CEREBRO FAVCIBVS VTERO
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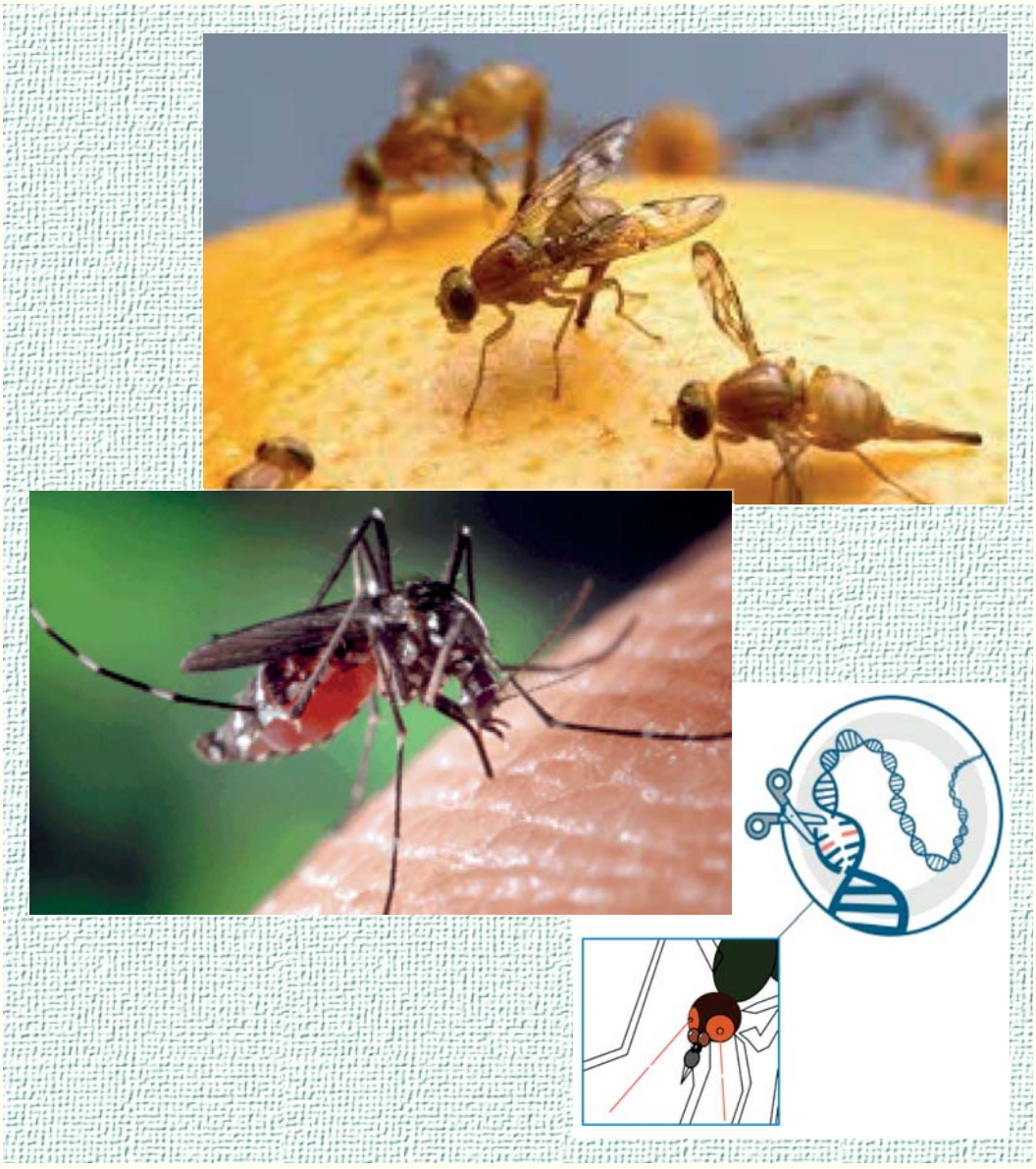
TENENT

Tavole Rotonde sui maggiori problemi
riguardanti l'Entomologia Agraria in Italia
Sotto gli auspici del MIPAAF

XXXIII.
APPROCCI GENOMICI E MOLECOLARI PER IL CONTROLLO DI SPECIE INVASIVE DI INSETTI DI INTERESSE AGRARIO E SANITARIO



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PRESENTAZIONE

Il tema oggetto della Tavola Rotonda odierna, promosso dalla Accademia Nazionale Italiana di Entomologia, considera alcuni aspetti delle ricerche attuali su nuove metodologie di controllo eco-compatibile delle popolazioni di insetti di importanza agraria e sanitaria. Il controllo degli insetti su base biologica rappresenta una delle maggiori sfide che la scienza e la società debbono affrontare per far fronte a possibili emergenze, senza tuttavia disattendere il rispetto dell'ambiente. L'uso di tecniche ecologicamente valide sono state introdotte nella seconda metà del XX secolo, sia con l'intuizione della Tecnica dell'Insetto Sterile (SIT), che con l'introduzione di nemici naturali di specie nocive.

L'avvento delle metodologie del DNA ricombinante e dell'ingegneria genetica, accompagnate dalle maggiori conoscenze delle specie considerate, ha stimolato miglioramenti nell'applicazione di SIT, e l'individuazione di strategie alternative che sfruttano fenomeni biologici atti a ridurre la potenzialità riproduttiva e la capacità vettoriale delle popolazioni.

Le specie considerate in questa Tavola Rotonda riguardano mosche della frutta e culicidi, specie che determinano drammatiche conseguenze di carattere socio-economico e sanitario, non solo in Paesi in via di sviluppo, ma anche in nuovi areali, recentemente occupati nel mondo a causa della globalizzazione dei traffici commerciali e dei cambiamenti climatici.

Le relazioni della Tavola Rotonda riportano studi innovativi e prove di applicazione di nuove strategie di lotta agli insetti: dalle modificazioni del genoma, all'uso di simbionti e all'identificazione di caratteristiche biologiche delle specie in funzione del loro controllo.

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Giuliano Gasperi, Accademico

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EDITING POPULATION GENETICS FOR VECTOR CONTROL

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Editing population genetics for vector control

Mosquitoes species of the genus *Aedes* and *Anopheles* are responsible for transmitting severe and life threatening diseases including a number of viral encephalitis, Dengue yellow fever, Malaria and more recently Zika. A few *Anopheles* species are responsible for causing 200 million cases of malaria every year and the death of half a million children under the age of five in less developed regions of Africa. During the last twenty years a worldwide concerted effort based on the use of bed nets, insecticides and drug treatment has halved malaria morbidity and mortality. The implementation of these control measures necessitates about 10 billion per year mostly in the form of donations thus questioning the long-term sustainability of this approach and its suitability for eradicating the disease in the next 30-40 years. The vectorial capacity of a mosquito species to transmit malaria depends on genetically determined traits such as feeding behavior, longevity, density and ability to support parasite development. Editing of the corresponding genes is anticipated to impair mosquito ability to transmit malaria. The recent development of CRISPR/CAS9 based gene drive technology has unlocked the possibility to selectively edit a mosquito population. Genetic modifications designed to either impair female fertility or interfere with mosquito ability to transmit the malaria parasite have been spread from few laboratory individual to large caged mosquito populations. These laboratory experiments have also supported mathematical modelling predicting how gene technology has the potential to eradicate malaria transmission in a span of few years from vast regions of Africa. Technical challenges in the development of a gene drive technology suitable for release include the development nuclease-resistant functional gene variant that would block the spreading of the drive as well as off target activity of the CAS9 nuclease that may generate undesirable mutations at other loci. We present here a number of solutions to overcome these problems.

KEY WORDS: vector-borne diseases; malaria control; gene editing; Anopheles; gene drive.

A BURDEN FOR HUMAN HEALTH

Mosquito-born diseases are a global burden for human health that is affecting billion of people, often exacerbated by climate change and global trade (MATHIEU & KARMALI, 2016; TJADEN *et al*., 2018). *Anopheles* and *Aedes* species (Diptera: Culicidae) transmit life threatening diseases such as malaria, yellow and dengue fevers, Chikungunya and Zika viruses (MAYER *et al*., 2017; BENELLI & MEHLHORN, 2018). For instance, half of the world population is at risk of malaria infection, which is caused by the protozoan human parasite *Plasmodium falciparum* and transmitted by infected *Anopheles* mosquitoes. (COWMAN *et al*., 2016). The disease affects 200 million people annually and still demands a death toll of over 400 thousand people every year, for the majority children in tropical areas of Africa and Asia, with dramatic and log-lasting impacts on socio-economic development of affected areas (SACHS & MALANEY, 2002; WHO, 2016; MCCORD *et al*., 2017). Control measures are focused on limiting exposure to mosquitoes through bed netting and insect repellents, managing mosquito populations using insecticides, and medical treatments, (CIBULSKIS *et al*., 2016). These management approaches have significantly helped limiting the global impact of malaria in the past few decades, but the high cost and the unavailability of high-efficacy vaccines make these options not fully sustainable in the long term and inadequate for eradicating the disease (HEMINGWAY *et al*., 2016; WHO, 2016; BENELLI & BEIER, 2017). This is urging the development of novel tools to tackle the emergency. The modification of mosquitoes through gene editing might be a game changer, opening new scenarios for managing vector-borne diseases through the control of vector populations (ALPHEY *et al*., 2002; GABRIELI *et al*., 2011; BURT *et al*., 2018).

DEFINING THE STRATEGY

We aim to provide a short overview of available approaches and propose a framework for imple-

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menting mosquito gene-editing technology, from idea to effective control, through distinct steps (Fig. 1).

The success of controlling disease vectors through genetic modifications depends on the effective suppression or replacement of vector populations following the release of modified insects. Various genetic modifications have been classified according to their expected dynamics and persistence into the target populations (ALPHEY, 2014). Self-limiting transgenes tend to decline in frequency and disappear rapidly from the population, unless maintained by the periodic release of additional modified insects. In contrast, some of the constructs are designed to be invasive and self-sustaining, spreading through the initial target population without further releases. Genetic constructs designed to be self-sustaining reduce costs of long-term programs, but impose a lack of post-release control (BURT, 2014).

Self-limiting strategies

The most conventional self-limiting strategies include the Sterile Insect technique (SIT), based on the release of radiation-sterilized males aimed to reduce the reproductive potential of the wild counterparts and decrease target mosquito populations. The release of transgenic sterile male mosquitoes carrying a dominant lethal genetic system (RIDL) have been used against the vector of dengue fever *Aedes aegypti* in the Caribbean, Brazil and Malaysia with mixed success (HARRIS *et al*., 2011; LACROIX *et al*., 2012; CARVALHO *et al*., 2015). Lab-reared RIDL *A. aegypti* males show high longevity but imperfect field mating competitiveness in comparison to wild-type populations, while SIT-developed sterilized *Anopheles* males display severe fitness costs in laboratory cages compared to the wild-type in terms of both adult longevity and mating competitiveness (HELINSKI *et al*., 2009). An approach based on male sterility induced by artificial trans-infection of maternally transmitted *Wolbachia* bacteria was successfully implemented for the suppression of *Aedes aegypti* populations and reducing dengue fever (WALKER *et al*., 2011). In *Anopheles,* native microbiota tends to inhibit vertical transmission of *Wolbachia* and elicits massive blood meal-induced mortality (HUGHES *et al*., 2014). Stable *Wolbachia* infections (*w*Anga strain) have been recently identified in natural populations of *An. gambiae* s.l. (BALDINI *et al*., 2014), and there is no evidence of cytoplasmic incompatibility (SHAW *et al*., 2016). Similar technical limitations have been observed for *An. gambiae* Dominant Sterile Male [Ag(DSM)] strains developed by SIT transgenic implementation based on Homing Endonucleases (HEGs) cleavage of the ribosomal rDNA repeats, which were exclusively located in the centromeric region of the X chromosome (KLEIN *et al*., 2012). In heterozygous Ag(DSM) males, the activity of beta2-tubulin promoter and consequently the expression of I-PpoI homing endonuclease during spermatogenesis induces the selective cleavage of the ribosomal rDNA repeats and the shredding of the paternal X chromosome in sperm cells (WINDBICHLER *et al*., 2008). The transmission of the I-PpoI enzyme via sperm to the embryos triggers a complete embryonic lethality as a consequence of the shredding of the maternal X chromosome.

Synthetic autosomal sex-ratio distorters that are

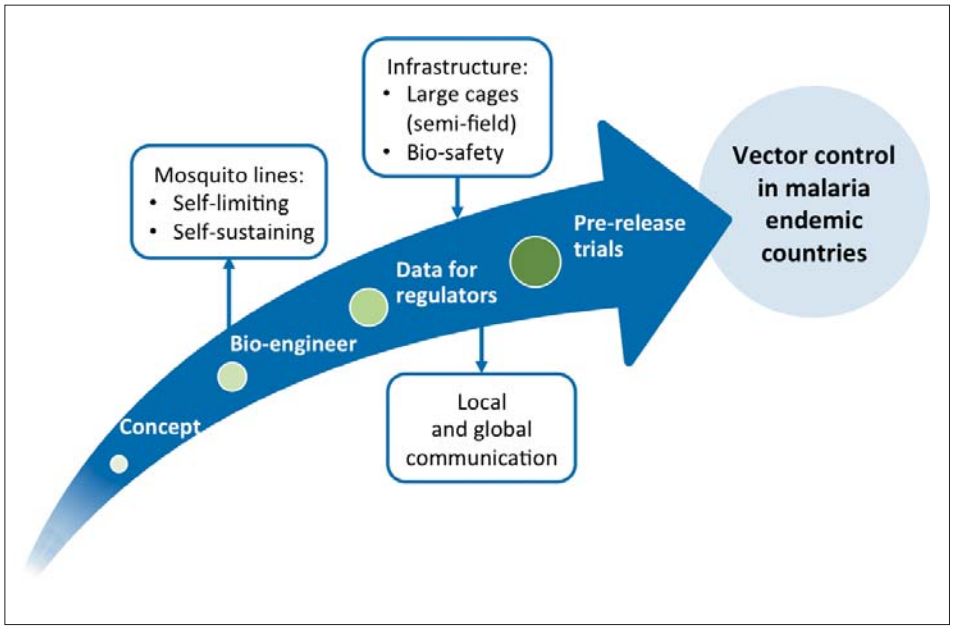

Fig. 1 – Gene editing approach for controlling vector-borne diseases: from idea to field.

fully fertile compared to Ag(DSM) have been generated using two alternative approaches. Engineering a reduction of I-PpoI *in vivo* half-life and restricting its activity to spermatogenesis using testis-specific promoters (beta2-tubulin) causes shredding of the X chromosome during male meiosis and leads to a fully fertile Paternal Male Bias transgenic line [Ag(PMB)1], with an almost 95% male offspring (GALIZI *et al*., 2014). Similarly, GALIZI *et al*. (2016) described a CRISPR-Cas9-based sex distortion system targeting X-linked ribosomal sequences; the expression of the Cas9 enzyme prevents the transmission of the X chromosome to their progeny, thus inducing 86% to 95% male bias. Both distorters can efficiently suppress wild-type mosquito populations in small cages, acting as powerful genetic tools for vector control.

