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SYMPOSIUM

The Development and Expansion of *in vivo* Germline Editing Technologies in Arthropods: Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control) and Beyond

Gerard Terradas, Vanessa M. Macias, Hillary Peterson, Sage McKeand, Grzegorz Krawczyk and Jason L. Rasgon [1]

Department of Entomology, Center for Infectious Disease Dynamics, and the Huck Institutes of the Life Sciences, Pennsylvania State University, University Park Pennsylvania, 16802, USA

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[1]E-mail: jlr54@psu.edu

Synopsis In the past 20 years, sequencing technologies have led to easy access to genomic data from nonmodel organisms in all biological realms. Insect genetic manipulation, however, continues to be a challenge due to various factors, including technical and cost-related issues. Traditional techniques such as microinjection of gene-editing vectors into early stage embryos have been used for arthropod transgenesis and the discovery of Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein (CRISPR–Cas) technologies allowed for targeted mutagenesis and the creation of knockouts or knock-ins in arthropods. Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control) acts as an alternative to embryonic microinjections, which require expensive equipment and extensive hands-on training. ReMOT Control's main advantage is its ease of use coupled with the ability to hypothetically target any vitellogenic species, as injections are administered to the egg-laying adult rather than embryos. After its initial application in the mosquito *Aedes aegypti*, ReMOT Control has successfully produced mutants not only for mosquitoes but for multiple arthropod species from diverse orders, such as ticks, mites, wasps, beetles, and true bugs, and is being extended to crustaceans, demonstrating the versatility of the technique. In this review, we discuss the current state of ReMOT Control from its proof-of-concept to the advances and challenges in the application across species after 5 years since its development, including novel extensions of the technique such as direct parental (DIPA)-CRISPR.

Introduction

The enormous growth that sequencing technologies have endured this century has led to fast-evolving biological fields, especially genetic engineering and its related disciplines. In entomology, there have been great advances due to the easy access to genomic data from nonmodel organisms that are relevant to public health, agriculture, and socioeconomics. In the past, *Drosophila* was used as the main insect model organism, and new developments were usually based on data generated in this organism first. Currently, however, >150 insect species have had their genomes publicly released and annotated (mostly Diptera and Hymenoptera; Li et al. 2019). From those, mosquitoes of human disease relevance have been researched extensively, not only in their biology and behavior but also looking into field population data, with one of its prime examples being the genomic diversity observed in *Anopheles gambiae* in Africa in an effort to control malaria (Ag1000G) through novel genetic technologies (Miles et al. 2017). These advancements affect not only mosquito research, as it is relatively straightforward to generate data on rare insects to target regions of the genome for the discovery of novel gene functions, genetic networks, or interactions between vectors and the pathogens they transmit. Using these data, targeted genetic

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manipulations can be generated that result in organisms that can carry desirable traits or be mutants for the genes targeted. Traditionally, vector genome editing has been achieved by transiently (somatic, no heritability) or stably (germline, heritability through generations) expressing foreign DNA in mosquitoes to study their biology, biocontrol, and production of nonnative gene products by using viral or plasmid expression vectors such as baculovirus (Maeda et al. 1985), Sindbis virus (Higgs et al. 1995), and plasmids (Cornel et al. 1997) or transposon-mediated integration such as *P-element* (Miller et al. 1987), *Hermes* (Jasinskiene et al. 1998), *Minos* (Catteruccia et al. 2000), and piggyBac (Kokoza et al. 2001). Of those, the most common in insect transgenesis are the piggyBac transposon (Handler and Harrell Ii 1999; Grossman et al. 2001; Kokoza et al. 2001) and the φC31 recombination system (Nimmo et al. 2006; Labbé et al. 2010). Both have proven highly successful for a range of species, and their reagents (lines that constitutively express pBac transposase, φC31 recombinase, or AttP-AttB docking sites) are available for a small number of selected species. Despite these capabilities, techniques, and knowledge, insect genetic manipulation of medical and economic importance has always been and continues to be a challenge in the field due to a variety of reasons, mostly technical and cost-related.

CRISPR–Cas9 paves the way

However, biology and medicine changed completely in 2012 with the discovery that the bacterial defense system CRISPR could be applied to different organisms as a cut-and-repair mechanism to create genetic edits (Jinek et al. 2012; Cong et al. 2013). The standard CRISPR/Cas system relies on the pairing of a DNA nuclease (spCas9; or CRISPR-associated protein 9 from *Streptococcus pyogenes*) with a customizable guide RNA (sgRNA; crRNA fused to the scaffold tracrRNA) that targets any genomic sequence of interest adjacent to a protospacer adjacent motif (PAM; -NGG in the case of spCas9). The Cas9 nuclease mediates cleavage of the sequence of DNA complementary to the sgRNA upon formation of the ribonucleoprotein (RNP) complex consisting of both elements. For genetic modification purposes, cleavage at a precise genomic location is the first step to targeted mutagenesis. The second is how the cellular machinery can repair the double-stranded DNA break. In most organisms, cellular repair occurs through nonhomologous end joining (NHEJ) or homology-directed repair (HDR), which are mutually exclusive. The former—NHEJ—is a faster process that does not depend on a template to repair the break and it often leads to indels creating shifts in the DNA-reading frame or missing codons, hence very useful to create gene knockouts (Gratz et al. 2013; Wang et al. 2013; Dong et al. 2015; Gilles et al. 2015). On the contrary, the latter—HDR—scans the DNA sequences flanking the break and finds the homology in the remaining chromosome, or in a donor template, to repair the DNA by copying in the missing sequences and thus being useful in genetic engineering to create knock-ins (Gratz et al. 2013; Gantz et al. 2015; Kistler et al. 2015).

