Genetic approaches to find innovative biotechnological strategies for an eco-sustainable fight against *Aedes albopictus* mosquito.

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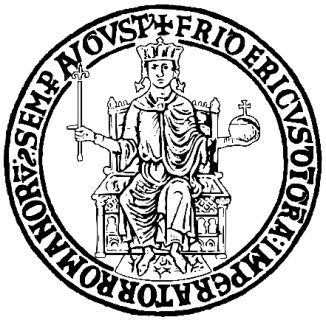

UNIVERSITY OF NAPLES
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Genetic approaches to find innovative biotechnological strategies for an eco-sustainable fight against *Aedes albopictus* mosquito.

Ph.D. in BIOLOGY

XXXIV Cycle

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One step at a time.

Abstract

The research of this doctoral project focuses on the study of the Asian tiger mosquito *Aedes albopictus*. This mosquito is an aggressive and epidemiologically imposing insect since its geographical area has considerably expanded in recent decades. It is one of the 100 most invasive species in the world and its territory is rapidly spreading due to its tolerance to egg drying, different environmental adaptations, photoperiodic diapause and resistance to insecticides (Li et al., 2014; Li et al., 2018; Xia et al. al., 2018).

The *A. albopictus* sex determination pathway has been investigated for its regulatory mechanisms. In addition, new gene targets have been identified in order to design novel sex separation systems that can contribute to the development of eco-sustainable strategies for the control and eradication of this invasive and dangerous mosquito species.

Finally, through field activities, it was possible to monitor the distribution of *A. albopictus* on the Island of Procida (NA) by actively involving citizens, according to the "*Citizen-science*" model.

Contents

CHAPTER 1

General introduction and thesis overview

THE ASIAN TIGER MOSQUITO *Aedes albopictus*

Aedes (Stegomyia) albopictus (Skuse, 1894), also known as *Stegomyia albopictus sensu* and as the "Asian tiger mosquito", is native to southeast Asia with the ability to move towards the Pacific and Indian ocean islands, to Cina, Japan and to Madagascar. The tiger mosquito is 4 to 10 millimeters long (the males are about 20% smaller respect to females) and is easily recognizable by its livery: the adults have a black body with transverse white bands on the legs and abdomen, which give the "striped" look (Figure n. 1.1).

Fig n.1.1- The Asian Tiger mosquito A. albopictus (Source: Carolina Nature)

Mosquitoes are "*holometaboli*" (*holo* = total, *metabolo*= metamorphosis or change) insects, so they present complete metamorphosis through a succession of larval stages that are very different from the adult from a structural, ecological and functional point of view. This cycle is called *multivoltine*, characterized by the egg stage (Figure n. 1.2-a), four larval stages (Figure n. 1.2-b), a pupal stage (Figure n. 1.2-c) and finally, an adult stage (Figure n. 1.2-d). Mosquitoes complete the life cycle mostly in water since only the adult form occurs in the air environment (Figure n. 1.2).

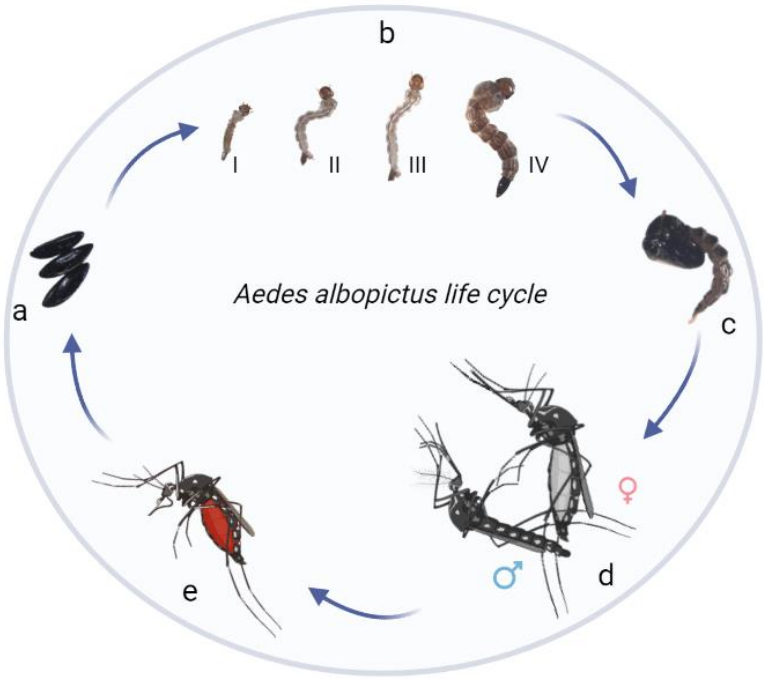


Fig n.1.2- A. albopictus life cycle
(a) Eggs; (b) larval stages (I-IV); (c) pupa stage; (d) adults; (e) female blood-fed. Schematics are created with BioRender.com.

Eggs of *A. albopictus* are generally 0.5 mm long with an elongated shape; they are white after being laid but become black after few hours (Figure n. 1.3). Eggs are usually laid on damp substrates above water level, but a diapause period may occur before the eggs hatch, consequentially they will not hatch until this resting period ends. Various stimuli, including a reduction in the oxygen content of water, changes in day length, and temperature, may be required to break diapause.

Fig n.1.3- Eggs of A. albopictus

The larval development consists of four stages in which the larva grows in size through 3 successive mouls, passing from a few millimeters in the first stage (L1), to just over one centimeter

in the fourth (L4) one. The larvae have a large head, globular thorax, and elongated abdomen (Figure n. 1.4).

They are related to tracheal respiration, like the adult, and that is why they must access atmospheric oxygen, which can be taken through an important anatomical conformation: the respiratory siphon (Mori., 1979).

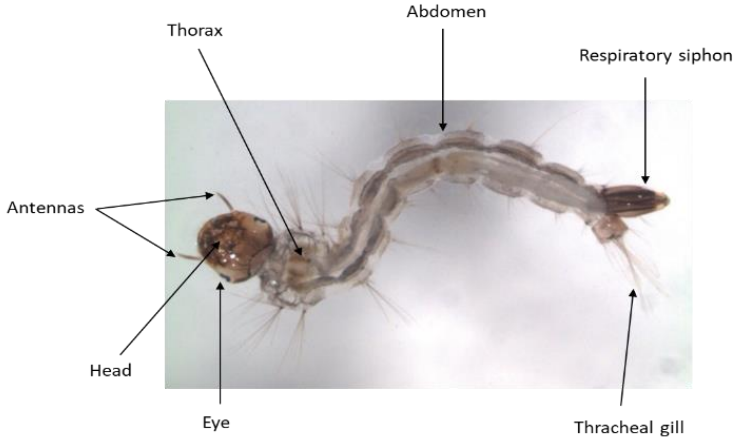


Fig n.1.4- Dorsal view of A. albopictus larva

The larvae eat by moving the brushes that act as vortices, filtering the water and bits of microorganisms. The larval stage takes a variable time, depending on the temperature and availability of food. Usually it lasts about 4-7 days in conditions of summer temperatures (25-30 °C).

At pupal stage (P), the insect stops eating and stays near the water surface where it only breathes. The pupa has a characteristic comma shape, in which it can be distinguished the head, the chest and an abdomen-tail (Figure n. 1.5). This stage generally lasts a couple of days, after which the tegument is torn and the adult emerges from the water.

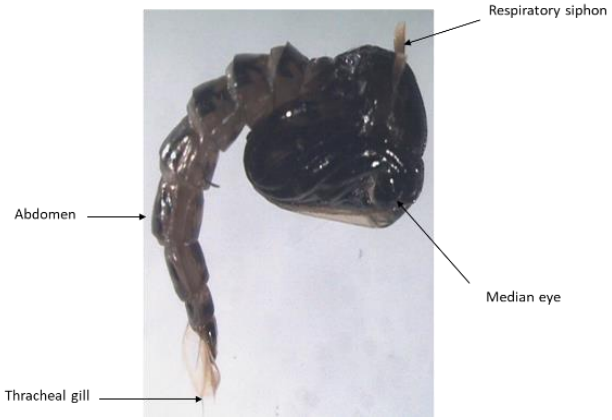


Fig n. 1.5- Pupa of A. albopictus

The adult's body is composed of three parts: head, thorax and abdomen (Figure n. 1.6). The antennae are evident on the head, elongated with numerous articles. In males, they are covered with numerous hairs, which give them a feathery appearance. In females, they are shorter and sparse. In both sexes, they have an olfactory function, while in males they also operate for the recognition of sex and species (Clements., 1996; Romi et al., 1997).

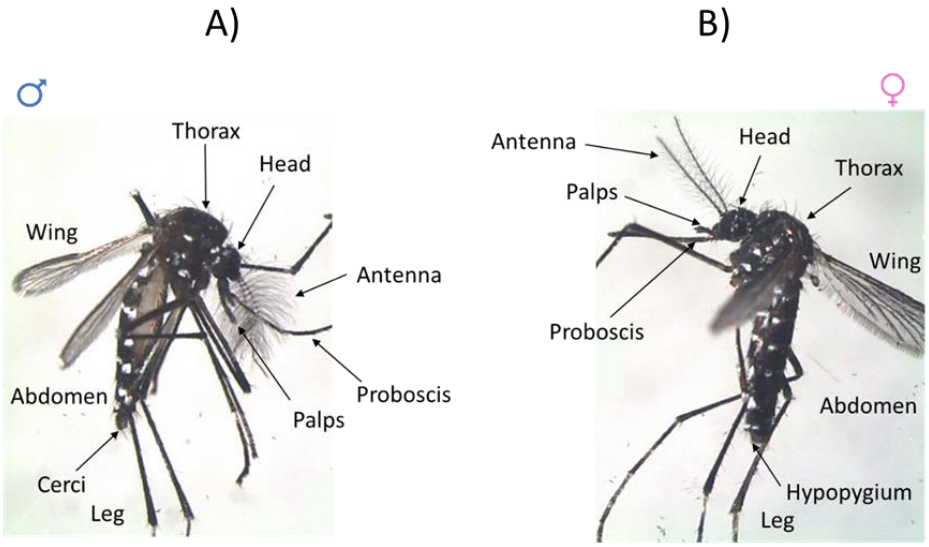


Fig n1.6- General morphology of adult mosquitoes.
Comparison between male (A) and female (B).

Adult mosquitoes, both male and female, eat vegetable juices and sugary substances. Only females need amino acids for the maturation of the eggs, which are found in the blood of vertebrates: batrachians, birds and mammals.

Fig n. 1.7- Females of A. albopictus before and after the bloodmeal

After making the blood meal, mosquitoes enter a digestion phase and they rest. The amount of eggs brought to maturity depends on the amount of blood that the mosquito has managed to suck, in general the number of eggs laid varies between 50 and 500.

ORIGIN AND DISTRIBUTION OF *A. albopictus*

The tiger mosquito has Asian origins but is currently considered an invasive insect as it has spread and adapted in different countries.

Today, *A. albopictus* is present in all countries along the Mediterranean, including parts of Turkey, Lebanon, Israel, and Syria in the Middle East, and is slowly making its way north (Medlock et al., 2012). Italy and southern France are the most infested countries (Medlock et al., 2012), but *A. albopictus* also have limited local distribution in southern Switzerland, The Netherlands, Bulgaria, Russia, Belgium, and Germany, confirming predictions of expansions based on climate changes (Caminade et al., 2012).

Once *A. albopictus* is established in an area it is very difficult to eradicate, and constant surveillance and appropriate control strategies are required (Holder et al., 2010). The widespread distribution of *A. albopictus* outside its native home-range is presumed to have been primarily human-mediated and accidental (Tatem et al., 2006).

In the Mediterranean basin, Europe offers the most ideal habitat for tiger mosquitoes (Mitchell et al., 1995). Already in 1979, its presence was reported in Albania (Adhami et al., 1998). This early date is due to the intense exchange of goods with China. Only 12 years later, in 1990, its presence was reported in Italy, in a nursery school in Genoa. In 1991 it was also found in Padua in a used tire warehouse that traded with the United States. It was possible to demonstrate that the tires from the United State were directly responsible for the introduction of the tiger mosquito into the deposit (Romi, 1999). In France, the tiger mosquito was found in 1999, in Normandy, in a deposit of old tires. In Spain *A. albopictus* was detected in 2004 in the Barcelona region (Aranda et al., 2006). The first discovery in Switzerland took place in Ticino in 2003.

The introduction in the various areas is dependent on climatic conditions which may prove to be more or less optimal for the mosquito: there is talk of a water requirement of about 500 mm of rain in the summer season and temperatures between 25 and 30 °C; however, thanks to the remarkable plasticity ecology, *A. albopictus* can withstand up to an optimal minimum of 11 °C and rainfall that is around 200-300 mm of rain (Ann. Ist. Super. Sanità, vol. 37, n. 2; 2001).

Distribution in Europe is observable from the data collected year by year by the *European Center for Disease Prevention and Control* (ECDC) (https://www.ecdc.europa.eu/en) (Figure n. 1.9).

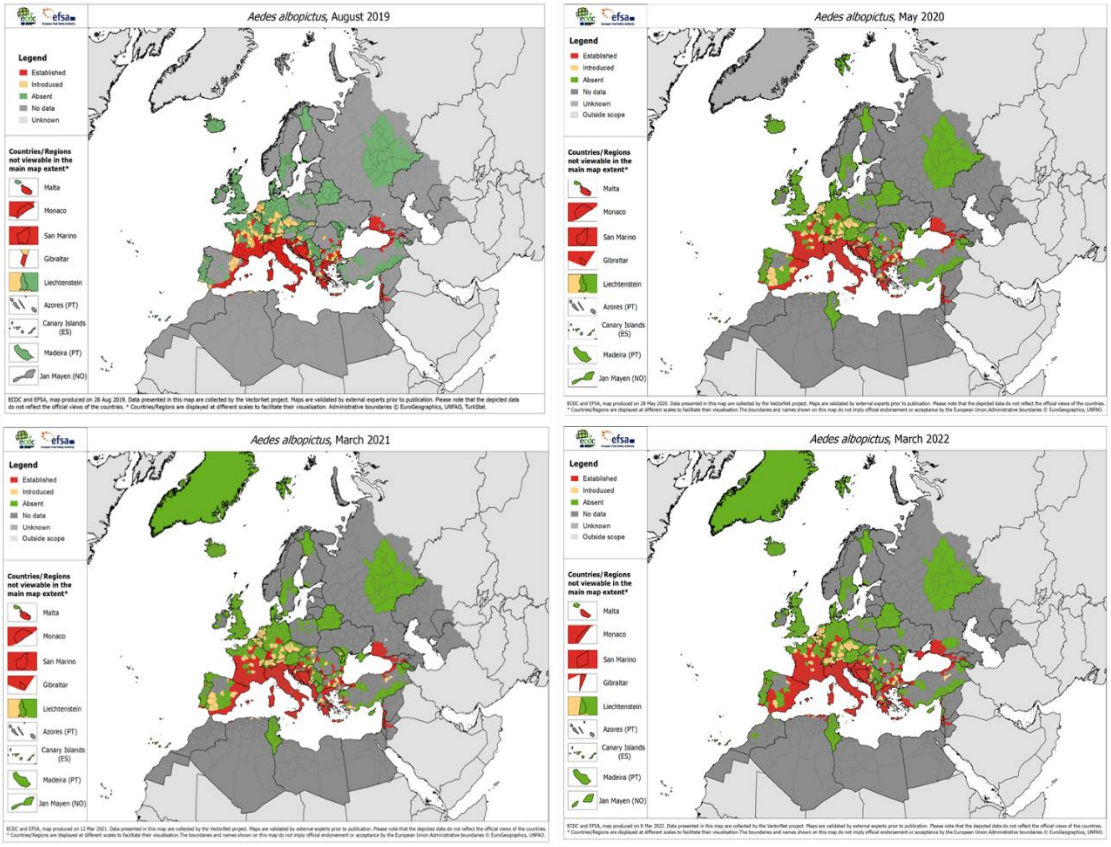


Fig n. 1.9- A. albopictus distribution in Europe during the last years (Source:* ECDC.*, 2019-2020-2021-2022)

In Italy, *A. albopictus* has been first discovered in Genoa in September 1990, when a few adult specimens were found in a nursery school (Sabatini et al., 1990). In August 1991, diurnal mosquitoes were identified in the southern part of the city of Padua, and the first breeding population was discovered (Dalla Pozza et al., 1992).

Dalla Pozza et al. (1994) stated that a shipment of used tires imported from Atlanta (Georgia, USA) was one of the sources from which the mosquitoes originated; Reiter (1998) specified that between 1988 and 1995 Italy had imported, on multiple occasions, several thousands of used tires from countries (USA, Japan and Taiwan) where *A. albopictus* was present.

By the end of 1995, the species had spread to scattered foci in 10 regions including 20 provinces (Romi, 1995; Romi, 2001) and some rural cities in North-eastern Italy (Veneto region) were heavily infested (Knudsen et al., 1996). Throughout 1997, a total of more than 20 provinces reported infestations (DiLuca et al., 2001; Romi, 2001). In 2003, however, the mosquito had been reported from several central mountainous provinces such as Perugia and Terni, many eastern

areas, covering almost the entire Adriatic coastal provinces southwards until Foggia (Puglia), and several southern areas in the provinces of Taranto, Cosenza, and Salerno (Romi et al., 2003).
In 2015, the presence of *A. albopictus* was confirmed on the three southern minor islands of Sicily: Lampedusa, Linosa and Pantelleria (Di Luca et al., 2017).

MEDICAL RELEVANCE

The sanitary significance of mosquitoes is linked to the ability to transmit pathogens to humans and other animals such as plasmodia, heartworms and arboviruses (Hapairai et al., 2013). The continuous evolutionary adaptation is particularly troubling considering *A. albopictus* competency for multiple arboviruses (Paupy et al., 2009) and emphasizes the importance of understanding and monitoring the competence of geographic populations for different arboviruses (Bonizzoni et al., 2013). In addition, *A. albopictus* exhibits ecological plasticity which makes it able to adapt and spread to new environments while interacting with other vector species. Diseases transmitted by mosquitoes to humans cause hundreds of thousands of deaths all over the world every year, so much to be considered the most lethal animal on the planet (McCarthy, 2014). Disease transmission occurs when the mosquito draws the blood of an infected individual. In this way, the virus enters the insect, arrives in the stomach and the rest of the organs, including the salivary system. When the animal stings a new host, the virus is transmitted through its saliva to the new host.

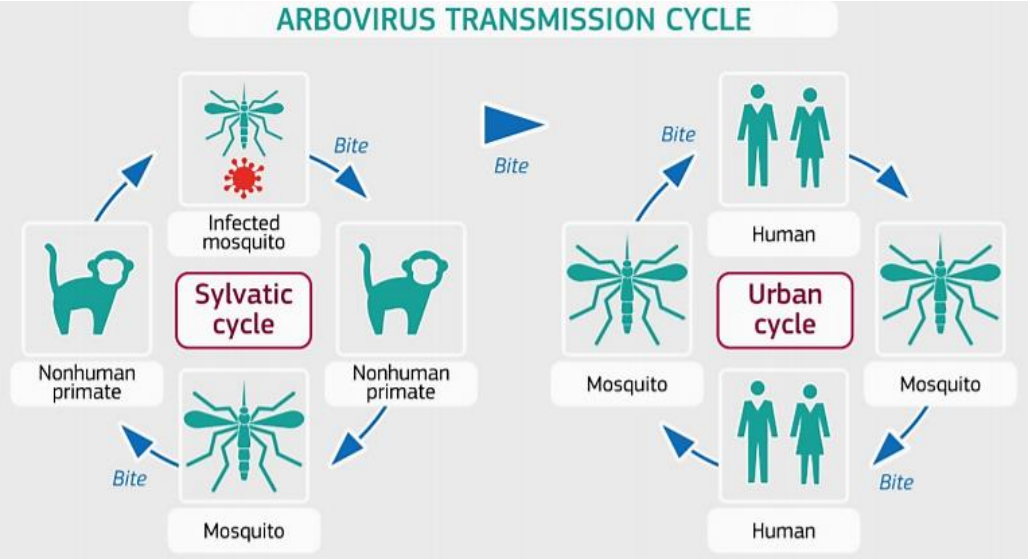


Fig n. 1.10- Arbovirus transmission cycle (Source: JRC)
In suburban and urban settings, infected Aedes mosquitoes spread the virus through a human–mosquito–human cycle. The virus can be acquired by mosquitoes from an infected person and then transmitted to other humans.

A. albopictus has been shown to be capable of transmitting 26 viruses belonging to the Flaviviridae, Togaviridae, Bunyaviridae, Reoviridae and Nodaviridae families (Paupy et al., 2009).

A. albopictus was the primary vector of recent (2001–2010) DENV outbreaks in Hawaii, Indian Ocean islands, Central Africa, and southern China (Rezza et al., 2012; Gasperi et al., 2012) and the first DENV autochthonous transmissions in Europe (Schaffner et al., 2013).