Following the guidance framework for testing genetically modified mosquitoes proposed by the World Health Organization (WHO-TDR & FNIH, 2014), we assessed the kinetics of the Ag(PMB)1 sex-ratio-distorter in large-cages that replicate "semi field" tropical environments (Fig. 1). We conducted: (1) Model parametrization based on experimental life history data of wild-type and transgenic Ag(PMB)1 mosquitoes, and (2) Model validation comparing semi-field conditions to model predictions. We validated an appropriate modelling framework to estimate (*i*) the effect of regular releases of hemizygous Ag(PMB)1 males and (*ii*) the transgene PMB1 persistence into age-structured, generation-overlapping populations of *An. gambiae*. Modelling outputs predicted that Ag(PMB)1 male releases can significantly reduce the number of females into wild type populations (FACCHINELLI *et al*., 2019), and that PMB1 transgene is likely to disappear rather quickly under confined conditions that induce a reproductive behavior (swarming) close to natural conditions (< 2 years) (POLLEGIONI *et al.,* in review).

Self-sustaining strategies and gene drive technology

The self-limiting PMB1 sex-ratio distorter is an attractive system because of the reversibility of its effects, but necessitates conspicuous and repeated releases to achieve significant epidemiological effects (BURT, 2014). Self-sustaining gene-drive systems are predicted to have a more robust effect on population suppression by inducing their own spread in a non-Mendelian manner (DEREDEC *et al*., 2011).

Gene drives increase the likelihood that a modified gene will be inherited by its offspring. In sexually reproducing diploid organisms that have two copies of each gene, any single copy normally has a 50% likelihood of being passed to the offspring. Highly invasive gene drives can promote an inheritance bias of nearly 100 percent (Fig. 2). Assuming a closed random mating population and no fitness costs, a gene drive element with 100% transmission to the progeny would increase its frequency up to 0.99 in just ten generations (BURT *et al*., 2018). Naturally occurring selfish elements such as transposable elements, heritable microorganisms, and homing endonucleases (HEGs) tend to spread into a target population over multiple generations, even when released at low frequencies (BURT, 2003, 2014; BURT *et al*., 2018). HEG genes, in particular, are selfish elements found in primitive single-celled eukaryotes (e.g. yeast and algae) that encode cutting -enzymes able to recognize and cleave a 20 to 30-bp

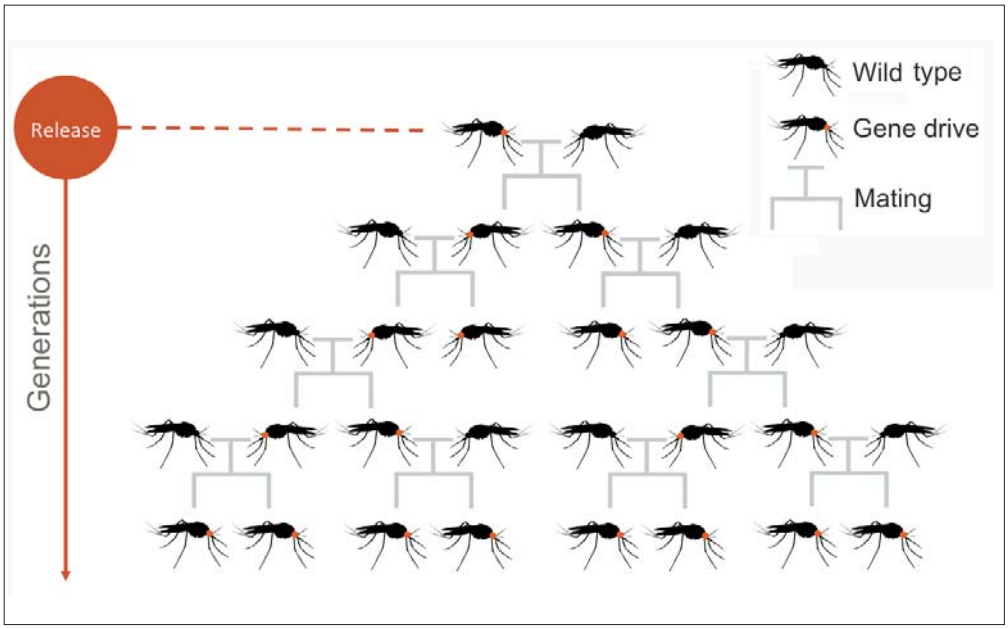

Fig. 2 – Spread of gene-drive systems in mosquito populations for vector control.

DNA sequence. HEGs are also located within the DNA recognition sequence, making it resistant to further cleavage. When HEG comes into contact with its intact homologous chromosome in heterozygous cells, then it cuts the target sequence. As a result of the chromosomal repair process (homology direct repair HDR mechanism) induced by the double-strand break, HEG can be copied converting heterozygous into homozygous cells. This mechanism is known as "homing" and promotes a rapid increase of HEG frequency in a population (Burt, 2003). HEGs are an ideal tool for disrupting suitable target genes essential for female fertility longevity, sex determination, host seeking or pathogen transmission (knock out scheme) and for spreading deleterious modifications that trigger population suppression (knock in scheme). In the last decade, an increasing number of artificial homing/gene drive systems based on modular nucleases have been proposed, including chimeric transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) (Gai *et al*., 2013). Although recent progress toward the development of both TALEN- and ZFN-based gene drive systems have been demonstrated in *Drosophila melanogaster*, difficulties in reprogramming DNA-binding modules and a low replication fidelity are limiting their application against malaria vectors (Simoni *et al*., 2014).

More recently, research conducted at the Imperial College in London sought to mimic natural gene drive processes and induce a Super-Mendelian inheritance of transgene/mutation in the offspring through CRISPR-Cas9 gene editing (Burt *et al*., 2018). These gene drives have been specifically designed to be active in the germline and disrupt genes essential for female fertility of *An. gambiae*, such as fertility gene AGAP007280 (Hammond *et al*., 2016). In CRISPR-mediated gene drives, the construct includes a single nuclease Cas9 and a small guide RNA that determines the DNA cleavage specificity of the target site. Once inserted in the genome and expressed within a germ cell, the Cas9 protein disrupts the AGAP007280 gene. In response to the double-strand breakage, the cell employs the HDR machinery to copy the construct, including any genetic cargo in the intact homologous chromosome. Although individual mosquito females with only one copy of the defective gene are still fertile, the gene drive ensures inheritance of the mutation in 99-95 % of the offspring and a rapid increase of AGAP007280 frequency in the population. As a result of this, the offspring has high probability to receive a copy of the gene drive construct from both parents, and all females homozygous for the mutation are sterile, while males continue to transmit the gene drive. In the best-case scenario, few generations may be required to spread the transgene/mutation from low frequency to near fixation through wild-type populations (Fig. 2).

This technology has the potential to induce a sustainable decrease of mosquito density by imposing a reproductive load on the population, although a strong selective pressure for resistant alleles, such as sequence variations of the target site of the Cas9 endonuclease, have been observed (Hammond *et al*., 2017). Allele variants might be pre-existing in the population or induced by the error-prone, non-homologous, end-joining (NHEJ) mechanism following Cas9 activity at the target site. The selection of in-frame-mutations, mainly indels of different length, was associated to the development of nuclear resistance to Cas9 cleavage, blocking the spread of gene drive after approximately ten generations in small cages (Hammond *et al*., 2017). Gene drive constructs that target highly conserved sequences, however, decreased the likelihood of nucleotide resistance.

The CRISPR-Cas9-targeted disruption of the *doublesex* gene (*Agdsx*) led for the first time to complete suppression of *An. gambiae* populations in small cages after 7-11 generations (Kyrou *et al*., 2018). The highly conserved *Agdxs* gene encodes two alternatively spliced transcripts, dsx-female (*Agdsx*F) and dsx-male (*Agdsx*M), and determines sexual dimorphism in anopheline mosquitoes. The CRISPR-Cas9 mediated disruption of the intron 4-exon 5 boundary in *Agdsx* inhibited the formation of functional *Agdsx*F transcript and induced female sterility, but did not affect male fitness. Homozygous null females ($dsxF^{-/-}$) show intersex phenotypes with male-specific traits including plumose antennae, abnormal proboscis and not-rotated claspers (Kyrou *et al*., 2018), and are unable to bite, feed on blood and lay eggs. Due to the functional constraint of *Agdxs* gene, no selection of nuclear resistant alleles has been observed in small cages after 14 generations. As recommended by the World Health Organization (WHO-TDR/FNIH, 2014) and the National Academy of Science of United States (2016), we are currently testing the doublesex gene drive in large cages, reproducing tropical environmental conditions to evaluate population dynamics and suppression potential of the gene drive construct in *Anopheles* mosquitoes.

Future directions

Gene drive technologies are a promising and powerful tool for controlling populations of malaria vectors, and further work should address and solve

technical challenges. For instance, the development of nuclease-resistant functional gene variants would block the spreading of the drive, and the off-target activity of the CAS9 nuclease may generate undesirable mutations at other loci (HAMMOND & GALIZI, 2018). Several strategies have been proposed to mitigate target site resistance: (*i*) Use of alternative tightly regulated germline-specific promoters to limit Cas9 activity and inhibit nuclear deposition into the embryo causing additional resistant mutations, (*ii*) Selection of novel or engineered endonucleases that show more tolerance to nuclear variation at the target site induced by non-homologous end-joining mechanism or naturally present in the wild-type populations, (*iii*) Exploring target sites within essential genes that are highly conserved in nature indicating selective pressure constrains and (*iv*) Multiplexing individual gene drives to recognize and target several short sequences in the genome offering a redundancy in terms of sites of cleavage. Few data are currently available about unintended off-target mutations generated by homing-based gene drives that might result in fitness costs. Ongoing gene drive research could generate effective genetic tools for malaria control, but the potential and the mechanism of self-sustaining gene-drive strategies as superior alternatives to self-limiting strategies remains to be fully tested in semi-field environmental settings.

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GENOMIC ENGINEERING OF INSECTS FOR SUSTAINABLE CROP PROTECTION

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Genomic engineering of insects for sustainable crop protection

Agriculture is under constant pressure to deliver safe products while reducing residues and environmental risks. One method contributing to integrated pest management, the Sterile Insect Technique, is in use for multiple species worldwide, residue-free and has developed novel technologies for improved strains over the last decades. Successful mass production of male-only populations, new developments in the field of genetically modified insects and enabling gene editing in the first insect pest species are presented and represent opportunities for developing sustainable crop protection.

KEY WORDS: Gene Editing, medfly, Sterile Insect Technique, CRISPR/Cas

Insects regularly attack agricultural products. They feed on plants and fruits, lay their eggs in parts of the plant that lead to the development of larvae which damage plants and crops. Several arthropods including the genera of Lepidoptera, Coleoptera, and Diptera are highly destructive pests in agriculture and can attack hundreds of crops worldwide. Therefore, preventional control or suppression programs are essential, but only effective in use in a few species such as the medfly, *Ceratitis capitata*. California alone can prevent estimated annual losses due to export restrictions of about $ 2 billion by using SIT programs (SIEBERT, 1994). Against other invasive pests, there are no sustainable, economical and effective control methods in place for a large-scale application. Thus, pest insects like the spotted wing drosophila (SWD), *D. suzukii*, which is a major invasive pest of many small and stone fruits (CINI *et al.*, 2012) or the Asian citrus psyllid, which carries a devastating plant virus, cannot be controlled adequately and sustainably at the moment. Those scenarios will lead to further uncontrolled proliferation and damage of crops, affecting not only the US but also Europe and Asia (WALSH *et al.,* 2011).

The available toolbox of control methods for the described pests consists mainly of physical, biological and chemical control. While physical and biological control is useful and relatively specific to the particular species, their widespread use in area-wide scenarios is often difficult for economic reasons. Instead, insecticides are used to respond in a fast, and for the farmers, established way of control option. This bears the risk of polluting ecosystems and ultimately also the food chain, destroying beneficial organisms, creating resistance to insecticides, requiring the development of ever more novel and effective insecticides. Also, long-term adverse effects on beneficial organisms are known, as shown by the example of neonicotinoids and bees. Some neonicotinoid substances with proven risks (clothianidin, thiamethoxam, and imidacloprid) have therefore been debated continuously and banned in the European market in 2018 (EFSA, https://www.efsa.europa.eu/en/press/news/180228). For other pesticides such as glyphosate, which are used on a large-scale, bans are still discussed (FINGER, 2018). While developing and assessing new control substances, bringing them on the market, and responding to any product that has scientifically proven adverse effects is a normal process, the more exciting and sustainable question should be - what other options are already available today or could be developed further?

In this respect, the goals should be defined upfront. Pest control is required on an area-wide basis using the least amount of harmful chemicals with the aim to preserve biodiversity, following the definition and principle of integrated pest management (IPM). The Sterile Insect Technique (SIT) is one strategy that can help the goals of IPM and is successfully used for several insect species. SIT decimates a pest population by mass release of sterile

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males leading to infertile matings with wild-type females in the field (see Fig. 1). Three main problems can be solved with this method: i) the species specificity is ensured by the mating action between individuals of only one species, ii) the use of insecticides can be significantly reduced; (iii) comprehensive, economical pest control is possible with insects that can be mass-produced in factories and are generally easy to grow.

