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A sustainable synthetic biology approach for the control of the invasive golden mussel (*Limnoperna fortunei*)

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The recent development of the CRISPR-Cas9-based gene drive has created the conditions to seriously consider this technology to solve one of the major environmental challenges in biodiversity conservation i.e. the control of invasive species. There is no efficient control method for golden mussel infestation available so far. Here we discuss the technical and economic feasibility of using a synthetic biology based approach to fight and control the invasive mussel *Limnoperna fortunei* in South American rivers and reservoirs.

Keywords: Golden Mussel, *Limnoperna fortunei*, Biological Invasion, Synthetic Biology, Gene drive, CRISPR-Cas9, South America
https://doi.org/10.7287/peerj.preprints.27164v1

1. THE INFESTATION CONTINUES

Almost 30 years after the golden mussel *Limnoperna fortunei* invaded Latin America (Uliano-Silva et al., 2013), there are no signs that this infestation is under control or even leveling off. A recent survey of all hydroelectric power plants (HPPs) in Brazil conducted by our research group found that **40% of the HPPs, representing 55% of Brazil's installed hydroelectric energy generating capacity, are infested**.

As far as we know, golden mussels have not reached the Amazon region, their advance probably slowed by the reduction of water levels in the Pantanal watershed. Nevertheless, golden mussels continue to spread; the latest reports include eastern segments of the São Francisco River, one of the great waterways of South America, which supplies water to more than 12 million inhabitants (Barbosa et al., 2016).

The harm caused by *Limnoperna fortunei* infestation includes economic losses, environmental degradation, and social disruption. The operator of the Sobradinho power plant declared at a conference in 2016 that the company (São Francisco Hydroelectric Company, CHESF) spent approximately USD 510,000[1] annually on the chemicals needed to keep the plant's pipes clean. Using data from another operator, CTG Brazil, the second largest private HPP operator in the country, we estimate that the cost of monitoring and maintenance due to golden mussel fouling HPP infrastructure in Brazil ranges from USD 6.9 to 8 million[1] annually. These expenditures, however, are dwarfed by the revenue HPP operators lose every time they halt a turbine – typically for three days – for mussel-related maintenance. Using a conservative selling price of USD 55 per MWh, we estimate that for all infested companies operating in the country, the opportunity cost (lost revenue) due to the aforementioned halts, is on the order of USD 120 million[1] a year.

There is also regulatory and civil litigation risk. The continuous application of hazardous chemicals to control mussel proliferation in the plant's pipes also puts the HPP operator's environmental operating license at risk. Several HPP operators have already spent millions defending themselves against lawsuits filed by public prosecutors and by non-governmental or-

[1] the contemporary exchange rate in 2012 was USD 1.00 = BRL 2.75

ganizations (NGOs) representing riverine communities affected by the loss of fisheries in the reservoirs and the unpleasant sequelae of decomposing mussels exposed when reservoir water levels fall. As a result of a civil suit in 2012 the São Paulo Energy Company (CESP) and the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA), Brazil's leading federal environmental regulatory authority (akin to the U.S. Environmental Protection Agency – EPA), were ordered to pay compensation of USD 10.9 million[1] to users of the Ilha Solteira reservoir (MPF, 2012).

Finally, there are concerns that the golden mussel may be shifting the balance among aquatic species. Evidence of such ecosystem disruption includes the toxic cyanobacteria blooms that follow *Limnoperna fortunei* infestations. Voracious filter feeding by this invasive species might even alter the relative proportions of river water nutrients (Boltovskoy & Correa, 2014).

In 2018, IBAMA chose the golden mussel as one of three priority invasive species for control, and published a control and eradication management plan, which was developed with input from the scientific community and industry. The plan established goals, including solving this biodiversity issue by 2030 (MMA, 2018). Without an effective technology against golden mussel proliferation and a sustainable business model to support control and eradication efforts over a period of years, we believe, such ambitious goals unlikely to be met by 2030.

We are inspired by the successfully application of the sex-distortion gene drive strategy to two malaria vectors: *Anopheles gambiae* (Hammond et al., 2016) and *Anopheles stephensi* (Gantz et al., 2015). We wonder whether a similar strategy based on the introduction of a Genetically Modified (GM) organism bearing self-limiting features would be a feasible strategy to control *Limnoperna fortunei* in South American rivers. If so, could the research and development costs of a genetic solution pay back? What might be risks to the environment and to society? Would a disinfestation business using a GM organism solution be viable? This article explores answers to these questions.