The ability to manipulate the genome at will has greatly advanced basic science in the insect biology field and led to the development of population vector control strategies. Insects are often the carriers and the spreaders of etiological agents of disease, causing incredible socioeconomic burdens in humans, either in healthcare with mosquitoes and ticks transmitting diverse arboviruses, bacteria, and parasites, and in agriculture with pests such as *Bemisia tabaci* or *Drosophila suzukii* destroying millions of dollars worth of crops. Traditionally, vector control efforts have been focused on chemically eliminating the vectors with insecticide-treated nets and broad-spectrum pesticides. However, the appearance of resistance to these compounds and the damage they cause to the environment, as well as the killing of nontargeted species, makes the need of alternative methods much more relevant. As such, there is ongoing research testing the feasibility and scalability of deployment of genetically modified insect strains that reduce total population numbers in a given area as well as the possibility of reversal of insecticide resistance (Kaduskar et al. 2022) in those populations and countries heavily affected by it.

Limitations of insect genetic transgenesis

A difficulty that most genome manipulation technologies face is how to deliver the correct quantities of donor DNA and complemental proteins specific to each transgenesis system (transposase, recombinase, Cas9, etc.) at embryonic stages where time and location are excruciatingly crucial. Transgenes are generally injected into the embryonic posterior pole during preblastocyst stages (Jasinskiene et al. 2007), and transformation efficiency is dependent on different parameters such as injection volume and pressure, desiccation, buffer pH and toxicity associated with the introduced substance on the target insect. Microinjection offers the capacity to incorporate precise minuscule amounts of these compounds into insect eggs with relatively high efficiency in comparison to other chemical {endocytosis (Colosimo et al. 2000), or physical (electroporation [Thomas 2003]), and gene gun (Kravariti et al. 2001)} manipulation techniques. However, there are different factors that can hamper or delay the success of obtaining

the desired transgenic individual. The proper and timed development of embryos is essential for genetic modification, and this may be reduced upon manipulation and injection, especially when injected embryos suffer enough physical damage to compromise their viability and, while microinjection methods can be modified to best suit each insect species of each insect, some non-model species suffer from their eggs getting too damaged during the procedure. This phenomenon can also be caused by intrinsic features of the target population as some are incapable of laying synchronous eggs (such as the burying beetle [Smiseth et al. 2006]), they give live birth to progeny—thus there are no eggs to inject—(e.g., tsetse fly [Benoit et al. 2015]), or that their eggs are inaccessible as they must be anchored to external tissues, such as the Asian citrus psyllids oviposition into host plants (Hall et al. 2013; Chaverra-Rodriguez et al. 2023). Reproducibility between experiments can also be hard to achieve due to environmental (temperature and humidity) or user factors (technique, DNA/protein purity).

The biggest barrier to using embryonic micromanipulation and microinjection is that required equipment is expensive and time-consuming for the user (training, injection, rearing, and husbandry). Therefore, the transgenesis field would highly benefit from the development of more straightforward, cheaper, and quicker methodologies. To get around these limitations, a few years ago, our laboratory developed a method that bypasses the need for embryonic microinjection to modify an individual genetically. Instead, it uses a natural ovarian delivery system to deliver Cas9 RNPs from the circulatory system to the developing oocytes of a vitellogenic adult female, resulting in the transformation and modification of the germline (Figure 1). The technique was named Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control; Chaverra-Rodriguez et al. 2018; Figure 1 and Table 1). In this review, we focus on the development and optimization of ReMOT Control and how far the technique has advanced in the 5 years since its conception, expanding from *Aedes aegypti* to its seamless transition to many different arthropod species and orders.

The origin of ReMOT Control

As mentioned, insect transgenesis has traditionally relied on the capacity to deliver exogenous nucleic acids to the embryo in a very time-consuming and expensive manner. However, endocytosis promoted by specific ligands has been assessed as a therapeutic technique capable of delivering various compounds (Wagner et al. 1994; Qian et al. 2002) to a desired cellular location. Transferrin is a prime example that a ligand molecule can be adapted to chemically bind specific molecular cargoes—liposomes (Hege et al. 1989), toxins (FitzGerald et al. 1983), proteins (Wagner et al. 1994), or DNA (Stavridis and Psallidopoulos 1982)—and be internalized by cells (Widera et al. 2003; Chen et al. 2013). The efficacy is determined by the capacity of these membrane-bound vesicles to release the cargo (Hege et al. 1989; Wagner et al. 1994; Widera et al. 2003). To aid in the internalization and release of the contents, membrane destabilizers can be used to slightly and transitionally disrupt or destabilize cellular membranes, inducing the formation of pores (Fuchs et al. 2013). Most chemical escape reagents that have been used are cell-penetrating cationic amphiphilic peptides (Huang et al. 2004), or nonpeptides such as amines, polymers (Liang and Lam 2012), monensin, saponin (Fuchs et al. 2009) or chloroquine (Wu 1997). Similar to transferrin, which naturally binds to iron and aids on its dispersal throughout the body, egg-laying animals translocate different kinds of proteins to the developing ovaries in a highly conserved process for egg formation and vitellogenesis. Yolk-protein precursors (YPPs) are synthesized in the arthropod fat body and are secreted into the hemolymph to bind to and be internalized through receptors located in the oocyte where they accumulate in endosomal vesicles to be sorted into yolk granules for nutrient storage. This pathway made the use of YPPs promising candidates to target gene-editing cargo to the developing germline, particularly as recombinant *Drosophila melanogaster* yolk protein 1 (DmYP1) had previously been shown by immunohistochemistry to be internalized into *An. gambiae* oocytes following intrathoracic injection (Bownes et al. 2002).