SIT programs are useful for pest control in agriculture, but also for mosquito control. Moreover, the SIT has another significant advantage. It can be used preventatively against possible invasions of insects, e.g., in harbors that are frequented with fruit shipments always at risk of spreading insect populations through infestations (Dowell *et al.,* 2000). However, to be effective, all steps in an SIT program must be carefully planned for each release round - from field data collection and monitoring to all processes during mass rearing, quality control, and release. While all topics are relevant, one is particularly important to enable SIT for a new species and to make it efficient and economical. These is the separation of males from females to release only males. Male-only releases are reported to be more efficient and female insects are biting (in case of mosquitoes) or laying eggs into fruits (even if sterile).

In species where sex separation is not yet possible, females can be sterilized and released with males, although this is costly and inefficient (Benedict and Robinson, 2003). Therefore, there is a need for sexing systems for large-scale operations. In principle, manual separation is possible, but only automated and inducible systems can produce several billion male insects per week, as is the case with the medfly-producing facility in Guatemala (Parker, 2005). Through the development of a heat-inducible genetic sexing strain in medfly, its control programs benefitted significantly (Robinson, 2002). The transfer

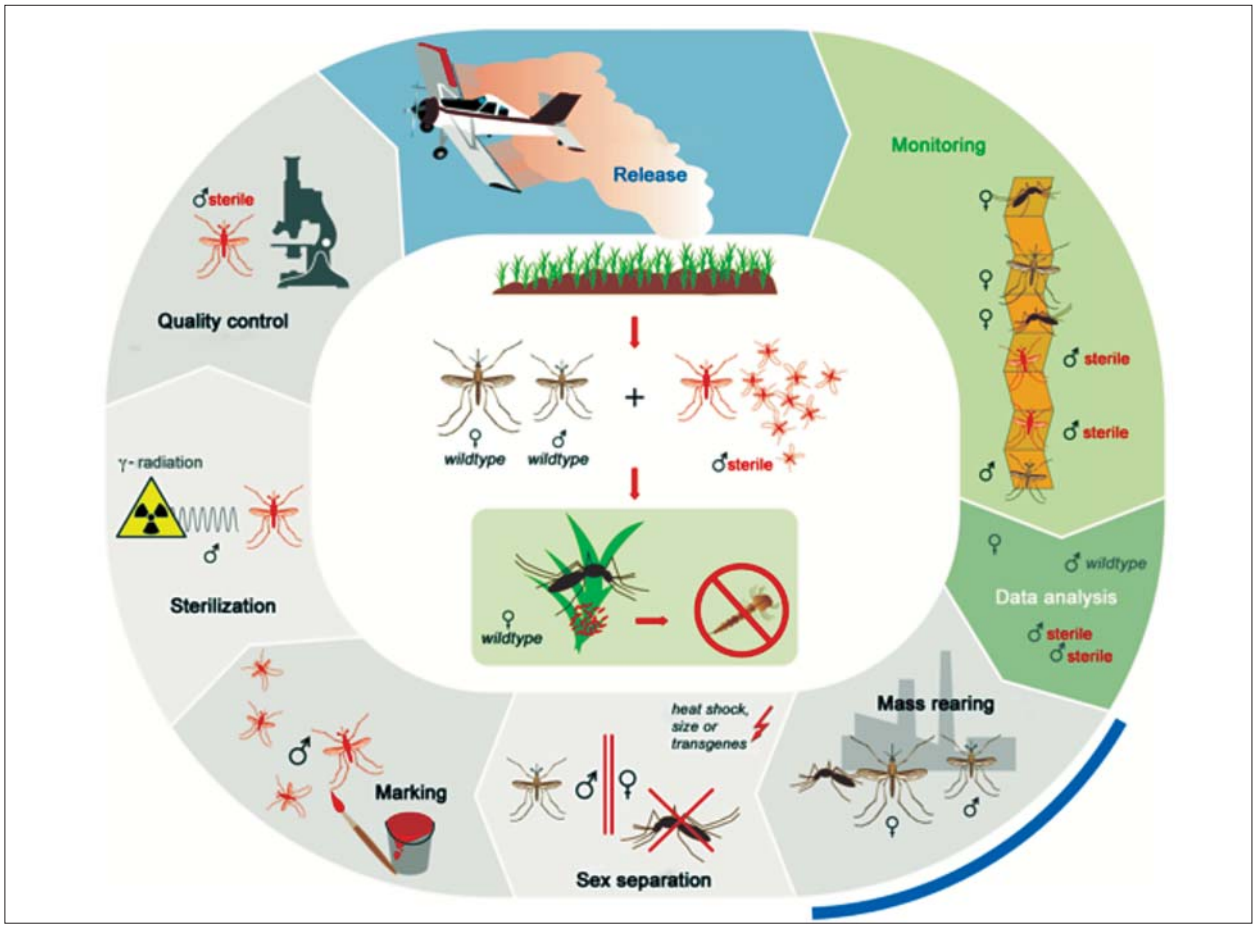

Fig. 1 – The principle of the Sterile Insect Technique. The Sterile Insect Technique uses a large amount of infertile male insects as a Trojan horse against their species. Sterile males released in overflowing amounts relative to the expected wild population, will mate to the majority of wild-type females and thus not produce offspring. Infertile males are produced in large factories, sex separated and marked. The sterilization is typically carried out by radiation. After that, they are transported to the target area, where millions of insects are released in aerial releases. The mass rearing facilities, especially the ones producing fruit flies like the Mediterranean fruit fly, have over 60 years of experience in the rearing of arthropods, developing mass rearing SOPs, and optimize equipment as well as quality standards to enable the production of high-quality insects.

of such large-scale and automated systems to novel crop pests is an important goal to enable the environmentally friendly SIT for those and allow the reduction of harmful substances used for crop protection - in line with the concept of integrated pest management.

Therefore, considerable efforts have been put into producing early embryonic sexing strains for pest species and transfer such technologies to invasive plant (Schetelig and Handler, 2012, Ogaugwu *et al.*, 2013, Schetelig *et al.*, 2016) and livestock pest insects (Yan and Scott, 2015, Yan *et al.*, 2017). All systems use genetically modified insects that produce only male offspring through a female-specific lethality. Combined with the safe sterilization method by radiation this could be a viable alternative for pest control of new species in the future. Because the insects are GMOs, their use in individual countries and markets could be difficult, even if they are sterilized. But they would lead to a reduction of chemicals that would otherwise be used for crop protection. Therefore, it might be essential to develop systems that are GMO-free to enable a holistic strategy for environmentally friendly control involving all parties - science, the public, agriculture stakeholders, and politics. GMO-free strains could be accomplished through new mutagenesis procedures like CRISPR/Cas (Heinze *et al.,* 2017, Kalajdzic and Schetelig, 2017, Li and Handler, 2017) that can create point mutations with no external genetic material added, comparable to classic mutagenesis techniques. Creating point mutations without transgenic technology or classical mutagenesis, was not possible until before CRISPR/Cas has been utilized in insect molecular biology. The technology has been adapted to important insect pests like the Mediterranean fruit fly, *Ceratitis capitata* (Meccariello *et al.,* 2017) and lately been optimized for high-efficiency genome editing that is able to create desired mutations in up to 90% of the progeny (Aumann *et al.*, 2018). Generating strains by CRISPR/Cas that carry only a small mutation to induce the desired effect would be a breakthrough for integrated pest management and mosquito control if regulatory issues could be overcome.

Outlook

More than 50 years of area-wide and large-scale SIT programs have yielded in a wealth of knowledge on the genetics of the Mediterranean fruit fly as a model organism for several other economically important pest insects. This knowledge, e.g., on sexing and marking strains accrued via classical genetics and mutagenesis and once biologically and biochemically fully understood, will be an essential hallmark of developing and bringing similar tools to new pest species and control them as efficient and sustainable as the SIT has pioneered it for this pest insects. The use of gene editing and genome engineering will boost this development further and allow the modification of more candidate insects for SIT programs in the long-term. The decision, if such strains qualify in the end, should be based on scientific facts. Critical for environmentally friendly pest control in agriculture will be products that help reduce the use of harmful substances applied for insect control to a minimum. It would be desirable if identical genetic changes, which were generated by different methods, will no longer be classified according to their production process, but based on the end product obtained. In this respect, a globally uniform regulatory process would be crucial, but at this time no harmonization is in sight. In the end, ideologies cannot be the driving factor for regulatory approval and policy, but evidence of scientific studies that aim for a safe and well-tested product. Expressing it according to the novelist Gertrude Stein: “a mutation is a mutation is a mutation” – no matter how it was created.

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SEX DETERMINATION AND GENE EDITING IN TEPHRITIDS: CONVERGING ON INNOVATIVE BIOCONTROL STRATEGIES

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Sex determination and gene editing in Tephritids: converging on innovative biocontrol strategies

The Tephritidae family includes more than 250 species of relevant economic importance in agriculture due to damage caused to a wide range of fruit hosts. Among them, *Ceratitis capitata* (Mediterranean fruit fly, medfly) is one of the top invasive and destructive species worldwide, affecting over 200 vegetal species alone. One of the most successful biocontrol strategies to fight the presence of the medfly in the orchads, to date, has been the Sterile Insect Technique (SIT). SIT consists of the continuous mass-release of laboratory-reared and sorted sterile males that through mating with wild females can suppress or even eradicate wild populations. A key component of the medfly SIT programs has been the close physical linkage of selectable traits to the Y-linked M factor, enabling male separation on massive scales (resistance of XY embryos to heat shocks or brown pupae colour of XY individuals versus respectively temperature sensitive lethality and white pupae colour of the XX individuals). An alternative to the male sex separation from females is the sexual transformation of XX individuals into males, which has been achieved only after isolating key sex determining genes of the medfly and using reverse genetic tools, such as RNAi and transgenesis. The recent emergence of the CRISPR/Cas9 not only opened new possibilities of functional genomics in non-model insect species, but also led to the development of novel gene drive strategies in the lab, including manipulation of sex ratio toward future suppression of infesting insect populations in the wild.

KEY WORDS: agricultural pest insects, sex determination, Sterile Insect Technique, transgenic, CRISPR/CAS9, gene drive.

SEX DETERMINATION IN TEPHRITIDS

True Flies (Diptera) are one of four super radiations of insects together with Coleoptera, Hymenoptera and Lepidoptera and consist of one of the most diverse animal orders on Earth, accounting for more than 124.000 species and sharing an approximately 240 mya old insect ancestor (WIEGMANN *et al*., 2011). Not surprisingly, sex determining mechanisms have been found to differ even within the same species (BACHTROG *et al*., 2014; BEUKEBOOM and PERRIN, 2014; BOPP *et al*., 2014; HAAG AND DOTY, 2005; SANCHEZ, 2008; SUZUKI, 2018). Interestingly, the different upstream primary signals seem to act throughout a conserved master gene for female sex determination, *transformer* (*Cctra*), which controls sex-specific expression of a widely conserved sex differentiation gene, *doublesex (Ccdsx*) (NAGARAJU and SACCONE, 2010). The *Drosophila melanogaster* orthologues, *tra* and *dsx*, are also needed for female sex determination (SANCHEZ, 2008). Sex determination studies of major agricultural pests, such as *Ceratitis capitata* and other Tephritidae species, is of economical relevance, because only females damage the fruit crop of hundreds of species by oviposition. In medfly, a deletion mapping localized the *Male determining* factor on the long arm of the Y chromosome nearby the centromere (WILLHOEFT and FRANZ, 1996). The *M* factor acts by repressing, either directly or indirectly the female determining master gene *transformer* (*Cctra*), during embryogenesis (PANE *et al*., 2002) (Fig. 1). In XY embryos, *Cctra* starts to be zygotically transcribed and male-specific longer isoforms appear at 5-6 h leading to translation of truncated non functional CcTRA isoforms (GABRIELI *et al*., 2010). Interestingly, fertile XX males, expressing male-specific *Cctra* transcripts, but lacking the Y chromosome, can be artificially obtained by transient embryonic RNAi targeting *Cctra* or *Cctransfromer-2* (*Cctra-2*), which is also required as an essential auxiliary factor for female sex determination (PANE *et al*., 2002; SALVEMINI *et al*., 2009). *Cctra* encodes a female-specific RS-type splicing factor and, differently from *Drosophila*, promotes its expression by female-specific alternative splicing of its own pre-mRNA, maintaining female sex determination. On the other hand, as in Drosophila, it induces female sexual differentiation throughout female-specific splicing of the downstream *doublesex* (*Ccdsx;* Fig. 1) (PANE *et al*., 2002;

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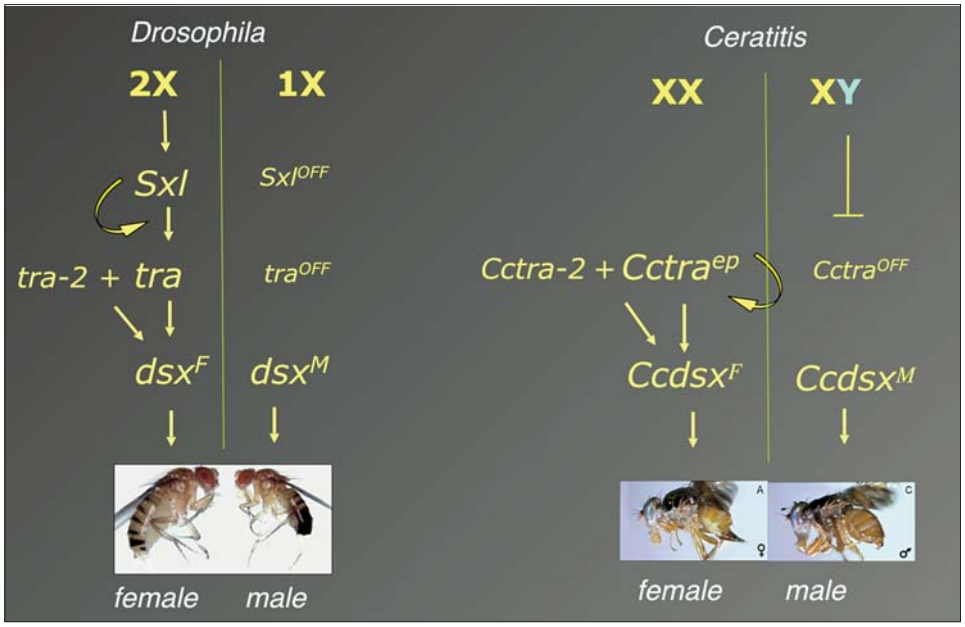

Fig. 1 – Evolutionary comparison of *Drosophila melanogaster* and *Ceratitis capitata* sex determination pathway. In Drosophila the primary signal is the number of X chromosomes and the master gene for sex determination is *Sex-lethal* (*Sxl*), which is able to autoregulate positively in XX individuals. In Ceratitis the primary signal is the Y-linked M factor repressing the establishment of the positive aturegulation of *transformer*, which plays the role of the master gene in the medfly. The Drosophila cascade is partially conserved in medfly, in other Tephritidae and many other insect species.

