR/Cas9 developments in Arthropods of Medical, Veterinary, and Agricultural Importance. Studies beyond the scope of this review (not in the past 2 years) are labelled with just the year in parentheses.

Species (common name)	Order	CRISPR Knockout (KO) Genes Targeted	Methodology	CRISPR Knock-in	Comments
C. hominivorax (New World Screwworm)	Diptera	*Yellow, transformer* (KO in 2019)	Embryo injection	No	U6 promoters tested in *L. cuprina*[16]
L. cuprina (Australian sheep blowfly)[16]		*Yellow* (KO in 2019)	Embryo injection (0.68-1.17%*)	No	*C. hominivorax* U6 promoters functional in *L. cuprina*[16]
Z. cucurbitae (melon fly)		*White*[32]	Embryo injection (66.7%*)	No	Percent of visible mutants out of surviving adults at G0. Unified protocol for tephritid species
B. dorsalis (oriental fruit fly)		*Sex peptide receptor*[33]	Embryo injection (75%*)	No	Percent of sterile females to total females at G0
L. hesperus (western tarnished plant bug)	Hemiptera	*White, cinnabar*, c*ardinal*[19]	Embryo injection (86%*)	No	
		β-tubulin[20]	Embryo injection	No	
R. prolixus (Kissing bug)[18]		*Rp-scarlet, Rp-white,* and *Rp-yellow*	ReMOT Control DIPA-CRISPR	No	ReMOT Control (with chloroquine) worked whereas DIPA-CRISPR did not work in this species
D. citri (Asian citrus psyllid)[22]		*White, kynurenine hydroxylase*	Embryo injection (1.4%*) ReMOT Control (3.1%*) BAPC (0%*) DIPA-CRISPR (8.8%*)	No	Methods used for the 1st time in this species and *in situ* microinjection on host plant
N. lugens (brown plant hopper)[24]		*NlugGr23a (putative fecundity-related Gr)*	Embryo injection	No	Caused sterility in mutant males but did not affect mating behavior or morphology
S. furcifera (white-backed planthopper)		*Tryptophan 2,3-dioxygenase*[14]	Thorax injection of the 3rd instar nymphs (19.76%*)	No	Nanoparticle-based CRISPR-Cas13d for disruption of mRNA expression
		Tryptophan 2,3-dioxygenase[23]	DIPA-CRISPR (56.7%*)	No	Highest gene editing efficiency was achieved 2 days posteclosion
H. vitripennis (glassy-winged sharpshooter)[21]		*White, cinnabar*	Embryo injection (58.9-80%*)	No	Methods used for the 1st time in this species and *in situ* microinjection on host plant
O. furnacalis (Asian corn borer)[29]	Lepidoptera	*Ovarian serine protease*	Embryo injection (28.42%*)	No	
		White[26]	Embryo injection (12-32%*)	No	

P. interpunctella (Indianmeal moth)		*White*[25]	Embryo injection (38%*)	Yes	A single-stranded oligo DNA nucleotide (ssODN) was used to rescue the one bp white spontaneous deletion
P. xylostella (diamondback moth)		*Yellow*[31]	Embryo injection (40%*)	Yes	1st single CRISPR/Cas9-based gene drive construct for this species and only non-mosquito/cell line example dsRNA knockdown of Ku70 to improve knock-in efficiency
		Ovary-serine protease[30]	Embryo injection	No	
S. frugiperda (fall armyworm)[28]		*Sfyellow-y, sfebony, sflaccase2, sfscarlet,* and *sfok*	Embryo injection	No	
T. absoluta (tomato leaf miner)[27]		*Cinnabar*	(Zygote) embryo injection (31.9%*)	No	1st CRISPR/Cas9-based genome editing in this species
T. castaneum (red flour beetle)	Coleoptera	*Vermilion*[34]	Embryo injection	Yes	Cas9 transgenic line having constructs pB-hs-Cas9-hs and pB-hs-Cas9-GFP-nanos
		Cardinal[10]	DIPA-CRISPR (53.2%*) ReMOT Control (0.26%*)	Yes	Knock-in of ssODN short homology arms (96 bp) introducing a restriction site and mutating the protospacer adjacent motif
H. hebetor (ectoparasitic wasp)[7]	Hymenoptera	*White*	Embryo injection	No	Combination of two sgRNA yielded higher mutation rate (84.62%*)
F. occidentalis (western flower thrips)[9]	Thysanoptera	*White*, *white-like*	BAPC + Saponin (SYNCAS) (24.07-32.05%*)	Yes	ssODN of 170 bp for *white* gene was used for knock-in
T. urticae (red spider mite or two-spotted spider mite)	Trombidiformes	*Phytoene desaturase* (KO in 2020)	DIPA-CRISPR (0.485%*)	No	
		Phytoene desaturase CYP384A1, Antennapedia[9]	BAPC + Saponin (SYNCAS) (5-6%*)	Yes	ssODN of 192 bp for *chitin synthase 1* gene was used for knock-in
I. scapularis (blacklegged tick or deer tick)[17]	Ixodida	*Proboscipedia, Chitinase*	Embryo injection (0.9%*) ReMOT Control (1.7-4.2%*)	No	First genetic transformation in any tick species

