Review

Toward invasive mussel genetic biocontrol: Approaches, challenges, and perspectives

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SUMMARY

Invasive freshwater mussels, such as the zebra (*Dreissena polymorpha*), quagga (*Dreissena rostriformis bugensis*), and golden (*Limnoperna fortunei*) mussel have spread outside their native ranges throughout many regions of the North American, South American, and European continents in recent decades, damaging infrastructure and the environment. This review describes ongoing efforts by multiple groups to develop genetic biocontrol methods for invasive mussels. First, we provide an overview of genetic biocontrol strategies that have been applied in other invasive or pest species. Next, we summarize physical and chemical methods that are currently in use for invasive mussel control. We then describe the multidisciplinary approaches our groups are employing to develop genetic biocontrol tools for invasive mussels. Finally, we discuss the challenges and limitations of applying genetic biocontrol tools to invasive mussels. Collectively, we aim to openly share information and combine expertise to develop practical tools to enable the management of invasive freshwater mussels.

OVERVIEW OF GENETIC BIOCONTROL STRATEGIES FOR INVASIVE SPECIES

As a result of intentional and accidental human activities (e.g., travel, tourism, trade, and so forth), invasive species have steadily gained ground by spreading in non-native ranges. These opportunistic organisms can damage ecosystems and infrastructure, and impair agriculture, water treatment, fisheries, forestry, recreation, and many other activities.[1,2] Although the full toll of damage caused by invasive species is challenging to calculate, their impacts are understood to be widespread. A wide variety of control measures have been developed, with varying efficacy depending on the technology and the target species. For invasive freshwater mussels, available treatments are generally limited to application in small-scale settings, such as controlling fouling of infrastructure. Technologies for the control or eradication of large populations in open water are not currently available. In recent years, genetic biocontrol techniques have become a focus of research and strategies for combating invasive species, pest species, and human disease vectors have been developed. Such genetic biocontrol approaches have the potential to allow for large-scale control of invasive mussel populations. In this review, we summarize the efforts to develop genetic biocontrol tools and how different groups are working to apply these tools with the ultimate goal of controlling the spread of invasive freshwater mussels.

Sterile male technique: the case of the New World screwworm

While many genetic biocontrol tools rely on relatively new technologies such as RNA interference or CRISPR/Cas9, practical genetic biocontrol methods have been successfully employed in the field for many decades now. During the last sixty years, several strategies have been developed to impair the reproductive performance of invasive insects.[3,4] One of the most successful such applications is the sterile male technique, which was used to eradicate the New World screwworm (*Cochliomyia hominivorax*) from the United States and continues to be applied to prevent the spread of screwworms north from the isthmus of Panama.[5-7] The sterile male technique involves the use of ionizing irradiation at sub-lethal doses to sterilize male insects prior to their environmental release.[8] In this pest control method, the γ-radiation induces sterility

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but does not significantly affect the ability of males to fly, mate, and transfer sperm to females.[9] However, the adaptation of this technique to other organisms has been challenging, principally due to the high costs of growing and distributing irradiated insects, the difficulty of isolating treated males, and difficulties in establishing doses of ionizing radiation that induce sterility but do not interfere with the ability to successfully mate in the wild.[10]

More recently, focused genetic biocontrol strategies have been developed using sequence-guided technologies to selectively sterilize pest insects. These next-generation approaches have several advantages over radiation-induced mutagenesis. Genetic biocontrol techniques for sterile insects are more scalable than radiation-mediated mutagenesis, more consistent in their results, and likely to produce cost savings in the long-term. Transgenic male-only strains have been obtained for screwworm control.[11] For this approach, a tetracycline-regulated female conditional-lethal system has been developed.[3] It is estimated that this transgenic strain could save as much as a million U.S. dollars per year in maintenance costs for the screwworm barrier zone.[11]

Among crustaceans, crayfish from the family Astacidea are recognized as one of the principal threats to freshwater ecosystems.[12] For many decades, efforts to control crayfish were based only on trapping this species at infested sites. Other approaches that have been explored for the management of these crustaceans include the sterile male release technique,[13] autocidal controls and sexual attractants,[14,15] monosex populations,[16,17] and RNA interference.[16,18]

RNA interference (RNAi) control

The RNA interference (RNAi) mechanism is hypothesized to have evolved as an immune pathway by which eukaryotic cells degrade viral transcripts to protect against infections,[19] but this system also contributes to many aspects of endogenous gene regulation. In the canonical RNAi pathway (Figure 1A), messenger RNA (mRNA) is inactivated by small RNA molecules produced from endogenous long double-stranded RNA (dsRNA) cleaved by dicer enzymes.[20] However, RNAi can be triggered by exogenous dsRNA, long single-stranded hairpin RNA, short hairpin RNA (shRNA), short interfering RNA (siRNA), and microRNA (miRNA).

RNAi has been adapted for use as a tool for post-transcriptional gene silencing in many eukaryotes.[21] But not every class of small RNA are found in every organism. For example, *Capitella teleta* lacks endogenous siRNA, so long dsRNA would presumably be a poor RNAi trigger.[22] This annelid has a dicer with a mutant helicase domain. Similar dicer variants are specific for miRNA and show reduced processivity and altered RNA binding specificities. Exogenous dsRNA is critical for inducing RNAi and there are differences in the processivity of dsRNA and the responses induced by different RNAi triggers. For example, studies conducted on the two-spotted spider mite, *Tetranychus urticae*, have revealed that the length of the exogenous dsRNA determines the ability to induce phenotypic changes *in vivo*.[23] Susceptibility to extra-organismal RNA found in an environmental medium, also known as environmental RNAi (eRNAi), can vary between species. For example, in herbivorous insects, eRNAi has been demonstrated to be effective in Coleopterans but not in Lepidopterans.[24]

A major area for the application of RNAi is the management of insect pest species, which adversely impact human health, agriculture, and the environment.[25] In North America, the emerald ash borer (EAB), *Agrilus planipennis,* is an invasive insect that has decimated forests and urban ash trees.[26] Methods for controlling EAB using double-stranded RNAi have been reported. EAB larvae process long dsRNA to produce siRNA. Oral delivery of dsRNA can silence the inhibitor of apoptosis (*IAP*) or COPI coatomer (*COP*) genes in EAB, resulting in larval mortality.[27] RNAi treatments have shown a high mortality rate in both EAB larvae and adults. The oral administration of dsRNA constructs targeting different genes is lethal to EAB after ingestion and no phenotypic effects have been observed for non-targeted organisms.[28,29] In other cases, RNAi approaches can silence gene expression but not elicit mortality, as was observed for paper wasp (*Polistes dominula*) control.[30]

In mosquitoes, the knockdown of target genes related to reproduction and survival has been widely tested with RNAi.[31] RNAi treatments reduced the expression of sex differentiation genes such as *doublesex* (*dsx*) in *Anopheles gambiae*, and the loss of *dsx* expression prevented female larvae from reaching adulthood.[32] Moreover, mosquito RNAi-based bioinsecticides have targeted important processes involved in larval survival, such as chitin synthesis.[33] Through expression in *E. coli*, dsRNA targeting chitin synthases (*CHs*) A and B can cause significantly lower viability in larvae associated with decreased expression of *CHs*. Other potential mosquito insecticides have been tested using micro-algae-based expression systems. Essential genes such as hydroxykynurenine transaminase (*3-HKT*) have been suppressed in mosquito larvae using both *Chlamydomonas* and *Chlorella* algal strains as vectors for dsRNA delivery.[34–36]