In order to check whether exogenous material was able to be efficiently delivered into the ovary using DmYP1, different iterations of the DmYP1 protein were fused to EGFP to check for internalization and then to Cas9 for editing (Chaverra-Rodriguez et al. 2018). When testing the different domains of the protein separately, the portion responsible for ovarian localization was identified and it is found in the N-terminal of DmYP1 (Chaverra-Rodriguez et al. 2018). This is important for engineering applications, as it greatly reduces the length of protein that needs to be genetically encoded for expression and purification (from 439 to 41 amino acids). The 41 amino acid peptide ligand is known as P2C (sequence: NLQQQRQHGKNGNQDYQDQSNEQRKNQRTSSEEDYSEEVKN; Chaverra-Rodriguez et al. 2018).

In general, injection of P2C-Cas9 ribonuclear complexes (P2C-Cas9 bound to a specific sgRNA) into the thorax of a mosquito female is required at 24–48 h

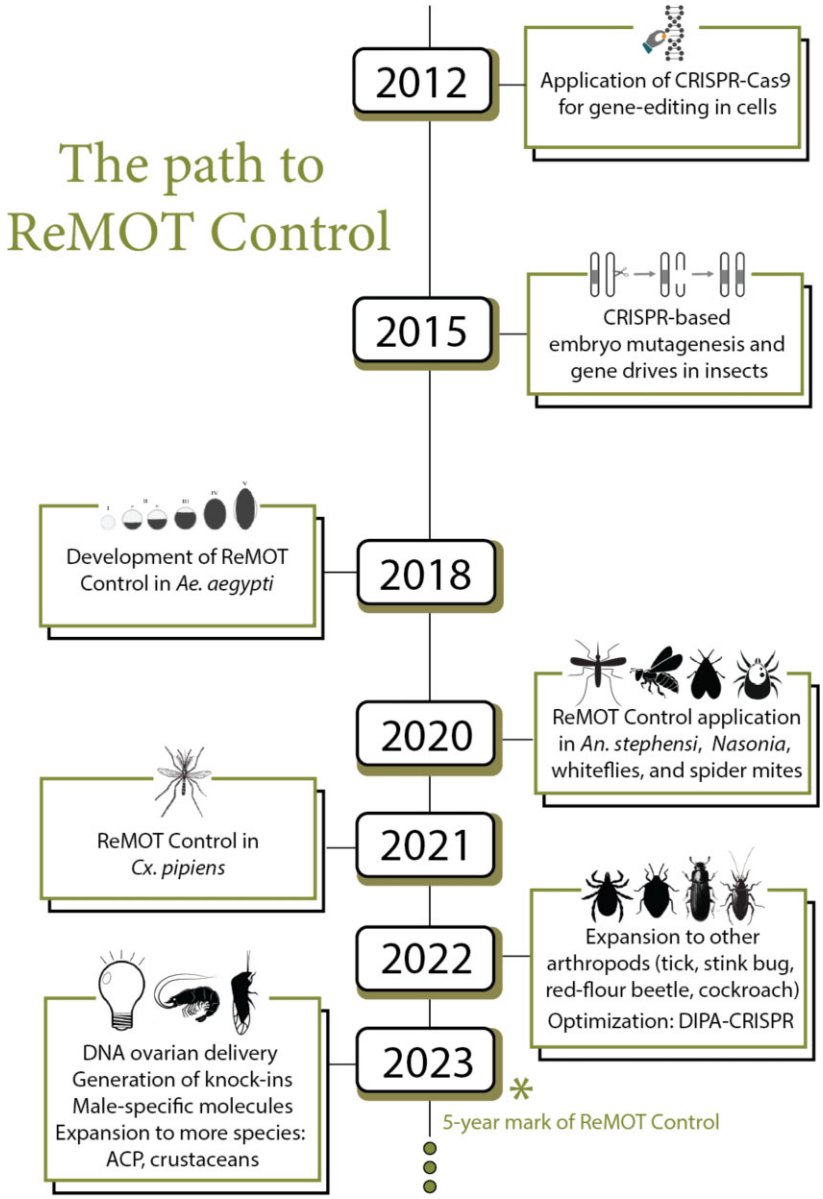

Fig. 1 Timeline of the development of *in vivo* targeted gene-editing technologies in arthropods.

post bloodmeal (vitellogenic stage; Macias et al. 2020). While there can be passive ovarian protein intake in some species, P2C guides the complex present in the hemolymph to localize into the ovaries with high efficiency. This is required in many species to achieve high enough levels of Cas9 RNP in the tissue needed for embryonic genome editing. Once localized, Cas9 cleaves the DNA of the embryo using the bound sgRNA, which determines the specific target site. The efficacy of the technique is similar to that of standard embryonic microinjection-based delivery of CRISPR components, with both producing heritable knockouts by the formation of NHEJ indels in the genome at similar rates (Chaverra-Rodriguez et al. 2018).

ReMOT Control, however, is not suitable for species in which vitellogenesis does not occur or species in which vitellogenesis is mainly an ovarian autosynthetic process such as in some higher Dipterans. In these cases, yolk proteins are produced and translocated from the nurse cells surrounding the oocytes, instead of extraovarian fat bodies (Brennan et al. 1982; Houseman and Morrison 1986).