Saccone *et al*., 2008). *Cctra-2*, encoding a non sex-specific auxiliary SR splicing factor, is required for *Cctra* autoregulation, as well as for *dsx* female-specific splicing. *Ceratitis transformer* orthologues, showing conservation of this epigenetic function of autoregulation in females, have been isolated in other Tephritidae species (Lagos *et al*., 2007; Luo *et al*., 2017; Morrow *et al.,* 2014a and 2014b; Peng *et al*., 2015; Permpoon *et al*., 2011; Ruiz *et al.,* 2007; Ruiz and Sánchez, 2010; Saccone *et al*., 2011), in other dipteran species (Concha and Scott, 2009; Li *et al.,* 2013; Hediger *et al.*, 2010; Petrella *et al.,* 2019) and in other different insect orders (Hymenopteran and Coleoptera) (Bopp *et al*., 2014; Verhulst *et al.,* 2010). Transient embryonic RNAi of *tra* or *tra-2* orthologues in several Tephritidae species, including medfly, the Mexican fly *Anastrepha suspensa* and the oriental fly *Bactrocera dorsalis* led to complete sexual transformation of XX individuals into phenotypically males (Salvemini *et al.,* 2009; Sarno *et al.* 2010; Schetelig *et al.,* 2014).

Interestingly, in lower dipteran mosquitoes species, which maintain the evolutionary conservation of the downstream *dsx* sexual differentiation regulator, the *transformer* orthologues have not been identified, either because highly divergent or absent (Suzuki, 2018). Recent genomic and transcriptomic approaches to other dipterans sex determination led to the discovery of unrelated Y-linked genes male determining master regulators, encoding either novel short proteins, such as *Yob* (*Anopheles gambia*e) and *Guy-1* (*Anopheles stephensi*), or splicing factors-related proteins, such as *Mdmd* in *Musca domestica* (Criscione *et al*., 2016; Krzywinska *et al*., 2016; Sharma *et al*., 2017). In *Aedes aegypti* the male determining factor *Nix*, encoding a putative *tra-2* related splicing factor, was also found on one of the homomorphic sexual chromosomes. *Nix* seems to be conserved also in the mosquitoes tiger *Aedes albopictus*, which recently invaded also Italy (Hall *et al*., 2015; Gomulski *et al*., 2018). However, the molecular mechanisms by which these primary signals act are still to be clarified. The search of male determining genes in the medfly and related Tephritidae is still on going, in spite of the definition of its draft genome (Papanicolaou *et al*., 2016).

Gene editing in Tephritids

Over the last two decades, novel genetic strategies in pest insect management have been developed to improve their effectiveness in the field (Dyck *et al*., 2005). Genetic technologies used thus far in the medfly are based on random integration of transposable elements into the genome, site-specific modification of the randomly integrated transgene and embryonic or transgene-mediated RNA interference (RNAi) (O'brochta and Handler, 2008). The CRISPR-Cas9 technology offers a novel approach also to stably introduce exogenous DNA sequences at pre-selected locations in the genome (Reid and O'Brochta, 2016). Cas9 is a bacterial protein evolved as part of an adaptive immune system able to recognize and cut viral DNA genomes (Doudna and Charpentier, 2014). The recognition is mediated by Watson-Crick base pairing of about 20 nt between a small bacterial RNA associated with Cas9 and the target DNA

sequence (Thurtle-Schmidt and Lo, 2018). This pairing is long enough to ensure its specific match and binding to a unique target DNA sequence because of the very highly specific combinatorial string (potential combinations are indeed 20^4). These small RNAs containing vital information for bacteria – namely the code of the enemy – are transcribed from specific "immune genes" present in bacterial genome and, only later, are complexed with Cas9 to form ribonucleoparticles (RNPs). A part of the small guide RNA (gRNA) is composed of a variable sequence necessary to recognize the viral DNA, while another part is common and gives rise to an hairpin structure recognized by the Cas9 protein. Hence, these small RNA genes are composed of a mix of DNA of bacterial origin and of viral origin: the bacteria indeed acquire the enemy "codes", integrating 20 nt long DNA fragments from the viral genome into their specific immune genomic regions. The viral fragments are generated during the "fight" between bacteria and viruses and integrated in the genomes of surviving bacteria. In the bacterial cells, the targeted viral DNA is cut and degraded. A key discovery was the observation that Cas9-gRNA complex can work also *in vitro*, as many other bacterial useful enzymes for molecular biology (Jinek *et al*., 2012). The second key experiment was conducted in living eukaryotic cells which confirmed gene editing events, even targeting multiple DNA sites (Cong *et al*., 2013). Cas9 to can be directed by the gRNAs into specific genomic regions with the only restriction that an NGG trinucleotide sequence (PAM) is found following the pairing of 20 nt long specific sequence. The efficient ability of Cas9 to scan the entire genome, to find specific desired target sequence and to induce DNA breaks, even in these evolutionarily distant mammalian cells, was revealed by the imprecise response of the cells in repairing them. Indeed, during DNA repair, the erroneous addition or deletion of few base pairs in the broken DNA site led to mutant alleles of that targeted gene, which was revealed by usual DNA cloning and sequencing techniques. The eukaryotic cells can respond also in a second way, if an exogenous DNA fragment which has homology to the targeted broken site, is co-delivered: the cells repair the DNA damage using the exogenous DNA sequence as template. Hence the donor DNA sequence can be designed to introduce specific nucleotide substitutions, which are indistinguishable from spontaneous mutations, or achieve high precise deletions or insertions of long genomic regions (Kim and Kim, 2014).

Then a plethora of methods were developed to delivery Cas9 and the gRNAs into cell lines and pluricellular organisms of any vegetal and animal species, provided that genomic and/or transcriptomic data are available and a specific gene to be targeted is identified. A convenient and widely used method to produce in vitro RNPs complexes is, for example, 1) to synthetize sequence-specific gRNAs by *in vitro* transcription of a synthetic DNA template (generated by PCR), 2) to express and purify recombinant Cas9 protein and 3) to pre-assemble them *in vitro*. The use of preloaded Cas9-sgRNA ribonucleoprotein complexes in embryos injections has already been successfully applied to target genes for disruption in a growing number of insect species (Reid and O'Brochta, 2016).

Also in *Ceratitis capitata* and other Tephritidae species, including the oriental fly *Bactrocera dorsalis* and Anastrepha species, the preferred methods are embryos injections of RNPs, or embryos co-injections with *in vitro* synthetized mRNA encoding the Cas9 (which it will be translated into protein by the insect cells once in the cytoplasm) and the gene-specific gRNA (Meccariello *et al*., 2017; Lee *et al*., 2014; Li and Handler, 2019; Sim *et al*., 2018; Zhao *et al*., 2018; Zheng *et al*., 2018). Gene specific mutations (mostly in a gene controlling insect eye colour) were introduced in the genomes of dividing embryonic cells of both somatic and germ lines, leading to adult flies with patches of cells (mosaics) which can show a mutant phenotype (caused by biallelic mutations in the same founder cells of that expanded cellular clone). These adult flies from treated embryos can be crossed and a second generation will lead to mutant flies bearing heteroallelic mutant alleles (loss of function alleles of the same gene showing different insertion/deletions in the specific targeted sequence). Considering that rearing Tephritidae is very laborious, the possibility to screen for mutant individuals in only 2 generations is a very attractive solution.

Converging toward biocontrol strategies

Ceratitis capitata apparently emerged in Africa and colonized other continents in the last centuries, expanding in those areas dedicated to extensive fruit crop production. Similarly other Tephritidae are also relevant agricultural pests and invades new regions (Qin *et al*., 2015). Very recently, for example the highly invasive oriental fruit fly, *Bactrocera dorsalis*, was detected for the first time in Europe (Nugnes *et al*., 2018).

As alternative to pesticides and pheromonal traps, it has been developed and successfully applied in many countries the sterile insect technique (SIT)

(Dyck *et al*., 2005). SIT consists of the continuous mass-release of males, following their mass-rearing, their sorting from females and their sterilization by X or gamma rays factory-reared. This genetic technique relies on the introduction of excessive numbers of only sterile males in infested areas and hence requires large-scale rearing and sorting of males and females prior to release. The sexing is necessary because the released males tend to mate with the release females and the released sterile females would contribute to the physical damage of the fruits by their telescopic ovipositors. Their mating with wild females can suppress or even eradicate wild populations and hence, SIT is eco-friendly and species-specific.

A key component of the modern medfly SIT programs has been the sexing of males before adult stage, achieved by serendipitous identification of 2 genetic autosomal linked mutations in the medfly (Caceres, 2002). Chromosomal reciprocal translocations were induced by X-rays between the autosome and the Y chromosome. A close physical linkage of two selectable traits to the Y-linked M factor, enabled male separation on massive scales (resistance to heat shocks of XY embryos and a XY-specific brown pupae colour). However, the export of this sexing strategy to other Tephritidae pest species also requiring an SIT control, was challenging. Molecular genetics and biotechnology have been proposed, almost 3 decades ago, to speed up the development of a "universal" solution for sexing in insect pests and improve it efficacy and stability (Louis *et al*., 1988). The development of gene transfer in the medfly (Loukeris *et al*., 1995; Zwiebel *et al*., 1995) and the exploitation of the regulatory region of *Cctra* responsible for female-specific splicing and expression of CcTRA protein led to develop both *Ceratitis* and *Drosophila* transgenic strains in which female-specific lethality is conditional and repressible by an antidote provided in the larvae and adult diet (Dafa'alla *et al*., 2010).

An alternative to the male sex separation by XX-specific lethality is the sexual transformation of XX individuals into XX males, which has been achieved only after isolating key sex determining genes of the medfly. RNA interference against *Cctra* or *Cctra-2* led to male-only progeny (showing both XY and XX karyotypes) with few XX intersexes (Pane *et al*., 2002; Salvemini *et al*., 2009). Interestingly, the XX males are also fertile, indicating the absence of major fertility factors on the Y chromosome. Similar results have been obtained in other Tephritidae species. Based on this knowledge, transgenic strains could be produced, in which an inducible transgene producing *Cctra* dsRNA into embryos can result in male-specific *Cctra* splicing and masculinization of XX individuals. The evolutionary conservation of Ceratitis *transformer* autoregulating master gene in other Tephritidae species, suggests that it is an ideal genetic tool to generalize a method for male-only production. However, the emergence of the CRISPR/Cas9 technology opened new opportunities for innovation and translational research. Indeed, Cas9 and gRNAs can be expressed stably in transgenic flies either during all development and in all cells, or in germ line specific pattern (Papathanos *et al*., 2009). If the transgenic construct bears also DNA homology arms flanking the Cas9-sgRNA transgenes and corresponding to the targeted endogenous sequence, then a specific integration of the entire artificial segment can be induced. Indeed, once the vector is injected into the embryos and it starts to be transcribed in the germ cells, the DNA breaks induced in a specific region and the homology arms paired to that region can lead to a DNA repair response by the homologous recombination pathway. The artificial construct including Cas9+gRNA is copied into the homologous cut DNA region and a transgene-bearing chromosome is generated. The process can be repeated by the dividing cells because the transgenic construct stably integrated in the specific genomic region can be transcribed and produce again Cas9 protein and sgRNA, which can be assembled in RNPs and cut the DNA target sequence present on the homologous chromosome. During cell divisions of the germ line, a "chain reaction" of this self-propagating cassette leads to the production of gametes which are all transgenic. If this transgenic insect is mated with a non transgenic one, generates a progeny of heterozygotes individuals. However, in the germ lines of these transgenic heterozygotes Cas9+gRNA starts again the cut and copy mechanism onto the homologous wild chromosome, during mitosis of the stem cells leading to homozygosity of the transgenes and, after meiosis, 100% super-mendelian inheritance of the transgenic cassette (Gantz and Bier, 2015). This mechanism could be exploited to manipulated sex bias in favor of males (Galizi *et al*., 2016) and to drive in wild populations of pest insect species, transgenes able for example to induce a reduction in their reproduction potential or "immunization" to pathogens. Gene drives were already developed in lab strains of malaria vectors including *Anopheles gambiae* (Hammond *et al*., 2016; Kyrou *et al*., 2018) and *A. stephensi* (Gantz *et al*., 2015) and induced a collapse in cage experiments after few generations. However, technical, ecological, political and social issues need to be addressed, before this technology using genetically modified

insects could move from the lab into the wild (Adelman *et al*., 2017; Karaminejadranjbar *et al*., 2018; Taning *et al*., 2017).

An appealing alternative to GM insects, based on gene editing by CRISPR/Cas9, is the development of non transgenic insects bearing highly specific nucleotides substitutions and able to produce conditionally male only progeny (Li and Handler, 2017; Aumann *et al*., 2018). In *Drosophila melanogaster* two temperature-sensitive (*ts*) mutations (each involving a single amino acid substitution) have been described in the gene *transformer-2*. *tra-2* encodes an auxiliary splicing factor required for female-specific *transformer* splicing. In these mutant strains, higher rearing temperature leads XX individuals to develop into XX male. First attempts have been made to replicate the very same *tra-2* mutations in the genome of the spotted wing *Drosophila suzuki*, an emerging agricultural pest (Li and Handler, 2017). Considering the high evolutionary conservation of *tra-2* sequence and function in Tephritidae, it is possible to develop novel sexing methods in different species, without integrating exogenous DNA sequences.

Conclusions

DNA-free genome modifications with the use of purified Cas9 endonuclease may be more acceptable to the public and considered legally legitimate by governments (Courtier-Orgogozo *et al*., 2017). This approach may in the long term help to gain social and political acceptance including even the release of genetically modified pest insects, as it was the case for *Aedes aegypti* in Brazil (Carvalho *et al*., 2015; Paes de Andrade *et al*., 2016). The recent availability of the medfly genome sequence and the ongoing genome projects in other Tephritids combined with the successful implementation of CRISPR/Cas9 genome editing technology open the road to rapidly gain more fundamental knowledge on genetics of sex determination and move toward translation research.