In 2005 and 2007 *A. albopictus* was the only or main vector for CHIKV outbreak, which occurred in all of the Indian Ocean islands and the surrounding countries. *A. albopictus* has also been confirmed as a vector during the first European outbreak reported in Italy in August 2007 (Angelini et al., 2007): Emilia-Romagna is the first European case of an autochthonous outbreak of a tropical disease transmitted by mosquitoes and more than 200 cases of infected have been recorded (Rezza et al., 2007; Dottori et al., 2008). In 2019, an outbreak of chikungunya, totaling more than 500 cases, occurred in Central and South Italy (Lazio and Calabria) (Riccardo et al., 2019; Vairo et al., 2018). However, in many temperate countries where *A. albopictus* is established, the species is primarily important for its ectoparasitic activity: in Italy, it has become one of the most important mosquito pest species (Romi et al., 2001).

STERILE INSECT TECHNIQUE (SIT)

Although mosquito control is critical to preventing mosquito-borne illnesses, the emergence of insecticide resistance, concern for the adverse effects of insecticides on non-target organisms, and a general lack of support for mosquito control programs compromise current mosquito control efforts (Benelli, 2015). Environmentally friendly alternatives have been proposed, such as *The Sterile Insect Technique* (SIT) (Knipling et al., 1955). SIT is emerging as a powerful alternative to most commonly-used approaches since it is ecologically benign, specific, and non-persistent in the environment if releases are stopped (Oliva et al., 2021).

The SIT is the first method involving insect genetics for population control of species. It has been used extensively against pest insects, in area-wide control programs of economically and medically important species, as an alternative to insecticides, promoted by the *International Atomic Energy Agency* (IAEA) and the *Food and Agricultural Organization of the United Nations* (FAO) (Dyck et al., 2005).

Since the early 1990s, the Joint FAO/IAEA Program has played a leading role in developing the ‘‘SIT package’’ for mosquitoes (Lees et al., 2015). SIT is a species-specific and environmentally friendly method for insect biological control (Figure n. 1.11 for the steps of the technique), based

on the release of sterilized (by X or gamma ray) insects within reasonable proximity of all native females to decrease the progeny, due to two different actions:

1 - The reduced mating between their fertile wild counterparts;

2 - Released insects are mobile and would actively seek mates.

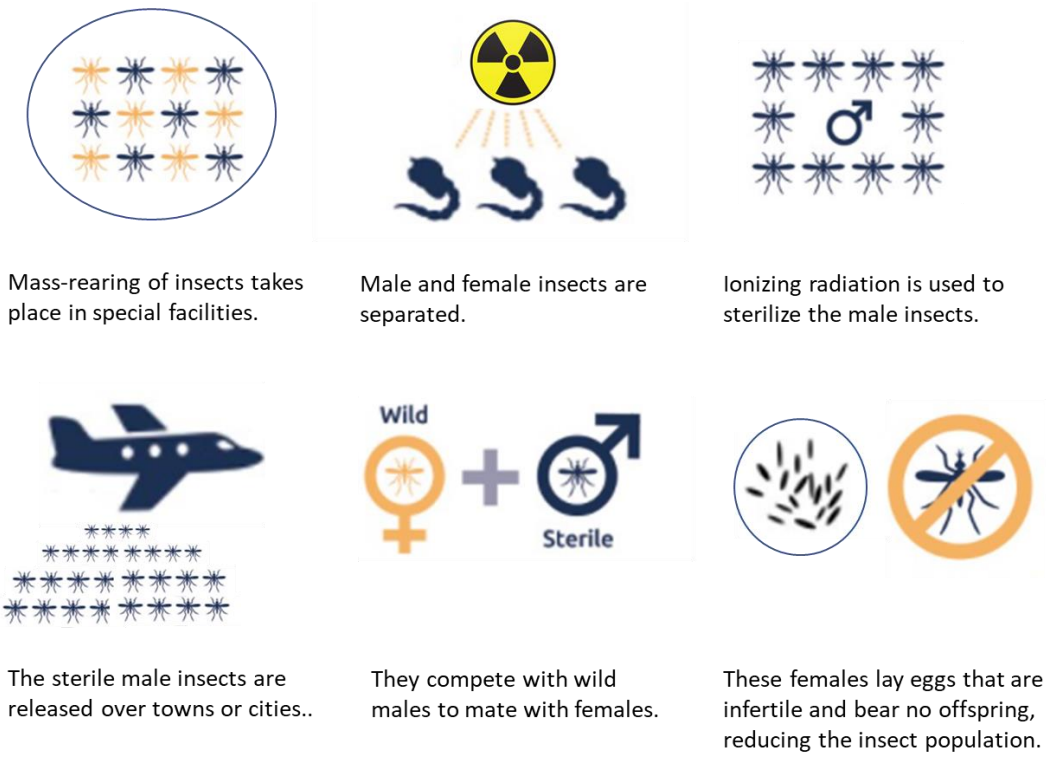


Fig n. 1.11- General scheme of a SIT program (Source: edit from TDR)

SIT has been already used successfully against several pest insects: it allowed the eradication of *Glossina austeni* from Zanzibar, *Cochliomyia hominivorax* from Libya and North and Central America, *Bactrocera cucurbitae* and *B. tryoni*, respectively from Japan and Australia and the Mediterranean fruit fly *Ceratitis capitata* from California, Florida, Mexico and Chile (Neil et al., 2010).

In species where females are harmful to human and animal health, such as mosquitoes, the release of only males is particularly critical. In order to ensure a high rate of male recovery and very few residual females (Gilles et al., 2014), an efficient sexing system is needed.

In 2007, an Italian group (Bellini et al., 2007) was among the first to revive the use of SIT against mosquitoes. They released around 1,000 irradiated *A. albopictus* pupae per hectare per week, resulting in a sterility rate up to 68% in populations between 16 and 45 ha (Bellini et al., 2013).

For pilot trials during this SIT application program, a mechanical sexing system has been designed and developed that exploits size dimorphism when the pupae are in the water (Bellini et al., 2013). Unfortunately, this system does not work well since it allows only 22-30% of males to be recovered while 0.5-1.0% of the females remain. In endemic countries, especially those with high disease rates, the low male survival rate has a severe impact on the production costs, and the presence of females is not acceptable due to the possibility of disease transmission from females. Sex separation, to be able to release only males, remains an important critical issue to be solved to apply SIT in mosquito suppression (Bellini et al., 2018). Besides the fundamental need to produce large quantities of males, these must be able to survive, actively disperse and compete for mating. Although the quantity of insects can be easily measured, assessing the quality is more challenging. Adult emergence rate, flight and dispersal capacity, longevity, location of mating, courtship, mating and sperm transfer are the most important parameters to be monitored and preserved during any campaign involving the release of sterile mass reared insects (Balestrino et al., 2017). .

INSECT SEX DETERMINATION CASCADE

During development, a complex network of genes is required to direct the growing embryo towards either a male or female pathway. Sex determination in insects is classified into three main categories depending on the different primary sex determining signals: 1) zygotic, 2) maternal, or 3) environmental (Sánchez., 2008). The mechanisms underlying sex determination in organisms are astoundingly diverse. There are numerous primary signals used by insects in sex determination pathways. A cascade of genes acts upon one another to carry the information from primary signal to terminal differentiation. The three critical components of the sex determination cascade are primary signal, key gene and final double-switch gene. Insects differ in their primary signal and keys genes, while the terminal gene is deeply conserved (Graham et al., 2003). The interplay between splicing regulators and a primary signal determines whether or not regulated splicing occurs in some sexes while default splicing occurs in others. As a result, terminal double-switch gene transcripts are formed that are sex-specific and cause secondary sexual characteristics. The key gene acts as a splicing regulator and thus splices its transcript leading to a differentially spliced product in the two sexes (Sawanth et al., 2016).

To become a successful reproductive organism, the individual must be assigned a sexual identity correctly. There are considerable differences between the 2 sexes in regard to the gamete-producing tissues, the ovaries in females and the testes in males, but they can also show

considerable differences in their morphology, anatomy and physiology as well. It is also thought that sex-specific behaviors are controlled by the neuronal circuits built by the central nervous system (CNS) as a result of the decision to become male or female (Bopp et al., 2014).

SEX DETERMINATION IN FRUIT FLY

Scientists have extensively studied *Drosophila melanogaster* to discover and determine the molecular and genetic mechanisms underlying sex determination. A major determinant of female sexual identity in *D. melanogaster* is the X:A ratio (the ratio between the number of X chromosomes and the number of sets of autosomes) (Salz., 2011). X:A ratios of 1.0 (2X:2A) dictate female development, while ratios of 0.5 (1X:2A) determine male development (Cline, 1993). Transcriptional factors on the X chromosome are capable of activating transcription of the sex determinant *Sex-lethal* (*Sxl*). The gene encodes an RNA-binding protein that regulates development by influencing the synthesis of essential genes for dosage compensation, germline homeostasis, morphology, and behavior (Graindorge et al., 2011; Venables et al., 2012).

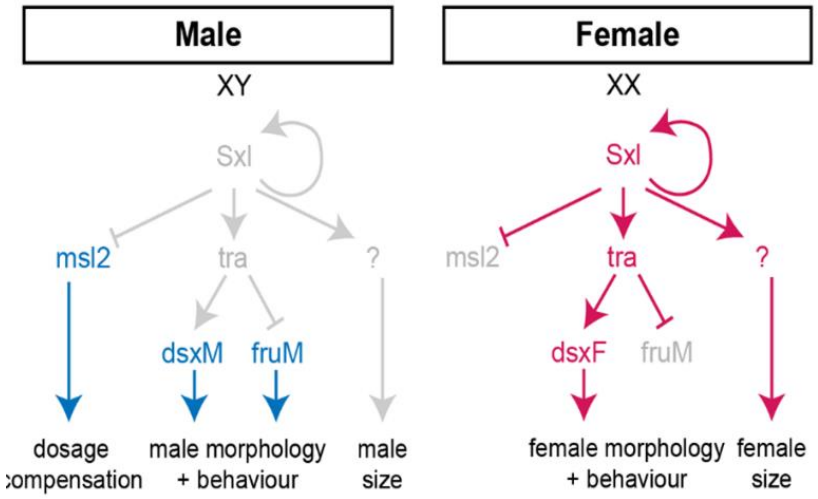


Fig n. 1.12 -Diagram of the sex determination pathway in Drosophila (Source: PLoS Biology).
In females, the presence of two X-chromosomes activates the expression of the splicing factor Sxl. TRA is only produced in females due to sex-specific splicing of Sxl. Tra functions as a transcription factor for the splicing of the transcription factors dsx and fru. X-chromosome dosage compensation complex assembly is limited in females due to the repression of the translation of the male-specific lethal 2 gene (msl-2).

During the early development, *Sxl* is active in females and inactive in males (Cline., 1983; Parkhurst and Meneely., 1994) and directly targeted by the X:A signal. The expression of *Sxl* is first transcriptionally controlled by an early promoter (Sxl^{Pe}) and later by differential processing

of RNA from a later promoter (Sxl^{Pm}). The Sxl^{Pe} is active only in XX embryos, and the Sxl^{Pm} is constitutively active in both XX and XY individuals (Keyes et al., 1992). In XX individuals, a double dose of X chromosome-linked factors activate *Sxl* producing a full-length SXL protein. In XY individuals, *Sxl* is inactive, and maleness follows (Erickson and Quintero., 2007).

The SXL functional protein can drive its splicing and direct the splicing of the downstream gene *transformer* (*tra*) in a female-specific manner. Only in females TRA functional protein is produced, which is able to control female-specific splicing of *doublesex* (*dsx*) and *fruitless* (*fru*) (Inoue et al., 1990; Camara et al., 2008). In male (absence of SXL protein), a non sex-specific splicing is followed by *tra* pre-mRNA, with production of shorter and non-functional TRA protein, leading also to a default male-specific splicing of *dsx* pre-mRNA.

The male-specific isoform DSX^M suppresses female-specific gene expression while activating male-specific gene expression. In females, the complex TRA-TRA2 and RBP1 recruits various splicing factors, known as SR proteins, to promote *dsx* female-specific splicing, and DSX^F directs female differentiation.

The sex determination pathways of the dipterans *D. melanogaster* and *Ceratitis capitata* (Wiedemann., 1824) have evolved separately for 120 million years and are consequently quite different (Beverley et al., 1984). In *C. capitata* the sex is determined by the presence of the Y chromosome (Saccone et al., 2002). Orthologues of *Sxl* and *dsx* have also been identified in *medfly* (Saccone et al., 1996; Saccone et al., 1998). In contrast to *Drosophila*, the orthologue of *Sxl (CcSxl)* in both XX and XY individuals expresses the same mRNA and protein isoforms (Saccone et al., 1998).

Different signals act on the same *transformer* gene in *Ceratitis* and *Drosophila*, determining sexual development in one direction or the other by transducing the decision in the female ($Cctra^{ON}$) or male ($Cctra^{OFF}$) sense (Graham et al., 2003; Willhoeft and Franz, 1996). Female-specific splicing control of the *Ccdsx* pre-mRNA is mediated by the medfly homologous TRA/TRA-2 protein (*Cc*TRA/*CcT*RA-2), which binds to dsxRE, a conserved splicing enhancer region, composed by clustered copies of a 13-nt long conserved sequence called TRA/TRA-2 binding site. The discovery of *dsx*RE elements inside and near male-specific exons of C*ctra* has revealed that, unlike *tra* in *Drosophila*, the *Cctra* gene is able of auto-regulation (Pane et al., 2002). *Cctra*'s pre-mRNA occurs alternative splicing in response to the presence or absence of a Y-linked masculinizing factor known as *Maleness-on-the-Y (MoY)*, which is the species' primary signal (Meccariello et al., 2019).

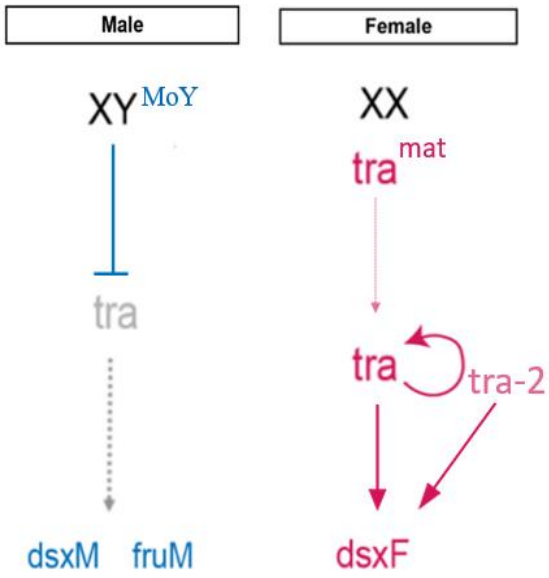


Fig n. 1.13 – Diagram of sex determination pathway in C. capitata
In blue are shown the genes responsible for the maturation of male-specific proteins; in pink are shown the genes responsible for the maturation of female-specific proteins.

CcTRA can also control the splicing of another downstream target gene, *fruitless (Ccfru)*, which is responsible for male sexual behavior (Salvemini et al., 2009). Similar to Drosophila, the *dsx* gene (*Ccdsx*), produces sex-specific transcripts through alternative splicing, implying that it functions as a regulator in sex determination (Saccone et al., 1996).
The MoY protein appears to block the link between the TRA/TRA-2 complex and the TRA/TRA-2 binding sites of the *Cctra* zygotic pre-mRNA in XY embryos. MOY causes default splicing in the male sense of both *Cctra* and the transcripts of the downstream genes *Ccdsx* and *Ccfru* in XY embryos in the first hours of embryonic development.

AIM OF THE RESEARCH

The main objective of the current thesis is to contribute to developing an innovative tool for an eco-sustainable control of the Asian Tiger mosquito *A. albopictus* by gaining more knowledge about the molecular regulation of the sex determination pathway and by identifying peculiar genes with possible biotechnological control functions.

Specifically, I first focused my research on the molecular and functional characterization of the downstream gene of the sex determination cascade, *fruitless*, involved in the control of courtship behavior in several insects. At the start of this project, little was known about the *fru* gene in the *Aedes* mosquitoes. This investigation explores the role of the *fru* gene in the sexual behavior of *A. albopictus*.

Another aim is to gain insight into new target genes that can be applied to develop innovative strategies to improve the application of the Sterile Insect Technique. The study identified two intriguing genes to focus on, the rhodopsin *GPROP3* and the *alpha-mannosidase.*

The last investigation is about the application of the SIT to control the population of *A. albopictus* on the Island of Procida. A monitoring and control program was designed through the active involvement of volunteers, using *Citizen-science* strategies.

THESIS OVERVIEW

During this research, I aim to answer the following questions: 1) Which genes are regulated by sex-specific alternative splicing?; 2) Is the function of the *fruitless* gene conserved in *A. albopictus*?; 3) Is it possible to develop an automated sexing system using genetic approaches?; 4) Could a citizen-based monitoring and control program against *A. albopictus* be realized?

Answering these questions will provide a better understanding of the sex-determining systems in *A. albopictus* and increase the possibility to realize a genetic sexing strain to obtain a good number of males to employ in a SIT program.

Chapter 2 described the experimental approaches and results toward the *in silico* identification and molecular characterization of genes (*fru, dsx, Sxl*) involved in the sex determination cascade. In this chapter the *fruitless* gene was studied through RNA interference experiments, to confirm its role in regulating sexual behavior.

Chapter 3 analyzed potential genes for improving the sexing stage for the application of SIT programs. In the first part of this chapter, were molecular analyzed the rhodopsin gene *GPROP3* during the different stages of development and through RNA interference

experiments were confirmed its role in the light response of larvae. The second part of chapter 3 examined the *alpha-mannosidase* gene expression during different stages of *A. albopictus and A. aegypti* mosquito development and found an unexpected male-specific expression.

Chapter 4 illustrates the data collected during the control and monitoring activities on Procida island.

Eventually, chapter 5 summarizes the conclusions of these data chapters and discuss future perspectives resulting from my Ph. D project. Moreover, the outcome from this study provides insight into the role of *fruitless* in male-specific behavior development, as it occurs in *D. melanogaster*.

In addition, the study of light response at the larval stage gives the possibility to identify the gene involved in this specific behavior and open the possibility to use this gene to create a genetic sexing strain regulated by the light.

CHAPTER 2

Sex determination pathway in *A. albopictus*

INTRODUCTION

Sex determination is a fundamentally vital process in the development of sexually reproducing organisms, but the underlying genetic mechanisms are remarkably diverse between species (Valenzuela et al., 2003; Beukeboom and Perrin., 2014). There are many genetic systems used by insects to determine sex (Sánchez., 2008). Systems range from male heterogamety (XX-XY), female heterogamety (ZW-ZZ) to haplodiploidy (male 1n-female 2n), and other systems (Beukeboom and Perrin., 2014; Blackmon et., 2015). Male heterogamety is most abundant, where male sex determination usually occurs by a dominant male-determining factor, located on the Y chromosome (Schmidt et al., 1997; Pane et a., 2002).

In insects, the primary instructive signals of the sex determination cascade appear to be highly divergent (Gempe and Beye., 2011; Bopp et al., 2014). As *Sxl* proved to have a sex-determination function in *D. melanogaster*, several studies investigated its orthologues in other species and reported no sex-determining role in non-*Drosophila* species (Zhang et al., 2014). The missing function of *Sxl* in the sex-determining pathway is especially striking because *doublesex,* the most downstream component of *Drosophila*'s sex-determining cascade, is functionally conserved in all insects investigated (Traut et al., 2006).

Mosquitoes use at least two types of sex chromosome karyotypes for determining sex. A heteromorphic species is *Anopheles gambiae* mosquito, a species in the subfamily Anophelinae, in which X and Y chromosomes are morphologically or karyotypically distinct (Krzywinski et al., 2004). Alternatively, homomorphy occurs in mosquitoes like *Aedes* spp., where the sex-determining chromosomes appear as autosomes but contain a Y-like chromosome region called the M-locus (Newton et al., 1974).

The first M-factor identified in insects was the *Nix* gene, discovered in *A. aegypti,* localizing on the homomorphic chromosome 1 M, in band 1q21 (Hall et al., 2015). NIX protein is predicted to have RNA-binding properties and is expressed at the onset of the maternal-to-zygotic transition before sex is determined. Knocked out of *Nix* using CRISPR/Cas9 technology, causes males feminization and development of female antennae and genitals with production of only the *fru* and *dsx* female isoforms. It was possible to conclude that *Nix* was necessary and sufficient to initiate male differentiation in *A. aegypti*.

Also in *A. albopictus, Nix,* acts as a male-determining factor (Gomulski, et al., 2018) although it is more complex in gene structure and splice isoforms than its *A. aegypti* homologue (Liu et al., 2020). As a result of the *Nix* disruption with CRISPR/Cas9, the *dsx* and *fru* splicing patterns shifted towards the female forms, consequentially female deformities and feminization were observed in

Nix knockout male mosquitoes (Liu et al., 2020).

Lutrat et al. (2022) illustrated that sex determination is tissue-autonomous in *A. albopictus*, due to co-existence of male and female tissue in the same individual in pseudo-males obtained after *Nix* transgenic expression in a females genomic background. *Nix* is necessary and sufficient to initiate the male sex determination cascade also in *A. albopictus* (Lutrat et al., 2022).