*Transformation efficiency data as reported in the cited paper. Note that calculation of efficiency varies widely by reporting study, ranging from transformation efficiency of all injected individuals to transformation efficiency of only the survivors of injections, to screening of progeny from maternal uptake transformation techniques.

References

[1] UN News: **Invasive pest spread another fallout from climate change, UN-backed study finds**. [December 19, 2023]; Available from: https://news.un.org/en/story/2021/06/1093202.

[2] **FAOSTAT**. [January 15, 2024]; Available from: https://www.fao.org/faostat/en/#home.

[3] **Vector-borne diseases**. [January 15, 2024]; Available from: https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases.

[4] Chen X, Palli SR: **Genome editing in pests: basic science to applications**. *J Pest Sci* 2024:1–18.

[5] Komal J, Desai HR, Samal I, Mastinu A, Patel RD, Kumar PVD, Majhi PK, Mahanta DK, Bhoi TK: **Unveiling the Genetic Symphony: Harnessing CRISPR-Cas Genome Editing for Effective Insect Pest Management**. *Plants* 2023, **12**:3961.

[6] Ashok K, Bhargava CN, Asokan R, Pradeep C, Kennedy JS, Rai A, Manamohan M: **First report on the utility of pupal case for early determination of CRISPR/Cas9 ribonucleoprotein mediated genomic edits in the oriental fruit fly, Bactrocera dorsalis (Hendel) (Tephritidae: Diptera)**. *Archives of Insect Biochemistry and Physiology* 2023, **113**:e22024.

[7] Bai X, Yu K, Xiong S, Chen J, Yang Y, Ye X, Yao H, Wang F, Fang Q, Song Q, Ye G: **CRISPR/Cas9-mediated mutagenesis of the white gene in an ectoparasitic wasp, Habrobracon hebetor**. *Pest management science* 2023.

[8] Takenaka A, Konno H, Kikuta S: **In vivo direct cell-penetrating peptide mediated protein transduction system in Acyrthosiphon pisum**. *BMC research notes* 2023, **16**:231.

[9] **Rouck S de, Mocchetti A, Dermauw W, van Leeuwen T: **SYNCAS: Efficient CRISPR/Cas9 gene-editing in difficult to transform arthropods**. *Insect biochemistry and molecular biology* 2024, **165**:104068.
Editing efficiency of Cas9 RNP by BAPC was significantly enhanced with endosome escape reagent Saponin in method termed SYNCAS. Authors also reported successful knock-in of white gene in the western flower thrips with ssODN of 170 bp and successful knock-in of chitin synthase 1 gene in the two-spotted spider mite with ssODN of 192 bp.

[10] **Shirai Y, Piulachs M-D, Belles X, Daimon T: **DIPA-CRISPR is a simple and accessible method for insect gene editing**. *Cell Reports Methods* 2022, **2**:100215.
First case of CRISPR-mediated knockin using DIPA CRISPR, but with low efficiency.