Aedes aegypti, the first success

The development and optimization of ReMOT Control occurred in the yellow fever mosquito *Ae. aegypti*, (Chaverra-Rodriguez et al. 2018). The choice was due to the organism being spread worldwide and being

Table 1 List of arthropods where *in vivo* editing technology has been applied. All species depicted in the table are currently being used and/or optimized for basic or applied entomological research.

Common name	Scientific name	Class (Order)	Target gene	Ligand	Efficiency	Modification	References
Yellow fever mosquito	*Ae. aegypti*	Insecta (Diptera)	*Kynurenine monooxygenase* (*kmo*)	P2C	1–2%	Gene editing	Chaverra-Rodriguez et al. 2018
Asian malaria mosquito	*Anopheles stephensi*	Insecta (Diptera)	*Enhanced Cyan Fluorescent Protein* (*ECFP*)	P2C	2–4%	Gene editing	Macias et al. 2020
Vivax malaria mosquito	*An. sinensis*	Insecta (Diptera)	*Kynurenine monooxygenase* (*kmo*)	P2C	>13%	Gene editing	Yang et al. 2023
Common house mosquito	*Culex pipiens*	Insecta (Diptera)	*Kynurenine monooxygenase* (*kmo*)	P2C	0.3–0.5%	Gene editing	Li et al. 2021
Silverleaf whitefly	*B. tabaci*	Insecta (Hemiptera)	*White* (*w*)	BtKV	1–3%	Gene editing	Heu et al. 2020
Brown marmorated stink bug	*Halyomorpha halys*	Insecta (Hemiptera)	*Kynurenine monooxygenase* (*kmo*)	P2C	2%	Gene editing	Terradas et al. 2022
Asian citrus psyllid	*Diaphorina citri*	Insecta (Hemiptera)	*White* (*w*)	P2C or None (DIPA)	1–10%	Gene editing	Chaverra-Rodriguez et al. 2023
Kissing bug	*Rhodnius prolixus*	Insecta (Hemiptera)	*Yellow* (*y*), *scarlet* (*sca*), *white* (*w*)	P2C and BtKV	0.68–3.95%	Gene editing	Lima et al. 2023
Kissing bug	*Triatoma infestans*	Insecta (Hemiptera)	–	P2C and BtKV	–	Ovarian entry	Lima et al. 2023
Parasitic wasp	*Nasonia vitripennis*	Insecta (Hymenoptera)	*Cinnabar* (*cin*)	P2C	9%	Gene editing	Chaverra-Rodriguez et al. 2020
Red flour beetle	*Tribolium castaneum*	Insecta (Coleoptera)	*Cardinal* (*cd*), *cinnabar* (*cin*)	P2C or None (DIPA)	0.3–3%	Gene editing	Shirai et al. 2020, 2022
German cockroach	*Blattella germanica*	Insecta (Blattodea)	*Cinnabar* (*cin*)	None (DIPA)	22%	Gene editing	Shirai et al. 2022
Two-spotted spider mite	*Tetranychus urticae*	Arachnida (Trombidifomes)	*Phytoene desaturase* (*tetur01g11270*)	None (DIPA)	0.5%	Gene editing	Dermauw et al. 2020
Black-legged tick	*Ixodes scapularis*	Arachnida (Ixodida)	*Proboscepedia* (*ProbP*)	P2C and IsVg8	2–4%	Gene editing	Sharma et al. 2022
Pacific white shrimp	*Litopenaeus vannamei*	Malacostraca (Decapoda)	–	Lv-Vmo1	–	Ovarian entry	Chen et al. 2023
Marbled crayfish	*Procambarus virginalis*	Malacostraca (Decapoda)	–	P2C	–	Ovarian entry	Stein et al. 2022
Freshwater prawn	*Macrobrachium rosenbergii*	Malacostraca (Decapoda)	Paired box 6 (*PAX6*)	VgP	87%	Gene silencing	Cohen et al. 2023

the vector of viral disease to more than 400M people yearly (Leta et al. 2018), mostly due to its geographic range with high capacity of transmitting viruses such as dengue, Zika, Chikungunya, and yellow fever (Souza-Neto et al. 2019). *Aedes aegypti*'s impact on socioeconomics and human health resulted in intensive research being done on this particular species (Matthews and Vosshall 2020). This species is also ideal to study genetic engineering technologies as egg development is synchronized by blood feeding and vitellogenesis (Raikhel 1984) and, for ReMOT, it shows a certain level of natural ovarian protein uptake (Noah Koller et al. 1989; Attardo et al. 2005). ReMOT's proof-of-concept study knocked out the kynurenine monooxygenase (*kmo*) gene (Han et al. 2003). *kmo* has been used extensively as a candidate for genetic engineering purposes due to its key role in the catabolism of tryptophan and ommochrome synthesis, where homozygous recessive mutants produce white-eyed (kmo^{w}) instead of typical black-eyed wildtype (kmo^{+}) mosquitoes. Thus, this allows for easy screening of the null phenotype at all live stages, from hatching to adulthood. Chaverra-Rodriguez and colleagues (Chaverra-Rodriguez et al. 2018) reared mutants for a specific *kmo* base pair position (kmo^{+}/kmo^{460}), which as heterozygous display a full black-eyed phenotype, to inject P2C-Cas9-sgRNA complexes that target a secondary nucleotide position (kmo^{519}). In the case of successful genome editing, the embryos would present white eyes (kmo^{w}/kmo^{w}) because of the complementation of the kmo^{460} and kmo^{519} mutations. After injection of P2C-Cas9-$sgRNA^{519}$ complexes and an endosomal release agent such as saponin, 1–2% of the hatched G_0 larvae showed a knockout phenotype. This represents an improvement compared to the efficiency of embryonic microinjections as the number of individuals required for ReMOT is significantly lower because they occur in the egg-laying female instead of single embryos.