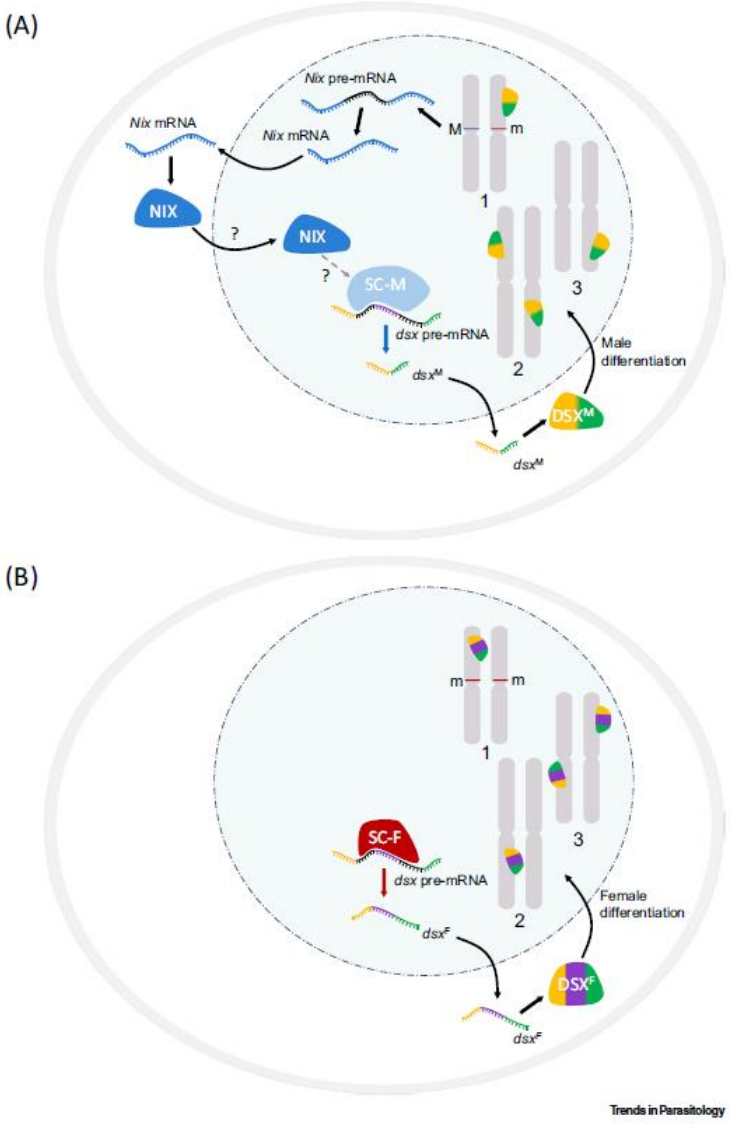


Fig. 2.1 – Simplified models of the sex-determination pathway in A. albopictus (Source: CellPress)
NIX is supposed to function as a splicing factor that somehow modulates an uncharacterized splicing complex in males (SC-M), ultimately affecting dsx splicing.

At the bottom of the sex determination cascade, *doublesex* is the final binary switch (Verhulst and van de Zande, 2015). *Dsx* has been characterized in mosquitoes belonging to three genera: *A. albopictus, A. aegypti, Culex quinquefasciatus* and *An. gambiae* (Price et al., 2015; Jin et al., 2020; Salvemini et al., 2011; Scali et al., 2005). Jin et al. (2020) analyzed the temporal and spatial

expression profiles of *dsx* in *A. albopictus* using not sexed embryos, larvae and pupae, and identified three different isoform for *dsx* gene called dsx^{M}, dsx^{F1}, dsx^{F2}, dsx^{F3} as a result of sex-specific splicing events. Interestingly, in the *dsx* gene sequence, there are several splicing regulatory elements, suggesting the presence of an upstream effector that regulates its splicing (Salvemini et al., 2011).

Also *fruitless* acts as an endpoint effector in the sex determination pathway, as a transcription factor that programs sexual behavior. Analysis of *A. aegypti* and *An. gambiae fru* genes indicate similar sex-specific alternative splicing and conserved function (Gailey et al., 2006; Salvemini et al., 2013).

Using *fru* mutant *A. aegypti* mosquitoes, Basrur et al. (2020) showed that males failed to mate, proving the ancestral role of this gene in determining male sexual behavior. Additionally, *fru* males become attracted to a live human host, a feature wild-type male mosquitoes never exhibit, suggesting that male mosquitoes possess the circuits that enable them to seek a host, and that removing *fru* reveals this latent behavior.

Based on the conservation of the functions of *dsx* and *fru*, it is reasonable to suggest that sex-specific splicing of these transcription factors drives sexual development in mosquitoes. However, this hypothesis has yet to be verified.

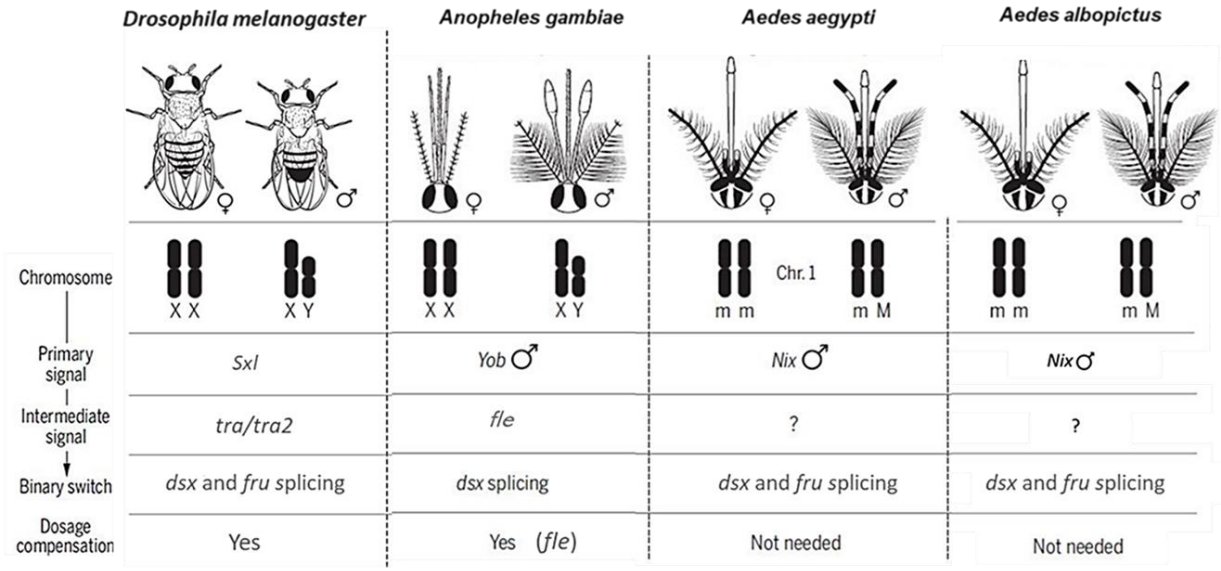


Fig. 2.2 – Comparative diagram of the sex determination pathway (Source: edit from SCIENCE)

Historical cytogenetic studies proved that the *Anopheles* Y chromosome is quite heterochromatic but contrary to *Drosophila,* and in line with mammals, it is partially homologous to the X chromosome and plays a male-determining role (Sakai et al., 1979; Fraccaro et al., 1976). Only

males have a gene called *Yob*, which is found on their Y chromosome. If *Yob* is injected into embryos younger than 2 hours, only male mosquitoes emerge, because *Yob*'s presence is lethal to female embryos. Recently, was demonstrated that the gene *femaleless* (*fle*) represents a sex determination pathway element regulating *dsx* and *fru* splicing in *An. gambiae* females (Krzywinska et al., 2021). Embryonic knockdown of *fle* is lethal to genetic females but apparently has no discernible effect on the development of genetic males. The level of masculinization of transgenic females was correlated with a substantially altered splicing of *dsx* and *fru*. Moreover, none of the transgenic females was attracted to a blood source, likely because they produce the male isoform of *t*he *fru* gene. Krzywinska et al. (2021) conclude that female-specific lethality in *Anopheles* indicates that dose compensation is activated in females in response to *fle* transcript depletion.

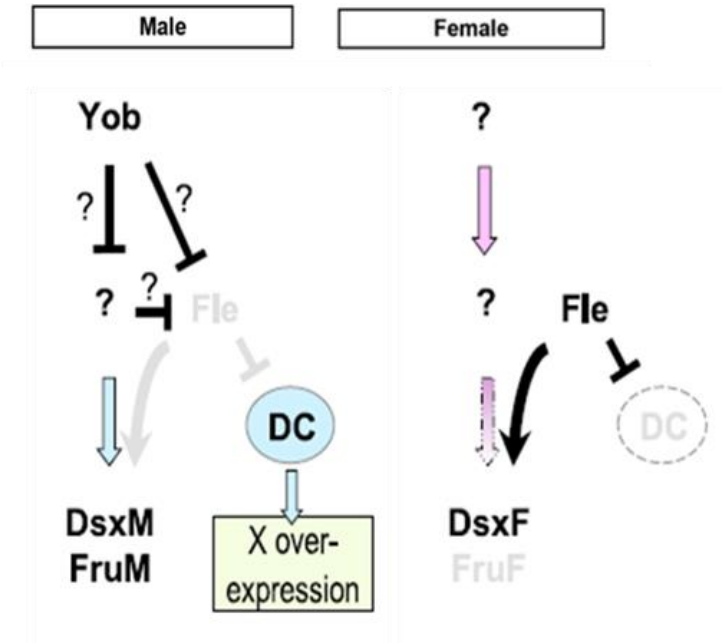


Fig n. 2.3 – Diagram of the sex determination pathway in Anopheles gambiae (Source: CellPRESS)
Fle is necessary for the splicing of dsx and fru into productive and non-productive forms, respectively, and for suppressing dosage compensation. Fle is not involved in the splicing of dsx or fru into male forms.

TRA apparently does not function in sex determination of mosquitoes (Kato et al., 2010). TRA is much less conserved than *dsx* or *fru* and cannot be found in all species of insects studied (Geuverink and Beukeboom, 2014). This absence may be due to divergent evolution beyond sequence recognition while retaining the functional capacity to regulate downstream effectors. Furthermore, no well conserved TRA/TRA-2 *binding sites* have been found in both *fru* and *dsx* genes in mosquitoes while they are highly conserved in many other dipteran species (Salvemini et al., 2011; Saccone et al., 2011). It remains possible that the M factor may directly or indirectly inhibit the TRA/TRA2 complex.

MATERIALS AND METHODS

A. albopictus strain

The *A. albopictus* strain originated from Naples, was reared under laboratory standards conditions, *i.e.* 26-27 °C, 60 % RH and 12:12 h light:dark regimen. Larvae were reared in plastic trays filled with deionized water and provided with TetraMin tropical fish food flakes (*Tetra Goldfish granules, Tetra GmbH, Melle, Germany*). The adults were kept in rearing cages (32,5 x 32,5 x 32,5 cm or 17,5 x 17,5 x 17,5 cm) (*Bug Dorm, MegaView Science Co., Ltd., Taichung, Taiwan)* with constant access to 10% glucose and females were blood-fed on swine blood using the Hemotek system (*Hemotek Membrane Feeding Systems, Blackburn, UK*). Eggs were laid three days later on wet kraft paper and allowed to develop for another five days before being hatched.

In silico analysis

BLAST analysis were conducted on **VectorBase**. Using the FRU protein of *A. aegypti* (Salvemini et al., 2013) as a query and aligning it on the transcriptomic assemblies of *A. albopictus*, were identified two different transcripts, the male "fru^{M}" and the female one "fru^{F}". Using the DSX protein of *A. aegypti* (Salvemini et al., 2011) as a query and aligning it on the transcriptomic assemblies of *A. albopictus*, it was possible to identify different transcripts, the male "dsx^{M}" and three female one "dsx^{F1}, dsx^{F2}, dsx^{F3}".

The SXL protein of *D. melanogaster Sxl* gene (NP_001027062.1) were used as a query to identify and reconstruct the same gene in *A. albopictus* (AALF000103) through the database **EnsemblMetazoa**.

RegRNA 2.0, available at http://regrna2.mbc.nctu.edu.tw/detection.html, was used to identify functional motifs and sites in an RNA sequence provided as input.

RNA isolation and cDNA amplifications

Total RNA was prepared from embryos ($E_{0\text{-}24h}$), larvae (L), pupae (P) and adult males (M) and females (F) with TRIzol Reagent (*Thermo Fisher Scientific, Waltham, MA, USA*) protocol and aliquots of 0,5 µg of each RNAs were retro-transcribed using LunaScript® RT SuperMix Kit (*NEB, Ipswich, MA, USA*) according to manufacturer's instructions. Total RNA from antennas (A), heads (H), body (B), thorax (T) and abdomen (ABD) was extracted and 0,8 µg of each RNAs was retro-transcribed as described above. RT-PCR were performed using Taq 2X MasterMix (*NEB, Ipswich, MA, USA*) according to manufacturer's instructions. Appropriate annealing temperatures and cycle numbers were adjusted empirically for each primer pairs.

The primers list is shown in Appendix 2.1. The primer pairs rp49+/rp49- were used as the positive control for the PCR.

Quantitative genes expression by Real time RT-PCR

Real Time RT-PCR was conducted using a 40-cycle, two step PCR in a QuantStudio™ 3 Real-Time PCR System (*Thermo Fisher Scientific, Waltham, MA, USA*) using PowerUp™ SYBR™ Green Master Mix (*Thermo Fisher Scientific, Waltham, MA, USA*). Expression levels were calculated using $2^{-\Delta\Delta Ct}$ method, with triple technical for each sample, and all data normalized to the relative gene target/rp49 expression in the samples of the wild-type samples.
The relative expression ratio (Rn) was calculated using the following calculation:

$$\mathrm{Rn} = (1 + \mathrm{Etarget}) - \mathrm{CTtarget} / (1 + \mathrm{Erp49}) - \mathrm{CTrp49}$$

where E is the PCR efficiency and CT the threshold cycle. The mean Rn and standard error (SEM) were calculated for each tissue and the statistical significance of the mean Rn differences among and between tissues was evaluated using the ANOVA test followed by the Tukey post hoc test or Dunnett's multiple comparisons test.

dsRNA in vitro synthesis and injection mix

The *fru* gene fragment with the T7 polymerase promoter site at both ends is amplified by PCR with primers that have T7 sequence at their 5' ends. This PCR product is used as a template in an *in vitro* transcription using the MEGAscript™ RNAi kit *(Invitrogen™, Waltham, MA, USA)* according to manufacturer's instructions. Similarly, a fragment of GFP gene was amplified by PCR from the pAct:dCas9 (*Addgene, Teddington, UK*) vector using e*GFP*-specific primers, each containing the T7 promoter sequence at the 5' end, and dsRNA was synthesized as described above.

Microinjection of dsRNA in embryos

To obtain the eggs for the microinjection, 5-8 blood-fed females are put into a *Drosophila* vial with a wet filter and placed in a dark place. After 1 hour the eggs are collected, selected, align, transfer on a glass and desiccated. The needle is fill with about 2 µL of a mix of dsRNA[1000 ng] and buffer solution (5M KCl; 0,1 M phosphate; pH 6,8) and the embryos are injected into the posterior pole.

Functional studies

Crosses

All crosses were composed of 1 male and 5 females using virgin individuals. All the experimental males and females came from the same cohort that emerged on the same day. A total of three types of crosses were realized:

(a) 1 wild-type male × 5 wild-type females (WT);

(b) 1 *GFP* male × 5 wild-type females (*GFP*);

(c) 1 *fru* male × 5 wild-type females (*fru*).

Eight replicates were conducted for the WT and *fru* crosses and four replicates for the *GFP* crosses. The crosses were realized in 17.5 cm x 17.5 cm x 17.5 cm cages (*BugDorm-4E1515*).

Mating and Feeding assay

The mosquitoes were allowed to mate for three days, under a climate-controlled insectary at 27 °C, RH: 60%, and 12L:12D regimen. The crosses a), b) and c) were observed in order to report the duration of the mating events.

Following a three-day mating period, the cages were examined during the feeding. The mosquitoes had constant access to 10% (*w*/*v*) sucrose and warm swine blood (put in a membrane to create a blood-sausage) was offered on the top of the cages for 30 minutes, then were scored if the feeding was good by visual observation of the abdomen of the animals.

Fecundity test

Three days after blood feeding, plastic cups containing deionized water and lined with germination paper were provided in each cage for 48h. The positive egg papers were then dried in controlled laboratory conditions and eggs were counted and examined under an optical microscope.

RESULTS AND DISCUSSION

Developmental transcriptional profiling of *fruitless (fru)*, *doublesex (dsx)* and *Sex-lethal (Sxl)*

The study of sex determination genes was performed by using several *in silico* strategies. Applying the *Trinity* software, we were able to create the *assemblies* of the transcriptomes of both sexes of the species, and then through BLAST analysis have been identified the sex-specific isoforms of the different studied genes. The gene structures were then reconstructed using the *Gene Structure Display Server 2.0* and shown in Figure n. 2.3

The principal genes investigated were: *fruitless* (*fru*), *doublesex* (*dsx*) and *Sex-lethal* (*Sxl*).

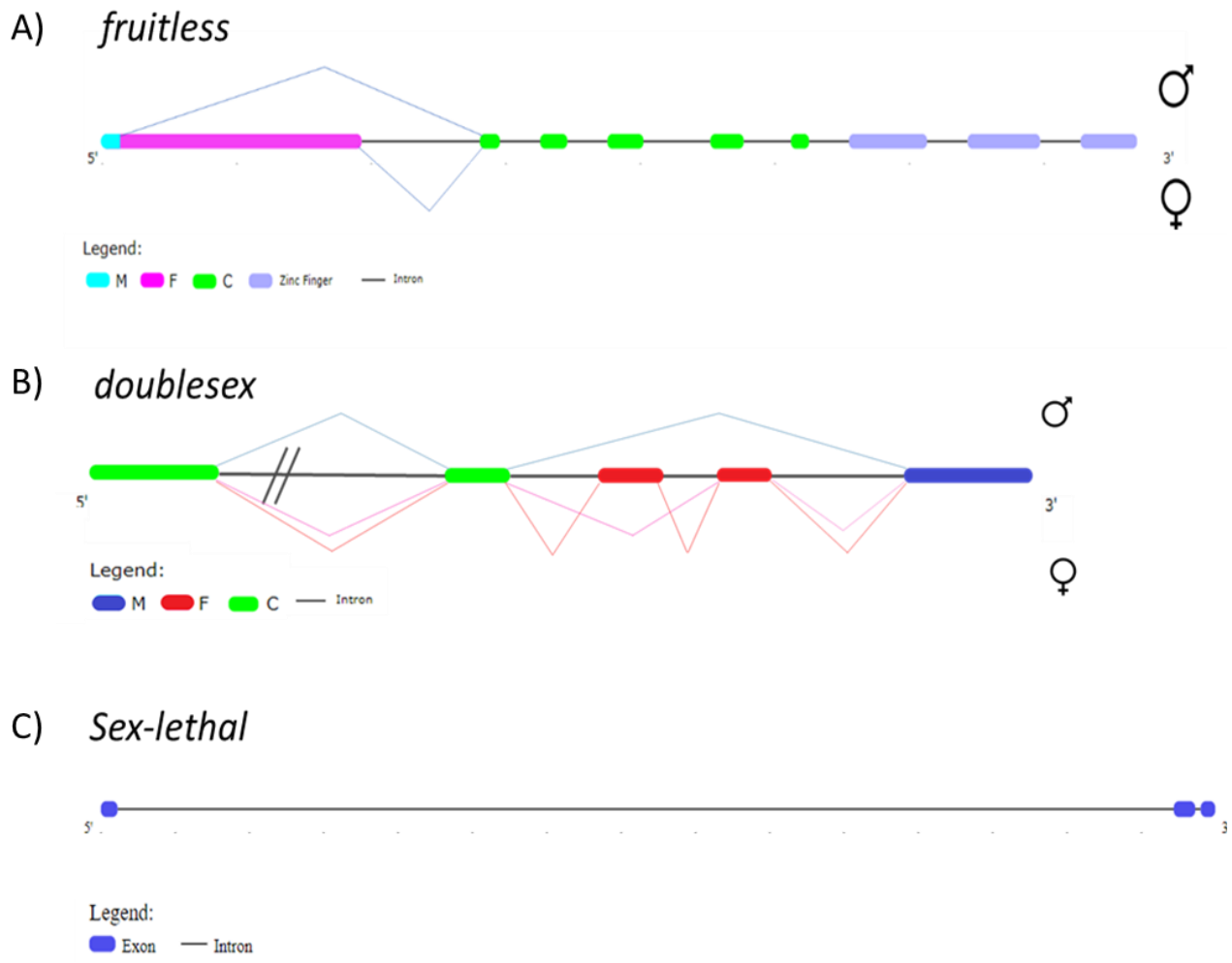


Fig n.2.3 – Sex determination genes structure in A. albopictus
A) The fruitless (fru) gene shows sex-specific splicing; B) The doublesex (dsx) gene shows sex-specific splicing; C) The Sex-lethal (Sxl) gene doesn't show alternative splicing.

Especially, sexually dimorphic traits, including behaviors, are the consequences of differential gene expression that originate during development and therefore these genes must be studied

during the vital development of the insect. In the current state of knowledge, it is unknown which genes regulate the development of sexually dimorphic traits in mosquitoes (Tomchaney et al., 2014).