RNAi has also been successfully applied in tephritid fruit flies such as *Bactrocera tryoni*. Strong RNAi phenotypes have been observed after RNAi-mediated knockdown of the *dsRNase* and *yellow* genes involved in dsRNA degradation and melanin synthesis, respectively.[37] Sterile insects can also be created using RNAi, without the need for irradiation. For instance, spermless male flies have been obtained by knocking down azoospermia and germ cell differentiation genes such as *boul, zpg, dsxM, fzo,* and *gas8*.[38] The downregulated expression induced by these dsRNA constructs has resulted in up to 85% male sterility in *B. dorsalis*.[38] In addition, using RNAi against transformer genes (*tra* and *tra-2*) in *B. dorsalis,* nearly 80% of the RNAi males mated, and the mated females laid eggs, but only about 50% of the RNAi males produced progeny.[39]

RNAi-based gene knockdown has been tested in a variety of aquatic organisms, such as sea lamprey (*Petromyzon marinus*[40]) and oysters (*Crassostrea gigas*[41–45] and *Crassostrea virginica*[46]). One representative study on RNAi knockdown in bivalves was conducted on *C. gigas*; these oysters were injected with HIF-α-targeted long dsRNAs, and after the injection, the knock-down of HIF-α resulted in the production of reactive oxygen species (ROS).[43] Another study utilized *Platymonas subcordiformis* and *Nitzschia closterium f. minutissima* as vectors to feed oysters with engineered *E. coli* to express dsRNAs targeting pigmentation genes. Feeding *C. gigas* dsRNA targeting *tyrosinase* resulted in the knockdown of this gene and hindered shell growth. Feeding oysters with dsRNA of the *peroxidase* gene showed a knockdown of the expression of the corresponding gene and caused diminished black pigmentation in the newly developed shell.[42] Aside from oysters, beetles
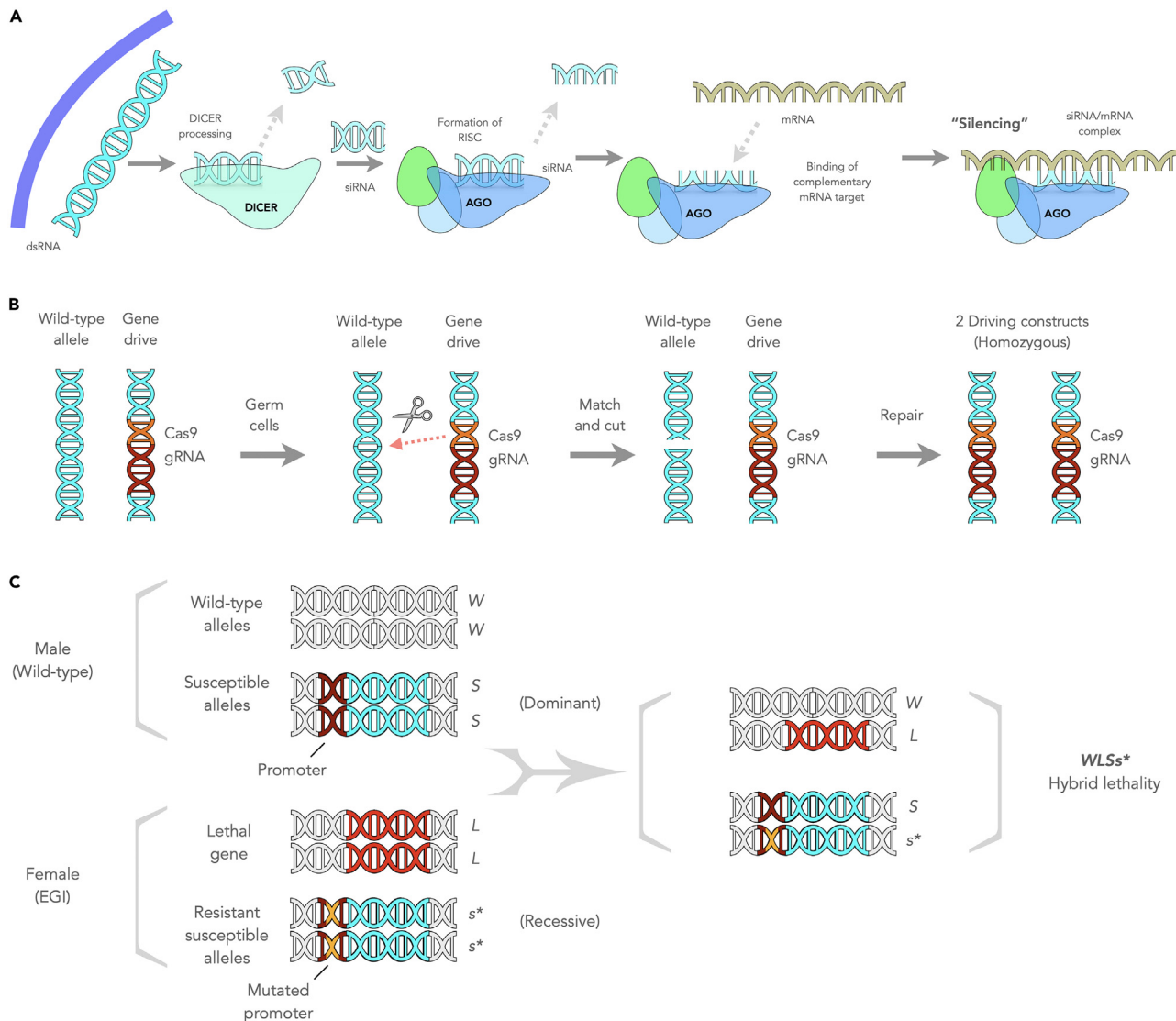
Figure 1. Approaches developed for the genetic biocontrol of invasive species

(A) RNA interference (RNAi). Silencing RNA precursors are trimmed into short double-stranded RNA (dsRNA) by a Dicer enzyme. An Argonaute protein binds and unwinds the short dsRNA and selectively retains one of the two RNA strands (the guide strand). A messenger RNA (mRNA)-silencing ribonucleoprotein complex, known as RNA-induced silencing complex (RISC), is formed between Argonaute proteins, the small single-strand guide RNA, and the mRNA target to induce mRNA silencing. RISC drives the silencing of a target mRNA via mRNA degradation or translational repression.

(B) Gene drive. CRISPR-Cas9-based gene drives utilize an expression cassette containing the Cas9 and guide RNA (gRNA) genes inserted into a target gene. The insertion disrupts the wild-type target gene and creates a self-propagating gene drive construct, where the gRNA directs Cas9 to cleave the wild-type target gene copy in the germ cells of heterozygous organisms. During the repair, the cleaved copy uses the driving construct as a template. This process converts the wild-type allele to the gene driving construct, resulting in the germ cells becoming homozygous for this allele and favoring its inheritance.

(C) Engineered genetic incompatibility (EGI). Outcrossing of wild-type male with Engineered Genetic Incompatibility (EGI) female strain produces hybrid lethality.

(*Leptinotarsa decemlineata*[47] and *Tribolium castaneum*[48]), aphids (Sitobion miscanthi,[49] *Sitobion avenae*,[50] and *Acyrthosiphon pisum*[51]), brown planthopper (*Nilaparvata lugens*[52]), western corn rootworm (*Diabrotica virgifera virgifera*[53]), among many other organisms have been subjected to RNAi studies.