[11] Chaverra-Rodriguez D, Macias VM, Hughes GL, Pujhari S, Suzuki Y, Peterson DR, Kim D, McKeand S, Rasgon JL: **Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing**. *Nat Commun* 2018, **9**:3008.

[12] Dermauw W, Jonckheere W, Riga M, Livadaras I, Vontas J, van Leeuwen T: **Targeted mutagenesis using CRISPR-Cas9 in the chelicerate herbivore Tetranychus urticae**. *Insect biochemistry and molecular biology* 2020, **120**:103347.

[13] Terradas G, Macias VM, Peterson H, McKeand S, Krawczyk G, Rasgon JL: **The Development and Expansion of in vivo Germline Editing Technologies in Arthropods: Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control) and Beyond**. *Integr Comp Biol* 2023, **63**:1550–63.

[14] *Ma Y-F, Zhang M-Q, Gong L-L, Liu X-Z, Long G-J, Guo H, Hull JJ, Dewer Y, He M, He P: **Efficient nanoparticle-based CRISPR-Cas13d induced mRNA disruption of an eye pigmentation gene in the white-backed planthopper, Sogatella furcifera**. *Insect science* 2023, **30**:1552–64.
Nanoparticle-based CRISPR-Cas13d for disruption of mRNA expression.

[15] **Dalla Benetta E, López-Denman AJ, Li H-H, Masri RA, Brogan DJ, Bui M, Yang T, Li M, Dunn M, Klein MJ et al.: **Engineered Antiviral Sensor Targets Infected Mosquitoes**. *CRISPR J* 2023, **6**:543–56.
This study used Cas13 line with a sgRNA targeting Chikungunya virus in Aedes aegypti mosquitoes. Compared to non-transgenic controls, the Cas13 transgenic line had reduced viral genome copies in infected mosquitoes and increased mortality (due to off-target RNA collateral degradation).

[16] Novas R, Basika T, Williamson ME, Fresia P, Menchaca A, Scott MJ: **Identification and functional analysis of Cochliomyia hominivorax U6 gene promoters**. *Insect Mol Biol* 2023, **32**:716–24.

[17] *Sharma A, Pham MN, Reyes JB, Chana R, Yim WC, Heu CC, Kim D, Chaverra-Rodriguez D, Rasgon JL, Harrell RA et al.: **Cas9-mediated gene editing in the black-legged tick, Ixodes scapularis, by embryo injection and ReMOT Control**. *iScience* 2022, **25**:103781.
This is the first genetic transformation of any tick species.

[18] Lima L, Berni M, Mota J, Bressan D, Julio A, Cavalcante R, Macias V, Li Z, Rasgon JL, Bier E, Araujo H: **Gene Editing in the Chagas Disease Vector Rhodnius prolixus by Cas9-Mediated ReMOT Control**. *CRISPR J* 2024, **7**:88–99.

[19] Heu CC, Gross RJ, Le KP, LeRoy DM, Fan B, Hull JJ, Brent CS, Fabrick JA: **CRISPR-mediated knockout of cardinal and cinnabar eye pigmentation genes in the western tarnished plant bug**. *Scientific reports* 2022, **12**:4917.

[20] Heu CC, Le KP, Gross RJ, Schutze IX, LeRoy DM, Langhorst D, Brent CS, Fabrick JA, Hull JJ: **β-tubulin functions in spermatogenesis in Lygus hesperus Knight**. *Journal of Insect Physiology* 2024, **152**:104598.

[21] Souza Pacheco I de, Doss A-LA, Vindiola BG, Brown DJ, Ettinger CL, Stajich JE, Redak RA, Walling LL, Atkinson PW: **Efficient CRISPR/Cas9-mediated genome modification of the glassy-winged sharpshooter Homalodisca vitripennis (Germar)**. *Scientific reports* 2022, **12**:6428.

[22] Chaverra-Rodriguez D, Bui M, Gilleland CL, Rasgon JL, Akbari OS: **CRISPR-Cas9-Mediated Mutagenesis of the Asian Citrus Psyllid, Diaphorina citri**. *GEN Biotechnology* 2023, **2**:317–29.