To examine when *fru*, *dsx* and *Sxl* are active during development, we monitored their expression at different developmental stages, for both sexes. The RNAs collected were analyzed (Figure n. 2.4) to confirm the good quality and retrotranscribed into cDNA.

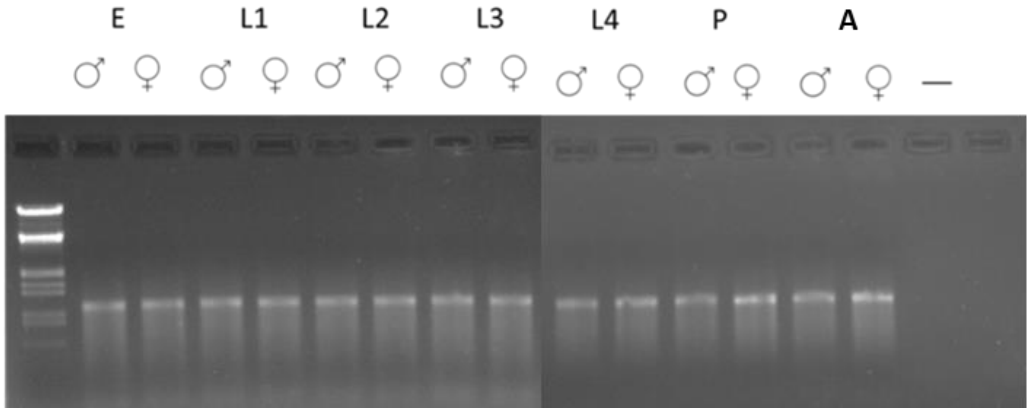


Fig n.2.4 – RNA gel electrophoresis
The 1% agarose gel electrophoresis shows RNAs extracted from different stages of development of A. albopictus.

At first, housekeeping gene *rp49* (Figure n. 2.5-A) and the male-specific gene *nix* (Figure n. 2.5-B) analysis confirm cDNA quality, genomic DNA contamination absence and molecular karyotyping.

The cDNA amplification shows the presence of two different bands in *fru* and four different bands in *dsx* gene (Figure n. 2.5-C and D), as observed in *A. aegypti* orthologs (Salvemini et al., 2013; Salvemini et al., 2011), derived from sex-specific splicing. It was possible to recognize that the sex-specific splicing event occurs from the early embryonic stage, in which we can already identify the sex-specific isoforms of the transcripts of both *dsx* and *fru* genes.

In *Sxl* gene no splicing event occurr in the transcript (Figure n. 2.5-E), as observed in *C. capitata* by Saccone et. al (1997), where *Sxl* expresses the same mRNAs and protein isoforms in both XX and XY flies.

We conclude that *fru, dsx* and *Sxl* are already active during the early development of *A. albopictus* so their functions need to be investigated.

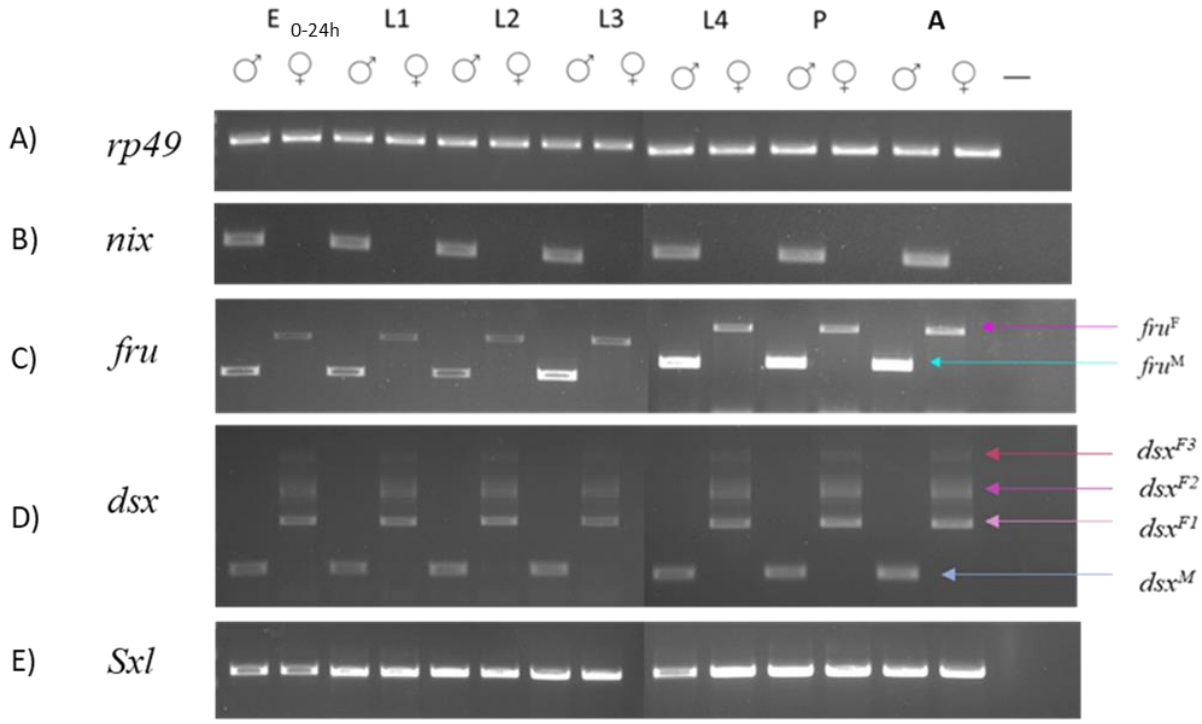


Fig n.2.5 – Developmental expression profiles of rp49, nix, fru, dsx and Sxl n A. albopictus.
The agarose gel electrophoresis shows different PCR amplified fragments: A) The housekeeping gene rp49; B) The male- specific gene nix; C) fru^M and fru^F transcripts; D) dsx^M, dsx^{F1}, dsx^{F2}, dsx^{F3} transcripts; D) Sxl trascript.

The master regulators of the somatic sex-determination pathway, *fru* and *dsx*, also control sexual behavior (Belote and Baker, 1987; McRobert and Tompkins, 1985; Taylor et al., 1994). In *D. melanogaster*, *fru* mutants exhibited a strongly reduced courtship toward females and they courted males. These mutants shown male courtship chains (the first male courting another male is followed by a second male counting another male, and so on) (Gailey and Hall, 1989; Ito et al., 1996; Ryner et al., 1996). Due to these first findings, *fru* was long believed to be the only regulator of male courtship behavior. Recently research has revealed that while *fru* plays a significant role in conferring male courtship ability on the nervous system, *doublesex* plays an equally critical role for normal male courtship as well (Dauwalder., 2011).

We decided to investigate the role of *fru* in *A. albopictus* mosquito, starting from the hypothesis that could be responsible for the male's behavior, as it occurs in *D. melanogaster* (Demir and Dickson, 2005).

The attention was focused on the study of the expression of *fru* in different tissues of the male insects: antennae, heads and carcasses, in order to identify tissue-specific or differential expressions. Real-time RT-PCR results are shown in Figure n. 2.6. *Fru* has a pretty evident differently expression, with a high expression in the antennae. These structures play a crucial role

in olfactory behaviors, especially in identifying the buzzing sounds of flying females (Roth., 1948); thus, the antennae' specific expression of *fru* suggests the gene could play a specific role in this sexual behavior.

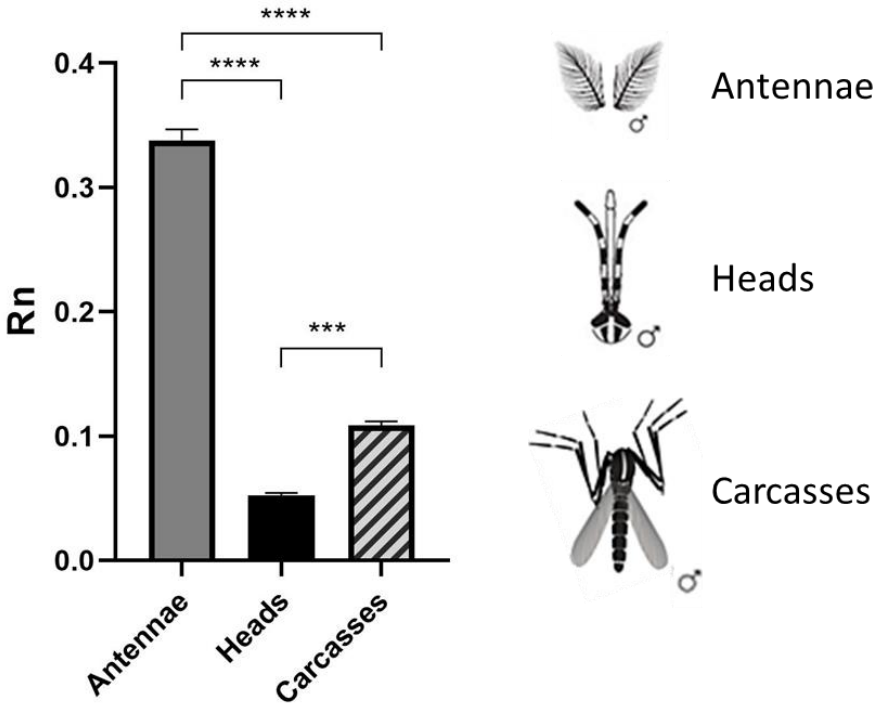


Fig n.2.6 – Differential expression of fru in male tissues

*Relative expression pattern of the fru gene in different tissue of A. albopictus studied by real-time RT-PCR. The expression is reported as Rn. The vertical bars represent the SEM of the biological and technical replicates. ANOVA test, p-Value **** <0.0001. Asterisks indicate statistically significant, as assessed by the Tukey post-hoc test.*

Sex-lethal in *Drosophila* works as the master switch and cellular memory of sexual identity, a role played by the *transformer* gene in other insects, though not yet found in mosquitoes. To figure out if *Sxl* perform a specific role in *A. albopictus* sex-determination cascade, were investigated its expression profile by real-time RT-PCR. Surprisingly, and observed for the first time in mosquitoes, a male-specific expression of *Sxl* is present in all the stages of development (Figure n. 2.7) and the highest expression occurs at the male embryonic stage.

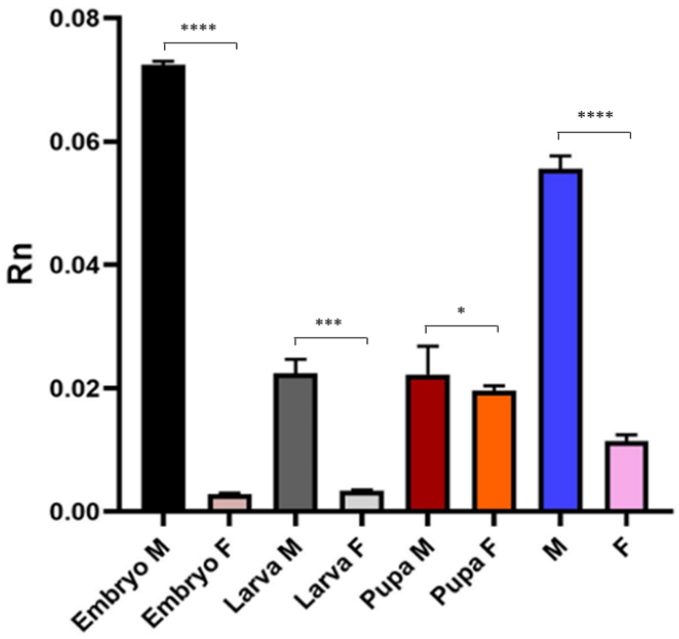


Fig n.2.7 – Expression trend of Sxl gene

*Relative expression pattern of the Sxl gene studied by real-time RT-PCR in different stage of development of A. albopictus. The expression is reported as Rn. The vertical bars represent the SEM of the biological and technical replicates. ANOVA test, p-Value **** <0.0001. Asterisks indicate statistically significant, as assessed by the Tukey post-hoc test.*

A better understanding of the differential expression of *Sxl* is mandatory to better assess the role of this gene. Consequentially, we dissected male and female insects into three-part: Head (H); Thorax (T) and Abdomen (A), to determine if *Sxl* localization varied among the different tissues, in which *Sxl* could have a specific role in the development of the insect.

Our analysis revealed a different expression of *Sxl* between male and female tissues (Figure n. 2.8).

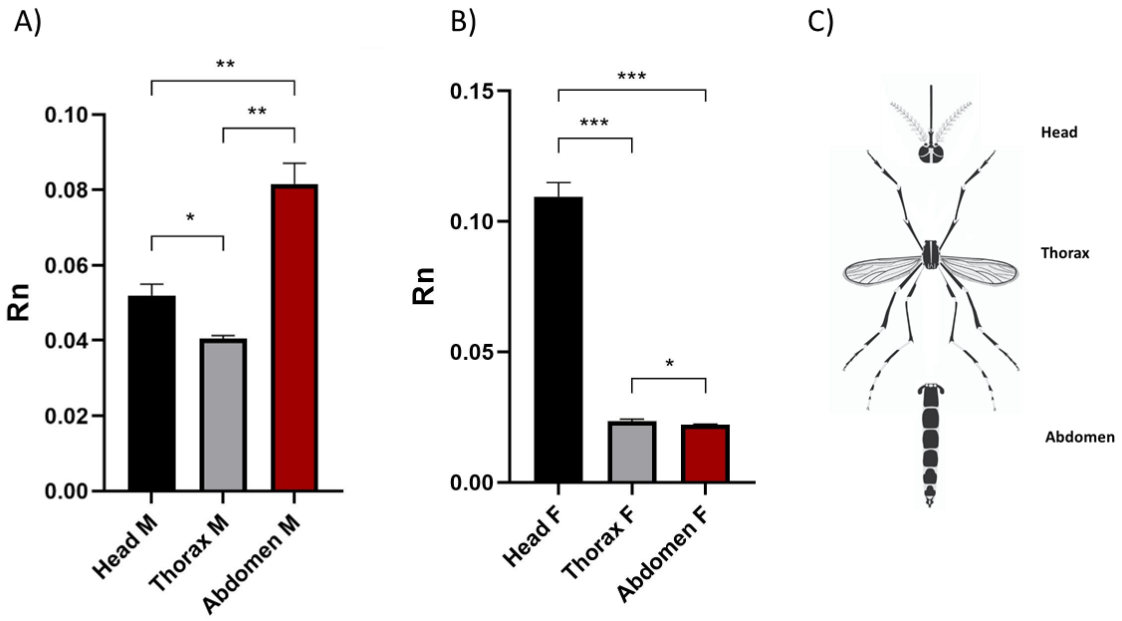


Fig n.2.8 – Tissues expression of Sxl gene

*Relative expression pattern of the Sxl gene in male and female tissues of A. albopictus studied by real time RT-PCR . The expression is reported as Rn. The vertical bars represent the SEM of the biological and technical replicates. Asterisks indicate statistically significant, as assessed by the Tukey post-hoc test. A) Male tissues, ANOVA test, p-Value *0.02, **0.0087; B) Female tissues, ANOVA test, p-Value *** <0.0007, *0.02.*

For male tissues, the highest expression of *Sxl* is found in the Abdomen, compared to the Head and Thorax (Figure n.2.8-A); for female tissues the high expression of *Sxl* is found in the Head and in Thorax and Abdomen is present the similar expression (Figure n.2.8-B).

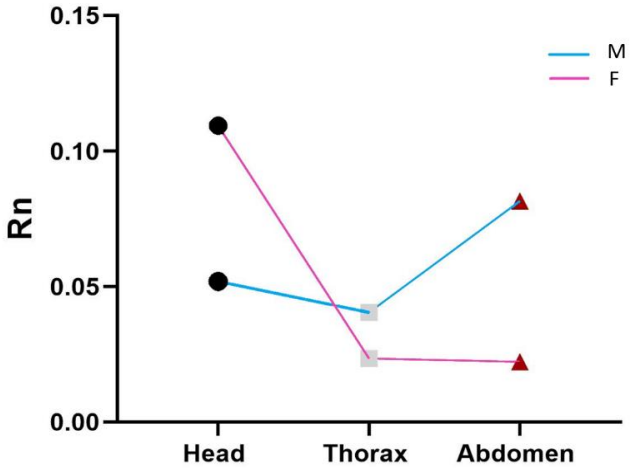


Fig n.2.9 – Male and female tissues expression trend of Sxl gene

For males, the peak of Sxl expression is in the abdomen. For females, the peak occurs in their heads instead. In both sexes, the thorax has the lowest level of Sxl.

Recently, was hypothesized that the ancestral function of *Sxl* might have changed during the evolution since in the Lepidoptera *B. mori* regulates spermatogenesis (Sakai et al., 2019) and, in

this work, we discovered in *A. albopictus* that *Sxl* has a male and abdomen-specific expression. It is supposed that in non-*Drosophilidae* insects *Sxl* may be involved in the genetic control of the germline development in male insects.

In addition, it was recently discovered in *D. melanogaster* that the *Sxl* gene acts as a remote stimulator of female body growth in specific groups of CNS neurons (Sawala et al., 2017). Sawala et al. (2018) discovered that RNAi knockdown of neuron-specific *Sxl* reduced female body size to that of males. Additionally, restoring *Sxl* expression in neurons improves female body size. These findings lend support to a relay model in which *Sxl* acts in the brain to promote female body growth.

It is common a sex difference in body size, called *sexual size dimorphism* (SSD) throughout the animal kingdom, although little is known about how the sex of an organism influences its growth (Sawala et al., 2018). These findings suggest that the high *Sxl* expression in *A. albopictus* females' heads could be related to the presence of specific female neurons responsible for females' body dimensions.

In silico search for binding sites: *putative Sxl binding sites*

For the purpose of collecting more information about the regulation of downstream genes of the sex determination cascade, the sequences of *fru* and *dsx* were investigated, looking for a possible regulatory motif for the sex-specific splicing events. For this analysis were used the **RegRNA 2.0** *in silico* tool and the output of the analysis identified an *intron splicing silencer (ISS)* sequence for the *Sxl* gene, in both *fru* and *dsx* (Figure n. 2.10; circled in red).

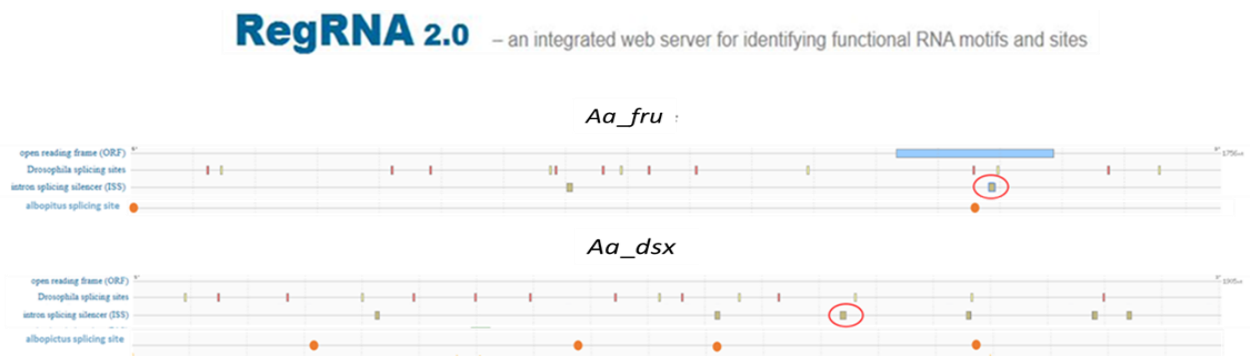


Fig n.2.10– RegRNA graphical interface
Schematic representation of putative Sxl binding sites distribution on fru and dsx gene.

The putative *Sxl binding sites* (red square) is composed by 9 bp and the sequence is "TTTTTTTTA". For *fru* gene this sequence is found between 1383-1391 bp, close to the splicing

site, at the end of the exon one; for *dsx* the putative sequence is found between 1238-1246 bp, between the third and fourth splicing sites, in the female-specific exon (Figure n. 2.11).

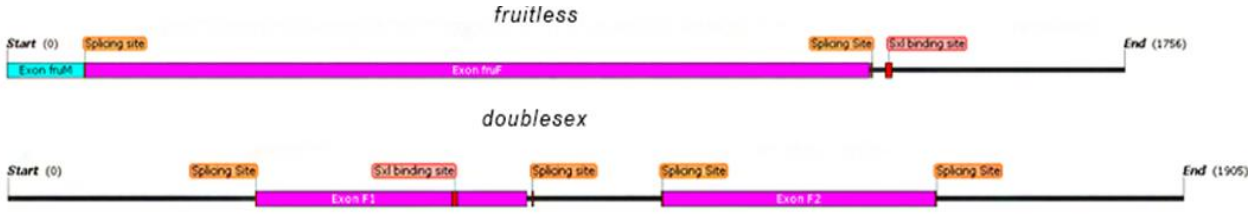


Fig n.2.11– Representation of fru and dsx gene
In light blue is the male gene region and in pink the females. Splicing sites are represented in orange, while putative Sxl binding sites are represented in red.

These results have stimulated much interest since it is known that in *D. melanogaster*, SXL proteins bind to an ISS in the *tra* transcript, preventing U2AF proteins from binding to the polypyrimidine tract. As a result, this junction cannot be used, shifting spliceosome binding downstream. The stop codon is removed as part of the intron during the splicing. An active TRA protein is produced, which is itself a transcription regulator for other sex-related genes (Black., 2003).