Gene drive: the case of the malaria-transmitting mosquito *Anopheles gambiae*

Gene drives can occur naturally. For instance, in several fly species, a naturally occurring gene drive acts during sperm development to abolish competition from non-driver carrying sperm.[54] A gene drive biases its inheritance, resulting in its transmission to offspring at frequencies

greater than 50%, a pattern known as super-Mendelian inheritance. As a result, the prevalence of the genetic element increases over generations, even in the absence of selective pressure.[55] Synthetic gene drives have been proposed to spread traits for controlling crop pests, disease vectors, and invasive species.[56] CRISPR/Cas9-based gene drives for population suppression require inserting an expression cassette containing the *Cas9* gene and single guide RNA (*sgRNA*) into a region of interest to impair reproduction, for example (Figure 1B). The insertion disrupts the wild-type target gene and creates a self-propagating gene drive construct, where the *sgRNA* directs Cas9 to cleave the wild-type target gene copy in heterozygous organisms. The homology-directed repair pathway repairs the cleaved copy using the driving construct as a template. This process, called *homing*, changes the wild-type allele to the gene drive construct, resulting in homozygosity for this allele and favoring its inheritance.[1]

Creating a gene drive requires a deep understanding of the species' life cycle, the ability to breed the organism in captivity, and the application of advanced genetic modification techniques to promote the site-specific insertion of a large construct, which will form the gene drive construct. This biocontrol technology also has additional specific prerequisites. To suppress invasive populations by disseminating sterility, it is crucial to maintain the fertility of heterozygous organisms, ensuring the transmission of the driving construct to offspring. To achieve this, the Cas9 gene in the gene drive construct must be placed under the control of a germline-specific promoter that restricts homing to gamete precursor cells. Additionally, the target gene must be expressed solely in somatic cells, where homing will not occur, and be haplosufficient, meaning that only one functional copy is required to maintain a fertile phenotype.[57] Consequently, heterozygotes remain fertile while only producing gametes with the driving construct, leading to the more frequent formation of sterile homozygous organisms and resulting in population decline over generations.

Currently, the most well-developed synthetic gene drives aim to suppress populations of *Anopheles gambiae*, malaria-transmitting mosquitoes, by disrupting a gene essential for female reproduction and restricting the homing process to the germ cells. The first generation of such a gene drive for *A. gambiae* demonstrated transmission rates of up to 99.6%.[58] However, the emergence and positive selection of cleavage-resistant variants prevented it from achieving population suppression in longer laboratory experiments.[59] To address this limitation, a second generation of gene drive targeted a highly conserved splice junction in the *doublesex* gene, which does not tolerate sequence variations due to functional constraints.[60] Large cage experiments resulted in the complete elimination of mosquito populations within a year (8–12 generations) without the emergence of gene-drive-resistant variants.[61]

The third generation of gene drive technology combines this *doublesex* gene strategy with the super-Mendelian transmission of the X-chromosome-shredding *I-PpoI* nuclease gene, thus shifting the sex ratio toward males. Experimental and modeling data suggest that this strategy outperforms the second generation in terms of speed to achieve population suppression.[62] To advance in the phased testing pathway proposed by the World Health Organization (WHO) for genetically modified mosquitoes, WHO recommends field tests that assess physical, geographical, or ecological containment of the GMO.[63] The main challenges in taking this next step are risk assessment, regulation, and addressing ethical and social concerns. The remarkable outcomes obtained thus far offer hope in the fight against malaria, a disease that causes over half a million deaths annually.[64] Success in laboratory and field trials with this method may help to pave the way for further development and application of gene drive technology to control other species.

Engineered genetic incompatibility methods

Engineered genetic incompatibility (EGI) methods represent another new approach for genetic population control. In this approach, genetically modified species express heritable genes that are incompatible with wild-type organisms, thus limiting the production or survival of their progeny upon mating (Figure 1C).

As an initial proof-of-concept for this approach, genetic barriers to sexual reproduction have been designed using CRISPR/Cas9 in *Saccharomyces cerevisiae*. Programmable transcription factors employing dCas9-based activators can induce the lethal overexpression of the *actin* gene (*ACT1*) in strains containing the wild-type actin promoter after mating with engineered strains.[65] In genetically engineered insects, such as *Drosophila melanogaster,* it has been possible to design proof-of-concept transgenes with dominant lethality and recessive resistance.[66] In *D. melanogaster*, EGI can be combined with conditional female lethality to create a sex-sorting incompatible male system. A tetracycline-controlled genetic construct controlled female viability,[67] while an independently functioning EGI construct targeted the *pyramus* gene to produce lethal ectopic expression in the hybrid offspring of the control agent and wild-type. The sex-sorted incompatible males can compete with wild-type males for mating and reproduction and were effective in population suppression in cage trials.[68] Strategies such as the field-amplified male sterility system (FAMSS), utilizing combined female lethality with EGI, could provide additional robustness. In simulations, FAMSS showed efficacy as a spatially and temporally self-limiting strategy for mosquito control.[69] Overall, modifying male genes by EGI methods in invasive species could lead to sterile offspring and the eventual control of these species.

IMPACTS OF INVASIVE MUSSELS

Invasive freshwater *D. polymorpha*, *D. rostriformis bugensis*, and *L. fortunei* species possess high fecundity.[70] They can disperse rapidly thanks to their free-floating larval stage.[71] The prolific zebra (*D. polymorpha*) (Figure 2A) and quagga (*D. rostriformis bugensis*) (Figure 2B) mussels native to Eurasia were likely introduced to the Laurentian Great Lakes in the 1980s, and they have expanded throughout the United States.[72] In South America, the Asiatic River golden mussel (Figure 2C) was first reported in 1991.[73] These problematic species have provoked widespread problems related to water quality, ecosystem imbalance, and macrofouling issues.

Invasive mussels have significant adverse economic and ecological impacts in ecosystems where they establish.[74] Users of freshwater, wastewater treatment, agriculture, industry, and hydroelectric generation can be directly impacted by macrofouling invasive mussels.
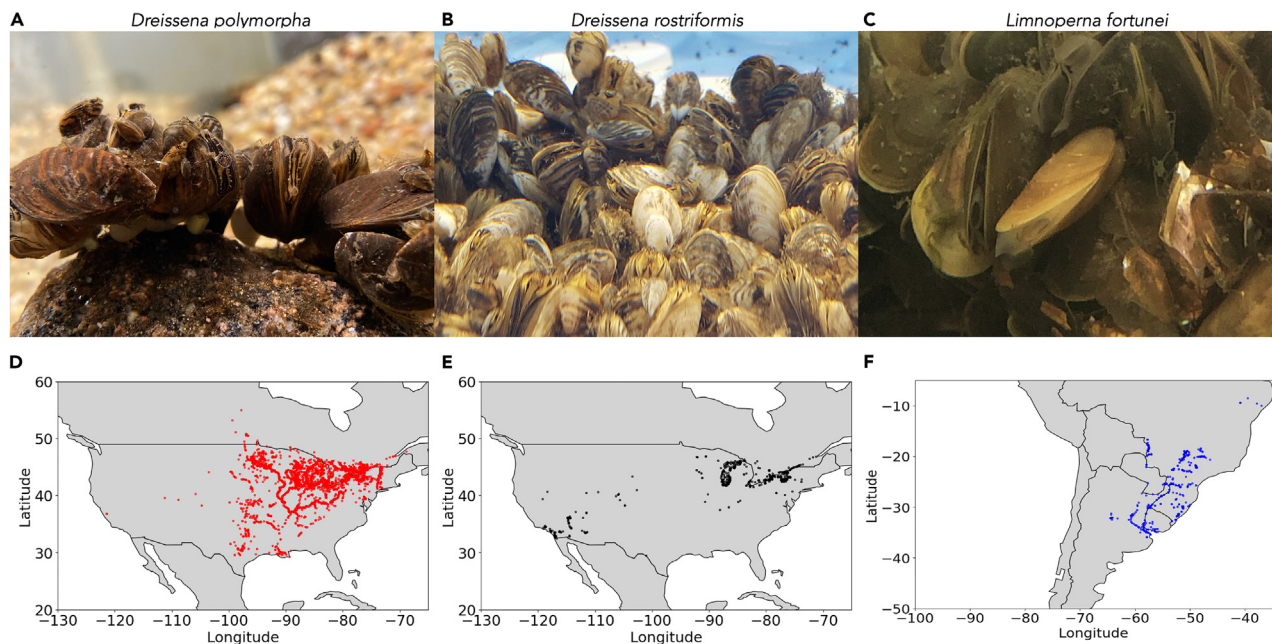
Figure 2. Zebra, quagga, and golden invasive mussel distribution in North and South America

(A) Zebra mussel (*Dreissena polymorpha*). Photo credit: Victor H. Hernandez Elizarraga, 2023.