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ANTIGENI SALIVARI QUALI STRUMENTI EPIDEMIOLOGICI PER LA VALUTAZIONE DELL'ESPOSIZIONE UMANA AD *AEDES ALBOPICTUS*

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Salivary antigens as epidemiological tools to evaluate human exposure to Aedes albopictus

Hematophagous arthropods during feeding inject into their hosts a cocktail of salivary proteins whose main role is to allow for an effective blood meal by counteracting host hemostasis, inflammation and immunity. However, saliva of blood feeders also evokes in vertebrates an antibody response that can be used to evaluate exposure to disease vectors. Salivary transcriptome studies carried out in different hematophagous species in the last fifteen years clarified the complexity of the salivary repertoires of blood feeding arthropods, pointing out that salivary proteins evolve at a fast evolutionary rate and highlighting the existence of family-, genus- and sometime even species-specific salivary proteins. Focusing on mosquitoes of the genera *Anopheles* and *Aedes*, which are important vectors of the human malaria parasite *Plasmodium falciparum* and of several arboviruses, we summarize here recent efforts to exploit genus-specific salivary proteins as biomarkers of human exposure to these vectors of large relevance for public health.

KEY WORDS: blood feeding, salivary proteins, biomarkers, *Anopheles*, *Aedes.*

INTRODUZIONE

La capacità di utilizzare una fonte alimentare ricca di aminoacidi quali il sangue dei vertebrati ha certamente conferito agli insetti ematofagi un considerevole vantaggio riproduttivo. Tuttavia, nutrirsi di sangue è un compito notevolmente impegnativo che ha richiesto l'evoluzione di complessi adattamenti comportamentali, morfologici e fisiologici tali da consentire all'insetto di localizzare l'ospite, attraversarne la barriera cutanea, quindi raggiungere, ingerire e digerire il sangue (LEHANE, 1991). È noto che il danno tissutale induce nei vertebrati risposte fisiologiche possenti e ridondanti atte a limitare le perdite di sangue, provvedere alla riparazione dei tessuti danneggiati e proteggerli dall'invasione di microorganismi. Per questi motivi, allo scopo di nutrirsi efficacemente di sangue, gli insetti ematofagi hanno evoluto un complesso armamentario di proteine salivari, parimenti possente e ridondante, capace di controbilanciare la risposta emostatica, infiammatoria ed immunitaria dell'ospite (RIBEIRO & ARCÀ, 2009). In effetti, la saliva di tutti gli artropodi ematofagi analizzati fino ad ora contiene almeno un inibitore piastrinico, un anticoagulante ed un vasodilatatore (Ribeiro, 1995); inoltre, nella saliva di queste specie si ritrovano anche numerose altre attività aggiuntive in grado di influenzare l'infiammazione e l'immunità dell'ospite (ARCÀ & RIBEIRO, 2018) ed eventualmente di interferire con la trasmissione di patogeni (FONTAINE *et al.*, 2011).

Grazie agli straordinari avanzamenti delle tecnologie di sequenziamento di acidi nucleici, nonché ai progressi delle tecniche proteomiche, la nostra comprensione della complessità, delle funzioni e dell'evoluzione delle proteine salivari di insetti ematofagi si è accresciuta in modo davvero considerevole negli ultimi quindici anni. Ad oggi gli studi di trascrittomica hanno consentito di ottenere informazioni sui sialomi (dal greco *sialo* = saliva) di almeno 49 specie di insetti ematofagi appartenenti a 3 differenti ordini (Ditteri, Emitteri e Sifonatteri). Queste analisi hanno rivelato che i repertori salivari della maggior parte dei Nematoceri ematofagi (come zanzare e flebotomi) includono ~100-200 proteine, mentre Brachiceri come la mosca tsetse ed Emitteri come la cimice Triatomina possono avere oltre 250 proteine salivari (ARCÀ & RIBEIRO, 2018). Attualmente, il ruolo fisiologico di numerose proteine salivari è stato chiarito attraverso studi funzionali oppure dedotto sulla base della similarità di sequenza a proteine note (NARASIMHAN *et al.*, 2017; RIBEIRO & ARCÀ, 2009). Tuttavia, è opportuno sottolineare che malgrado i considerevoli progressi ancora non abbiamo alcuna idea sulle possibili funzioni di circa il 30-40% delle putative proteine salivari identificate da insetti ematofagi.

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Antigeni salivari come strumenti epidemiologici

Le secrezioni salivari di artropodi ematofagi, a parte ed indipendentemente dalle proprietà biochimiche e farmacologiche delle proteine che le compongono, stimolano l'immunità umorale dei vertebrati. Di conseguenza, anticorpi circolanti diretti contro componenti salivari possono essere rivelati e misurati nei sieri di individui ripetutamente punti da artropodi. Numerosi studi hanno evidenziato come questa risposta anticorpale anti-saliva possa essere sfruttata per valutare l'esposizione dell'ospite a punture di artropodi vettori anche molto diversi fra loro come zecche, flebotomi, zanzare, mosche tsetse e cimici Triatomine (vedi Rizzo *et* al., 2011 per le referenze originali). Al momento, la valutazione dell'esposizione umana a Culicidi vettori si basa su misure entomologiche classiche della densità vettoriale, ed eventualmente sulla propensità ad effettuare il pasto di sangue sull'uomo (trappole, catture al piretro, catture sull'uomo, etc.). Tuttavia, queste metodologie forniscono soltanto misure indirette del grado di esposizione umana a punture del vettore. Inoltre, richiedono un notevole impegno lavorativo, sono relativamente costose ed in alcune circostanze possono essere di difficile o impossibile implementazione (bassa densità vettoriale o costrizioni logistiche). A questo riguardo la disponibilità di semplici saggi immunologici per misurare direttamente il contatto uomo-vettore rappresenterebbe uno strumento addizionale estremamente utile. Infatti, la valutazione della trasmissione/rischio di malattie trasmesse da Culicidi vettori si basa frequentemente su misurazioni serologiche delle risposte anticorpali ad antigeni virali o parassitari; quindi, la disponibilità di antigeni salivari vettore-specifici consentirebbe la valutazione simultanea della circolazione del patogeno e dell'esposizione umana al suo vettore. Infine, ma non meno importante, la messa a punto di marcatori salivari sarebbe estremamente utile anche per la valutazione dell'efficacia di misure anti-vettoriali (per es. zanzariere impregnate o interventi con larvicidi e/o adulticidi). Se da un lato la misurazione della risposta anti-saliva rappresenta un utile strumento epidemiologico, dall'altro lato l'uso della saliva o di estratti salivari è problematico. Innanzitutto, ottenere grandi quantità di saliva o di estratti è una procedura laboriosa, difficile da standardizzare e scarsamente riproducibile. In secondo luogo, la saliva di artropodi ematofagi, come in precedenza specificato, è una miscela complessa e può determinare fenomeni di cross-reattività che possono essere fuorvianti. Per questi motivi un saggio immunologico per valutare l'esposizione umana a vettori di malaria o di arbovirosi dovrebbe essere idealmente basata su singoli antigeni specifici rispettivamente di zanzare *Anopheles* o *Aedes*.

Diversità delle proteine salivari di insetti ematofagi

Abbiamo già accennato alla grande quantità di informazioni ottenute negli ultimi dieci o quindici anni sui sialomi di insetti e artropodi ematofagi. Per quanto concerne i Culicidi attualmente sono disponibili trascrittomi salivari da 12 differenti specie appartenenti a 5 diversi generi (Arcà & Ribeiro, 2018). Inoltre, il recente sequenziamento completo dei genomi di 16 specie di *Anopheles* da diversi continenti ha fornito una opportunità unica di studiare l'evoluzione dei geni codificanti proteine salivari in un arco temporale che abbraccia circa 100 milioni di anni di radiazione delle anofeline (Arcà *et al.*, 2017; Neafsey *et al.*, 2015). Questa larga quantità di informazioni ha permesso di chiarire che i geni salivari delle anofeline, e verosimilmente degli artropodi ematofagi più in generale, evolvono ad un tasso accelerato, forse sotto la pressione selettiva dei sistemi immunitari degli ospiti (Arcà *et al.*, 2017; Arcà *et al.*, 2014; Neafsey *et al.*, 2015). Questo tasso evolutivo insolitamente elevato, insieme all'osservazione che l'ematofagia si è evoluta indipendentemente numerose volte (convergenza evolutiva), giustifica l'osservazione che i repertori salivari di insetti ematofagi consistono sia di proteine che sono ampiamente condivise fra differenti famiglie di insetti ma anche di proteine salivari famiglia-, genere- e persino specie-specifiche (Arcà & Ribeiro, 2018; Ribeiro & Arcà, 2009). Più specificamente, analisi trascrittomiche comparative hanno evidenziato l'esistenza di gruppi di proteine salivari specifiche dei Culicidi e ristrette a specie dei generi *Anopheles* o *Aedes* (Arcà *et al.*, 2007; Arcà *et al.*, 2005; Ribeiro *et al.*, 2007; Ribeiro *et al.*, 2010). Queste proteine genere-specifiche, se immunogeniche, rappresentano dei candidati ideali per lo sviluppo di saggi ELISA (Enzyme-Linked ImmunoSorbent Assay) per valutare l'esposizione umana a vettori di malaria (zanzare *Anopheles*) o di arbovirus (zanzare *Aedes*).

La proteina gSG6 di *Anopheles gambiae*: una prova di principio

Analisi comparative hanno consentito di identificare un gruppo di 18 polipeptidi salivari, appartenenti a 9 differenti famiglie proteiche, che si ritrovano esclusivamente nella saliva di anofeline e sono privi di similarità con proteine note (Arcà *et*

al., 2005; Ribeiro *et al.*, 2010). Le risposte IgG alle proteine gSG6 e cE5 del vettore afrotropicale di malaria *Anopheles gambiae* sono state analizzate in individui naturalmente esposti a punture di zanzare *Anopheles* da una zona ad iperendemia malarica del Burkina Faso, Africa occidentale. gSG6 (AGAP000150) è una piccola proteina di ~10 kDa espressa specificamente nelle ghiandole salivari di femmine adulte e relativamente abbondante nella saliva. Il preciso ruolo funzionale di gSG6 è ancora da chiarire, sebbene la sua deplezione dalla saliva mediante RNAi aumenti il tempo di *probing* e diminuisca l'efficienza del pasto di sangue (Lombardo *et al.*, 2009). Anche cE5 è un piccolo polipeptide (82 aa, AGAP008004) ma la sua struttura e funzione sono stati accuratamente determinati: si tratta di un potente inibitore trombinico appartenente alla famiglia dell'anofelina e lo si ritrova esclusivamente nella saliva di zanzare *Anopheles* (Arcà *et al.*, 2017; Pirone *et al.*, 2017; Ronca *et al.*, 2012). Queste due proteine salivari genere-specifiche di *An. gambiae* sono entrambe immunogeniche ma è interessante notare che stimolano risposte immunitarie sostanzialmente differenti in individui ripetutamente esposti ad alti livelli di punture anofeliche. Infatti, la proteina gSG6 induce una risposta IgG di breve durata, come indicato dalla brusca diminuzione dei livelli anticorpali durante la stagione secca, dopo un periodo di ~3-4 mesi di esposizione scarsa o nulla (Rizzo *et al.*, 2011). La proteina cE5, che esibisce una maggiore immunogenicità in confronto a gSG6, evoca invece una risposta IgG di più lunga durata, come mostrato dall'assenza di variazione significativa dei livelli anticorpali fra la stagione delle piogge, ad alta densità vettoriale, e la stagione arida, a bassa densità vettoriale (Rizzo *et al.*, 2014). La risposta a queste due proteine è risultata differente anche (i) per la sottoclasse IgG predominante (IgG4 ed IgG1 rispettivamente per gSG6 e cE5) e (ii) per la capacità di indurre tolleranza immunologica, suggerendo che inducano risposte immunitarie di differente polarità negli individui esposti: di tipo Th-2 per gSG6 e di tipo Th-1 per cE5 (Rizzo *et al.*, 2014). La natura a più lungo termine della risposta IgG anti-cE5 indica una limitata adeguatezza di questa proteina quale marcatore di esposizione; tuttavia, la sua elevata sensibilità potrebbe essere sfruttata per valutare l'impatto di interventi di controllo vettoriale sul contatto uomo-vettore, e quindi per stimarne l'efficacia (Marie *et al.*, 2015). Dall'altro lato, la proteina gSG6 (o il peptide gSG6-P1) sono stati validati quali marcatori di esposizione umana a vettori di malaria in un'ampia varietà di condizioni epidemiologiche in Africa (Angola, Benin, Burkina Faso, Costa d'Avorio, Kenya, Senegal, Tanzania ed Uganda) e, più recentemente, in Asia (Cambogia, confine Tailandia-Birmania) e Polinesia (Vanuatu) (Badu *et al.*, 2012; Drame *et al.*, 2010; Drame *et al.*, 2015; Idris *et al.*, 2017; Poinsignon *et al.*, 2008; Proietti *et al.*, 2013; Rizzo *et al.*, 2011; Stone *et al.*, 2012; Traore *et al.*, 2018; Ya-Umphan *et al.*, 2017). Questi dati forniscono una chiara prova di principio che antigeni salivari da artropodi ematofagi possono essere usati in maniera affidabile per valutare l'esposizione umana a vettori di patogeni e che rappresentano utili strumenti per studi epidemiologici e per la stima dell'efficacia di interventi di controllo vettoriale.

Antigeni salivari per la valutazione dell'esposizione a zanzare *Aedes*

Zanzare appartenenti al genere *Aedes* sono vettori di arbovirus di grande rilevanza per la salute umana quali i virus della dengue (DENV), Zika (ZIKV), chikungunya (CHIKV) e febbre gialla. *Aedes aegypti* ed *Aedes albopictus* sono certamente i vettori più rilevanti, con *Ae. aegypti* che rappresenta il principale vettore nelle aree tropicali e subtropicali, mentre *Ae. albopictus* sta rapidamente guadagnando l'attenzione generale in virtù della sua rapida diffusione globale e della sua competenza alla trasmissione di numerosi arbovirus (Kraemer *et al.*, 2015). Inoltre, i recenti casi di trasmissione autoctona di chikungunya e dengue causati da *Ae. albopictus* in Italia, Francia e Croazia hanno evidenziato che il continente europeo è vulnerabile a infezioni trasmesse da *Ae. albopictus* (Gossner *et al.*, 2018), sottolineando la necessità di un migliore monitoraggio e controllo di questo rilevante vettore.