This discovery is intriguing since the early expression of *Sxl* could be involved in the splicing regulation of *fru* and *dsx* genes, which are found sex-spliced from the embryonic stage. It is essential to conduct further research to determine if there is a specific interaction between these genes and whether or not *Sxl* could be considered a missing gene in the sex determination pathway of the A. *albopictus* mosquito.

Functional analysis of *fru* in *A. albopictus* by embryonic RNAi

In order to identify the role of *fru* in *A. albopictus* mosquito, functional analyses were essential.

A dsRNA was designed in the male region of the exon one (dsRNA_*fru*) and injected into 1-hour old embryos. As control was injected a dsRNA against the *GFP* gene (dsRNA_*GFP),* which is not present in the mosquito genome and is expected to do not have any effect on the development of the insects.

The following tables report the sets of injection data (Tables n. 2.1-2.2).

SET	Nr Inj eggs	Hatched eggs	Larvae	Adults	Males	Females	Crosses
Set 1	619	5	1	4	3	1	3
Set 2	160	2	0	2	2	0	2
Set 3	184	5	5	0	0	0	0
Set 4	230	2	0	2	2	0	2
Set 5	180	8	7	1	1	0	1
tot	**1375**	**22**	**13**	**9**	**8**	**1**	8

Table n.2.1 – dsRNA_fru injection data

SET	Nr Inj eggs	Hatched eggs	Larvae	Adults	Males	Females	Crosses
Set 1	346	3	0	3	1	2	1
Set 2	170	4	0	4	1	3	3
tot	**516**	**7**	**0**	**7**	**2**	**5**	4

Table n.2.2 – dsRNA_GFP injection data

The number of hatched eggs is pretty low, both in dsRNA_*fru* and dsRNA_*GFP* microinjection. This low efficiency is due to the type of mosquito strain used in the technique and it is not attributable to a gene effect on the embryo's development.
A possible explanation for the variation in the survivability in the different sets of injections could be due to the difference in the egg's developmental biology and also the variability in needle shape or volume of material injected (Sampath et al., 2012).

Much attention has been drawn to identifying phenotypical alteration by observing the interfered insects, during development, using the stereomicroscope. All the injected *fru* individuals developed as normal mosquitos, except for little difference in the size of the body and the tail at pupal stage (Figure n. 2.12-A), and size and plumage of the antennae at adult stage (Figure n. 2.12-B).

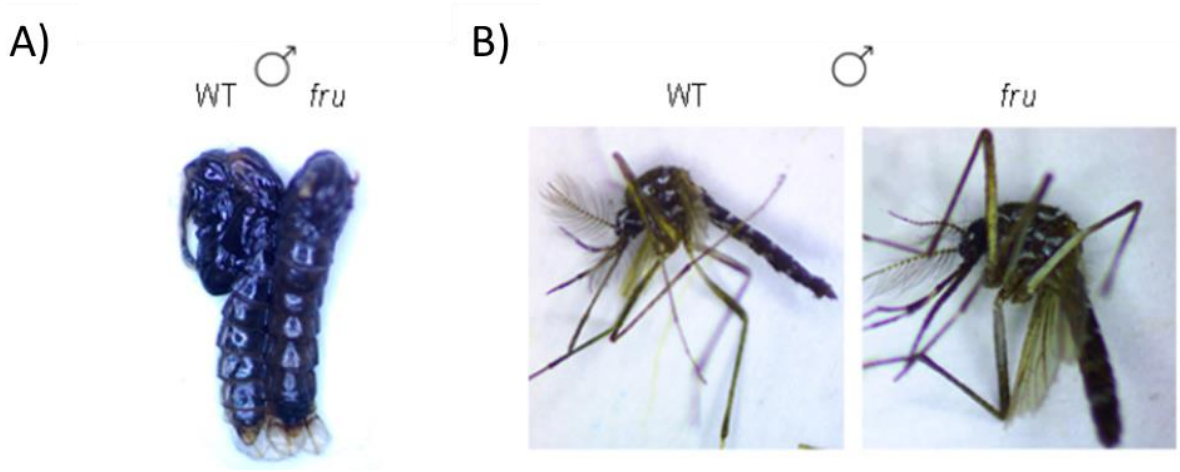


Fig n.2.12 – Microscopy images of A. albopictus fru-interfered males
A) Wild-type and fru-interfered male pupae are compared to analyze the size; B) Wild-type and fru-interfered male adults are compared to analyze the different morphology of the antennae.

Previously, was found a differential high expression of *fru* in the antennae of wild-type males (Figure n. 2.6) and note that the males use their antennae to sense the buzz generated by the females in flight, the alteration in antennae morphology in the *fru*-interfered males could be evidence of change in the male's behavior.

Thus, RNAi silencing of *fru* let us to hypothesize that *fru* is required for correct male structures development, which may have a significant effect on reproductive success.

Embryonic silencing of *fru* in *A. albopictus* causes change in male behavior

To analyze different aspects in the behavior of *fru*-interfered male, were realized different crosses (Figure n. 2.13). As control were realized two type of crosses: the "wild-type" one, composed of 1 wild-type male and 5 wild-type females (total 8 crosses); the "*GFP*" one, composed of 1 *GFP* interfered-male with 5 wild-type females (total 4 crosses). The cross of interest, "*fru*" cross, was composed of 1 *fru*-interfered male with 5 wild-type females (total 8 crosses). All the insects used in these crosses were virgins.

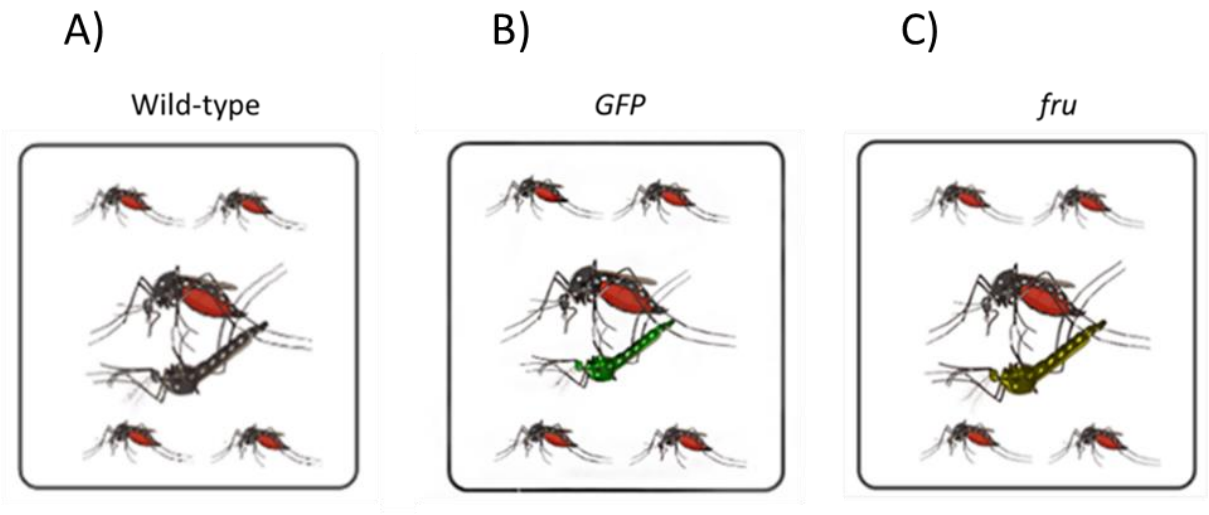


Fig n.2.13 – Cages realized for the crosses
Each cages were composed of five females and one male. A) Wild-type cross; B) GFP-interfered male cross; C) fru-interfered male cross. Schematics of panel A, B and C are created with BioRender.com.

Given the wide range of neural expression and sexual dimorphism in *fru* circuits of several insects, we wondered if sexually dimorphic feeding and mating behaviors were affected in *fru*-interfered males. Consequentially, we observed mating and feeding behavior during the crosses.Observing males mating with females allows identifying that the *fru*-interfered males cannot fully mate since females quickly run away. Figure n. 2.15-A reports the mating times, compared to the control crosses. It appears that the mating period, for *fru*-interfered males, lasted only 2-3 seconds. During this short mating, the *fru*-interfered males assumed an unusual position during the copulation, as shown in Figure n. 2.14, where the female was in opposition to the male. Similarly, *A. aegypti fru* mutated males could contact females but were unable to successfully inseminate wild-type females (Basrur et al., 2020).

This mating failure is compatible with the role of *fruitless* in *Drosophila* male sexual behavior (Demir and Dickson, 2005; Ryner et al., 1996).

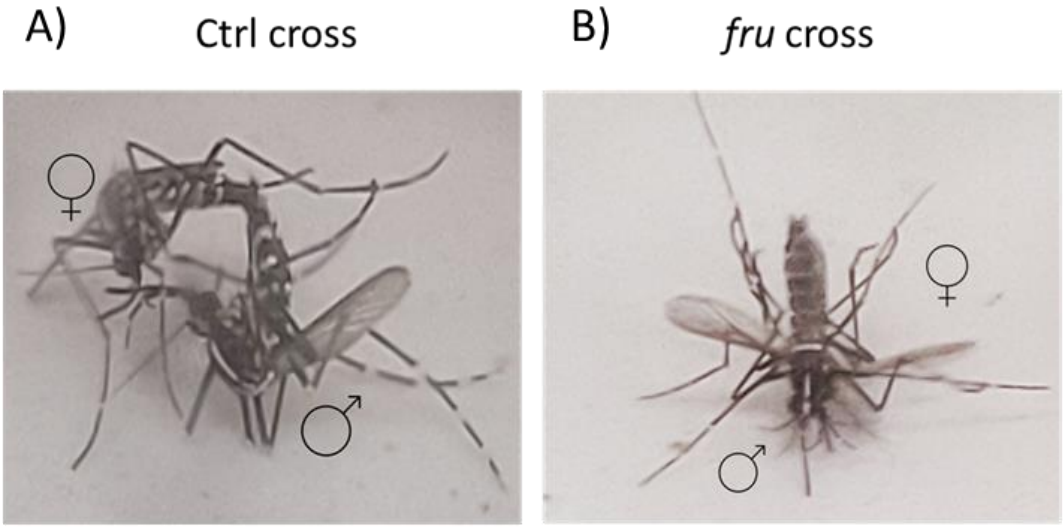


Fig n.2.14 – A. albopictus adults during the mating
The images show male and female A. albopictus mosquito during the mating. A) In control mosquitoes during the mating the female is above the male; B) Fru male mosquitoes assume an atypical position during the mating.

This first observations allow to hypothesize a possible effect on the male fertility.

When a blood-sausages were put on the top of the cage (Figure n. 2.15-B), 3 *fru*-interfered males were attracted to the blood and they went on the top of the cages. Note that only the females need blood to develop eggs, this behavior in the *fru* males needs to be investigated. In addition, Basrur et al. (2020) showed that *A. aegypti fru*-mutated males became unable to mate successfully, but differentially from *A. albopictus fru*-interfered males, they still preferred sugar water over blood when feeding. However, these male mosquitoes exhibit similar female behaviors of attraction to the body odor of a person's arm.

As a control, we monitored sucrose feeding too, but no differences were found (Figure n. 2.15-B).

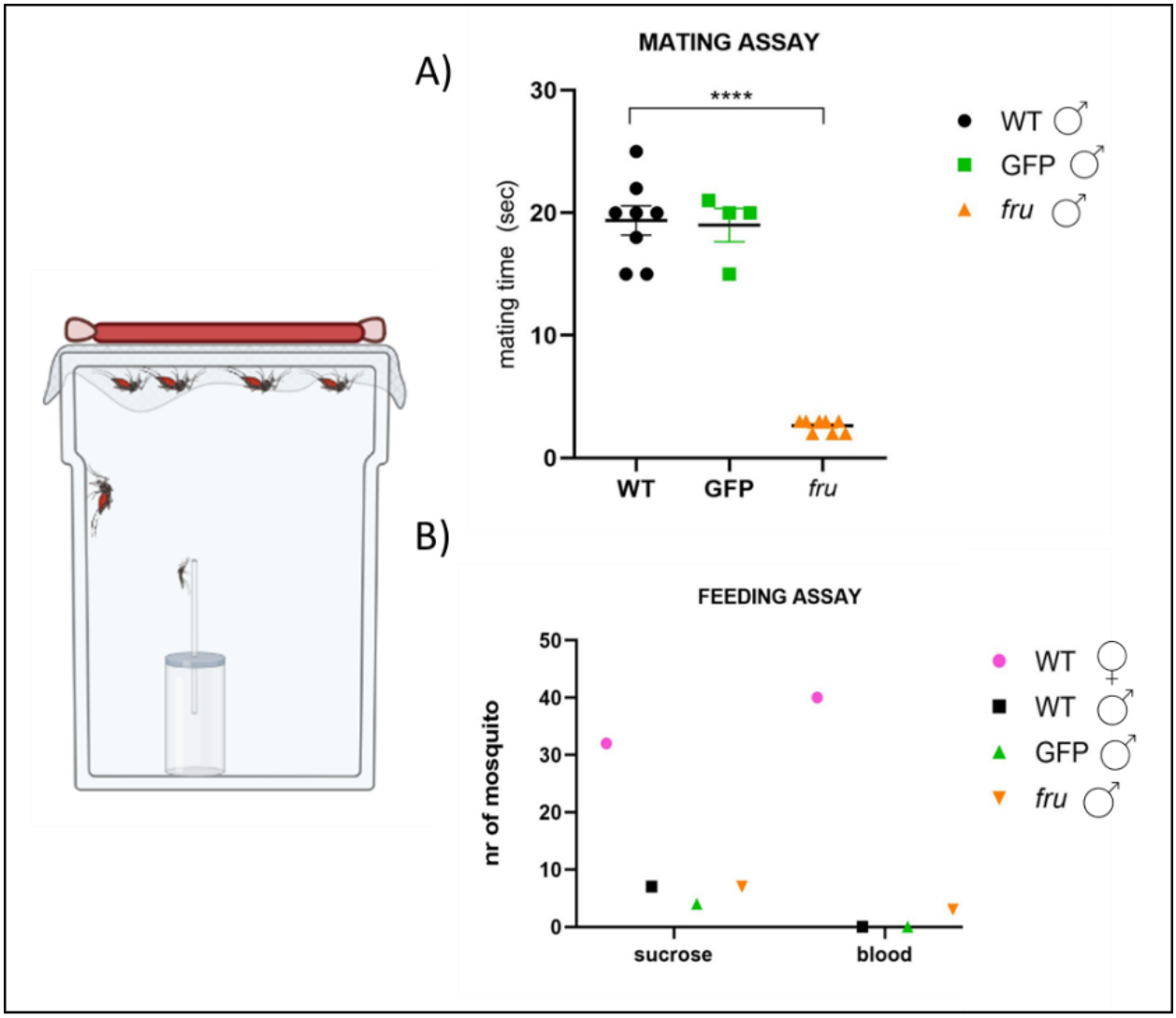


Fig n.2.15 – Behavioral tests in A. albopictus adults
*A. albopictus adults are analyzed during the three type of crosses: WT, GFP and fru. A) Mating time analysis: p-Value *** <0,0001 ANOVA test; B) Feeding analysis on sucrose and blood.*

Moreover, after the blood-feeding, the eggs were collected in order to evaluate the fecundity of the insects. The number of eggs collected from *fru* crosses is 9 times lower than the controls, as shown in Figure n. 2.16. This lower number of eggs validates our hypothesis about the change of fecundity in the *fru* interfered male, possibly after the interference of *fru* the males are unable to reproduce successfully.

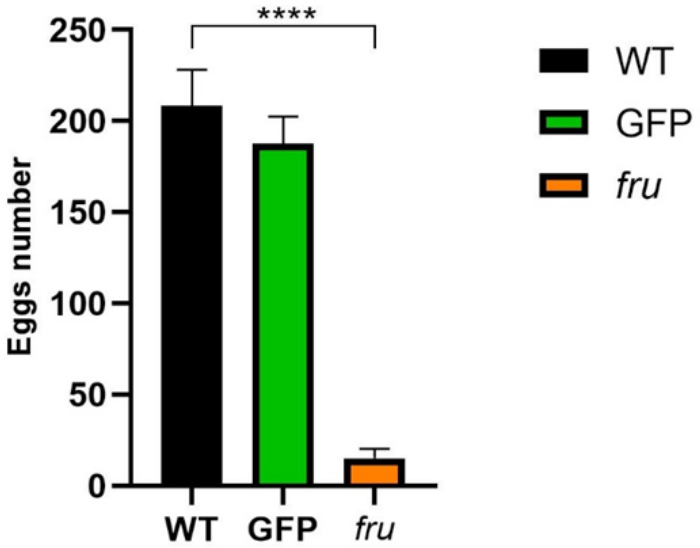


Fig n.2.16 – Fecundity analysis
*Number of eggs collected from WT, GFP and fru crosses. p-Value ****<0,0001, ANOVA test.*

The best confirmation of this hypothesis was obtained analyzing the eggs morphology that appears degenerated, making the embryos unable to survive (Figure n. 2.17-B).

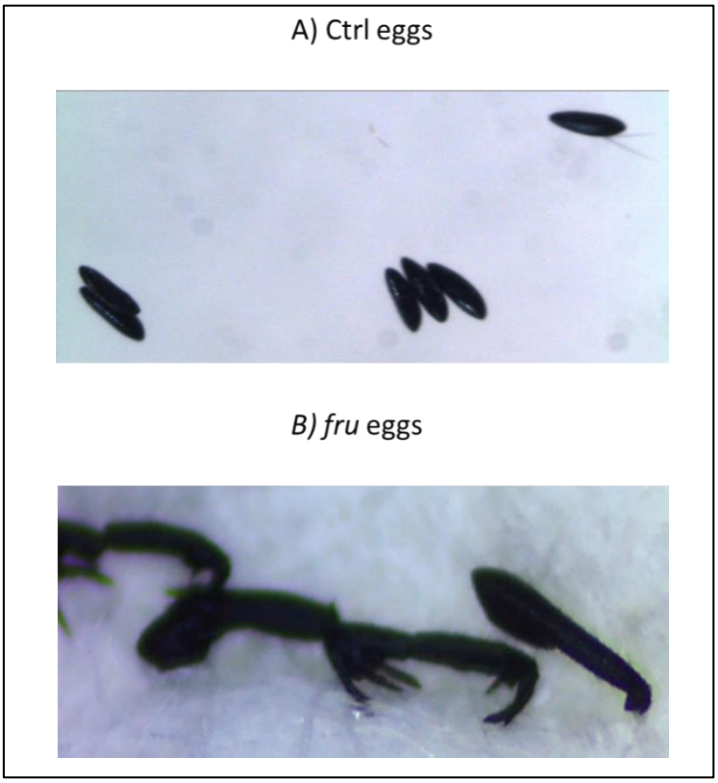


Fig n.2.17 – A. albopictus eggs comparison
Microscope image of A. albopictus eggs collected from: A) ctrl crosses and B) fru crosses.

Expression levels of *fru* after RNAi

To examine the RNAi effects on *fru* expression, was measured the relative transcript levels of *fru* at third or fourth instar stage of development, after dsRNA injections. Relative level of the gene transcription was determined by normalization to ribosomal housekeeping gene *rp49*.

A significant reduction of *fru* transcript levels is observed in *fru*-interfered larvae (Figure n. 2.18), compared to the control larvae.

This result indicates that the different phenotypes identified in the interfered mosquitoes are due to the reduction expression of *fru* gene.