(B) Quagga mussel (*Dreissena rostriformis bugensis*). Photo credit: Steve Suhr, 2023.

(C) Golden mussel (*Limnoperna fortunei*). Photo credit: Fábio Sendim, 2023.

(D) Zebra mussel invasion in North America. Red dots indicate where the occurrence of this species has been confirmed according to the USGS database: https://nas.er.usgs.gov/viewer/omap.aspx?SpeciesID=95 (Accessed May 2023).

(E) Quagga mussel invasion in North America. Black dots indicate where the occurrence of this species has been confirmed according to the USGS database: https://nas.er.usgs.gov/viewer/omap.aspx?SpeciesID=5 (Accessed May 2023).

(F) Golden mussel invasion in South America. Blue dots indicate where the occurrence of this species has been confirmed according to the GISD and the CBEIH databases: http://www.iucngisd.org/gisd/species.php?sc=416 (Accessed May 2023), https://base.cbeih.org/index.php?lan=en (Accessed May 2023).

Biofouling reduces water flow through intake pipelines and cooling systems and causes the deterioration of surfaces. Costs for treatment plants in the United States ranged from ¢ 0.0001 to ¢ 0.0013 per gallon, depending on the location and extent of the mussel infestation.[75] In addition, invasive freshwater mussels have negative impacts related to the prevention and control of these organisms that infest hydropower facilities, where annual costs for increased maintenance can reach approximately $464,000 per affected facility.[76]

Modeling work has shown that the zebra mussel invasion in the United States presents a high risk of continuing spreading across the Midwest, and it is likely throughout the Southeast and along the eastern seaboard.[77] Comparable with what has been observed for zebra mussels, the quagga mussel has expanded rapidly in non-indigenous regions, including the United States, Western Europe, and the Netherlands.[78]

Dreissenids are efficient filter feeders that can outcompete many native species for food resources while shifting energy pathways from the pelagic to benthic zones.[79–81] These shifts can reduce phytoplankton production[82] while promoting cyanobacteria, potentially harmful algal blooms,[83] and filamentous nuisance algae, such as *Cladophora*.[84–87] Dreissenid Invasive mussels have been shown to induce high mortality in native freshwater mussel (Unionidae) populations. Particularly, they have also significantly reduced and even extirpated native unionid mussels.[88,89] Invasive Dreissenids can encrust unionid surfaces using their byssal threads, preventing the opening and closing of valves, and limiting feeding in native mussels, resulting in decreased energy reserves and causing the arrest of essential processes for survival such as reproduction and shell formation.[90] In lakes and rivers from North America, zebra mussels can reach high densities (>3000 organisms per square meter), which leads to the extirpation of Unionoida native mussels within 4–8 years after the invasion.[91] Besides, *D. polymorpha* has contributed to native mussels' population decline in Europe. Some affected native species are *Anosonta anatina, Anodonta cygnea, Pseudanodonta complanata, Unio tumidus,* and *Unio pictorum*.[92] As per the IUCN red list (https://www.iucnredlist.org/), many native mussels, such as *Pseudanodonta complanata,* are currently recognized as vulnerable species that can be seriously threatened by invasive mussels. However, the impacts of invasive mussels on Unionoida populations are not fully understood.

The golden mussel (*Limnoperna fortunei*) (Figure 2C) is the most aggressive invasive freshwater species in Latin America (Figure 2D). It alters both biotic and abiotic factors, leading to significant impacts on freshwater ecosystems and biota.[79,93] Since its initial detection in Argentina in 1991, it has spread to Uruguay, Paraguay, Bolivia, and Brazil, where its northernmost range is in the northeast.[94] Predictive models suggest that by 2050, there is a risk that the golden mussel may extend its range to the Amazon basin.[95] The species reproduces year-round[96] and can reach high densities of up to 200 individuals/m^2, particularly on man-made structures such as those in hydroelectric power plants and fish farms.[97] Golden mussel biofouling has caused significant economic impacts, with estimated losses of up to $128 million annually in the

Brazilian electric sector alone.[98] Controlling the golden mussel remains an unsolved challenge. While existing methods can mitigate biofouling in man-made structures,[97] they are unable to eliminate the species from the environment or prevent further geographic spread. This is evidenced by the lack of reports of eradication in areas where the golden mussel has been established and the continued emergence of new occurrences.

OVERVIEW OF EXISTING CONTROL METHODS FOR INVASIVE MUSSELS

Invasive bivalves are spreading quickly across freshwater ecosystems (Figures 2D–2F).[99] To mitigate the impacts elicited by non-native mussels, a wide assortment of treatments and molluscicides have been tested for controlling these problematic species in closed systems. Efforts to eradicate or reduce invasive mussels have used chemical, physical, and biological tools.

Regarding chemical methods, chlorine compounds, such as hypochlorite and chlorine dioxide, are substances capable of killing mussels. These agents disrupt cell integrity through a general denaturation of proteins and the arrest of cellular enzymatic activity.[100] Chlorides have shown a dose-dependent effect in mussels, but their effectiveness depends on the right exposure concentration and duration.[101] Studies have shown that larval and juvenile freshwater mussels can have low susceptibility to chlorination.[102] Additionally, chlorine compounds can generate harmful byproducts such as trihalomethanes, pose a human safety risk, and involve neutralization steps before discharge into the environment.

In addition to chlorine compounds, non-chloride oxidizing compounds such as ammonia, activated bromine, and potassium permanganate, can be used for population suppression of invasive mussels. These chemicals are mainly used for the prevention of biological fouling on the surfaces of boats, docks, or other infrastructure. There are many oxidizing agent treatments for the control of mussel macrofouling within piping systems. Several control tools for internal piping systems have been developed for golden,[103] quagga,[104] and zebra mussels.[105]

The non-oxidizing molluscicides are a group of chemicals that mainly consist of commercial and non-generic formulations (e.g., BULAB, MEXEL, EVAC, Clam-Trol CT-2, VeliGON, among many others[105]). Non-oxidizing treatments are more selective than oxidizing molluscicides, do not have a general mechanism of action, do not readily react with organics, and target specific cellular components. Mulloscicides are applied at regular intervals. Non-oxidizing molluscicides are generally compatible with materials and metallurgies found in pipe and water systems. These molluscicides are also non-corrosive, non-volatile, non-flammable, easy to transport, and safe to handle compared with oxidizing agents. High variability in the efficacy of these formulations has been observed against mussels, and their application is limited due to several factors such as formulation type, mode of action, feasibility of application in waterways, cost of treatment, effective concentrations, regulatory restrictions, toxicological data, and precautions on product handling.[106]

The copper-based molluscicides EarthTec QZ and Natrix are also EPA-registered for invasive mussel control in open water. Although effective for killing invasive mussels, copper can be toxic to aquatic plants, algae, fish, and other invertebrates and persists in the environment. Potassium chloride (KCl), or potash, is not a registered molluscicide, but has also been used in open water treatments with regulatory exemption.[107] Similar to copper, potassium can harm non-target species and does not degrade in the environment. Carbon dioxide, currently registered as a piscicide, can prevent the settlement and attachment of dreissenids[108] and readily off-gasses, but application methods in open water are in the early stages of investigation.

Physical control strategies include hand removal, desiccation by water drawdown, and anoxia with benthic mats. Mussels can be removed manually from water bodies by scraping and suction removal. Manual removal is highly resource- and time-consuming but effectively withdraws live mussels and dead shells. Desiccation exposes the shorelines and decreases the ability of the water to retain heat. Depending on the drawdown duration, the water can reach freezing temperatures, causing the mussels to die. Benthic mats kill mussels by withholding oxygen and food and restricting the water flow. In addition, these systems block light to prevent photosynthesis. Benthic mats may also prevent larval mussels from spreading.