Expansion to other mosquitoes of interest, *Anopheles* and *Culex*

Anopheles: The proof-of-concept paper of ReMOT Control already demonstrated the uptake of P2C in alternative mosquito species including multiple *Anopheles* and *Culex* mosquitoes, to display the potential adaptability of the technology to multiple organisms. However, the technology was applied more extensively in these other blood-sucking species later on—*An. stephensi* (Macias et al. 2020) and *Cx. pipiens* (Li et al. 2021). The capacity to apply ReMOT Control to these less researched species represents an improvement because their embryos are more difficult to synchronize and manipulate as well as a certain intrinsic refractoriness to editing. For *An. stephensi* (Macias et al. 2020), the Indo-Pakistan main malaria vector and first non-*Aedes* arthropod to be edited by ReMOT Control, the authors knocked out the ECFP (Enhanced Cyan Fluorescent Protein) marker from a double-marked line, also containing DsRed (VgCp26.10 [Gantz et al. 2015]). This was the gene of choice because *kmo* has shown important homozygosis-associated fitness costs in the species, leading to female mortality upon blood feeding (Pham et al. 2019). In *An. stephensi*, 4% of the available alleles ended up being edited and presenting stable $ECFP^{-}$ mutations after injection of transgenic adult females with RNP complexes with saponin. These $ECFP^{-}$ alleles were inherited in a Mendelian fashion, showing the stability of the germline edits and not transient mutations. The editing percentage in the species is higher than expected by embryonic microinjection and also high enough to be considered for an edition of those genes that do not show any visible phenotype and whose G_0 edited alleles need to be detected via polymerase chain reaction (PCR).

Similar results were obtained when ReMOT Control was applied to the vivax malaria vector *Anopheles sinensis* (Yang et al. 2023). P2C delivered the fluorescent protein DsRED to the ovaries with 100% efficiency after injection into bloodfed females. Using a guide RNA targeting the *kmo* gene and coinjecting with saponin, editing efficiencies of >13% were observed in offspring injected with P2C-Cas9 (Yang et al. 2023). These results demonstrate that the application of ReMOT Control to Anopheles mosquitoes is likely to be generalizable across the genus.

Culex: Similarly to *Ae. aegypti*, the study in *Cx. pipiens* (Li et al. 2021) demonstrated that targeting the *kmo* gene is an approach easily transferrable to other mosquito species and allows for easy quantification of technique's efficacy across species. The injection of $sgRNA^{kmo}$ RNPs, complemented with either chloroquine or saponin, resulted in the efficient generation of mosaic and diallelic knockout individuals. Most species tolerate saponin well (Chaverra-Rodriguez et al. 2018), however in some cases the injected adults do not survive. For those, an alternative chloroquine-based treatment (Li et al. 2021), the use of low concentrations of saponin or even complete avoidance of these endosomal escape reagents is needed, especially for some species where the treatment may lead to editing inhibition (e.g., saponin in *B. tabaci* Heu et al. 2020). Research in *Culex* is more challenging than with the other mosquito families previously described, as they are harder to rear for transgenesis due to the problems on obtaining enough injectable eggs from the iconic *Culex* egg rafts, and injecting those embryos is difficult without disrupting the stability of the raft that usually leads to higher embryo

mortality. Thus, the ability to use ReMOT Control for producing knockout transgenic lines have allowed basic research in this species and broadened its gene-editing capacities.

Other human health relevance vectors

Ixodes scapularis: I. scapularis, commonly known as the black-legged tick, is the primary vector of significant public health concern in the United States due to its ability to transmit various pathogens, including bacteria, viruses, and protozoa (Hinckley et al. 2014). Among these, Lyme disease is the most prevalent vector-borne illness in the United States, which is caused by the spirochete *Borrelia burgdorferi* and transmitted by *Ixodes* ticks. Injecting tick embryos is an exceptionally challenging task due to various factors (Santos et al. 2013). These include the high pressure within the egg, the chorion and a layer of hardened wax surrounding the embryo that must be removed before injection because a glass needle is unable to penetrate it. After optimization, ReMOT Control components were injected on the tick's right spiracle (Nijhof et al. 2007). Both P2C and a peptide (IsVg8; NFTKTKNY) based on the ticks endogenous vitellogenin protein were able to localize GFP to tick ovaries. To test ReMOT Control for gene-editing, P2C-Cas9 was injected with sgRNAs targeting *Proboscepedia* (*ProbP*), which had been proved a visual mutant in a different arachnid (Schwager et al. 2007), and saponin. Upon injection, a total of 11 mutants were isolated and confirmed, with deletions of over 100bp followed by GC-rich regions as flanking sequences being present in most of those individuals (Sharma et al. 2022). The gene-editing efficiency of tick ReMOT is comparable to that observed for mosquitoes (∼2–4%) and experimentally higher than embryo microinjections (1.7% vs 4.1%) in the same species.

Triatome bugs: The triatome bug *R. prolixus* is the kissing bug vector of Chagas disease, which is caused by the protozoan parasite *Trypanosoma cruzi*. Chagas disease affects up to 8 million people in the Americas, and treatments for chronic disease are lacking (Lima et al. 2023). Genetic tools to study the triatome bugs are lacking, primarily limited to RNAi. Lima et al. tested both the P2C ligand and the hemipteran ligand BtKV (originally isolated from *B. tabaci*—see below) for their ability to deliver fluorescent cargo to developing triatome ovaries. They found that both P2C and BtKV were functional in both *R. prolixus* and the related species *Triatoma infestans*, with BtKV more effective than P2C. When fused to Cas9 and injected into bloodfed *R. prolixus*, offspring gene edits were detected using both P2C and BtKV, but similar to the ovary entry experiments, BtKV was much more effective in generating genetically modified offspring with altered eye color and cuticle color phenotypes. Direct parental CRISPR (DIPA-CRISPR; see below) was not effective in *R. prolixus* (Lima et al. 2023).