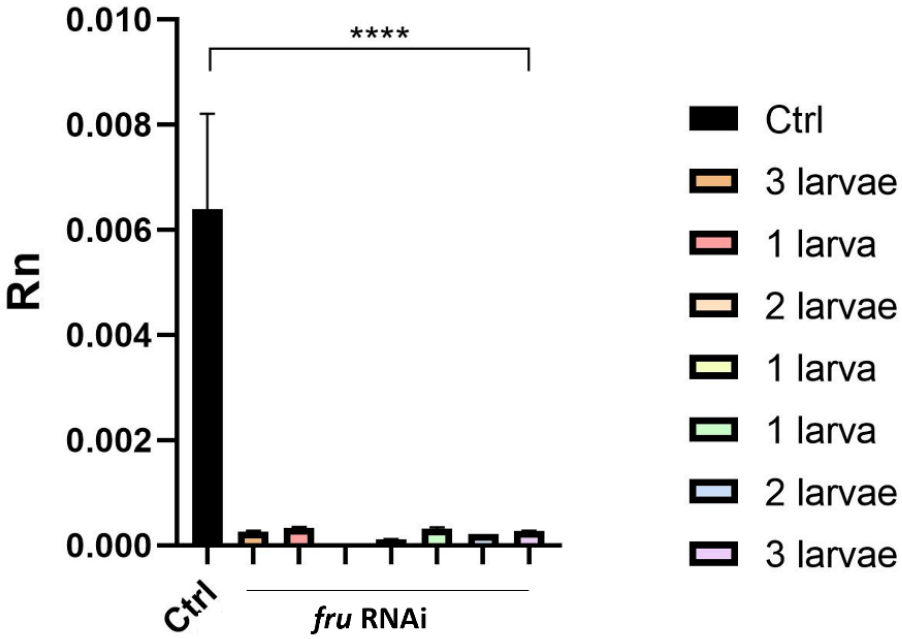


Fig n.2.18 – Fruitless RNAi expression studied by real time RT-PCR

*Relative expression pattern of the fru gene studied by real time RT-PCR in different larvae of A. albopictus. Ctrl: not interfered L; fru RNAi: fru-interfered L3 from different sets of injection. The expression is reported as Rn. The vertical bars represent the SEM of the biological and technical replicates. ANOVA test- Dunnett's multiple comparisons test, p-Value **** <0.0001.*

APPENDIX 2.1

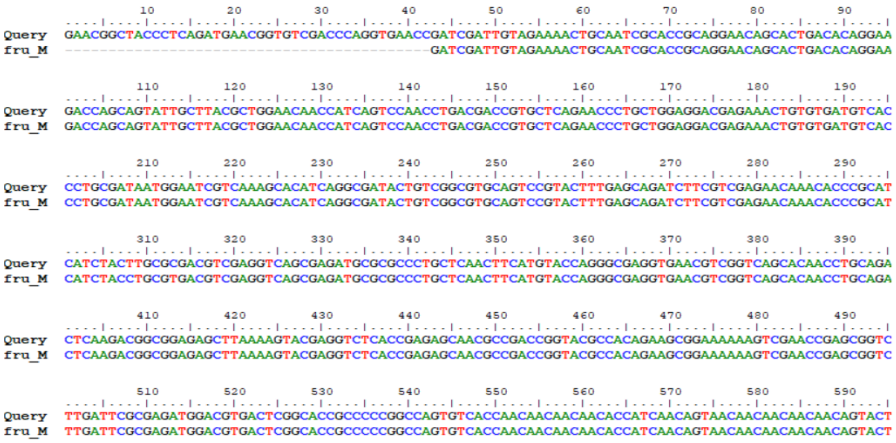
Primer sequences

PRIMER NAME	PRIMER SEQUENCES (5'-3')	PURPOSE
rp49+	GACGAAGAAGTTCATCCGCC	cDNA amplifications
rp49-	GTTCTGCTGCGAGCGCAG	cDNA amplifications
nix+	GTTGTTCGTTACAGACTGATG	cDNA amplifications
nix-	CAAAGCTAATGTAAACCATGAC	cDNA amplifications
*fru*_M2+	GAACGGCTACCCTCAGATGA	cDNA amplifications
*fru*_C3-	ACCTGCGATTCGTATCCACC	cDNA amplifications
*dsx*_C+	GTGACCCCAACAAGCAG	cDNA amplifications
*dsx*_M-	CAATGGTGGTCTGCTGGTTC	cDNA amplifications
Sxl+	CCCGAATGTTTGACATGCCT	cDNA amplifications
Sxl-	GCATTCGTACTCTACCTCGG	cDNA amplifications
*fru*_MB+	CGGCAATGCCCATCTACAG	qRT-PCR
*fru*_MB-	TCAGATGAACGGTGTCGACC	qRT-PCR
Sxl+_real	CCCGAATGTTTGACATGCCT	qRT-PCR
Sxl-_real	GACGATCAGATTGGTTCCCG	qRT-PCR
rp49+_real	AGAAGTTCCTGGTCCACAAC	qRT-PCR
rp49-_real	GTTCTGCTGCGAGCGCAG	qRT-PCR
*fru*_MB_T7+	taatacgactcactagggAGTTCTCAATAGTATGTCATCG	dsRNA synthesis
*fru*_MB_T7-	taatacgactcactagggGCGGTGCGATTGCAGTTTTC	dsRNA synthesis
e*GFP*_T7+	taatacgactcactagggGGTGAACTTCAAGATCCGCC	dsRNA synthesis
e*GFP*_T7-	taatacgactcactagggGCATGGACGAGCTGTACAAG	dsRNA synthesis

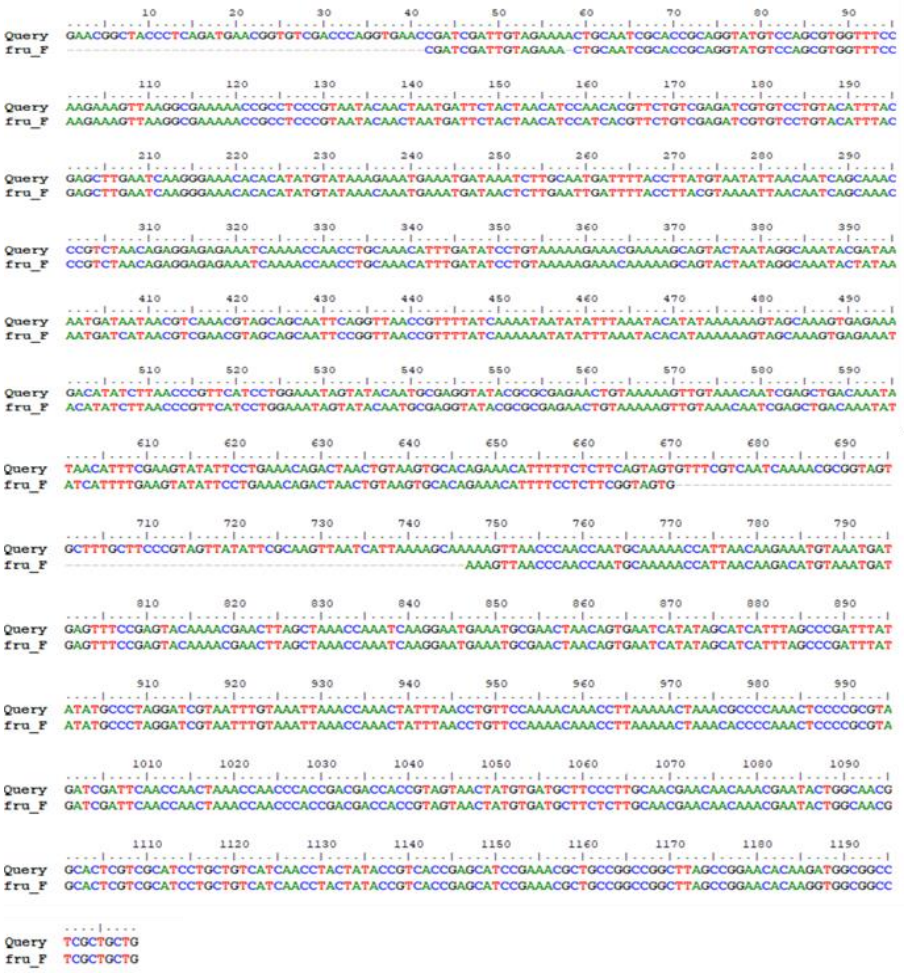
APPENDIX 2.2

Nucleotide sequences alignment

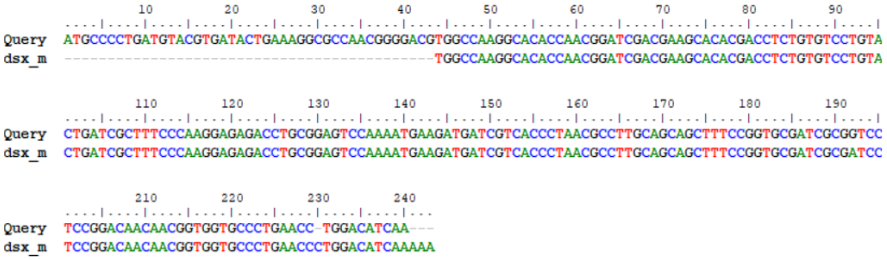
1) Fruitless male sequence



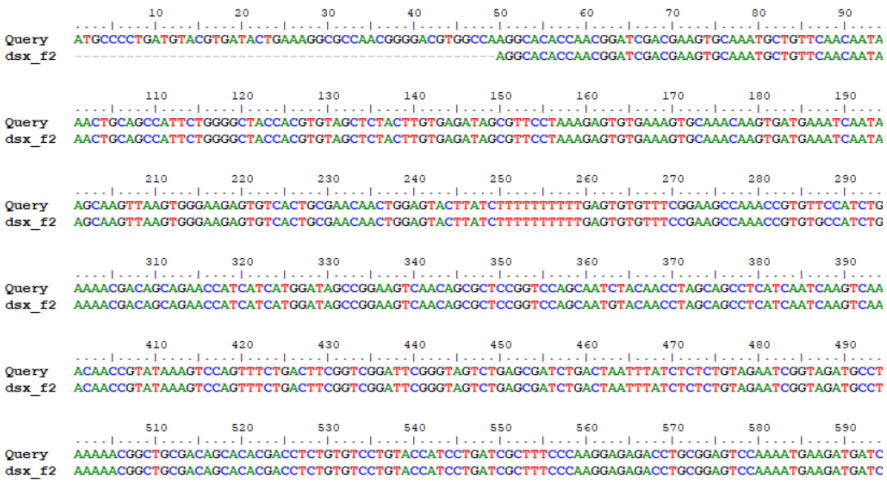
2) Fruitless female sequence



3) Doublesex male sequence



4) Doublesex female sequence "f2"



CHAPTER 3

Novel genes with potential functions for vector control

INTRODUCTION

Vector-borne diseases (VBDs) are infections caused by pathogens carried by arthropods such as mosquitoes, triatomine bugs, blackflies, tsetse flies, sand flies.

Globally, VBDs such as malaria, dengue, and leishmaniasis cause high morbidity and mortality, especially in the poorest countries of the world. The primary method of controlling these diseases is vector control. The selection of insecticide-resistant vectors (Sokhna et al., 2013), the need to enact more vector control interventions, and the rise of vector-borne diseases, make evidence-based vector control a necessary investment. There is a need to return to vector control approaches based on a thorough knowledge of the determinants of pathogen transmission, which utilize a range of insecticide and non–insecticide-based strategies in a locally tailored manner for more effective and sustainable vector control (Wilson et al., 2020). The principal control method for many VBDs – both historically and today – is vector control. Currently, vector control is the only method of preventing certain diseases, such as dengue, chikungunya, Zika, and West Nile disease.

A vector control technique aims to reduce or eliminate human contact with vectors to limit pathogen transmission. WHO defines environmental management as the modification or manipulation of environmental conditions, or their interaction with the human population, to prevent or minimize vector propagation and reduce human–vector–pathogen contact (World Health Organization., 1980).

A wide range of vector control tools exists, generally classified into chemical- and non–chemical-based tools. Control of vector larvae is possible with tools such as chemical or biological larvicides and predator species, or by removing their habitats (e.g., habitat modification and manipulation). Tools targeting the adult vectors function by killing the vector (e.g., indoor residual spraying, space spraying) and/or reducing vector contact (blood-feeding success) with human and/or animal reservoir hosts (e.g., topical repellents, house screening, insecticide-treated bed nets, insecticide-treated dog collars). Furthermore, several new vector control tools exist, including genetic manipulation of mosquitoes, a bacterial infection of vectors (e.g., Wolbachia), the release of several sterile males (SIT), and insecticide-treated eaves.

The key aspect in the applicability of SIT programs is the efficient separation of the sexes, for mosquitoes the separation strategies are mechanical and with a residual % of females from 0.5 to 1; therefore, it is essential to develop industrially applicable and automated systems for the separation of the sexes (Bellini et al., 2018).

Genetic approaches, studying genes with particular functions can give the way for the development of innovative genetic and eco-sustainable strategies.

FIRST PART

LIGHT RESPONSES IN *A. albopictus* LARVAE

Mosquitoes have two compound eyes located on either side of the head (Figure n. 3.1).

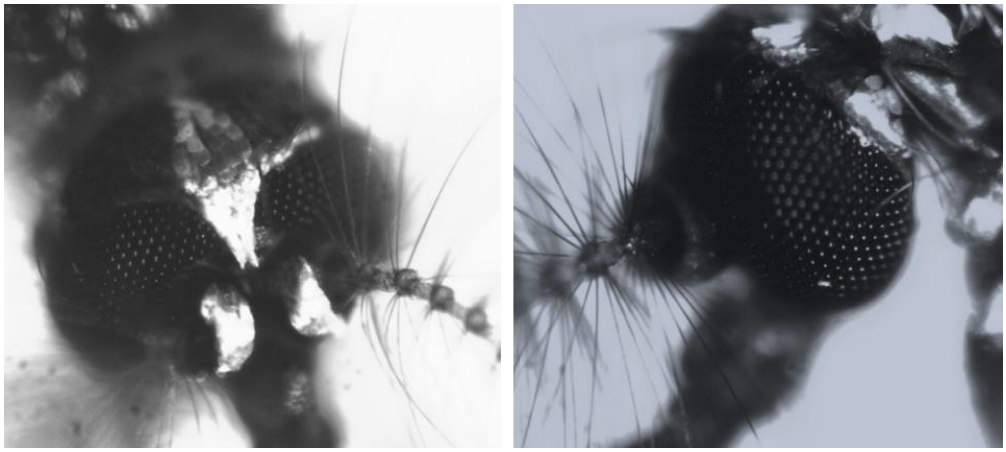


Fig n.3.1 – A. albopictus adult eyes
Microscope images of compound adult eye, front and side view.

These eyes are covered with special lenses called *ommatidia*, which essentially function like single eyes. The compound eyes sense movement very well and allow the mosquito to see in multiple directions. They also have simple light-sensitive eyes, the *ocelli*, on the top of their head that allow them to sense changes in light. The eye of an adult mosquito contains about 300-400 ommatidia, which consist of eight photoreceptors (R1-R8). Many mechanisms contribute to a photoreceptor's ability to adapt to light conditions. *Aaop1* is the major rhodopsin and is expressed in all R1-R6 cells and most R8 cells. In daylight, most of *Aaop1* is located in the multivesicular bodies in the cytoplasm. When mosquitoes are moved to darkness, *Aaop1* migrates back to the rhabdomeric membranes and is thus positioned for light perception (Hu et al., 2012).

The simple eyes of larvae are called *ocelli* (Figure n. 3.2), divided into two distinct groups, *ocelli* and *stemmata*, that are innervated from separate parts of the brain. A group of *stemmata* is usually found on each side of the head, innervated laterally from the optic lobes and used for horizon detection (Stehr., 2009). S*temmata* contain two photoreceptor types, distinguished by the expression of different rhodopsins.

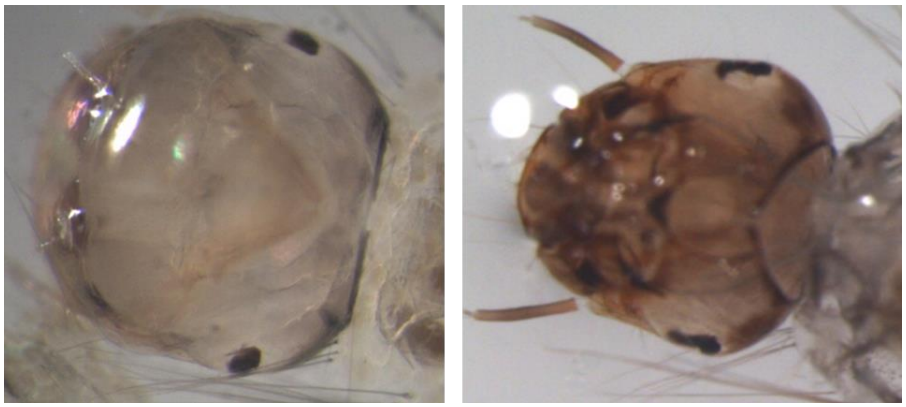


Fig n.3.2 – A. albopictus larval eyes
Microscope images of simple larval eye, front view.

The ocellar nerve, formed by the axons of the receptor cells, extends from the eye to the brain. From the 1st instar, the visual system consists of a set of five trunks arranged laterally on each side of the head (Sato., 1953; White., 1961). In the middle of the 1st instar, the adult compound eye begins to develop in the epidermal cells located in front of and next to these trunks. In the 3rd larval instar, a cellular sheath develops around the ocellar nerve. At this stage of development, the axons of the sensory cells of the differentiating compound eye begin to grow along the ocellar nerve toward the brain (White and Sundeen, 1967). Recruitment and differentiation of adult retinal cells occur from two sites so that the two distinct pigmented eye fields are visible in the early 4th instar larva and continue to expand anteriorly to form the fully formed adult eye by the middle of the pupal stage (Rocha et al., 2015).
In the swimming larvae and pupae of *Aedes* and other mosquitoes, a shadow moving across the visual field triggers an avoidance response consisting of a downward movement from the water surface (Kasap., 1978). To distinguish the role of larval stems and the adult compound eye in this behavior, Kasap (1977a) used black paint to cover the stems, compound eye, or both. In the 4th instar *Aedes* larvae and pupae, only painting over both the stems and the compound eye resulted in statistically significant defects in light-triggered behavior.

Light absorption by rhodopsin triggers the activation of G proteins in the photoreceptor cell (Kiselev et al., 1994). The photoreceptors of some invertebrates exhibit a remarkable diurnal cycle in which the light-sensitive rhabdomeric membranes are degraded at dawn and rebuilt at dusk (Blest et al., 1978; Chamberlain and Barlow., 1984; Naessel and Waterman., 1978; Williams and Blest., 1980).
Under dark conditions, rhodopsin accumulates in the light-sensitive rhabdomeres of the photoreceptor. However, light treatment results in extensive movement of rhodopsin into the cytoplasmic compartment. Rhodopsin has been found to exist in two distinct forms: the larger

immature form, which is de-glycosylated during the post-translational maturation process to generate the smaller mature form. The immature form is maintained at a constant level regardless of light conditions. These results suggest that rhodopsin biosynthesis and movement into rhabdomeres occurs at a constant rate. Rhodopsin-mediated processes could be targeted to disrupt mosquito mating, host-seeking, and oviposition (Gloria et al., 2017).

Rocha et al. (2015) found that a member of the long-wavelength rhodopsin *Aaop3* (*GPROP3*) is detectable in all five stemmata structures. In light-treated larvae of *A. aegypti*, the rhodopsin *Aaop3* is largely excluded from the rhabdom structures and is found scattered in the cell body of the majority of stemmata photoreceptors. Indeed, in larval retinas under dark conditions, *Aaop3* is sequestered in the rhabdomeres and found in very low amounts in the cell bodies of the photoreceptors (Rocha et al., 2015).

Our observations revealed that *A. albopictus* larvae have a specific response when illuminated with direct light. Under illumination, larvae quickly move to a shaded area to avoid the light stimulus source (see Figure n. 3.3, A-F).

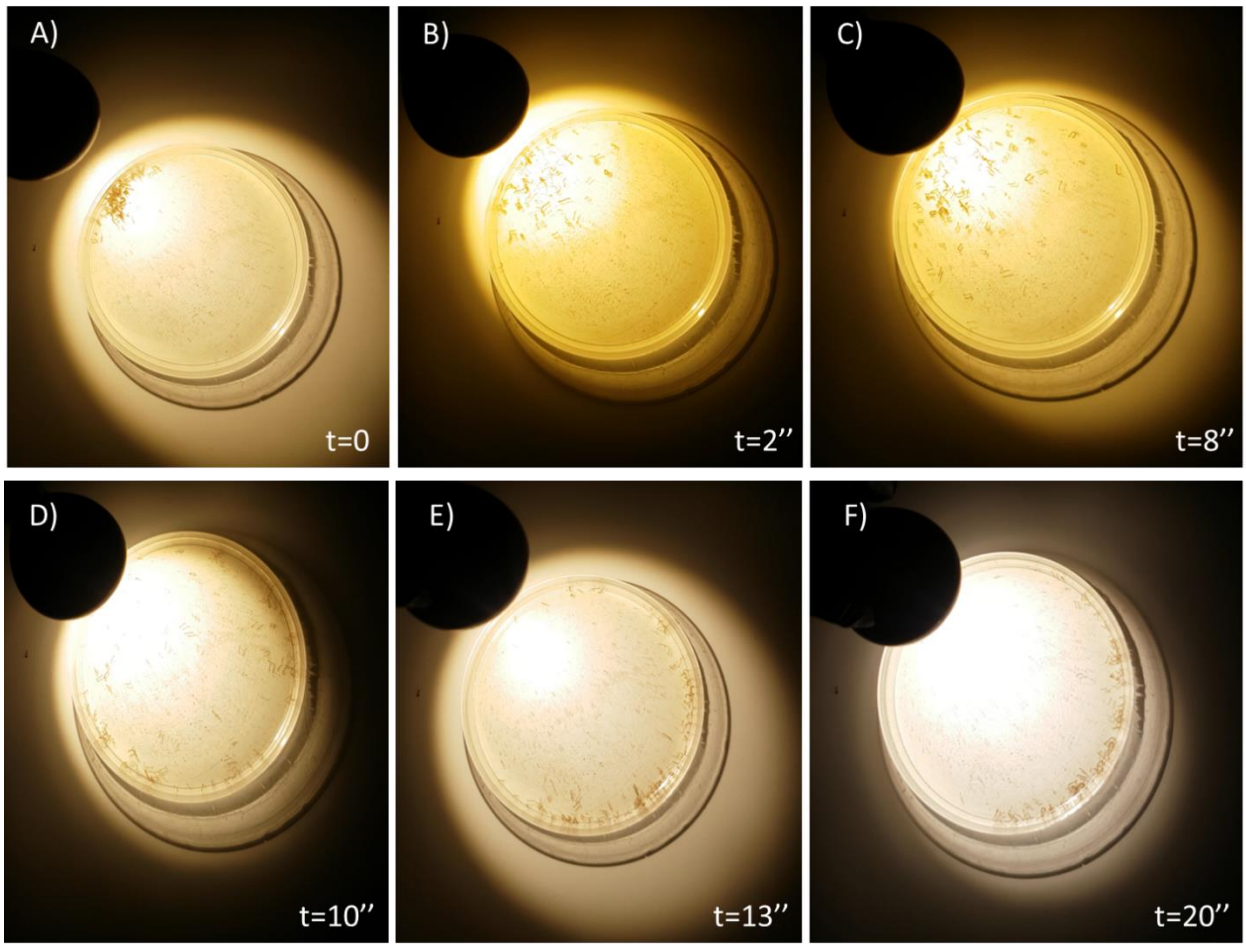


Fig.3.3 – A. albopictus larvae response to light

Experimental observations on A. albopictus larvae illuminated with a direct source of light. The images show how the larvae progressively move away from the source of the light (t=time in seconds).