The naturally derived pesticide, Zequanox, is an approved biopesticide by the U.S. Environmental Protection Agency (EPA) for zebra and quagga mussel control.[109] The active ingredient in Zequanox is killed *Pseudomonas fluorescens* strain CL145A cells[110,111] which cause the degradation of the digestive system epithelium.[112] This biopesticide can reduce invasive mussel biomass, degrades rapidly, and is relatively selective, but it can affect water quality, and when it is applied in large doses can be harmful to some fish species and other organisms.[113]

In summary, invasive mussel control technologies that are effective, specific, safe for the environment, and scalable for large water bodies are currently lacking. Furthermore, control tools for invasive mussels in open water are more limited than those used in closed systems because of the large treatment areas, associated environmental concerns, and costs for materials and application. These gaps could be addressed with novel biocontrol tools, as reviewed here.

CURRENT APPROACHES IN DEVELOPMENT FOR THE GENETIC BIOCONTROL OF INVASIVE MUSSELS
RNA interference for the control of zebra mussels

Zebra mussels (Figure 2A) have caused significant changes in food webs, including the loss of diversity of zooplankton and phytoplankton in invaded waters, impacting aquatic ecosystems across North America (Figure 2D).[114] Chemical control methods have thus far been insufficient to eradicate mussels from infested water and to prevent additional water bodies from becoming infested, and such control methods often have non-specific toxic effects on a wide range of other organisms.[115–117] RNAi represents a strategy for controlling invasive mussels that has the potential to be highly species-specific, deployable in both closed and open systems, and that does not require genetic modification of the target invasive mussel species.
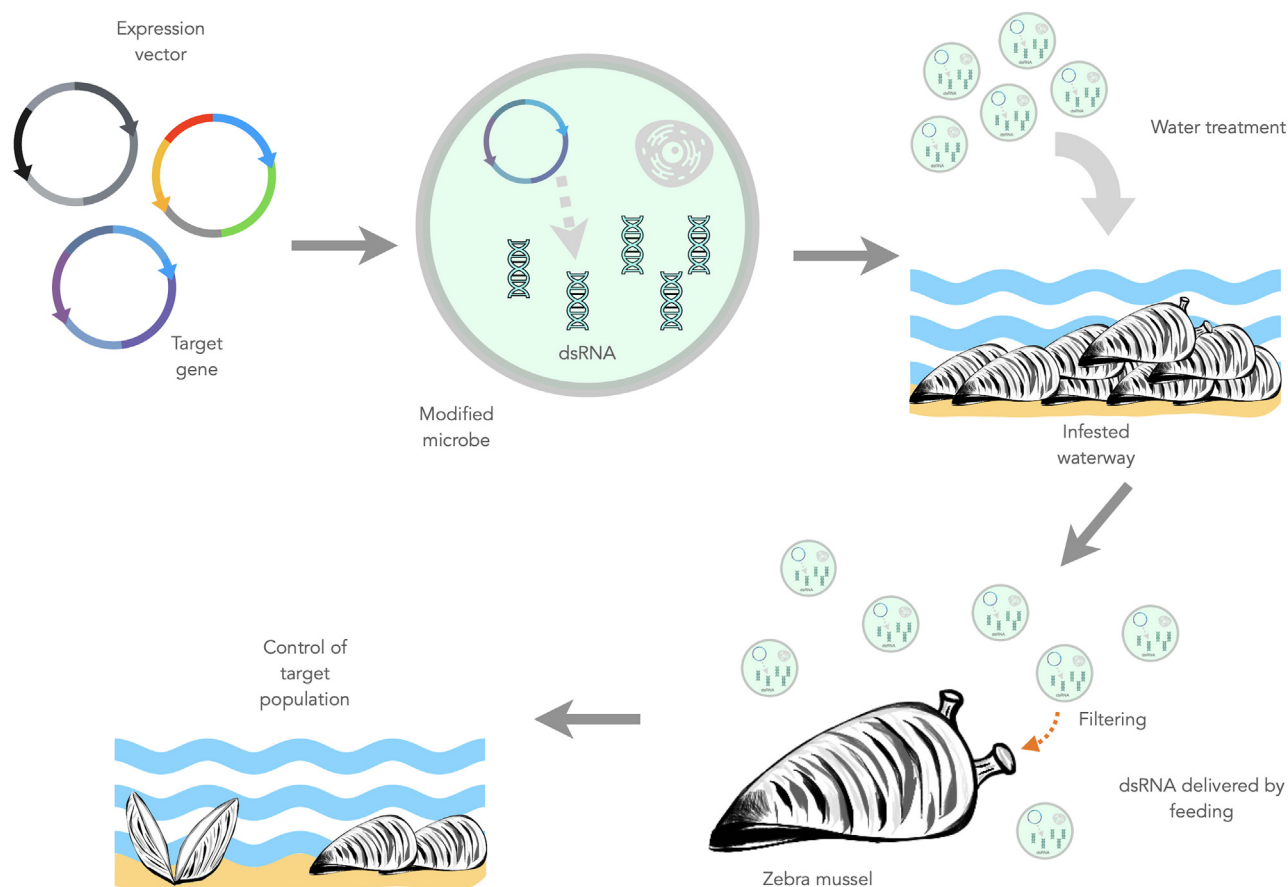
Figure 3. RNA interference (RNAi) for control of zebra mussels

RNAi can potentially be employed to create species-specific biocides that selectively inhibit essential gene functions in zebra mussels. Engineered microbes, such as bacteria and algae, could be utilized to treat infested waterways to produce and deliver dsRNA to zebra mussels through feeding.

A critical constraint in developing genetic biocontrol tools for zebra mussels at present is the lack of robust laboratory culture methods. The inability to culture zebra mussels in the lab throughout their full life cycle and over multiple generations precludes the use of tools such as gene drives or EGI that require the construction and maintenance of strains with heritable genetic modifications. One strategy that has been employed for RNAi-based pest control, which does not require breeding or transgenesis, is feeding with genetically engineered microbes (e.g., RNAi feeding). The RNAi feeding method was first demonstrated in the nematode *C. elegans*,[118] but has been applied in a wide variety of organisms. Feeding with modified microbes that express dsRNA can be effective only if the target species internalizes the genetic material through the digestive system.[119]

As filter feeders, mussels are known to consume bacteria in addition to algae and other phytoplankton.[120] It may be possible to knock down molecular targets essential for survival, growth, reproduction, attachment, or other key processes in invasive mussels using RNAi triggers delivered by genetically modified microorganisms (Figure 3). Bacteria, such as *E. coli*, have been widely used to produce and deliver silencing RNA by feeding.[118] *E. coli* is relatively easy to genetically modify, methods for maximizing the expression are well established, and it grows quickly to high cell densities.[121] At the same time, *E. coli* bacteria is an acceptable food source for many animals and has been found in the zebra mussel gut.[122] Several alternative microbial vectors have also been tested as vectors for feeding RNAi in other systems, including bacterial symbionts,[123] yeast,[124] and microalgae.[35,36] Due to the variation in eRNAi susceptibility between species and the fact that zebra mussels can selectively filter different particles when feeding,[87,125] it will be important to determine whether the feeding of RNAi trigger-expressing microorganisms is a viable delivery strategy for zebra mussels or if the amount of consumed microbe in the wild is enough to induce RNAi phenotypes.

Recently, the sequence of the *D. polymorpha* genome was published.[126] This resource, together with tissue-specific transcriptomic data, provided a rich starting point to identify genes involved in key processes that could be potential targets for RNAi-based genetic biocontrol. To begin, we identified nearly two hundred zebra mussel candidate biocontrol genes that were predicted to impact survival (housekeeping genes involved in basic cell biological functions), as well as other processes such as shell formation, feeding, byssal thread formation/surface attachment, reproduction, and stress response. We have begun to clone these target genes into *E. coli* expression vectors and test their

effects on zebra mussel gene expression and other phenotypes. Our ongoing project aims to test whether RNAi can be used to manipulate the expression of genes in zebra mussels through the delivery of RNAi triggers such as dsRNAs or shRNAs, delivered either by direct injection or by engineered bacteria or algal expression strains. By identifying RNAi reagents that disrupt key processes, we aim to develop RNAi tools that could be deployed to safely and specifically eradicate zebra mussels from non-native waterways.