2. WHAT CAN WE DO?

We performed a technical and economic cost-risk/benefit assessment using conventional financial parameters to critically evaluate the proposed business. We also employed Steve Blank's Lean Startup method (Blank, 2007) to consolidate primary and secondary market data and information about prospective clients.

We started mapping the development of the solution using the widely acknowledged Technology Readiness Scale (TRL) (NASA, 2016), presented in Table 1.

There is significant evidence that synthetic biology enables safe and effective manipulation of genetic elements to create novel transgenic organisms with self-limiting demographic capability. The Oxitec mosquito strain OX513A that expresses a conditional lethality trait (Phuc et al., 2007) has been characterized for more than a decade. More recently Hammond et al (2016) developed a so-called "sex-distortion gene drive" in the malaria mosquito vector Anopheles gambiae highlighting the tremendous versatility and precision of CRISPR-Cas9, even in non-traditional model organisms.

Other recent scientific milestones support us in our claim that we can transpose the mosquito model to mussels. The first and most important was the publication of the golden mussel genome last year by our group (Uliano da Silva et al., 2018). This enabled us to identify genes related to reproduction and infertility phenotypes. To date we have identified 26 genes related to reproduction that will be further validated biologically. It also allowed us to design plasmid and CRISPR constructs to edit the genome. The modification of a mollusk genome using CRISPR-Cas9 by Perry Henry in 2015 had already demonstrated the applicability of the widely used genome editing technology to our target organism.

Over the past two years, we have accomplished most of TRL#3 goals, obtaining genetically modified *L. fortunei* sperm cells (Figure 1).

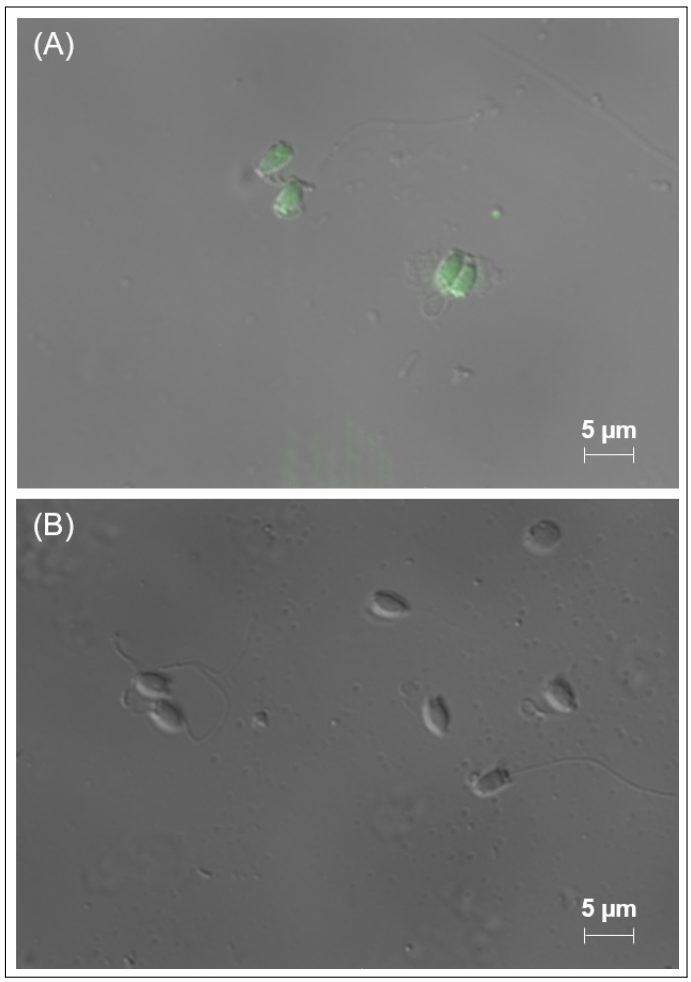

Fig. S1. Glowing golden mussel sperm, result of a transfection assay performed in sperm cells with a gene reporter vector (pcDNA3.1+C-eGFP backbone), where the eGFP gene was placed under the control of vasa, a germline-specific promoter. Transfected sperm cells are shown in (A), while (B) shows negative control. These images are the result of a successful transfection experiment that is currently being repeated in our laboratory. The full article describing the methodology and results is being prepared for submission.