ReMOT Control in agricultural and household pests

The effectiveness of CRISPR–Cas technologies in genetic manipulation of nondipteran species of agricultural and health interest, such as whiteflies, hymenopterans, and mites, needs to be optimized. Although population and evolutionary research has been conducted on many of these species, the difficulty in creating mutant lines hinders the identification of potential genetic targets for pest control strategies. All these species face similar challenges when performing successful microembryonic injection, such as extreme mortality rates, embryo size, and host dependency. ReMOT Control provides a crucial technical solution for generating biological reagents without the need for embryo injection.

Bemisia tabaci

The silverleaf whitefly, scientifically known as *B. tabaci* [cryptic species Middle East-Asia Minor I (MEAM1)], is a polyphagous agricultural pest that poses a significant economic threat. At all life stages, this insect feeds on phloem sap using piercing-sucking mouthparts, causing direct harm to plants. Furthermore, it is a vector for a range of viruses, including begomoviruses (Czosnek et al. 2017), which cause damage to important crop species. Currently, the primary control methods for *B. tabaci* are insecticides and predators in greenhouses (Faria and Wraight 2001; Gerling et al. 2001).

Embryo microinjection is highly challenging for *B. tabaci* (small embryo size–0.2 mm). Although the P2C ligand is effective for mosquito species, it does not have the same effect in whiteflies. To address this issue, ReMOT Control was developed via a vitellogenin-based peptide (BtKV; KPYGVYKTMEDSV Heu et al. 2020) in *B. tabaci*. Unlike blood-sucking insects, ovarian development in *B. tabaci* is asynchronous because their oocytes are continuously developing. However, endogenous vitellogenin has been shown to be upregulated and endocytosed by phase II oocytes (Guo et al. 2016), making it suitable as an ovary transducer. *Bemisia tabaci white* (*w*) was targeted with multiple sgRNA. In most insects, *w* encodes for an ABC transporter protein that is responsible for transporting ommochrome pigment into the eyes, thus its null mutants display altered eye color phenotypes (Morgan 1910; Chaverra-Rodriguez

et al. 2018; Feng et al. 2021). *w* mutants were recovered from 7 out of 9 experiments using BtKV-Cas9 RNP complexes without or with very low concentrations of saponin as an endosome escape agent, as higher concentrations of saponin inhibited the editing process. *Bemisia tabaci* females can control the sex ratios of their offspring, and all survivors that reached adulthood were haploid males, which is a benefit as recessive mutations cannot be masked by any wildtype allele. Editing efficiency in whitefly females was approximately two-fold higher than that of mosquitoes (Chaverra-Rodriguez et al. 2018; Heu et al. 2020), and germline editing was confirmed by sequencing and genetic crosses. The inheritance of the trait did not follow Mendelian ratios due to fitness costs associated with carrying a null phenotype. However, this successful application of ReMOT Control in *B. tabaci* offers possibilities for future research and genetic studies in economically relevant agricultural pests, which can lead to the development of species-specific biocontrol measures instead of broad-spectrum chemical agents. The BtKV ligand has also been demonstrated to be highly effective for both ovary targeting and gene editing in other hemipteran species such as Triatome kissing bugs in the genera *Rhodnius* and *Triatoma* (Lima et al. 2023; Table 1) and may act as a general ovary targeting ligand for hemipterans.

Diaphorina citri

Another important agricultural pest worldwide is the Asian citrus psyllid, or *D. citri*, because of its invasiveness and it is role as the main vector of *Candidatus liberibacter*, the etiological agent of Huanglongbing (HLB), a disease that destroys citrus crops rapidly. Genetic control methods are being developed against *D. citri* because of the inability to control the propagation of the insect via insecticides or natural predators that generate resistance and the difficulties for effective application (Tiwari et al. 2011; Milosavljević et al. 2021). However, delivering exogenous sequences into early embryos has been proven extremely challenging mainly due to their attachment to host plant tissues that facilitate water exchange, making it impossible to collect and inject the eggs without affecting their survival. Thus, ReMOT control was adapted and optimized for the species in order to bypass embryonic microinjections (Chaverra-Rodriguez et al. 2023). The authors targeted and edited visible eye phenotypes (*w* and *kh*) with good success in the former (1–10% efficiency, depending on the sgRNA used), but not the latter (Chaverra-Rodriguez et al. 2023). DIPA-CRISPR (see below) was also successful for editing in this species (Chaverra-Rodriguez et al. 2023).