One set of molecular targets that are particularly intriguing from the standpoint of RNAi-based zebra mussel biocontrol are genes controlling reproductive capacity. For example, silencing sex determination genes, such as *doublesex* (*dsx*)[32] or *transformer* (*tra*)[39,48] have been used to generate sterile males in insect species. However, this strategy may be challenging due to the lack of information on the mechanisms of sex determination in zebra mussels. Another option to impair reproduction is to silence spawning-related genes. It is known that serotonin triggers spawning in zebra mussels.[127–131] However, while the silencing of zebra mussel genes involved in serotonin signaling could limit their spawning, impairing this critical pathway may also elicit more widespread effects.

Other delivery mechanisms, such as synthetic RNA, can also be employed for gene silencing.[132] Synthetic RNA can directly induce RNAi responses in organisms without using a live genetic vector, which makes it easy to analyze standard toxicology, dosing, and effectiveness. Thanks to advances in RNA synthesis and stabilization, driven partly by agricultural applications for RNAi, synthetic RNA production costs have significantly decreased and yields have significantly increased in recent years.[133] However, it likely remains cost-prohibitive to deliver synthetic RNA in open water systems. First, it would be challenging and extremely expensive to reach effective concentrations of synthetic RNA in treated waterways. Second, "naked" dsRNA is unstable and susceptible to environmental nucleases. Thus, more sophisticated mechanical delivery routes are required to ensure the effectiveness of synthetic RNA or other toxicants against mussels. Techniques such as biocide microencapsulation may present a solution that makes synthetic RNA delivery more feasible, and microencapsulation approaches have shown promising results for invasive mussel control.[116,117]

The molecular mechanisms of the RNAi pathways employed in zebra mussels are not yet well characterized. For instance, the susceptibility or effectiveness of RNAi at different life stages has not been assessed. In addition, we are still assessing the effectiveness of different RNAi triggers and delivery methods. Insights into the function of the RNAi system in zebra mussels will shed new light on a relatively underexplored facet of mussel biology and may ultimately yield practical tools for highly species-specific genetic control of zebra mussels.

Quagga mussel control by disseminated neoplasia

In some marine bivalve species, there is a naturally occurring disease known as *disseminated neoplasia* (DN) that has been associated with large die-offs of farmed shellfish. DNs are very rare in nature and are a type of cancer where the cancer cells themselves are transmitted from one animal to another of the same species, producing lethality.[134] DN in molluscs was first described in the late 1960s and has since been studied by marine biologists concerned with the preservation of farmed marine molluscs of commercial importance.[134] DNs are transmitted from bivalves in close proximity through seawater. In the laboratory, transmission can occur by injecting DN cells from an infected bivalve into an uninfected one or by simply co-housing infected and healthy animals together in the same tank (Figure 4).[135,136]

One common perturbation of molluscan DNs is the alteration of the cell cycle and cell death master regulating protein p53.[137–139] p53 is the subject of hundreds of studies for its role in cancer in many organisms, and mutations in p53 are widely considered to be the most common mutation in human cancers.[140] Based on published reports linking changes in p53 to molluscan/mussel DN and the known role of p53 in neoplasia of mammals from mouse to man, it is feasible that the mutation of the tumor suppressor p53 within the mussel genome also has a high probability of producing culturable cancer cells.

The conversion of healthy primary cells to cancerous, "transformed" cells, can be achieved by targeting cancer-causing oncogenes that knock-out cell cycle-regulating genes. This has proved to be a difficult task with dreissenid mussels because methods of live animal culture, live cell culture, and gene transfer are not at an advanced stage compared to other commonly researched species. Nevertheless, using microinjection, it might be possible to introduce DNA encoding known oncogenes, such as SV40 Large-T antigen (Tag), into the quagga mussel genome to induce a cancer cell phenotype. The Tag protein has been shown to work through multiple cellular pathways to induce cellular transformation, most notably through the inhibition of cell cycle control proteins such as p53 and tumor suppressive factor Retinoblastoma-1 (Rb).[141] Tag has been used successfully for decades to induce the transformation of mammalian, reptile, and amphibian cells. We predict that Tag could have the same properties of cellular transformation in invasive mussel cells as well.

Engineered DN could possess several advantages over other possible genetic biocontrol solutions. The DN requires a compatible host to survive and replicate, so once the last dreissenid mussel in a target waterway has died, the agent itself is completely removed from the environment. Additionally, DN is progressively toxic and should kill invasive mussels of breeding age at a rate that does not put an undue burden on the environment due to the accumulation of dead mussel biomaterial. Because the anti-dreissenid DN should spread through host populations of any size, a single deployment of the DN may be all that is required, even in the largest water body. Finally, with no capacity for sexual reproduction, prolonged nutrient uptake, or survival in the wild unless engrafted within the body of a host mussel, the DN agent could avoid some of the risks commonly associated with genetically modified organisms.

Gene drive for the control of the golden mussel

Advances in DNA sequencing and genome editing technologies have made it possible to consider using gene drives for non-model species, such as the golden mussel (Figure 5). A genome sequencing program was initiated within our collaborative group to address the knowledge gap for the golden mussel and to identify candidate target genes and promoters required for a gene drive strategy (Figure 1B). We used genome and transcriptome sequencing to identify genes[142,143] and RNAseq analysis to find sex-biased genes potentially involved in sex
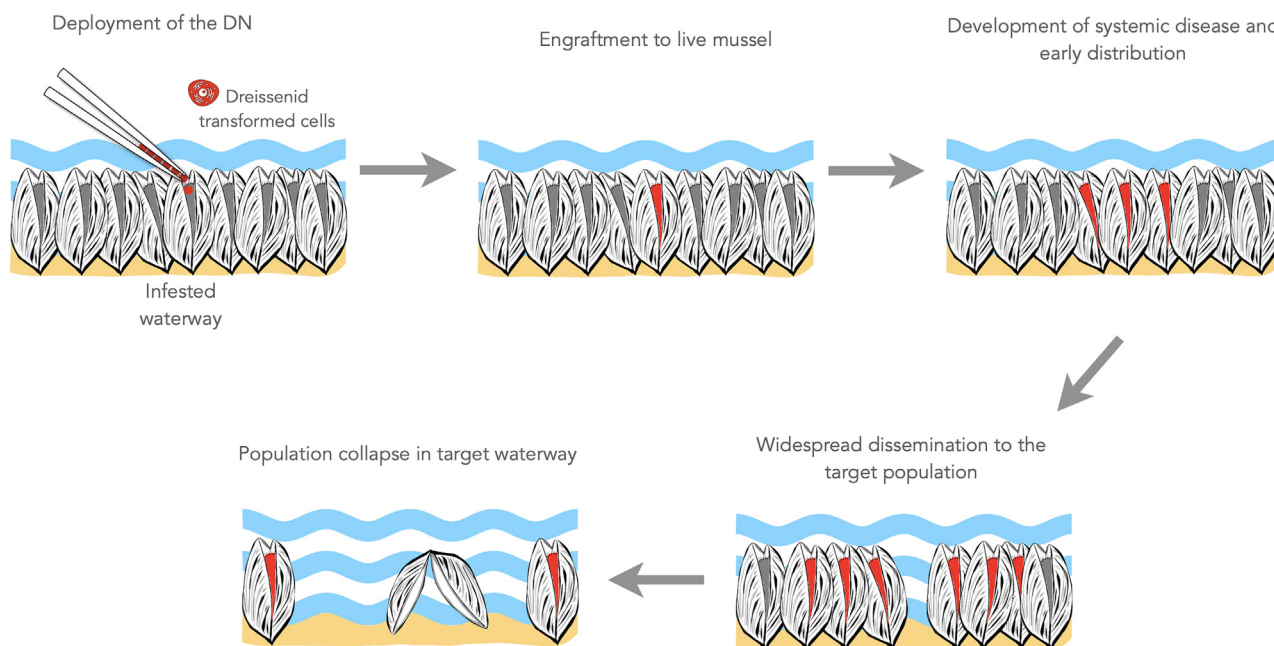
Figure 4. Quagga mussel control by disseminated neoplasia (DN)
Disseminated neoplasias (DNs) can be transmitted between bivalves in close proximity through seawater. Given the absence of natural pathogenic DNs in quagga mussels, it may be possible to engineer a DN from healthy quagga mussel cells. After the development of systemic neoplasia, the widespread dissemination of cancer-causing oncogenes could occur. Mussels growing at high density in inland lakes and reservoirs could be highly susceptible to the toxic effects of DNs resulting in the collapse of the target population.

determination and differentiation in golden mussel gonads.[144] Although much about these processes in bivalves is still unknown, it is believed that they are oligogenic and influenced by environmental factors.[145] Therefore, gene-environment relationships appear to be key.