The principal question addressed in this chapter is the study of genes underlying the photoreception and light response mechanism.

We try to answer this question by functional analyzing the role of the long-wavelength rhodopsin *GPROP3*, in order to provide the molecular basis for developing an innovative sexing system based on the light response. Indeed, through genetic manipulation, would be possible to create a sex-separation system based on the differentially perception of light at the larval stage; this system could be applied on a massive scale for the automatic production of large numbers of insects for SIT programs.

MATERIALS AND METHODS

A. albopictus strain

Previously described in Chapter 2.

In silico analysis

The GPROP3 protein of *Aaop3* gene (AAEL006484) of *A. aegypti* (Rocha et al., 2015), was used as a probe in tBLASTn to identify and reconstruct the rhodopsin *GPROP3* gene in *A. albopictus* (AALF009531). By BLASTp analysis was possible to confirm the protein's identity. MEME-Suite online tool (https://meme-suite.org/meme/) was used to identify different motif on the protein sequences.

RNA isolation and cDNA amplifications

Previously described in Chapter 2.

Sequencing and transcript analysis

The sequencing reactions were generated using by Mix2Seq Kit (*Eurofins genomics, Ebersberg, Germany*), using 10 µM primers forward and reverse (see appendix 3.1) in two different tubes, using as a template the PCR gel-purified product.

Quantitative genes expression by Real time RT-PCR

Previously described in Chapter 2.

dsRNA in vitro synthesis and injection mix

Previously described in Chapter 2.

Microinjection of dsRNA in embryos

Previously described in Chapter 2.

Light test

Post-injection larvae were singularly placed in a Petri dish (*Thermo Fisher Scientific, Waltham, MA, USA*) to estimate the larval light response through the development.

The assay was performed in a dark room maintained at a constant temperature of 26 ± 1 ° C. The "Light test" consists of positioning a light source (*phone's flash*) at a 2 cm distance from the larvae and registering the orientation of the larvae movements.

RESULTS AND DISCUSSION

Phylogenetic analysis

Since in *A. aegypti*, under light conditions the rhodopsin *GPROP3* localizes to cytoplasmic vesicles in the photoreceptor body, and under darkness, it moves into the rhabdom, this rhodopsin seems an ideal candidate for regulating the larval response to light.

tBLASTn of the *A. albopictus* transcripts and genome using *GPROP3* of *Ae. aegypti* as a query was performed to identify the putative orthologue of the rhodopsin in *A. albopictus*. The gene is 1197 bp long, consists of two exons and one intron (Figure n. 3.4), and it encodes a protein of 374 amino acids. The *in silico* assembled sequence was confirmed by sequencing (Appendix 3.2).

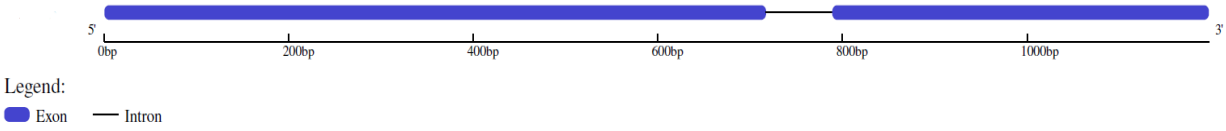


Fig n.3.4 – GPROP3 gene structure in A. albopictus

The protein sequences of the rhodopsin GPROP3 of *A. albopictus (Aa)* and *A. aegypti (Ae)* were aligned (Figure n. 3.5) using BLASTp tool and was found 94.92 % of identity.

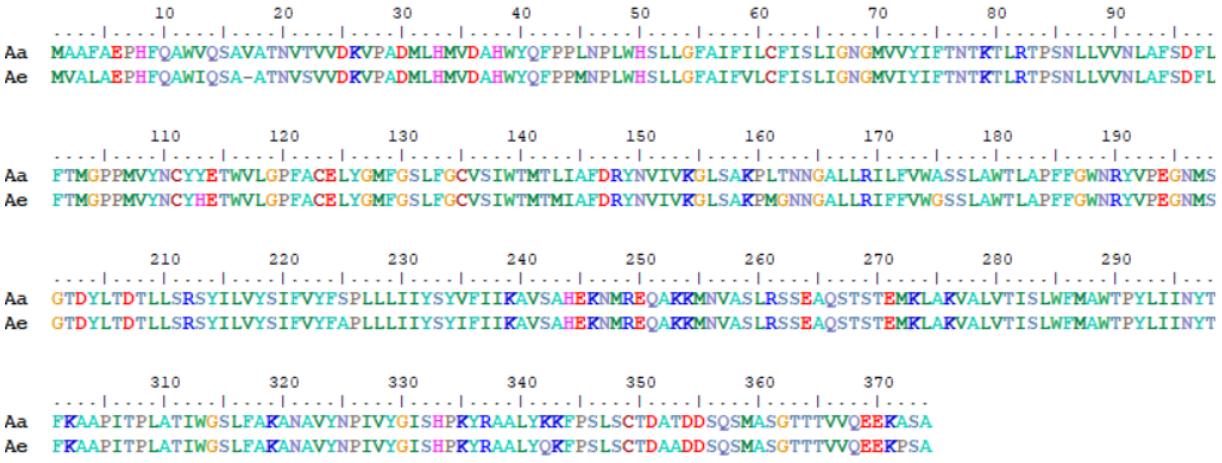


Fig n.3.5 – BLASTp of rhodopsin GPROP3
Amino acid alignments between GPROP3 proteins in A. albopictus and A. aegypti.

These protein sequences were also characterized by using the online tool **MEME-suite**, identifying ten amino acid conserved motifs distributed on the protein sequences of these two mosquito species (Figure n. 3.6).

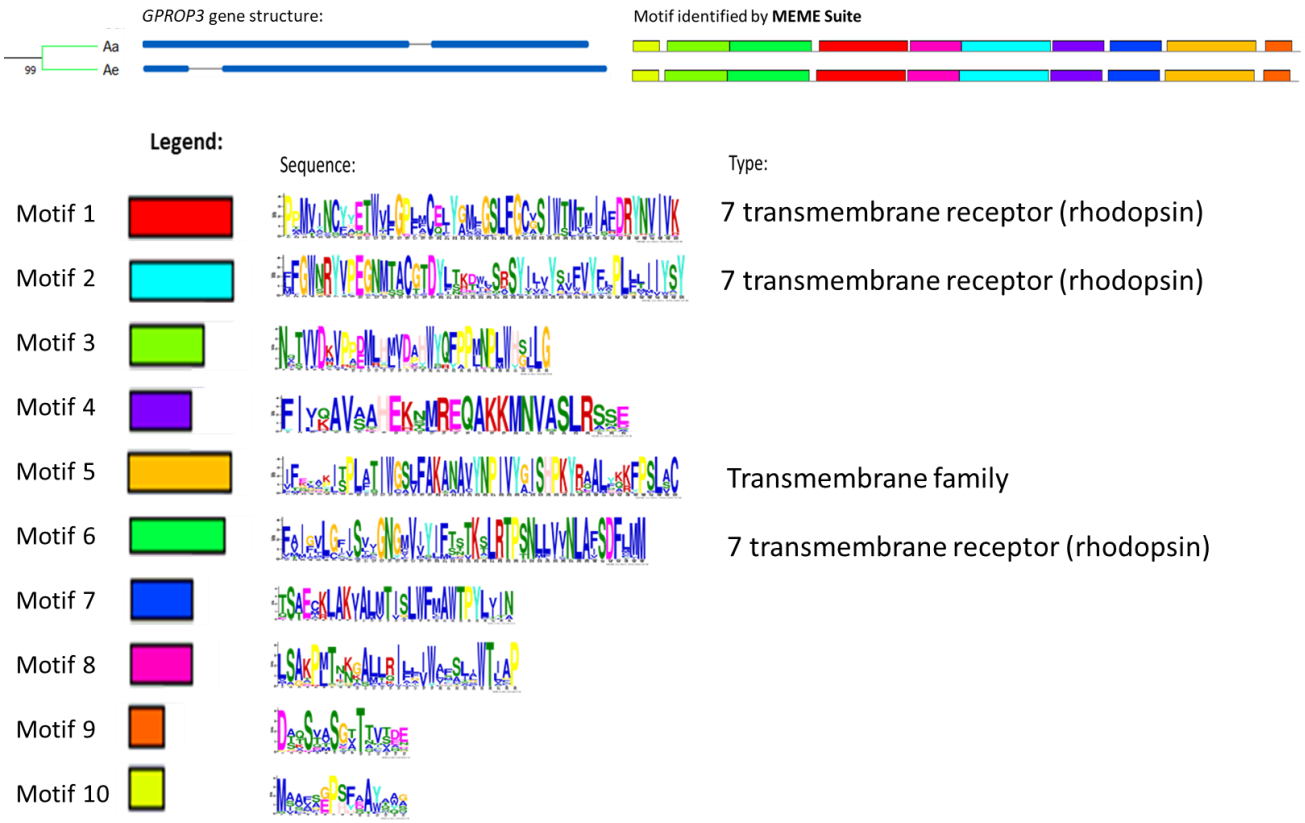


Fig n.3.6 – MEME-Suite analysis
GPROP3 *proteins of A. albopictus and A. aegypti are analyzed using MEME-Suite. The tool identified ten amino acid motif conserved in this two mosquito species.*

The Motifs 1 (in red), 2 (in light blue) and 6 (in green) are part of the *7 transmembrane receptor* (rhodopsin), the Motif 5 (in orange) is part of *Transmembrane transporters family*, while the others have unknown function. Presumably, motifs 1, 2 and 3 are essential for the photoreceptive function of rhodopsin.

Rhodopsin "*GPROP3*" developmental expression in *A. albopictus*

The *GPROP3* gene expression profile was investigated by RT-PCR in samples of *A. albopictus* collected at different developmental stages, from embryos to adults, of both sexes.
Indeed, cDNA amplifications show a common amplification of the total transcript for all samples but a significant decrease in expression from the pupal stage (P) (Figure n. 3.7- C).
Since the light response is observed only at the larval stage, this high expression found specifically at larval stage, suggests that *GPROP3* could be responsible for light behavior.

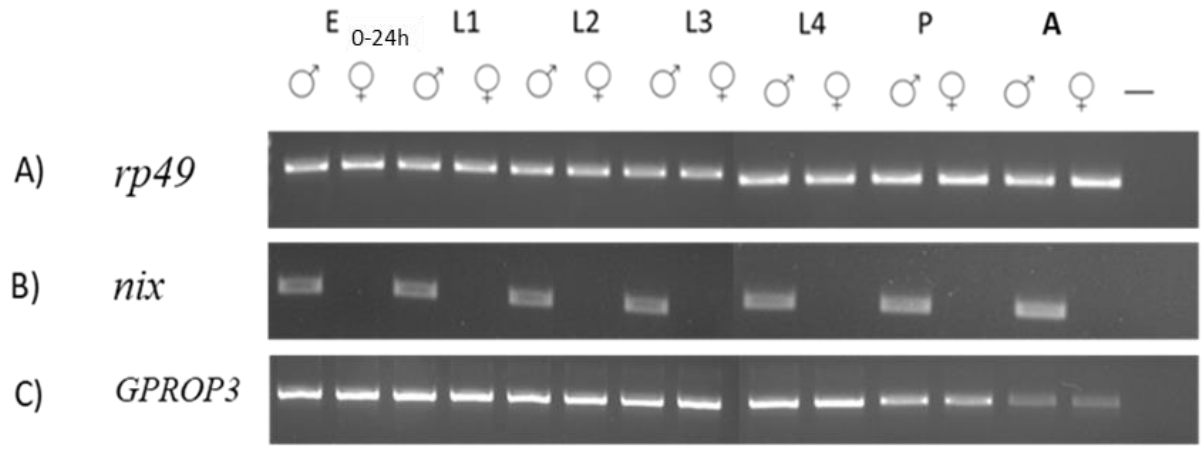


Fig n.3.7 – Developmental expression of GPROP3

The 2% agarose gel electrophoresis shows PCRs amplified fragments: A) The housekeeping gene rp49; B) The male-specific gene nix; C) The rhodopsin gene GPROP3.

To quantitative examine *GPROP3* activity during development, was monitored its expression by real-time RT-PCR at different developmental stages: a pool of embryos ($E_{0\text{-}24h}$), larvae (L3), pupae (P), male (M) and female (F) adults. Based on the following graphs (Figure n. 3.8), it is clear that the gene has specific embryonic and larval expression, with a decreasing trend occurs after the larval stage.

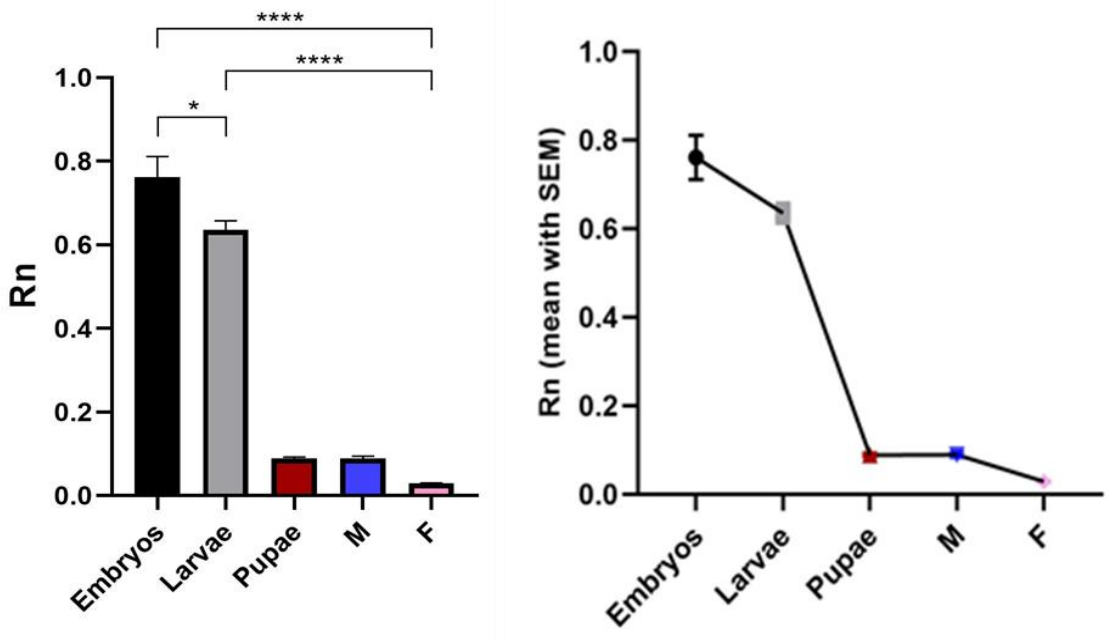


Fig n.3.8 – Expression trend of the GPROP3 gene

*Relative expression pattern of the GPROP3 gene studied by real time RT-PCR in different stage of development of A. albopictus. The expression is reported as Rn. The vertical bars represent the SEM of the biological and technical replicates. ANOVA test, p-Value **** <0.0001. Asterisks indicate statistically significant, as assessed by the Tukey post-hoc test.*

Embryonic RNAi silencing of *GPROP3* in A. *albopictus* causes change in larval light-response

Starting from the hypothesis that *GPROP3* could be responsible for the larvae's response to light, the functional characterization of this gene was essential.

A dsRNA was designed between the first and the second exon of the gene (dsRNA_ *GPROP3*) and injected into 1-hour old embryos. As control was injected a dsRNA against the *GFP* gene (dsRNA_*GFP),* which is not present in the mosquito genome and is expected to do not have any effect on the development of the insects.
The following tables report the sets of injection data (Tables n. 3.1-3.2).

SET	Nr Inj eggs	Hatched eggs	Larvae	Light positive larvae
Set 1	90	3	3	2
Set 2	250	6	6	5
Set 3	150	8	8	8
Set 4	89	6	6	6
tot	**579**	**23**	**23**	**21**

Table n.3.1 – dsRNA_GPROP3 injection data

SET	Nr Inj eggs	Hatched eggs	Larvae	Light positive larvae
Set 1	346	3	3	0
Set 2	170	4	4	0
tot	**516**	**7**	**7**	**0**

Table n.3.2 – dsRNA_GFP injection data

The number of hatched eggs is pretty low, both in dsRNA_*GPROP3* and dsRNA_*GFP* microinjection. This low efficiency is due to the type of mosquito strain used in the technique and it is not attributable to a gene effect on the embryo's development.
The most probable cause of this low efficiency is the physical insult to embryos during injection, the needle penetration and the injection setting. The number of hatching embryos can go as low as 4.8 per cent with the highest efficiency being 17.5 per cent (Sampath et al., 2012).

We tested the effectiveness of the dsRNA in the interference of *GPROP3*, analyzing single *GPROP3*-interfered larvae response to light. In the experiment called "*Light test*", each larva was exposed to a source of light and was observed its behavior (Figure n. 3.9).

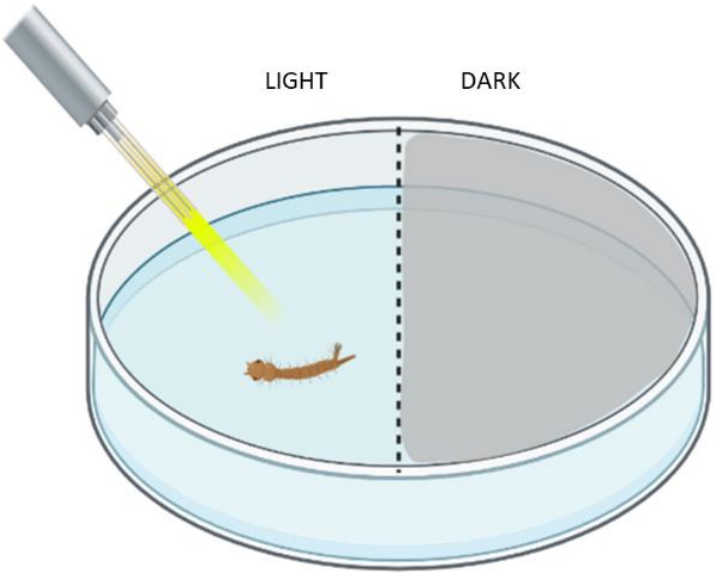


Fig n.3.9 – Graphic representation of the Light test
The experiment illustration is created with BioRender.com.

The analysis was focused on the observation of the larvae movement from light to a dark place. Each larva was put in a petri dish partially covered to create a shadow area and on the opposite side of the petri dish, a flash was placed.

For each larva was reported if it can stay at light or if it runs away to the shadow area, the graph in Figure n. 3.10 shows the number of larvae distributed to shadow or light areas. The 91,3 % of *GPROP3*-interfered larvae (in light blue) can stay at the light and only 8,69 % showed the same behavior as the control larvae (WT larvae are in black and *GFP*-interfered larvae are in green), fleeing to the shaded area.

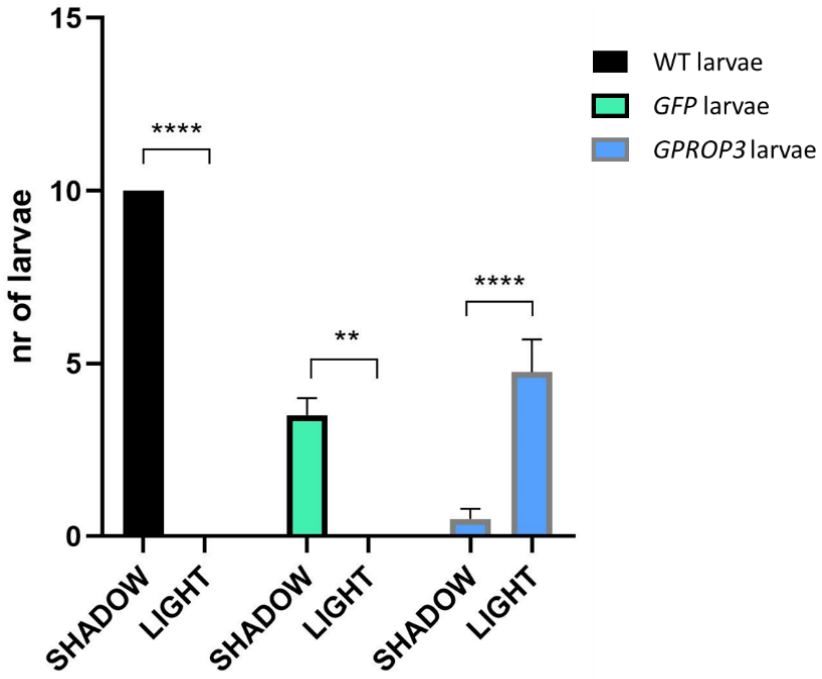


Fig n.3.10 – Light test analysis in A. albopictus larvae.
*Single larvae were analyzed in a petri dish using a source of light as stimulus. Larvae were distributed in two area: light and shadow. The vertical bars represent the SEM of the biological replicates. ANOVA test, p-Value **** <0.0001.*

As a result of RNAi of the rhodopsin *GPROP3*, larvae begin to change their behavior when exposed to light: they can stay at the light without running away, or swim from the light zone to the shadow one and return back to the light again.