Nasonia vitripennis

Nasonia vitripennis is a parasitic wasp that has been extensively studied as a model organism in various fields, including speciation, sex ratios (Werren 1983; Parker and Orzack 1985), sex determination (Beukeboom and Kamping 2006; Beukeboom et al. 2007), and evolution (Breeuwer and Werren 1990; Bordenstein et al. 2001) and its parasitic behavior makes it useful for biocontrol of unwanted insects (Werren and Loehlin 2009). Genetic manipulation using CRISPR/Cas9 has proven challenging as microinjection techniques rely on *Nasonia* eggs (small, ranging from 0.08 to 1.16 mm (Lalonde 2005) to be dissected from host pupae, injected with editing reagents through a viscous cytoplasm and then transplanted into a recipient blowfly pupa to ensure their proper development (Li et al. 2017). Despite these difficulties, several genes that produce visible phenotypes have been successfully mutated using CRISPR/Cas9 in *Nasonia*. RNP complexes consisting of P2C-Cas9 and a sgRNA targeting *cinnabar* (*cin*) were delivered to late-stage black pupae of Nasonia to test for gene editing in the species (Chaverra-Rodriguez et al. 2020). Saponin had no effects on the survival of G_0 offspring. Null mutations in *cin* produce red eye phenotypes in *Nasonia* instead of wildtype black eyes (Li et al. 2017). Using high concentrations of RNP (≈3 μg/μl) resulted in 8.8% of the egg-laying females (4/45) producing independent *cin*-mutating events, with three G_0 individuals displaying a bright red eye phenotype and one showing a milder red phenotype (Chaverra-Rodriguez et al. 2020). The presence of cin^- phenotypes in the G_2 male progeny could only indicate that germline gene editing had taken place, as was the case for males with bright red eyes. No mild phenotypes were observed over generations, thus indicating that the mild phenotype observed in G_0 was likely caused by somatic mosaicism rather than germline editing (Chaverra-Rodriguez et al. 2020).

Tribolium castaneum

Tribolium castaneum (red flour beetle) is a globally distributed major agricultural pest of stored grain that has been used as an experimental model in genetics and developmental biology for decades. Knockouts of the *Tr. castaneum cardinal* (*cd*) gene were achieved by injecting P2C-Cas9 $sgRNA^{cd}$ RNP complexes into 55 adult females (62% survival) resulting in one cd^- male found in the progeny of those that laid progeny (Shirai and Daimon 2020). The mutagenesis efficiency was 3% based on the number of surviving females (1/34), or 0.2% based on the number of hatched embryos (1/383). The knockout individual presented a 4bp deletion with the mutation occurring early in development during

oogenesis due to its lack of mosaicism (Shirai and Daimon 2020).

Halyomorpha halys

ReMOT Control can also be used to edit the genomes of species are not in laboratory culture. In fact, material directly from the field can be edited using this technique. This makes ReMOT Control a useful tool for studying and manipulating wild populations. As a proof of principle, ReMOT Control was used to generate CRISPR-edited field-collected *H. halys* (brown marmorated stink bug). At the time, the stink bug genome had not yet been made available (Sparks et al. 2020) and thus the study also proved that gRNAs designed from transcript data can be utilized for field-captured insects. *Halyomorpha halys* is a member of the family Pentatomidae, which include many problematic pest species worldwide and itself is an Asian invasive species to USA, Canada, Europe, and South America (Leskey and Nielsen 2018). The species is known to cause significant crop loss to specialty crops (Leskey et al. 2012a) and demonstrated certain resistance to broad-spectrum insecticides like pyrethroids and neonicotinoids (Leskey et al. 2012b). P2C was found to be functional in this species (Terradas et al. 2022). To investigate the capacity for CRISPR-mediated gene editing, the *H. halys kmo* ortholog was targeted for ReMOT Control (Terradas et al. 2022). Eleven independent egg masses were laid by wild-caught female *H. halys* injected with a solution containing RNP complexes formed by $sgRNA^{kmo}$ and P2C-Cas9. Out of those nymphs that hatched, 4/194 (2%) displayed kmo^{mos} phenotypes. This highlights the capacity of ReMOT Control to be applied for stink bug research with an efficiency similar to that observed with microembryonic injections (also tested in Terradas et al. 2022) and importantly, empirically demonstrates that field material can be edited.

ReMOT Control in crustaceans

As observed throughout the review, the P2C and BtKV ligands have been used to localize Cas9 to the ovaries of many arthropods, from non-mosquito dipterans to hymenopterans to hemipterans. However, ligand-based techniques would benefit from experimental testing on the breadth of use of the current ovarian delivery molecules and explore and characterize molecules capable of editing the male germline or complementary to the current female-specific. New or orthogonal ligands have been developed for crustaceans and shown proper delivery of cargo to the target tissue. GFP entry to the ovaries has been achieved for the Pacific white shrimp (via newly developed Lv-Vmo1; Chen et al. 2023) and the marbled crayfish (via original P2C; Stein et al. 2022). In both cases, this is just the first step to editing, as gene targets and resource availability increase. A step further than these two species is the freshwater prawn (*Macrobrachium rosenbergii*), with dsRNA against *PAX6* being delivered with great efficiency to the ovaries through VgP, which is an ortholog of vitellogenin, and resulting in gene silencing in 87% of the offspring (Cohen et al. 2023).

DIPA-CRISPR

In some arthropod species, it has been demonstrated that the developing ovaries can take up material from the hemolymph without a targeting ligand. Indeed, in the original ReMOT Control study in *Ae. aegypti*, mutants were obtained after injection with unmodified Cas9 RNP, albeit at low efficiency (Chaverra-Rodriguez et al. 2018).