The transcription factor families Drmt (doublesex and mab3-related-transcription factor) and Fox (forkhead-box) are involved in sex determination and differentiation in model organisms.[146] Evidence also suggests their involvement in bivalves. *Dmrt1* and *Foxl2* have shown sex-biased expression patterns in several bivalve species, including *L. fortunei*.[144,147–149] The knockdown of *Foxl2* and *Dmrt1* genes by RNAi silencing in the oyster *C. gigas* resulted in a reduction in gonad volume and the failure of gonadal differentiation into females and males, respectively.[150] Starvation has also been shown to alter the expression of these genes in *C. gigas*, leading to a delay in gonadal development and a bias toward a higher proportion of males in the sex ratio.[151] This provides further support for the gene-environment hypothesis for sex determination and differentiation in bivalves and for the involvement of these two antagonist genes in these processes. Therefore, in the search for candidate target genes to disseminate sterility in the golden mussel, the focus has been on the *Dmrt* gene family, which also includes the *doublesex* gene successfully used as a target for gene drive in *A. gambiae*.[60] The recent assembly of a chromosome-level genome for the golden mussel[152] has allowed us to identify and characterize the Dmrt family in *L. fortunei*, including the main candidate target gene, *Dmrt1l* (unpublished results). However, it is still necessary to experimentally validate the knockout phenotype and haplosufficiency of this gene in *L. fortunei*.

To make gene drive viable, it is also crucial to identify and validate functional promoters for obtaining the gene drive construct. In our ongoing project, we are using a GFP-based reporter system to evaluate candidate promoters, including endogenous promoters, which are expected to be specific to germ cells, as well as exogenous promoters such as CMV and U6. We are testing these promoters by transfecting naked plasmid DNA into *L. fortunei* embryos obtained through induced spawning in captivity. We are also delivering the plasmid DNA into adult mussels through injection in the adductor muscle, which has been shown to work for the delivery of nucleic acid (dsRNA) in other bivalves.[43,153] These plasmid DNA delivery methods are also being used to test genetic modification protocols, particularly for genomic insertion via the CRISPR/Cas9 system.

Due to the length of time required for gene drive development, the complexity of population dynamics, and the regulatory challenges involved, mathematical models are commonly used to test scenarios, anticipate risks, and assess requirements associated with gene drive technology.[154,155] While most current models tend to focus on gene drive efficiency, the inclusion of other important ecological aspects would enhance assessments of gene drive risk. Informative aspects include organisms life histories and those required by the European Food Safety Authority (not regionally applicable) and WHO guidelines for genetically modified organisms.[156] We are currently building a model specific for the golden mussel using the SLiM framework,[157] which allows for the customization of reproductive strategy and is considered one of the most powerful models available for gene drives.
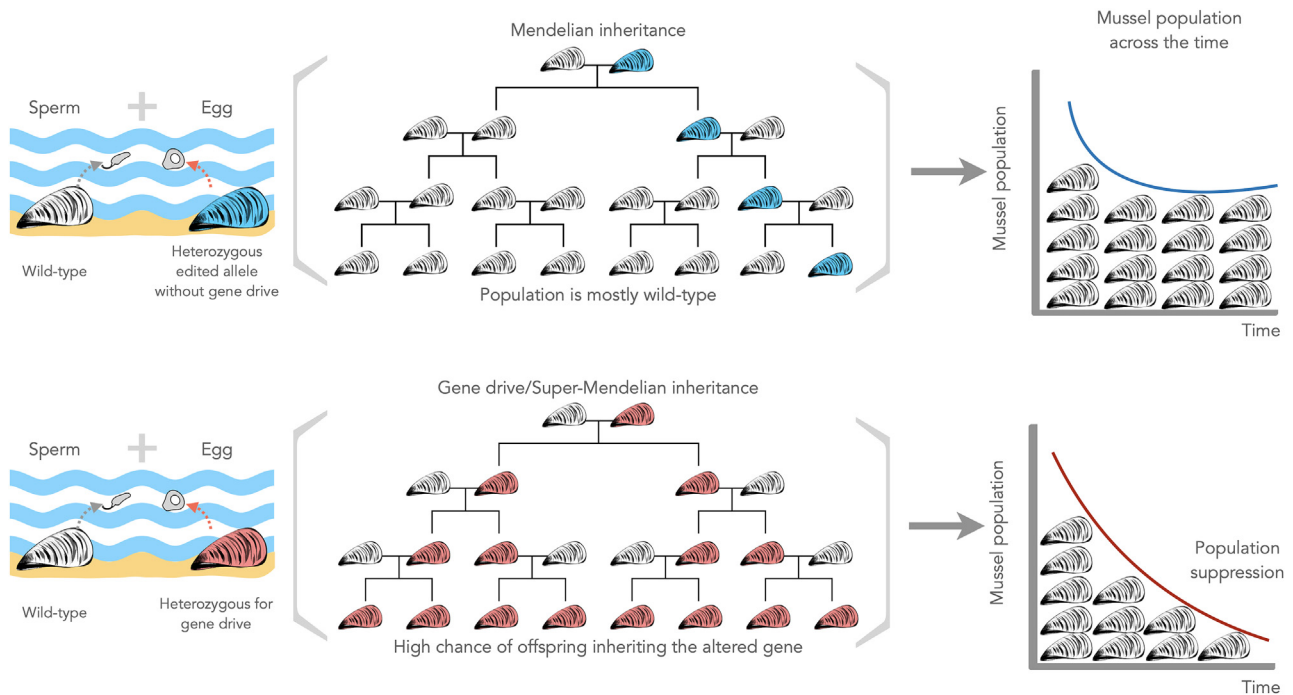
Figure 5. Gene drive for the control of the golden mussel

Heterozygous edited alleles without gene drive have a 50% chance of being inherited by offspring when crossed with a wild-type organism. Gene drives increase the probability of transmitting the modified genes to offspring at frequencies greater than 50%, a pattern known as super-mendelian inheritance. Heterozygote offspring remain fertile while only producing gametes with the gene drive construct, leading to the more frequent formation of sterile homozygous organisms, and resulting in population decline over generations.

Despite all the challenges, gene drive technology offers a promising avenue for the control of the golden mussels and the reduction of associated environmental and economic impacts. Further research would improve our understanding of this mussel's biology and inform effective gene drive strategies to control its populations. These efforts will be enhanced by engaging multidisciplinary groups in golden mussel research and collaborating within the community of researchers currently addressing invasive freshwater mussel challenges worldwide.