The next step will be to integrate the CRISPR-Cas9-based gene drive construct into the mussel genome and reach a viable adult organism that carries this modification. The principal challenge has been the gaps in knowledge when working with a non-model species such as the mussel. It is important that environmental and medical scientists start to use the golden mussel as a model species in the lab, so we produce the body of knowledge necessary to resolve technical problems as they arise

Table 1. Technology Readiness Level of the biotechnological disinfestation service project

Technology Readiness Level	Accomplishment description
TRL #1 Basic Principles	• Open field performance of engineered Aedes aegypti mosquitoes for the control of dengue and other diseases (Harris et al., 2011). • Scientific evidence that GM organisms bearing a gene drive can limit the population growth for malaria vector mosquitoes under laboratory conditions (Windbichler et al., 2011; Hammond et al., 2016). • The genome of the golden mussel is published (Uliano da Silva et al., 2018).
TRL #2 Conceptual model	• In this article we conceptualize a GM mussel with self-limiting demographic features based on CRISPR-Cas9 gene drive sex-distortion: GM females are infertile while GM males spread female infertility in the population. • Technical and economical viability and sustainability studies are made.
TRL #3 Proof of concept	• Identification of haplo sufficient, somatic female-fertility genes in the Limnoperna fortunei genome. • Computer simulations of Homing Endonuclease Genes (HEG) dynamics along with mussel demography. • Construction of CRISPR-Cas9 gene drive vectors targeting female fertility genes. • Genetic transformation of mussel cells.
TRL #4 Lab scale prototype	• Generation of genetically modified mussels bearing the CRISPR-Cas9 allele. • Genotyping, phenotyping and demonstration of haplosufficiency. • GM mussels laboratory testing in experimental aquaria under highly controlled conditions according to the phase 1 of World Health Organization (WHO) guidelines.
TRL #5 Field scale prototype	• Breeding of GM mussels with wild-type specimens in controlled mesocosm experiments. • Empirical evaluation of homing rate and CRISPR homing allele frequency in 4 generation experiments (phase 2 of WHO guidelines).
TRL #6 Scale-up	• Production of large (109) quantities of GM embryos that can stand transportation over several hours (12–24h) to be seeded in laboratories by a reservoir.
TRL #7 Pilot test	• Open field performance of GM mussels (phase 3 of WHO guidelines). • Disinfestation of a reservoir branch upstream of a power plant using caged GM mussels, in conjugation with chemical disinfestation of the power plant. • Monitoring of reinfestation and transgenic DNA using quantitative PCR (phase 4 of WHO guidelines).
TRL #8 Regulatory step	• Biosafety and efficacy assessment of the introduced genetic elements. • Standard Operational Procedures to produce GM embryos, GM adults, raise seeds, cage GM mussels, etc. • MAPA (Brazilian Ministry of Agriculture, Livestock and Food Supply), IBAMA, CTNBio (Brazilian Technical Biosafety Commission) licenses, certificates and permits.
TRL #9 Go to market	• Successful disinfestation of an entire hydroelectric reservoir. • Disinfestation as a service available to the market.

during development. This will become mandatory, as a much better understanding of the organism and its gene expression-environment relationships is necessary if we want to comply with the guidelines on the use of gene-drive systems of the US National Academy of Sciences (NASEM et al., 2016).

We also need to start working now to address the challenges that will come up in TRL#6 as we scale up production of embryos, in order to accelerate the deployment of the solution. We can use the non-modified invasive organisms to establish protocols to massively produce and transfect embryos, larvae and seeds. A model laboratory can be established in a reservoir inlet to develop the tools, such as probes, cages and monitoring tools that would allow a pilot-scale field experiment when the first batch of the genetically modified organisms (GMOs) is ready for field testing.

Of course the release of a GMO into the environment to control an invasive species carries technical, commercial and environmental risks and has to be done responsibly. We are closely following the discussions stemming from concerns raised over CRISPR and gene drive such as those published by NASEM et al (2016) and following World Health Organization (WHO) recommendations of the 4-phase testing pathway for GM mosquitoes that constitutes an interim standard of practice for gene drive development procedures (WHO, 2014). We are keeping abreast of studies that address problems associated with the technology, such as the recent release of CRISPR-Cas12 (Strohkendl et al., 2018).

In 2005 Brazil enacted exemplary biosafety legislation, Law Nº 11105 of 2005. It established a National Commission for Biosafety (CTNBio) as the governmental entity responsible for safeguard the environment. Regulations promulgated by the CTNBio require many tests and validations in an extremely rigorous process before a GMO can be deemed safe for consumption or release into the wild.

We believe that the application of sex-distortion gene drive to the *Limnoperna fortunei* infestation problem is a bold solution justified by the absence of alternative technologies with the potential to protect our hydroelectric energy infrastructure and the environment from this highly invasive species.