[23] Zhang M-Q, Gong L-L, Zhao Y-Q, Ma Y-F, Long G-J, Guo H, Liu X-Z, Hull JJ, Dewer Y, Yang C et al.: **Efficient DIPA-CRISPR-mediated knockout of an eye pigment gene in the white-backed planthopper, Sogatella furcifera**. *Insect science* 2023.

[24] Zhang M, Hu Y, Liu J, Guan Z, Zhang W: **CRISPR/Cas9-mediated genome editing of gustatory receptor NlugGr23a causes male sterility in the brown planthopper Nilaparvata lugens**. *International Journal of Biological Macromolecules* 2023, **241**:124612.

[25] Heryanto C, Hanly JJ, Mazo-Vargas A, Tendolkar A, Martin A: **Mapping and CRISPR homology-directed repair of a recessive white eye mutation in Plodia moths**. *iScience* 2022, **25**:103885.

[26] Shirk BD, Shirk PD, Furlong RB, Scully ED, Wu K, Siegfried BD: **Gene editing of the ABC Transporter/White locus using CRISPR/Cas9-mediated mutagenesis in the Indian Meal Moth**. *Journal of Insect Physiology* 2023, **145**:104471.

[27] Ji S-X, Bi S-Y, Wang X-D, Wu Q, Tang Y-H, Zhang G-F, Wan F-H, Lü Z-C, Liu W-X: **First Report on CRISPR/Cas9-Based Genome Editing in the Destructive Invasive Pest Tuta Absoluta (Meyrick) (Lepidoptera: Gelechiidae)**. *Frontiers in genetics* 2022, **13**:865622.

[28] Cheng Y, Wang P, Zeng Y, An W, Wang T, Xiao Y: **Characterization of five pigmentation genes as transgenic markers in Spodoptera frugiperda (Lepidoptera: Noctuidae)**. *International Journal of Biological Macromolecules* 2023, **242**:124981.

[29] Zhang P, Jialaliding Z, Gu J, Merchant A, Zhang Q, Zhou X: **Knockout of ovary serine protease Leads to Ovary Deformation and Female Sterility in the Asian Corn Borer, Ostrinia furnacalis**. *International Journal of Molecular Sciences* 2023, **24**.

[30] Asad M, Liao J, Chen J, Munir F, Pang S, Abbas AN, Yang G: **Exploring the role of ovary-serine protease gene in female fertility of diamondback moth using CRISPR/Cas9**. *Pest management science* 2024.

[31] *Asad M, Liu D, Li J, Chen J, Yang G: **Development of CRISPR/Cas9-Mediated Gene-Drive Construct Targeting the Phenotypic Gene in Plutella xylostella**. *Frontiers in physiology* 2022, **13**:938621.
1st single CRISPR/Cas9-based gene drive construct for this species and only non-mosquito/cell line example dsRNA knockdown of Ku70 to improve knock-in efficiency.

[32] Paulo DF, Cha AY, Kauwe AN, Curbelo K, Corpuz RL, Simmonds TJ, Sim SB, Geib SM: **A Unified Protocol for CRISPR/Cas9-Mediated Gene Knockout in Tephritid Fruit Flies Led to the Recreation of White Eye and White Puparium Phenotypes in the Melon Fly**. *J Econ Entomol* 2022, **115**:2110–5.

[33] Chen H, Sun H, Xie J, Yao Z, Zheng W, Li Z, Deng Z, Li X, Zhang H: **CRISPR/Cas9-induced Mutation of Sex Peptide Receptor Gene Bdspr Affects Ovary, Egg Laying, and Female Fecundity in Bactrocera dorsalis (Hendel) (Diptera: Tephritidae)**. *Journal of insect science (Online)* 2023, **23**.

[34] *Rylee JC, Nin-Velez A, Mahato S, Helms KJ, Wade MJ, Zentner GE, Zelhof AC: **Generating and testing the efficacy of transgenic Cas9 in Tribolium castaneum**. *Insect Mol Biol* 2022, **31**:543–50.
Authors have generated the first *Tribolium castanaeum* lines expressing Cas9 endogenously with endogenous promoters through a piggyBac transposable element.