Studi precedenti hanno chiaramente mostrato che la risposta IgG alla saliva di *Ae. aegypti* o di *Ae. albopictus* può essere impiegata per valutare l'esposizione umana a questi vettori (Doucoure *et al.*, 2012a; Doucoure *et al.*, 2012b; Mathieu-Daude *et al.*, 2018; Orlandi-Pradines *et al.*, 2007). Allo stesso tempo le analisi trascrittomiche su differenti specie di zanzare *Anopheles*, *Aedes* e *Culex* hanno portato all'identificazione di un gruppo di almeno una decina di proteine salivari tipiche di culicine e con limitata identità aminoacidica fra specie di *Aedes* e *Culex* (Arcà *et al.*, 2007; Arcà *et al.*, 2005; Ribeiro *et al.*, 2007; Ribeiro *et al.*, 2004; Ribeiro *et al.*, 2010; Ribeiro *et al.*, 2018). Queste osservazioni, insieme alla prova di principio ottenuta con gSG6/gSG6-P1 per vettori di malaria, incoraggiano gli sforzi per la messa a punto di saggi simili per la valutazione dell'esposizione a zanzare *Aedes*. Fra i candidati idonei ci solo le proteine salivari

di *Ae. albopictus* 23.4 kDa (AAV90700), 27 kDa (AAV90698), 30.5 kDa (AAV90697), 34k1(AAV90689), 34k2 (AAV90690), 62k1 (AAV90683), 62k2 (AAV90682), HHH (AAV90655), W-rich (AAV90636), hyp8.2 (AAV90696); inoltre, alcune indicazioni preliminari di immunogenicità per l'uomo sono state ottenute per alcuni di questi candidati mediante approcci di immunoproteomica (Doucoure *et al.*, 2013).

L'unico antigene salivare *Aedes*-specifico utilizzato finora con qualche successo è il peptide Nterm-34 kDa che è disegnato sulla regione N-terminale della proteina salivare 34k1 di *Ae. aegypti* (ABF18017). In uno studio effettuato in 7 differenti villaggi del Benin meridionale (Africa occidentale) si è visto che la risposta IgG anti-Nterm-34 kDa aumenta in bambini esposti a zanzare *Aedes* passando dalla stagione secca (bassa esposizione) alla stagione delle piogge (alta esposizione) (Elanga Ndille *et al.*, 2012). Inoltre, ci sono alcune evidenze che il peptide Nterm-34 kDa potrebbe essere utile per valutare la qualità di interventi di controllo vettoriale (Elanga Ndille *et al.*, 2016; Sagna *et al.*, 2018). Questi risultati, sebbene necessitino di più estesa validazione, sono certamente promettenti ed incoraggianti ma l'analisi di ulteriori candidati potrebbe consentire la messa apunto di marcatori più efficaci. Da questo punto di vista è opportuno considerare come l'utilizzo di peptidi sintetici, pur presentando alcuni rilevanti vantaggi, abbia anche alcune limitazioni. Infatti, da un lato l'impiego di peptidi consente di evitare le laboriose procedure di espressione, purificazione e rinaturazione delle proteine ricombinanti, peraltro non sempre coronate da successo, e può garantire una minore variabilità da preparazione a preparazione dell'antigene. D'altro canto, i peptidi sintetici hanno spesso una sensibilità limitata, dovuta alla perdita degli epitopi conformazionali delle proteine native, e richiedono l'impiego di sieri più concentrati, il che può essere un problema in alcune situazioni o condizioni epidemiologiche. Ci si aspetta che ulteriori progressi verso la messa a punto di marcatori sensibili ed efficaci di esposizione a zanzare *Aedes* possa venire dall'espressione in forma ricombinante e dalla validazione di proteine salivari *Aedes*-specifiche. Un passo importante in questa direzione può venire dall'utilizzo di sistemi sperimentali animali (topo, coniglio o cavia) che consentano un rapido screening preliminare di numeri relativamente elevati di candidati. Un approccio di questo tipo, pur necessitando di validazione finale sull'uomo in condizioni naturali di esposizione, ha numerosi vantaggi. Innanzitutto, il regime di esposizione può essere strettamente controllato ed è possibile impiegare protocolli di esposizione a punture di differenti zanzare vettrici (*Anopheles*, *Aedes*, *Culex*): questo può consentire di valutare la genere- ed eventualmente la specie-specificità della risposta anticorpale. In aggiunta, il prelievo di piccoli volumi di siero a tempi definiti (prima, durante ed a tempi diversi dal termine dell'esposizione) può fornire informazioni dettagliate sulla cinetica di comparsa e decadimento della risposta, che è un parametro cruciale per la selezione di marcatori efficaci. In un sistema di questo tipo si può immaginare che lo screening iniziale di peptidi disegnati su idonee proteine salivari *Aedes*-specifiche possa guidare la selezione di candidati ottimali per la successiva espressione dell'intera proteina in forma ricombinante. In conclusione le prospettive per lo sviluppo di marcatori di esposizione a zanzare *Aedes* sono certamente molto incoraggianti: ci si attende che questi possano rappresentare degli strumenti addizionali estremamente utili per stimare il grado di esposizione umana a questi importanti vettori di arbovirus con implicazioni rilevanti per studi epidemiologici, per la valutazione del rischio e per il miglioramento degli interventi di controllo antivettoriali.

RIASSUNTO

Gli artropodi ematofagi, durante il pasto di sangue, iniettano nei loro ospiti un cocktail salivare il cui ruolo principale è di consentire un'efficace assunzione del sangue controbilanciando le risposte emostatica, infiammatoria ed immunitaria dell'ospite. Le proteine salivari di ematofagi, tuttavia, inducono negli ospiti vertebrati una risposta anticorpale che può essere sfruttata per valutarne l'esposizione a vettori di importanti malattie. Studi di trascrittomica effettuati negli ultimi quindici anni su differenti specie di insetti ematofagi hanno consentito di chiarire la complessità dei loro repertori salivari mettendo in rilievo come le proteine salivari mostrino un accelerato tasso evolutivo ed evidenziando l'esistenza di proteine salivari famiglia-, genere- e talvolta anche specie-specifiche. Focalizzando l'attenzione su zanzare dei generi *Anopheles* ed *Aedes*, importanti vettori rispettivamente del parassita malarico *Plasmodium falciparum* e di numerosi arbovirus, riassumiamo qui recenti studi finalizzati all'utilizzo di proteine salivari per la messa a punto di saggi atti a valutare l'esposizione umana a questi vettori di notevole rilevanza per la salute pubblica.

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I BATTERI SIMBIONTI NEL CONTROLLO DELLE MALATTIE TRASMESSE DA INSETTI VETTORI

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Symbiotic control of insect pests

It is well known that the microbiota of insects has a key role in their evolutionary success. Examples of bacterial symbiosis are remarkably common in insects, in particular in species involved in the transmission of diseases: numerous studies were carried out to reveal the basic mechanisms of the host–symbiont relationships and to develop alternative strategies to control vector borne diseases. The 'Symbiotic control', a new multifaceted approach that uses symbiotic microorganisms to control insect pests or reduce vector competence, has attracted much interest in the last few years. Three such approaches currently at the cutting edge are: (1) the disruption of microbial symbionts required by insect pests by intervening of their fitness; (2) the manipulation of symbionts expressing anti-pathogen molecules within the host; and (3) the introduction of exogenous microbes affecting life-span and vector capacity of the new hosts in insect populations. This work reviews the current knowledge on microbial symbiosis in mosquitoes that holds promise for the development of interventions of symbiotic control for mosquito borne diseases.

KEY WORDS: Microbiota, Symbiotic Control, Paratransgenesis, Insect pests, Insect vectors.

INTRODUZIONE

Considerando la loro biodiversità e abbondanza, gli insetti possono essere considerati tra gli animali di maggior successo sulla Terra. Delle tantissime specie di insetti descritte, molte sono vettrici di agenti patogeni per gli animali e le piante. In questo contesto, le zanzare contribuiscono in modo significativo alla biodiversità e alla biomassa degli insetti. Al momento ne sono state descritte circa 3500 specie, alcune delle quali pongono seri problemi di natura sanitaria e socio-economica in molte regioni, con particolare riguardo all'Africa sub-sahariana. Le zanzare possono trasmettere tra i tanti, i patogeni responsabili della malaria, della febbre gialla, della dengue, della febbre del Nilo occidentale, della chikungunya e di alcune filariosi. Di conseguenza più della metà della popolazione umana globale è a rischio di esposizione alle infezioni trasmesse dalle zanzare e alcuni milioni di infezioni umane sono registrate ogni anno. Le zanzare sono state descritte in quasi tutti i continenti, adattandosi ad una grande varietà di habitat diversi nei quali svolgono importanti ruoli ecologici e funzionali.

I simbionti microbici, in particolare i batteri, possono influenzare tratti specifici della biologia dell'insetto-ospite, quali per esempio il riconoscimento per l'accoppiamento specie- specifico, come riportato in *Drosophila paulistorum* (MILLER, 2010), o la resistenza ad alcuni insetticidi, come riportato in *Riptortus pedestris* (KIKUCHI, 2012), o ancora, avere effetti sulla fitness, la nutrizione, la difesa da predatori, e la riproduzione, in un numero significativo di specie di insetti (SIMON & VAVRE, 2011; FERRARI, 2011). Così come per molte specie di insetti, le relazioni simbiotiche tra zanzare e diversi microrganismi hanno avuto, molto probabilmente, implicazioni importanti nel loro successo evolutivo, incidendo positivamente anche sulla loro diffusa distribuzione geografica. In particolare, alcune componenti del microbiota associato a zanzare vettrici possono inibire lo sviluppo di agenti patogeni bloccandone la trasmissione (PUMPUNI *et al.*, 1993); pertanto diversi microbi potrebbero potenzialmente essere utilizzati per manipolare la capacità delle zanzare di trasmettere agenti patogeni all'uomo, agli animali o alle piante. Conseguentemente, negli ultimi anni si è registrata un'intensificazione degli studi mirati alla descrizione del microbiota in diverse specie di zanzare. Difatti, sebbene i primi studi sulla relazione tra batteri e le zanzare risalgano alla metà degli anni '20, quando HERTIG & WOLBACH (1924) descrissero per la prima volta la presenza di batteri della specie *Wolbachia pipentis* all'interno degli organi riproduttivi della zanzara *Culex pipiens* (HERTIG, 1936), solo di recente lo studio della simbiosi microbica nelle zanzare ha trovato un forte interesse risultante in un numero rilevante di pubblicazioni nel settore.

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La manipolazione di alcuni simbionti di zanzara può offrire nuovi metodi di controllo che sono uniformemente definiti come Controllo Simbiotico (CS). In questo contesto, a seguire descriverò quei rapporti tra simbionti e zanzare vettrici che sembrano aprire le migliori prospettive applicative per il controllo delle malattie trasmesse da zanzare.

Wolbachia e zanzare: un rapporto antico con nuove prospettive per il controllo di malattie trasmesse da insetti

I batteri del genere *Wolbachia* rappresentano un gruppo di batteri intracellulari, ereditati per *via materna*, e descritti per la prima volta in *Culex pipiens* (Hertig,1936). Più recentemente è stato riscontrato in un numero elevato di specie di insetti. In zanzara, *Wolbachia* è stato rilevato in diversi generi tra cui *Aedes*, *Culex*, *Coquillettidia* e *Mansonia*, ma solo negli ultimissimi anni in alcune popolazioni di specie del genere *Anopheles* (Baldini *et al.*, 2014; Jeffries *et al.*, 2018; Baldini *et al.*, 2018; Niang *et al.,* 2018) che comprende le circa sessanta specie coinvolte nella trasmissione della malaria umana. *Wolbachia* inoltre non è mai stata rilevata in popolazioni naturali della specie *Aedes aegypti*, principale vettore di dengue e febbre gialla. La trasmissione materna di *Wolbachia* avviene attraverso il citoplasma dell'uovo e causa diversi disturbi riproduttivi nell'ospite dell'insetto, comprensivi dell'incompatibilità citoplasmatica, della partenogenesi, della femminilizzazione e del "male killing" (Stouthamer *et al.*, 1999). Attraverso l'incompatibilità citoplasmatica, i batteri sono in grado di diffondersi nelle popolazioni delle zanzare, conseguentemente la *Wolbachia* è stata proposta come un "gene drive system" con la finalità di ridurre la dimensione della popolazione di zanzare e per interferire con la struttura della popolazione con possibili effetti sulla riduzione della trasmissione della malattia ad essa associata (Hoffmann *et al.*, 2011).

Recentemente, diversi studi hanno dimostrato il notevole potenziale dell'uso di *Wolbachia* per il controllo delle malattie trasmesse dalle zanzare. In particolare, l'introduzione "forzata" di alcuni ceppi di *Wolbachia* in *Ae. aegypti* provoca una serie di fenomeni tra i quali la riduzione della longevità della zanzara (McMeniman *et al.*, 2009) e un potenziamento della risposta immunitaria tale da rendere le zanzare refrattarie alle infezioni da dengue e quindi incapaci di trasmettere il virus (Kambris *et al.*, 2009). Le popolazioni di *Ae. aegypti* rese resistenti alle infezioni da dengue tramite l'uso di *Wolbachia* sono in grado di sostituire rapidamente le popolazioni naturali e sensibili dimostrando la fattibilità di questa strategia nel contrasto alla dengue nel mondo.

Il controllo biologico della trasmissione delle zanzare mediante *Wolbachia* è stato dapprima validato con successo con piccoli test sul campo, per poi essere esportato con successo in trials più estesi a Townsville, in Australia, ed è stato poi applicato con risultati positivi in più di 10 paesi diversi attraverso il programma *Eliminate Dengue*. La sua più ampia applicazione, oltre a limitare la trasmissione della dengue e includere altre specie di zanzare, ha visto il programma crescere in un'iniziativa globale senza fini di lucro, nota come World Mosquito Program.