Estimates vary, but the costs involved in RD and regulatory compliance could easy surpass USD 100 million. Oxitec, a company at the forefront of insect control through genetic engineering, received over USD 13.8 million in venture capital and government funding to finance the costs of technological development of its main products (GeneWatch, 2010). Phillips McDougall (2011) reported that the average cost associated with the research, development, and licensing of biotech crops that were marketed from 2008 to 2012 by companies such as Bayer, Monsanto, and Syngenta was USD 136 million, with 25.8% (USD 35 million) of these costs related to compliance with biosafety regulation.

Readers will note that we did not estimate a time frame, as it is extremely difficult to establish how long it is going to take to develop a product that is still at Technology Readiness Level 1 or 2. If we look at drug development benchmarks in the pharmaceutical industry, it is reasonable to assume that RD may take 10 to 12 years (Grabowski, 1991). After 10 years, the benchmark in Brazil's GMO sector for a technology to be approved by CTNBio is less than a year (BRASIL, 2011), but that is just the time needed for data and compliance evaluation by the regulatory agency. Recently this time has been increasing (Phillips McDougall, 2011). Whereas CTNBio approval can take one year, the execution of safety experiments can take up to 10 years, as shown by the benchmark of the Brazilian Agricultural Research Corporation (Embrapa) of a genetically modified bean (BRASIL, 2011). In addition to CTNBio approval, IBAMA must issue a license authorizing its commercialization and specifying how the product must be controlled (Decree 4074 of 2002).

3. MODELING THE BUSINESS

Given this scenario, we decided to analyze the economic viability of the business that would be possible were we to reach TRL#9. The Business Canvas Model (Osterwalder Pigneur, 2013) is a tool to consolidate all the information from the customer development phase in which you validate, in a hypothesis-driven 'scientific' way, several key assumptions of your business model.

To draw our business canvas model, we interviewed more than a hundred stakeholders of HPPs and identified the main customer segments as maintenance and operations engineers, environmental managers responsible for licensing and monitoring, financial managers responsible for the budget and the legal department head that oversees regulatory affairs, compliance, and litigation.

Given the range of problems that HPPs face due to golden mussel infestation described above, it is not surprising that the main value proposition is risk reduction, followed by cost reduction and performance gain. Assuming that all RD activities will be completed, we estimated the Capital Expenditure and Operational Expenditure (CAPEX/OPEX) investments that will be required. Considering a laboratory with a capacity of 100,000,000 seeds (Blacher, 2012), we estimated a CAPEX investment of USD 640,000[1] for each laboratory construction and an OPEX investment of USD 3 million[1] annually. The cost of land was excluded from this model, because we assume that the contracting HPP will be responsible for providing an area for the establishment of a cultivation support laboratory. Depreciation and amortization of the investments in infrastructure and equipment were set at 20% per year over five years. Revenue was estimated based on two hypothetical services: a subscription to a disinfestation maintenance service (that includes infestation monitoring) and a stand-alone disinfestation service (with microencapsulated pre-disinfestation such as the one proposed by Calazans et al., 2013).

4. A DEVELOPMENT WORTH PURSUING

Brazil has 12% of the world's freshwater (Shiklomanov et al., 2000). The ecological benefits to society of these freshwater resources and the biodiversity they are home to, are hard to quantify, and even harder to value in monetary terms. Thus they typically are not considered in calculations of the operational expenditure (OPEX) of most economic activities including electric energy generation. Our preliminary evaluation suggests that the cost of developing a definitive solution for the invasive golden mussel is small compared to the benefits of protecting and conserving these environments. The cost of developing this original and high tech solution could be offset, i.e. financed by those institutional players who would reap the greatest economic and environmental benefits of controlling the invasion, through compulsory RD investment of the electric energy sector or similar instruments. Over the last 20 years, more than USD 3 billion was invested in RD projects and we are sure that this innovative project complies with the requirements of the regulatory agency ANEEL. Our financial figures have shown that, using a discount rate of 20%, we could estimate a Net Present

Value (NPV) of USD 857,000[1], a value that would be considered modest for most investment vehicles. However, the Internal Return Rate (IRR) reaches 63.0%, which is substantially higher than 17%, the mean IRR of US equity funds. Moreover, business is estimated to reach breakeven in three years with cumulative cash flow exceeding free cash flow (payback) in the fourth year. Thus, the disinfestation business is not only viable, but it seems a good business. Therefore, together with the exciting initial results that we have obtained, we are ready to move on to the next milestone on the TRL scale, building a definitive solution for the golden mussel infestation, adhering to the best practices in biosafety and respect for the environment.

5. ACKNOWLEDGEMENTS

The authors are indebted to dr. Andrea Crisanti from Imperial College for his review of the manuscript and his commitment to provide technical support throughout the project.

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