LIMITATIONS AND BARRIERS TO APPLYING GENETIC BIOCONTROL TOOLS IN INVASIVE MUSSELS

Genomic resource tools

Knowledge of the genome is essential for the design of new targeted genetic biocontrol techniques to eradicate invasive mussel species.[158] Recently, the *D. polymorpha*,[126] *D. rostriformis bugensis*,[159] and *L. fortunei*[152] genomes were reported. Some features of these genomes, such as their large size and a high proportion of repetitive DNA regions, large-scale structural variants, and high levels of heterozygosity reflect a very complex genomic architecture of bivalves.

These genomes, along with a large number of other recently published bivalve genomes, provide a resource for comparative genomic studies that could shed light on common mechanisms of response against stressors, pesticides, or biocontrol treatments. Nevertheless, current methods for sequence annotation are imperfect and may produce errors in the accurate identification of exons and untranslated mRNA regions. We encountered limitations in automated annotation when characterizing the *D. polymorpha* byssus genes.[126] The primary amino acid sequences of these byssal proteins were known, but in many cases the genome annotation algorithms failed to predict the corresponding transcripts. For this reason, manual annotation was required to identify many of the exons that encode the known byssal proteins.[160]

Transcriptomic data from adult tissues and developing embryos have also been used to annotate these mussel genomes, but these transcriptomic datasets and the resulting annotations are far from complete. Regardless, a diverse transcriptome repertoire that comprehensively represents the expressed RNA during different life stages and in different tissues is desirable for modern genetic biocontrol strategies that rely upon genome editing. Small RNA (~20–30 nt) is found in all eukaryotes and is processed through RNAi pathways. Small RNAs and their regulatory regions are not currently well annotated in invasive freshwater mussel genomes. A better understanding of small RNA pathways could contribute to developing efficient inhibitory mechanisms such as RNAi.

Furthermore, because it is imperative that any developed RNAi control tool is highly specific to the target species, it is also important to have genomic data for cohabiting non-target species that could be exposed to the treatment. Often, these species can be

underrepresented in available genomic repositories, which can make interfering RNA design challenging and may necessitate more extensive specificity testing after candidates are produced.

Lack of robust lab culture methods

Although a vast literature exists describing the reproduction and developmental processes of diverse mussels,[161] few laboratories have successfully cultured larvae of *D. polymorpha*, *D. rostriformis bugensis*, and *L. fortunei* through metamorphosis and settlement due to the need for space, equipment, and expert staff to (1) properly care for systems, (2) rear algae as food and ensure adequate larvae nutrition, (3) prevent the contamination of culture water by predators or bacteria, and (4) keep water quality conditions optimal.[130,162–166]

To begin rearing, adult mussels can be encouraged to release gametes in the lab with a temperature increase, the introduction of a gonad slurry, or the addition of serotonin hydrochloride for *D. polymorpha* and *rostriformis bugensis*[128,166] and Spectrus CT1300 for *L. fortunei*,[167] which results in the highest spawning rate in reproductively ripe individuals. Females typically take twice as long to spawn compared to males and fresh sperm results in the best rates of fertilization. Thus, successful fertilization in the lab requires proper timing and planning.[128,166,167] The settlement of planktonic larvae is highly variable and depends on temperature and nutrition. For instance, if larvae are held at 22°C, settlement occurs at 21 days for *D. polymorpha* and 32 days for *D. rostriformis bugensis*[163,168], while *L. fortunei* settles in 11 days at 28°C.[167] Larvae develop best in the absence of turbulence and other hydrodynamic forces that cause mechanical damage, particularly during the trochophore, D-shaped veliger, veliconcha, and pediveliger stages, prior to metamorphosis.[169,170] Preventing interactions with other veligers is also important and a density of <1 larva/ml is recommended.[163,166]

Moreover, significant mortality occurs during the transitions between the D-shaped, umbonal,[171,172] and the veliconcha stages[166] likely due to shifts in metabolic demand toward organ development as seen in marine bivalves.[173,174] To meet nutritional needs, larvae require a diet of algae that are the appropriate size (<6 μm) for selection by gill cilia and ingestion at each developmental stage and are comprised of a diverse fatty acid composition, particularly polyunsaturated fatty acids.[168,175–178] To reduce the presence of predators or competitive protozoa and rotifers, broodstock can be treated with sodium hypochlorite solution.[163,167] The impacts of fouling bacteria can also be reduced by the addition of antibiotics.[163,166]

Larval mussel development in static or recirculating systems is further supported by high-quality water, defined as as low total ammonia nitrogen (NH_4^+), hardness ($Ca(HCO_3)_2$) above 31 ppm,[179] pH 7.4–9.4,[180] Ca^{2+} concentrations above 12 mg/L,[172,179,180] and Na^+, Cl^-, K^+, and Mg^{2+} above a minimal threshold[181] to maintain conditions viable for respiration, waste abatement, and growth of shell material.

These exacting requirements, along with the need for a large space and the staff time to culture food and maintain larval tanks makes successful culture of invasive mussel larvae challenging.

Lack of existing molecular genetic tools and transgenesis methods

In general, non-model organisms lack advanced molecular techniques. The sequences that control fundamental processes such as DNA replication, transcription, RNA processing, and translation have not been well defined for these organisms. Regulatory sequences that could be used to control reporter gene expression have not been characterized. There are developed techniques for monitoring endogenous mRNA, but there are no established reporter genes or cell lines that allow for more rapid gene expression studies.

There are currently no established methods for transgenesis or targeted mutagenesis in invasive mussels. There has been some progress in developing techniques for monitoring and manipulating genes in commercially important marine bivalves (i.e., introducing nucleic acid via injection[182] or soaking[183], as well as CRISPR[184] gene editing). However, at present, most techniques for the genetic manipulation of invasive mussels are being developed *de novo*. Current culture limitations may require alternative transgenesis approaches.[185]

Phenotypic assays for assessing effects of manipulations

Phenotypic screening is an essential aspect of assessing the effects of genetic manipulations. A wide variety of methods have been developed to assess toxicology in dreissenid mussels and efforts are currently underway to harmonize these methods so that comparisons can be made more easily between laboratories.[186] Our groups are also currently working on establishing quantitative phenotypic assays for attachment, filtration of food particles, growth, reproduction, and survival. In addition, the assessment of molecular phenotypes by RT-qPCR or RNA-sequencing will also provide valuable insights into the efficacy of genetic manipulations. Moreover, methods for the robust testing of potential off-target effects on native mussels or non-target organisms will help to establish that the observed changes in the phenotype are specific to the disruption of the target gene and not due to off-target effects, and that the genetic biocontrol tools under development will not have undesired impacts on other organisms.

CONCLUDING REMARKS

Here we summarize existing methods for genetic biocontrol and our efforts to apply these tools to control freshwater invasive mussels. We aim to overcome the many challenges to developing effective, specific, and safe genetic biocontrol tools through collaborative interactions amongst our groups and by employing the diverse, multidisciplinary approaches described above. In addition to surmounting the many

technical hurdles to demonstrate laboratory-based proof-of-concept, defined regulatory pathways and stakeholder engagement and education will be needed to enable the safe and responsible field deployment of these potentially powerful tools.

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AUTHOR CONTRIBUTIONS

Conceptualization, V.H.H.E and D.M.G; writing – original draft, V.H.H.E., S.B., L.G., J.A.A., S.T.S., M.C.S., B.M., C.M.M., T.M.E., K.K., C.A.R., and D.L.W.; writing –review & editing, V.H.H.E, Y.J.P., M.F.R., and D.M.G.; supervision, D.M.G.

DECLARATION OF INTERESTS

S.T.S and M.C.S. are co-founders of Biomilab LLC and are members of its scientific advisory board and have management/advisory roles and a financial interest. Biomilab LLC has patents related to this work. Bio Bureau Biotecnologia is a private, for-profit company with commercial interests in invasive mussel biocontrol. J.A. A. is a shareholder of Bio Bureau Biotecnologia and its Scientific Director. M.F.R. is a shareholder, founder, and member of the scientific advisory board of Bio Bureau Biotecnologia. The remaining authors have no competing interests to declare.

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