Difatti, esperimenti simili sono stati condotti in *An. gambiae*, il maggior vettore di malaria Afro-tropicale, dimostrando che un'infezione indotta da *Wolbachia* può inibire lo sviluppo del parassita malarico *Plasmodium* nella zanzara (Hughes *et al.*, 2011; Gomes *et al.*, 2017). È opportuno ricordare che non tutti i ceppi di *Wolbachia* esercitano un fenotipo protettivo nel proprio ospite come mostrato da uno studio comparativo volto a valutare l'effetto di due ceppi di *Wolbachia*, wAlbB (isolato da *Ae.albopticus*) e wMelPop (isolato da *Drosophila melanogaster*), sulla competenza vettrice di *An. gambiae* (Hughes *et al.*, 2011). Il ceppo wAlbB aumenta in modo significativo i livelli di oocisti di *Plasmodium* nel midgut delle zanzare, mentre wMelPop inibisce moderatamente lo sviluppo delle oocisti. Inoltre, il ceppo wAlbB non è virulento per le zanzare, al contrario del ceppo wMelPop. Tutto ciò dimostra che differenti ceppi di *Wolbachia* possono avere differenti tipi di interazione con l'ospite e con i patogeni che esso può trasmettere. Questi studi, seppur preliminari, dimostrano quindi il potenziale utilizzo di *Wolbachia* anche nella lotta al controllo della malaria, confortando le evidenze che indicano che la presenza di *Wolbachia* in popolazioni naturali di *An. gambiae* determina un marcato effetto deleterio sugli sporozoiti e conseguentemente sulla trasmissione della malaria.

Manipolazione genetica di simbionti: cavalli di Troia per esprimere molecole "anti-patogeno" all'interno dell'insetto vettore

Con il termine paratransgenesi si intende una metodica mirata all'eliminazione di agenti patogeni da popolazioni di insetti vettori/dannosi attraverso la manipolazione genetica di simbionti dell'insetto. Si tratta di una metodica che ha già avuto numerosi riscontri positivi: uno dei migliori esempi di fattibilità dell' uso della paratransgenesi per il controllo delle malattie trasmesse da vettori riguarda la malattia di Chagas, ovvero la tripanosomiasi americana (Durvasula *et al.,* 1997). La malattia di Chagas è causata dal protozoo parassita *Trypanosoma cruzi*, trasmesso dal cosiddetto *kissing bug*, un insetto appartenente alla famiglia delle

Reduviidae (sottofamiglia Triatominae). Il vettore *Rhodnius prolixus,* si nutre per tutto il suo intero ciclo di sviluppo di sangue del vertebrato ospite. Nell'intestino esso alberga batteri simbionti della specie *Rhodococcus rhodnii* che producono sostanze nutritive, come le vitamine, che consentono all'insetto di compensare una dieta costituita esclusivamente da pasti sangue. È stato possibile coltivare popolazioni di questo simbionte, modificarle geneticamente per produrre molecole effettrici anti-tripanosoma e reinserirle nella triatomina per esprimere la/e molecola/e antiparassita nell'intestino degli insetti. Tale metodica ha effettivamente dimostrato il blocco della trasmissione del patogeno attraverso l'inibizione dello sviluppo del parassita nel vettore (Beard *et al.,* 2002). Inoltre, prove di campo hanno dimostrato la diffusione controllata di simbionti geneticamente modificati in popolazioni naturali di vettori di triatomine, rafforzando quindi la fattibilità della paratransgenesi come parte di un programma di controllo integrato (Durvasula *et al.,* 1999; Hurwitz *et al.,* 2011).

Un altro esempio di successo, riguarda una strategia paratransgenica mirata all'interruzione della trasmissione di *Xylella fastidiosa*, uno dei principali agenti patogeni batterici in agricoltura, trasmesso da *Homalodisca vitripennis* (noto con il nome di "Glassed-Winged Sharpshooter", GWSS), utilizzando *Pantoea agglomerans*, un simbionte batterico del GWSS come agente di controllo paratransgenico. Ceppi di *P. agglomerans* geneticamente modificati per esprimere due peptidi antimicrobici, un'(AMP)-melittina e una molecola simile alla scorpina (SLM), che hanno un'attività anti-*Xylella* a concentrazioni non letali per *P. agglomerans*, sono risultati capaci di interrompere la trasmissione del patogeno dagli insetti alle piante d'uva (Arora *et al.*, 2018).

Questi risultati in insetti vettori diversi dalle zanzare, hanno fortemente incoraggiato i ricercatori a sviluppare approcci simili per controllare le malattie trasmesse da zanzare, indicando che per la messa a punto di un efficiente approccio paratransgenico al controllo delle malattie trasmesse da zanzare (e più generalmente di insetti vettori/dannosi), microrganismi simbionti con peculiari e ben definite caratteristiche, devono essere identificati e caratterizzati a livello microbiologico e genetico. Difatti occorre che i simbionti selezionabili siano coltivabili e geneticamente modificabili in modo stabile per esprimere e secernere la/le proteine/e anti-patogeno. Inoltre la fitness del simbionte non deve essere compromessa dal processo di manipolazione.

Un altro elemento molto importante riguarda la definizione di un efficiente mezzo di diffusione del simbionte manipolato nelle popolazioni di insetti *targets*. Ad oggi, alcuni batteri sono già stati progettati per esprimere molecole anti-patogeno in zanzara. Riehle e collaboratori (2007) hanno ingegnerizzato batteri della specie *Escherichia coli* capaci di esporre due molecole effettrici anti-*Plasmodium* sulla loro membrana esterna. Utilizzando entrambe le molecole effettrici si é registrata una significativa inibizione dello sviluppo di *Plasmodium berghei*, allorquando le zanzare sono state alimentate con i batteri ingegnerizzati, ventiquattro ore prime del pasto di sangue infetto. Nonostante il numero e la prevalenza dei batteri ricombinanti aumenti significativamente dopo il pasto di sangue, *E. coli* sopravvive con difficoltà nelle zanzare ospiti. Conseguentemente altre specie di batteri simbionti delle zanzare sono state isolate. Tra questi, ottimi candidati per applicazioni paratransgeniche per il controllo delle malattie trasmesse da zanzare sono state giudicate le specie *Enterobacter agglomerans* e *Pantoea agglomerans*.

Uno studio recente ha dimostrato che ceppi di *P. agglomerans* ingegnerizzati per esprimere alcune molecole effettrici anti-*Plasmodium,* hanno inibito lo sviluppo del parassita umano della malaria (*Plasmodium falciparum*) e del parassita malarico dei roditori (*Pl.* B*erghei*) fino al 98% in vivo. Questa è una ulteriore dimostrazione che l'approccio paratransgenico può essere uno strumento potente per il controllo della malaria (Wang *et al.*, 2012).

In questo contesto, un simbionte che negli ultimi dodici anni circa ha attratto grande interesse, è il batterio acetico *Asaia* (Favia *et al*., 2007). Questo alfa-proteobatterio ha stabilito una peculiare relazione simbiotica con molte specie di zanzare e, più in generale, con molte specie di insetti. Questa peculiarità si riferisce a diversi elementi: prima di tutto *Asaia* si localizza nell'intestino, nelle ghiandole salivari e negli organi riproduttori delle zanzare di entrambi i sessi in molte specie dei generi *Anopheles*, *Aedes* e *Culex* (Favia *et al*., 2007; Damiani *et al*., 2010, Crotti *et al*., 2009; DeFreece *et al*., 2014). In secondo luogo, *Asaia* è presente in tutte le fasi dello sviluppo delle zanzare vettrici studiate, con prevalenze variabili all'interno delle popolazioni ospiti, ma spesso risultando il batterio dominante (a volte raggiungendo il 100% di prevalenza). L'*Asaia* è facilmente coltivabile fuori dall'ospite in "cell-free media" ed è stata geneticamente trasformata per esprimere proteine esogene. La re-introduzione dei batteri modificati, tali da esprimere molecole fluorescenti nelle zanzare, ha mostrato inequivocabilmente la loro capacità di colonizzare massivamente gli organi originali, in un numero rilevante di zanzare delle popolazioni destinatarie (Fig. 1). È inoltre da sottolineare che *Asaia* utilizza diversi percorsi di trasmissione all'interno e tra le popolazioni di zanzare. Può essere trasmesso verticalmente alla discendenza per via materna o paterna e in maniera trans-stadiale, può inoltre essere trasmesso orizzontalmente tra individui mediante copula o "co-feeding". Questo offre grandissime possibilità per l'introduzione e la diffusione

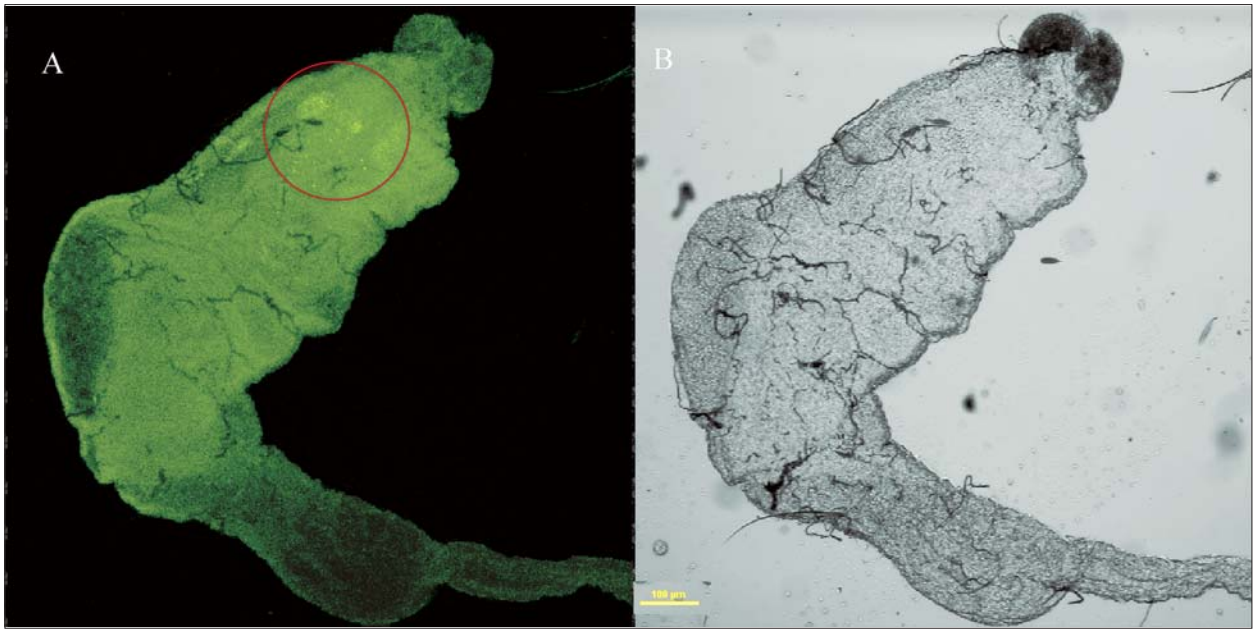

Figura 1 – A. Colonizzazione dell'intestino di zanzara della specie *Anopheles stephensi*, da parte di batteri del genere *Asaia* modificati allo scopo di produrre la Green Fluorescent Protein. Nel cerchio rosso è possibile vedere una intensa colonizzazione di batteri esprimenti la proteina ricombinante. B. Immagine dell'intestino colonizzato a contrasto di fase.

delle molecole effettrici anti-patogeno nelle popolazioni naturali. Recentemente l'*Asaia* è stata ingegnerizzata per esprimere condizionalmente la proteina scorpina ad azione antiplasmodiale una volta avvenuto il pasto di sangue. Questi ceppi di *Asaia* hanno mostrato di inibire l'infezione da *Plasmodium* aprendo la possibilità di applicare questa strategia per il controllo della malaria su campo (Shane *et al.*, 2018.).

Variabilità del microbiota delle zanzare

Tra gli artropodi, il microbiota dell'intestino di molte specie/popolazioni di insetto è stato ampiamente studiato, fornendo informazioni cruciali sul ruolo e le implicazioni delle relazioni simbionte-ospite. Questi studi hanno ampiamente rilevato come popolazioni di una stessa specie ma provenienti da aree geografiche differenti, si caratterizzino per una composizione del microbiota "popolazione-specifica". Un esempio tra i tanti riguarda le variazioni del microbiota in *Culex nigripalpus*, vettore del virus del Nilo occidentale e dell'encefalite di Saint Louis, in popolazioni provenienti da diverse località geografiche (Duguma *et al.*, 2019).

Un numero limitato di studi ha invece avuto come obiettivo lo studio della composizione del microbiota delle ghiandole salivari e degli organi riproduttori, entrambi organi cruciali per l'invasione dei patogeni e per l'eventuale trasmissione verticale di microrganismi simbiotici. Ciononostante, la caratterizzazione del microbiota dell'intestino, delle ghiandole salivari e degli organi riproduttori di diverse specie di zanzare, rappresentative di alcuni dei principali vettori di malattie, allo scopo di descrivere le dinamiche delle comunità batteriche all'interno delle differenti popolazioni e dei differenti organi, ha evidenziando che le zanzare sono caratterizzate da un microbiota distintivo in diversi organi, probabilmente riflettendo diverse funzioni e/o processi di adattamento di tipo organo-specifico, specie-specifico o addirittura popolazione-specifico (Mancini *et al*., 2018).

In definitiva, molteplici fattori, come la condizione dell'habitat e la specie/popolazione di appartenenza delle zanzare possono influenzare la composizione complessiva della comunità microbica e quindi fornire una base per ulteriori indagini sulle interazioni tra i vettori, le loro comunità microbiche, l'ambiente e gli ospiti vertebrati che in ultimo, da un lato possono influenzare il rischio di malattia trasmessa da vettori, dall'altro offrire possibili "tools" per il contrasto alle malattie trasmesse dalle zanzare.