Dermauw et al. (2020)used this technique to edit the two-spotted spider mite (*Te. urticae*). *Tetranychus urticae* feeds on 1100 different plant species and is resistant to >90 pesticides. It is also considered an adaptation model due to its generalist diet and acaricide resistance (Grbić et al. 2011; Wybouw et al. 2015; Villarroel et al. 2016; Bui et al. 2018). Direct injection of Cas9RNP was attempted as there is a high mortality rate associated with injected eggs (Garb et al. 2018). The phytoene desaturase gene, encoding an enzyme essential for red body pigmentation (Armstrong et al. 1990), was targeted by RNP complexes (Dermauw et al. 2020). The number of albino males recovered from the progeny was low (0.4–0.6%) and some died during development, but one edited male was obtained for each injection, isolated and crossed to obtain two homozygous lines with distinct phenotypic patterns. Line A displayed complete albinism in all life stages, while B presented it only in immature stages and adult legs. Both lines had mutations in the targeted gene (A—frameshift, complete disruption of the enzyme; B—hypomorphic, partial loss of function) that failed to complement each other (Dermauw et al. 2020). Although the transformation efficiency was low, the injection of RNP complexes induced two independent events in spider mites, which opens the possibility for future optimization of mutagenesis and transgenesis in the system.

This technique was further extended by Shirai et al. (2022), who termed it "direct parental CRISPR", or "DIPA-CRISPR", and used it to edit cockroaches and *Tribolium* beetles. Cockroach females fertilize and encapsulate their oocytes into an ootheca in their genital atrium, where they will remain until hatching (Cornwell 1968). Because of this unique reproduction system, it is impracticable to inject materials into early embryos of this global urban pest. Thus, genetic manipulation

of cockroaches had not been achieved until the use of DIPA-CRISPR against *cinnabar* in *Blattella germanica* (German cockroach; Shirai et al. 2022). In the study, the authors injected RNP complexes into adult females at different stages of their reproductive cycle, achieving an impressive 57 gene-editing events out of 262 hatchlings (22% efficiency) at 4 days after female ootheca drop. From those, some produced biallelic *cin*$^{-}$ mutations, showing that editing occurred at early embryogenesis. Furthermore, mutated alleles were transmissible to progeny in all cases, either from mosaic or complete knockout individuals, demonstrating that the generation of mutants could also aid in the development of knockout stable cockroach lines.

Similarly, DIPA-CRISPR was also used in the red flour beetle *Tr. castaneum*. Importantly, three independent knock-in events (3/817–0.36% knock-in efficiency) were produced in the study by coinjecting the RNP complex with ssODNs containing homology arms to the cutsite and a 3 bp mutation (Shirai et al. 2022). This is a breakthrough for the species as the capacity to generate knock-ins, albeit small, expands the genetic toolbox immensely.

While DIPA-CRISPR is an attractive method to attempt genome editing in arthropods, it should be noted that it does not always work. For example, in *R. prolixus* and *B. tabaci*, DIPA-CRISPR injections did not result in any modified offspring (Heu et al. 2020; Lima et al. 2023). Similary, in *An. stephensi* DIPA-CRISPR was not effective (Macias et al. 2020) and in *An. sinensis* it was significantly less efficient compared to ReMOT Control (Yang et al. 2023).

Future directions *in vivo* editing

The main advantages of using ReMOT Control and other *in vivo* techniques over other targeted gene-editing approaches such as embryonic microinjection is the ease of use, cost-effective and translatable across species in a relatively easy manner. However, there are certain caveats to the technique that must be addressed in order to make them mainstream tools, mainly the capacity to generate targeted knock-ins of medium to large-sized cargoes. To do so, the delivery of a DNA template into the ovaries is essential and which, upon DNA cleavage of the desired genomic location, would insert seamlessly via HDR. This template could be presented in any stable form, be single or double-strand DNA, in a linearized or plasmid form and, hypothetically, of any length. However, the translocation of DNA into the ovaries is not a natural pathway, contrary to what happens with proteins. Thus, certain mediators or cellular components would have to be paired to the template in order to facilitate ovarian access or proper DNA delivery. This line of research is essential, ongoing and will expand as the field would catalogue it as a major breakthrough.

Despite not yet being able to generate large knock-ins, it is important to understand that many species still lack transgenics or even basic genetic research methods, hence ReMOT Control aids on the capacity to create those resources in house. Nevertheless, the optimization of the technique is dependent on visual markers and the search for those is highly valuable, among other obstacles. Visual markers are usually well-conserved, validated for genetic manipulation, and often associated with eye pigmentation (e.g., *white* [Chaverra-Rodriguez et al. 2018; Feng et al. 2021], *cardinal* [Carballar-Lejarazú et al. 2020; Feng et al. 2021], *cinnabar* [Sethuraman and O'brochta 2005; Chaverra-Rodriguez et al. 2020], *kmo* [Han et al. 2003; Gantz et al. 2015; Feng et al. 2021], *yellow* [Feng et al. 2021], and *ebony* [Feng et al. 2021]). If no visual marker is available or known for a particular species, a dominant nonlethal mutation presenting a phenotype can be suitable for G_0 screening. However, these dominant nonlethals are difficult to find, as most of them are highly detrimental to fitness and thus probably also not available for use. Thus, other nontargeted techniques could be used to generate the needed fluorescent transgenic lines in order to evaluate whether ReMOT Control is efficient in the species of interest. Some other challenges are common to all types of genetic manipulation, such as difficulties in rearing the organisms or that not all areas of the genome are equally accessible, particularly for Cas9 cleavage (Jensen et al. 2017), and repairing DNA with HDR can be less efficient than other repair mechanisms, which may lead to random mutations in the targeted sequence without inserting a cargo. However, some obstacles are specific to each genus and even species. Among these, it is important to consider the optimal timing of injection, particularly for genera with lengthy or poorly understood oogenesis, in order to ensure the survival of injected adults and successful DNA cleavage and repair. Furthermore, it is essential to perform such injections without interfering with the laying capacity of females.

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Conflict of interest

J.L.R. has applied for patent protection on the ReMOT Control technology.

Data availability

No new data were generated in this study.

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