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THE ROLE OF THE ENDOSYMBIOTIC BACTERIUM *WOLBACHIA* IN THE CONTROL OF *AEDES ALBOPICTUS*-BORNE HUMAN DISEASES

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The role of the endosymbiont bacterium Wolbachia in the control of Aedes albopictus*-borne human diseases*

In recent years, the endosymbiotic bacterium *Wolbachia pipientis* acquired a leading role in the context of the biotechnological tools available for the development of innovative control strategies against vector mosquitoes. This success is mainly due to two biological properties shown by certain strains of this bacterial endosymbiont: i) the ability to induce a post-mating mechanism of reproductive incompatibility, known as Cytoplasmic Incompatibility (CI); ii) the induction of a reduced vector competence for various severe viruses once an appropriate *Wolbachia* infection has been established in suitable vectors. These properties, both alone or combined, can be exploited to reduce the epidemiological role of a target vector species through the trans-infection of opportune *Wolbachia* strains. This objective was pursued at ENEA focusing on *Aedes albopictus* through the replacement of its native *Wolbachia* infection with specific foreign strains. Herein, we review all of the results achieved by this research program and discuss them in the context of developing opportune strategies of field application. Specifically, we highlight the potential of a method of *Ae. albopictus* suppression based on the exploitation of *Wolbachia* also pointing out the needed safety and sustainability issues.

KEY WORDS: Incompatible Insect Technique, Cytoplasmic Incompatibility, vector competence, population replacement

INTRODUCTION

Aedes (*Stegomyia*) *albopictus* (Diptera: Culicidae), commonly known as the Asian tiger mosquito, is currently among the most invasive insect species of the world. Following the outbreaks of chikungunya virus in the Indian Ocean islands in 2005-2006 (ZELLER *et al.*, 2018) and in Italy in the summer of 2007 (207 cases) (ANGELINI *et al.*, 2007), the European Centre for Disease Prevention and Control (ECDC) closely collaborated with experts in entomology to ensure a comprehensive understanding of the vector-related risk for introduction of the virus in Europe (ECDC, 2009). In fact, due to the effects of global warming (BARCLAY, 2008), re-emerging arboviral pathogens such as dengue and chikungunya viruses (respectively, DENV and CHIKV) have been becoming an increasing threat also in temperate regions (GASPERI *et al.*, 2012), as recently confirmed by a second Italian CHIKV outbreak occurred in summer 2017 in the Southern province of Rome (MANICA *et al.*, 2017).

The concept that vector control remains a key option in the general strategy to reduce the incidence of vector mediated diseases in humans is now consolidated in the scientific community. This is a strong incentive to focus research efforts towards the development of innovative technologies for vector control.

THE GENETIC CONTROL METHODS: A POWERFUL WEAPON AGAINST MOSQUITO VECTORS

Current control methods against *Ae. albopictus* (insecticide spraying, larval control, source removal, community participation) show unsatisfactory results in terms of a sustainable reduction of vector density (RITCHIE and JOHNSON, 2017). It could be argued that the main reason for the weakness of conventional control practices lies in the eco-ethology of the species that makes it very adaptable to colonize a number of artificial habitats. Also, mosquitoes showed remarkable capacity to acquire resistance to several insecticides (MOYES *et al.*, 2017) and breeding sites are often located in private properties, thus making control by public institutions extremely difficult because of limited access to private properties and to the high costs of running control programs.

This, combined with recent enabling technical advances in mosquito genetics, provides the underlying motivation for the development of new genetics-based approaches that basically are imple-

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mented as "population suppressors and/or pathogen blockers".

Among genetic control strategies, the Sterile Insect Technique (SIT) exploits the mutagenic properties of ionizing radiations (gamma and X rays) to induce male sterility (Lees *et al.*, 2015), thus exploiting classical genetic principles, while more recent genetic systems such as innovative recombinant DNA methods (Alphey *et al.*, 2010) and gene drive (Kyrou *et al.*, 2018) (Hammond *et al.*, 2016) have opened the door to the development of powerful genetics-based tools with which to fight major vector-borne diseases.

Endosymbiotic control: *Wolbachia* as a means to induce egg sterility

A third way, the exploitation of endosymbiotic bacteria, in particular *Wolbachia*, has been progressively affirming in the last decade. *Wolbachia pipientis* is a widespread intracellular bacterium, carried by an estimated 60% of insect species as well as by some crustaceans, mites and filarial nematodes (Zug and Hammerstein, 2012). This bacterium acts like a real manipulator of host reproduction (Werren *et al.*, 2008) but some strains also inhibit pathogenic viruses replication in the vector mosquitoes representing a very promising powerful tool for controlling vectors of human diseases (Bourtzis *et al.*, 2014) (Sinkins, 2013).

Cytoplasmic incompatibility (CI) is the most frequently found *Wolbachia*-induced phenotype and has been described in several arachnids, isopods and insect orders (Werren, 2008). Sperm from *Wolbachia*-infected males is incompatible with eggs from females that do not harbour *Wolbachia* or are infected with a different *Wolbachia* strain (or strains). *Wolbachia*-mediated CI acts through two distinct mechanisms: sperm modification during spermatogenesis and rescue of this modification in eggs infected with the same *Wolbachia* strain. In the absence of compatible *Wolbachia* strains, the fertilisation fails and development of the embryo is disrupted. The molecular mechanisms that underlie CI are still not known with certainty, despite considerable work carried out on the phenomenon and various proposed mechanisms (Bossan *et al.*, 2011) (Le Page *et al.*, 2017) (Beckmann *et al.*, 2017).

Exploiting *Wolbachia*-induced sterility to enhance suppression strategies against *Ae. albopictus*

Modern biotechnology techniques allow us to artificially transfer this endosymbiont bacterium from a species to the other, offering the possibility to generate new patterns of CI and enlarging the list of target species for *Wolbachia*-based control strategies. The technology used for this purpose, namely transinfection, is based on the microinjection of *Wolbachia* infected ooplasm containing the appropriate *Wolbachia* strain into recipient insect embryos.

In 2008, a new *Ae. albopictus* line was established in ENEA Casaccia Research Center (Roma) through the replacement of the wild-type *Wolbachia* infection (wAlbA and wAlbB strains) with a *Wolbachia* strain caught from *Culex pipiens molestus* (Calvitti *et al.*, 2010). The new mosquito line, named ARwP, was characterized in the subsequent years and selected to eliminate the fitness costs initially associated with the new infection (Calvitti *et al.*, 2012). ARwP *Ae. albopictus* displays a bidirectional CI pattern with the wild-type populations and shows other biological traits which were found favourable for mass production and field application as a suppression tool against this vector species (Puggioli *et al.*, 2016).

The *Wolbachia*-based Incompatible Insect Technique (IIT), relies on the release of large numbers of "incompatible males" to significantly reduce the mean egg fertility of the target population. It may offer a highly efficient approach to reach this objective because it can conjugate high efficacy and specificity with sustainable costs and negligible side-effects (Brelsfoard and Dobson, 2009) (Jeffries *et al.*, 2016). The efficiency of the approach is starting being demonstrated in the field with *Ae. albopictus* in USA (Mains *et al.*, 2016) and, more recently, ENEA obtained the authorization by the Italian Ministry of Health to carry out similar pilot trials also in Rome (Italy).

The advantage of incompatible over irradiated males, is due to the fact that the achievement of a 100% unconditional sterility, by irradiation, is generally associated with significant somatic effects which may negatively affect their male mating competitiveness and survival (Bellini *et al.*, 2013). In contrast, ARwP incompatible males preserve actually a status of healthy fertility (Moretti and Calvitti, 2013). Basically, they are sterile only when necessary, that is when they mate with wild-type females. Consequently, incompatible males preserve a natural level of mating competitiveness as demonstrated in trials performed both in small laboratory cages (Atyame *et al.*, 2016) and even more evidently under large enclosures in the field (Puggioli *et al.*, 2016).

Managing the lack of a perfect method for sex separation

If on the one hand incompatible males may enhance the traditional SIT approach, on the other, the lack of

effective methods for sex separation remains a major constraint even more pressing in the case of IIT (Calvitti *et al.*, 2015) (Zhang *et al.*, 2015). Indeed, in large scale operations, millions of male adult mosquitoes are released each time and even a small percentage of residual females would mean that thousands of females are released. It is certain that releasing additional disease vectors in areas subjected to epidemics would not be acceptable.

While innovative methods to achieve a perfect sexing are under investigation, it is important to note that different *Wolbachia* infection types may lead to two main incompatibility patterns which drastically affect the possibility to manage the females possibly escaping from the sexing procedures.

In the case of an incompatibility pattern based on unidirectional CI (i.e releasing a triple infected *Ae. albopictus*), once contaminant females have been released into the field, they can produce progeny also by mating with wild-type males. This reproductive advantage over the wild-type females would lead to a progressive replacement of the local mosquito population by the new infection type which would reduce the effectiveness of population suppression and rule out any possibility of eradication. In addition, the new infection might spread through spatial waves gradually leading to a widespread replacement phenomenon (Dobson *et al.*, 2002) (Jiggins, 2017). Combining CI with irradiation at doses only capable to sterilise females has been proposed as a mean to reduce this risk and allow to co-releases incompatible males and a few sterile females (Zhang *et al.*, 2015).

In the case of bidirectional CI patterns, simulation models (Dobson *et al.*, 2002) and recent validation experiments (Moretti *et al.*, 2018a) support the conclusion that repeated releases of incompatible males with small percentages of contaminant females have few chances of inducing population replacement except when environments are confined or isolated and thus closed to migration. The latter condition does not impedes the occurrence of spatial waves of spreading of the new infection type that could preclude eradication purposes of isolated target populations. In contrast, when targeting population suppression in areas open to the a constant input of wild-type mosquitoes (i.e. operating in heavily infested urban areas), low rates of female co-release could be managed to target high efficiency and sustainability in wild-type suppression. In fact, the temporary establishment of conditions of coexistence between two incompatible populations (Keeling *et al.*, 2003) may enhance the effectiveness of the incompatibility in reducing the overall mean egg fertility. Because of a high rate of unproductive matings (due to bidirectional CI), these mixed populations would slow down their growth rate, until the restoration of the predominance of the wild-type population (Moretti *et al.*, 2018a).

In this context, the costs for sterile males production could be also decreased as sexing protocols could be less severe and males to be released could be reduced with respect to other approaches.

Exploiting an *Aedes albopictus* line producing incompatible males and showing a strongly reduced vector competence

In addition to the suppression effects associated to the release of incompatible males, establishing the coexistence of two bi-directionally incompatible populations means to extend longer the slowing effect on the population growth rate, also once male releases have been stopped. In order to apply this suppression strategy we need that co-released females show a not increased or better a significantly reduced vector competence.

To generate an *Ae. albopictus* line with such characteristics, we recently microinjected the *w*Mel strain of *Wolbachia* in ARwP embryos. A new *Ae. albopictus* line was established (ARwP-M) which combines the remarkable suitability to the mass rearing protocols and male mating competitiveness shown durably by ARwP, with a reduction in the vector competence of females (Moretti *et al.*, 2018b).

Currently, this new line is under testing in laboratory to monitor the stability of the double *w*Pip and *w*Mel *Wolbachia* infection over the generations and under various rearing conditions.

Future perspectives and conclusions

After a long phase of laboratory and semi-field studies, aimed at verifying the long-term stability of the "*w*Pip" infection in *Ae. albopictus* and its related effects on host, ARwP *Ae. albopictus* is ready for open field evaluation. In the summer of 2018 we long last obtained the permission from European and National authorities to start the first European field trials in Rome involving a mosquito line with manipulated *Wolbachia* infection. Incompatible male releases started in a limited green area of Rome (Villa Mirafiori) at the University "La Sapienza" of Rome. The project is being conducted in cooperation with the Department of Public Health of the Sapienza University of Rome and the Centro Agricoltura e Ambiente (CAA) G. Nicoli of Crevalcore (BO). The collection of data is still being completed and results, now under analysis, will be published shortly.

We hope to expand the experimental areas, as early as next year, in order to consolidate the data and obtain valuable indications on how to implement the operating issues.

RIASSUNTO

Negli ultimi anni, il batterio endosimbionte *Wolbachia pipientis* ha acquisito un ruolo di primo piano nel contesto degli approcci biotecnologici e molecolari disponibili per lo sviluppo di strategie innovative di controllo di zanzare vettori. Tale successo è dovuto principalmente a due proprietà biologiche mostrate da alcuni ceppi di questo batterio: i) l'induzione di un meccanismo di sterilità condizionale in post-copula, noto come Incompatibilità citoplasmatica (CI); ii) l'interferenza negativa con i processi di replicazione e trasmissione di importanti virus patogeni per l'uomo.

La combinazione di queste due caratteristiche, attraverso l'impiego di linee di *Ae. albopictus* con adeguate simbiosi batteriche, può essere la base per sviluppare nuove strategie di controllo dei vettori ad elevata sostenibilità, integrabili con altre tecniche e con effetti nel lungo termine.

Nel caso di *Ae. albopictus*, modelli teorici e dati sperimentali di validazione suggeriscono che in aree target ad elevata densità di infestazione e con flussi attivi di popolazione (caratteristica di molte aree urbane), il rilascio di maschi incompatibili può agire efficacemente, secondo i canoni classici della tecnica di lotta col maschio sterile, riducendo la fertilità media della popolazione selvatica e rallentandone così il tasso di accrescimento. Tuttavia, in aree non confinate ad alta densità di zanzare, ci si aspetta che la pressione migratoria renda improbabile l'eradicazione e che, in caso di interruzione dei lanci di maschi incompatibili, la popolazione selvatica riacquisisca immediatamente il naturale tasso d'accrescimento.

Gli attuali protocolli di separazione dei sessi della zanzara tigre non consentono di escludere in modo assoluto la presenza di femmine residuali (0,2 - 1%) tra i maschi sterili da rilasciare. Nel caso si disponga di linee di *Ae. albopictus* in grado di produrre maschi incompatibili e femmine con attenuata competenza vettoriale, il ripetuto co-rilascio di femmine può determinare locali e temporanei episodi di coesistenza tra popolazione selvatica e popolazione simbioticamente modificata che, essendo tra loro sterili in modo bidirezionale, generano accoppiamenti improduttivi. Simulazioni modellistiche e verifiche sperimentali hanno evidenziato che queste condizioni inducono un significativo rallentamento della ripresa demografica della popolazione target che si protrae nel tempo anche una volta terminati i lanci di maschi. Questo si traduce in evidenti benefici per quanto riguarda la sostenibilità a lungo termine degli approcci di controllo basati su *Wolbachia* e la loro integrabilità con altri metodi di lotta.

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