Expression levels of *GPROP3* after RNAi in larvae

To examine the RNAi effects on *GPROP3* expression, we measured the relative transcript levels of *GPROP3* at third instar stage of development after dsRNA injections. Relative level of the gene transcription was determined by normalization to ribosomal housekeeping gene *rp49*.
A significant % reduction of *GPROP3* transcript levels is observed in *GPROP3*-interfered larvae, compared to the control larvae (Figure n. 3.11).

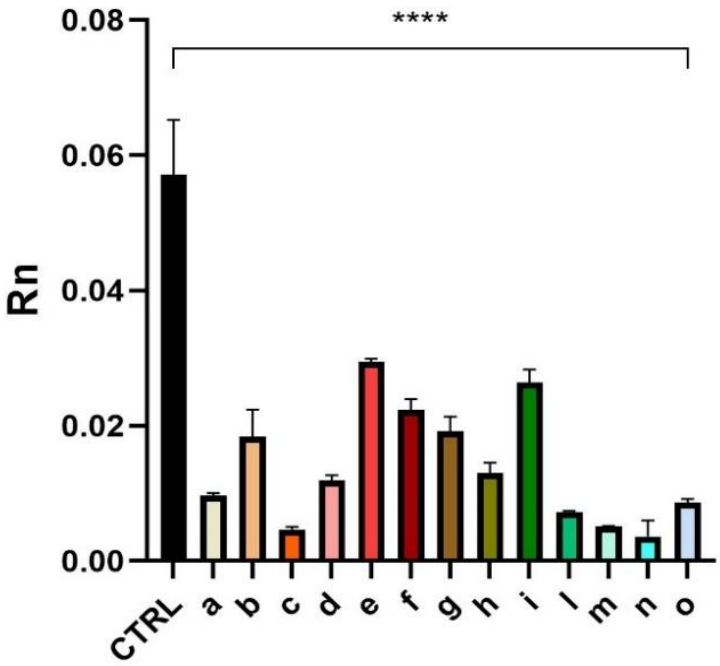


Fig n.3.11 – GPROP3 RNAi expression studied by real time RT-PCR

*Relative expression pattern of the GPROP3 gene studied by real time RT-PCR in different larvae of A. albopictus. Ctrl: not interfered L;, a-o: GPROP3-interfered L3 from different sets of injection. The expression is reported as Rn. The vertical bars represent the SEM of the biological and technical replicates. ANOVA test, p-Value **** <0.0001. Asterisks indicate statistically significant, as assessed by the Tukey post-hoc test*

According to the evidence of GPROP3's down-regulation, it is possible to say that the observed light-positive larvae are due to the reduced expression of the gene.

Based on these findings, we can conclude that the *GPROP3* gene is responsible for the light perception in *A. albopictus* larvae and might represent a new target for developing a new mosquito genetic sexing strain system.

In the wild population, the rhodopsin *GPROP3* is not expressed differently between the sexes but with innovative genetic engineering strategies, it is possible to make this gene sex-specific expressed. In this way, light may be able to separate mosquito sexes at an early larval stage resulting in an eco-sustainable and automatized biological tool for the application of the SIT.

SECOND PART

Lifespan regulation in *A. albopictus*

A variety of mechanisms have been proposed to control ageing, including oxidative damage caused by reactive oxygen species, alterations in genomic integrity, and gene regulation that affects reproduction, soil tolerance, and nutrient utilization (Zhang & Herman, 2002; Lombard et al., 2005; Partridge et al., 2005; Sinclair, 2005; Lim et al., 2006). During natural ageing, stress-responsive genes become more prominent in long-lived individuals, increasing their resistance to external stress (Vermeulen & Loeschcke, 2007; Gems & Partridge, 2008; Rattan, 2008). In nematodes, stress increases longevity, a process known as hormesis state (Cypser & Johnson, 2002), responsible for lifespan extension (Gems & Partridge, 2008).

alpha-mannosidase 1 (*mas1*) is a member of the class I glycosidases, involved in N-linked glycosylation (Herscovics, 2001) and it is expressed in the ER, the Golgi apparatus, and the lysosome. *Mas1* removes mannose from permanently unfolded proteins, then the de-mannosed proteins are recognized by ER degradation-enhancing alpha-1,2-mannosidase-like protein (Edem), and degraded by ER-associated degradation (ERAD) (Hosokawa et al., 2001; Ellgaard & Helenius, 2003; Olivari & Molinari, 2007) but abolishing N-glycosylation results in unfolded protein accumulation and ER stress (Elbein., 1991; Lawson et al., 1998).

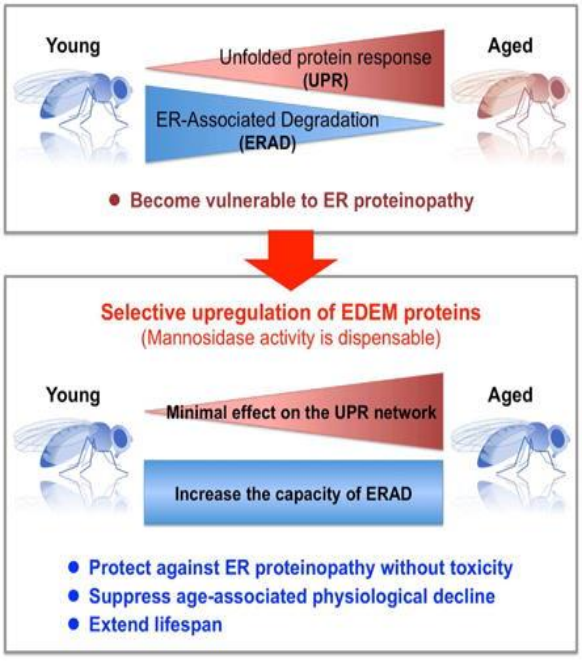


Fig n.3.12 – Age-related physiological decline in Drosophila (Source: Developmental Cell., 2018)
ERAD activity in the brain decreased with aging, and upregulation of EDEMs suppressed age-dependent behavioral decline and extended the lifespan without affecting the UPR gene expression network (Sekiya et al., 2017).

Several evidence indicate that *mas1* is important during the aging process. First, altered N-linked glycosylation affects the maturation rate of proteins that influence longevity. Furthermore, the expression of *mas1* is decreased in aging and oxidatively-stressed *Drosophila* (Zou al., 2000) as well as in the livers of aging mice and humans (Cingle et al., 1996; Zhu et al., 2006).

Liu et al. (2009) demonstrate that down-regulation of *mas1* can significantly extend longevity in *Drosophila* and *C. elegans*, suggesting *mas1* plays a conserved role in regulating longevity. An RNAi line of transgenic flies was generated to knock-down *mas1* expression, and the average lifespan of the progeny was 39% longer than that of control flies, suggesting that *mas1* is an important determinant of lifespan. As in the fly, the knockdown of *mas1* also led to an extension of lifespan in *C. elegans*, with 9% longer mean life expectancy (Liu et al., 2009).

Since the mutants with a silencing of *mas* displayed better resistance to individual stress, oxidative stress and starvation, combined with an increase in longevity, although the life (Liu et al., 2009), this gene appears to be the ideal candidate for further investigation in order to produce more resistant and long-lived sterile males for use in SIT programs.

MATERIALS AND METHODS

A. albopictus and *A. aegypti* strains

Previously described in Chapter 2.

In silico analysis

MAS protein of *An. gambiae mannosidase* gene (AGAP004032) was used as a probe in tBLASTn to identify and reconstruct the homologous gene in *A. albopictus* (LOC109401057). The *A. albopictus* MAS protein was used as a probe in tBLASTn to identify and reconstruct the same gene in *A. aegypti* (AAEL004389). By BLASp analysis was possible to confirm the protein's identity.

MEME-Suite online tool (https://meme-suite.org/meme/) was used to identify different motif on the protein sequences.

RNA isolation and cDNA amplifications

Previously described in Chapter 2.

Quantitative genes expression by Real time RT-PCR

Previously described in Chapter 2.

RESULTS AND DISCUSSION

alpha-mannosidase gene structure in *A. albopictus* and *A. aegypti*

Because *mas* down-expression extends longevity in flies and worms, investigating this gene in *Aedes* mosquitoes became quite intriguing. Specifically, this gene is interesting because of its potential to create long-lived and stress-resistant sterile male mosquitoes.

tBLASTn of the *A. albopictus* transcripts and genome using *mas* of *An. gambiae* as a query was performed to identify the putative orthologue of the *mas* gene in *A. albopictus.* The *mas* gene reconstructed in *A. albopictus* is composed of 369606 bp, consists of six exons and five introns (Figure n. 3.13), and it encodes a protein of 1228 amino acids.

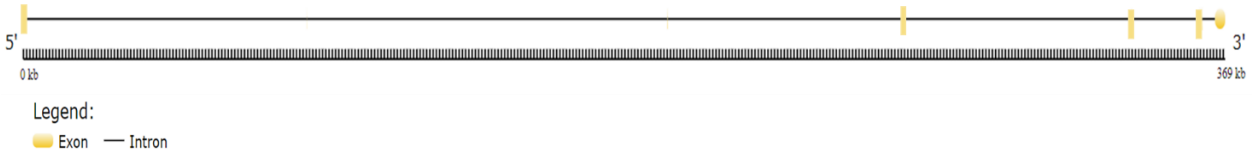


Fig n.3.13 – mas gene structure in A. albopictus

Moreover, to identify the putative orthologue of the *mas* gene in *A. aegypti*, tBLAST was performed using *mas* of *A. albopictus* as a query. The *mas* gene in *A. aegypti* is 330366 bp long, consists of six exons and five introns (Figure n. 3.14), and it encodes a protein of 1226 amino acids.

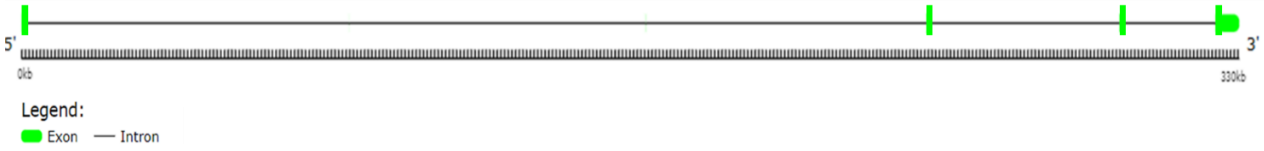


Fig n.3.14– mas gene structure in A. aegypti

The protein sequences of the MAS of *A. albopictus* and *A. aegypti* were aligned (Figure n. 3.15) using BLASTp tool and was found 95.05 % of identity.

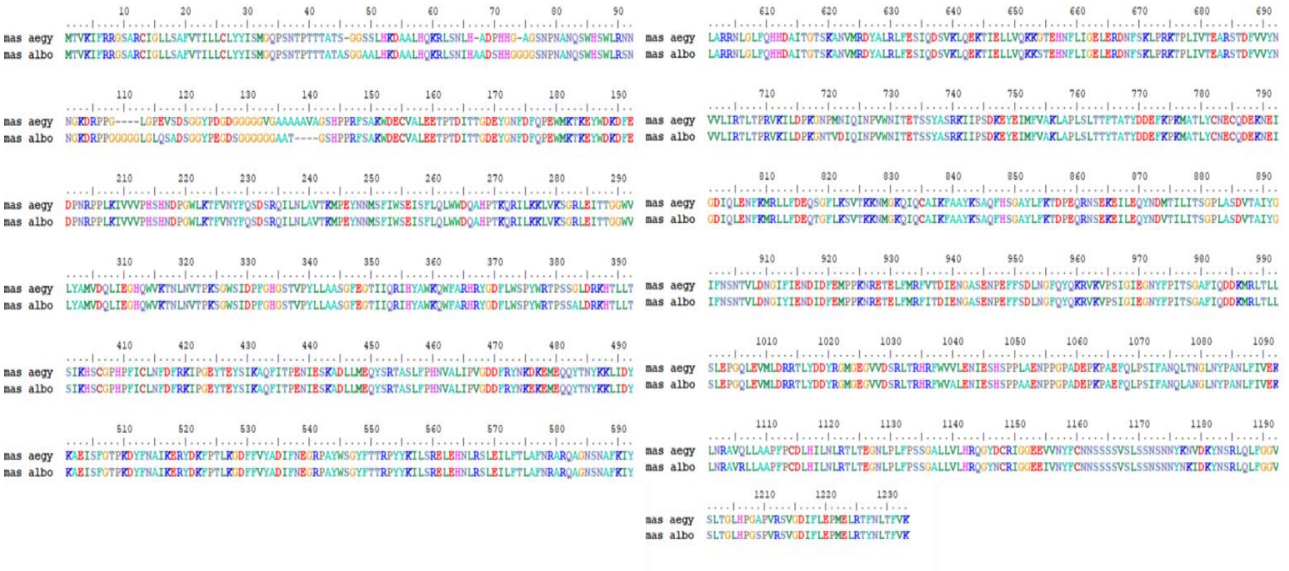


Fig n.3.15 –BLASTp of alpha-mannosidase in Aedes mosquitoes
Amino acid alignment of MAS proteins in A. albopictus and A. aegypti.

These protein sequences were also characterized by using the online tool **MEME-Suite**, identifying ten amino acid conserved motifs distributed on the protein sequences of these mosquito species (Figure n. 3.16).

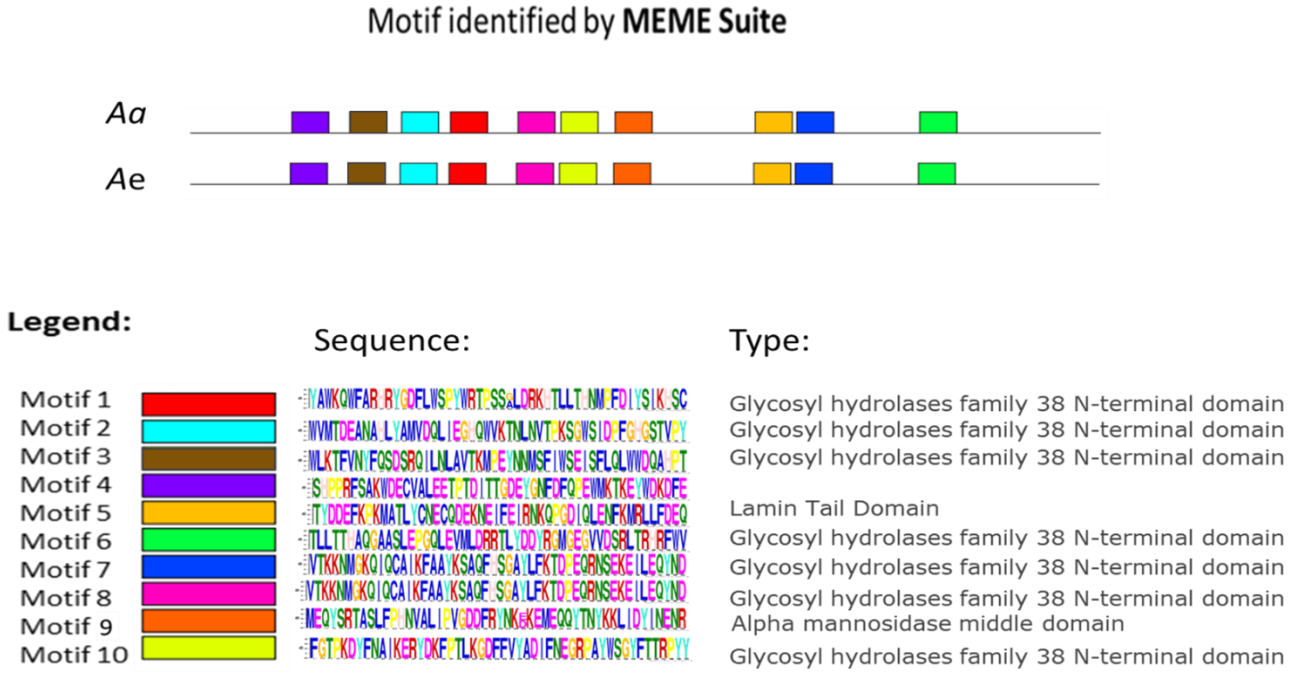


Fig n.3.16 – MEME-Suite analysis
MAS *proteins of A. albopictus and A. aegypti are analyzed using MEME-Suite. The tool identified ten amino acid motif conserved in this two mosquito species.*

The Motifs 1 (in red), 2 (in light blue), 3 (in brown), 6 (in green), 7 (in blue), 8 (in pink) and 10 (in yellow) are part of the *Glycosyl hydrolases family 38 N-terminal domain. Glycoside hydrolases of family 38* are class *II α-mannosidases*, belonging to Golgi, Lysosome and cytoplasm. Physiological roles are known for the Golgi enzyme in the protein N-glycosylation pathway and lysosomal mannosidases, in general, are likely to be involved in scavenging degraded glycoproteins. Functions for the cytoplasmic subclass are not yet certain, but they may play a role in protein recognition or signalling.

The Motif 5 (in orange) is a *Lamin Tail Domain*, involved both in protein and DNA binding; the Motif 9 (in dark orange) is the *alpha-mannosidase middle domain*, where defects in this structure cause lysosomal alpha-mannosidosis (AM), a lysosomal storage disease characterized by the accumulation of unbranched oligo-saccharide chains.

The Motif 4 (in violet) is the only one with an unknown function.

The two MAS proteins analyzed in *A. albopictus* and *A. aegypti* are composed of the same motif, distributed in the same way.

alpha-mannosidase developmental expression in *A. albopictus* and *A. aegypti*

The purpose of this section was to assess how the *mas* gene is expressed during the development of *Aedes* mosquitoes. Using real-time RT-PCR were analyzed embryos (E0-24h), larvae (L3), pupae (P), male (M) and female (F) adults of *A. albopictus*, for both sexes.

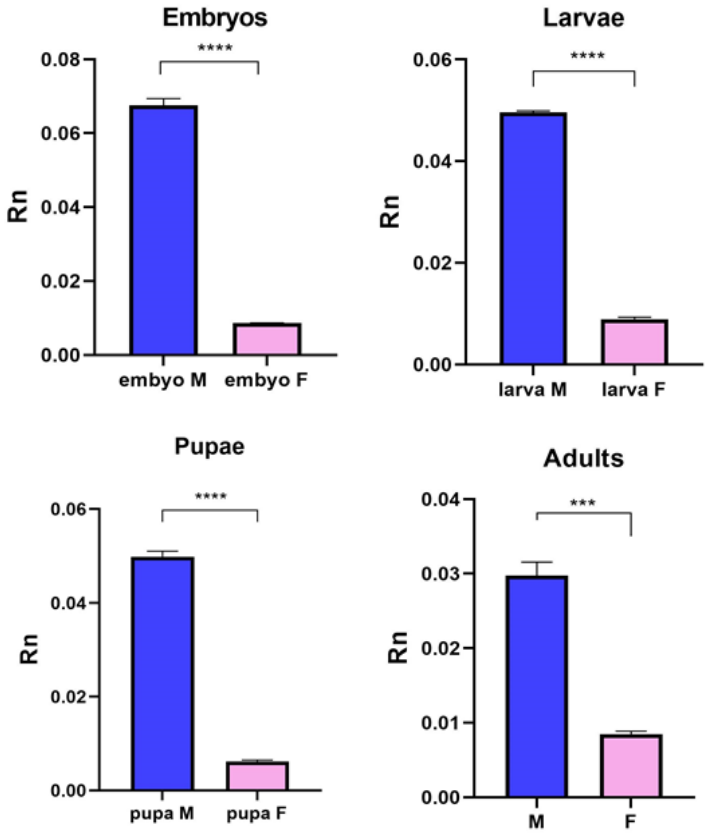


Fig n.3.17– mas sex specific expression studied by real time RT-PCR
*Relative expression pattern of the mas gene studied by real time RT-PCR in different stage of development of A. albopictus. The expression is reported as Rn. The vertical bars represent the SEM of the biological and technical replicates. t-test, p-Values **** <0.0001, *** <0.0003.*

According to the previous graphs (Figure n. 3.17), the gene has a male-specific expression throughout development, with a higher level during embryonic development.

Additionally, *mas* expression was examined in *A. aegypti*, using larvae (L3), pupae (P) and adults (M and F) of both sexes. Even in this mosquito species, the *mas* gene has a male-specific expression during the development (Figure n. 3.18).

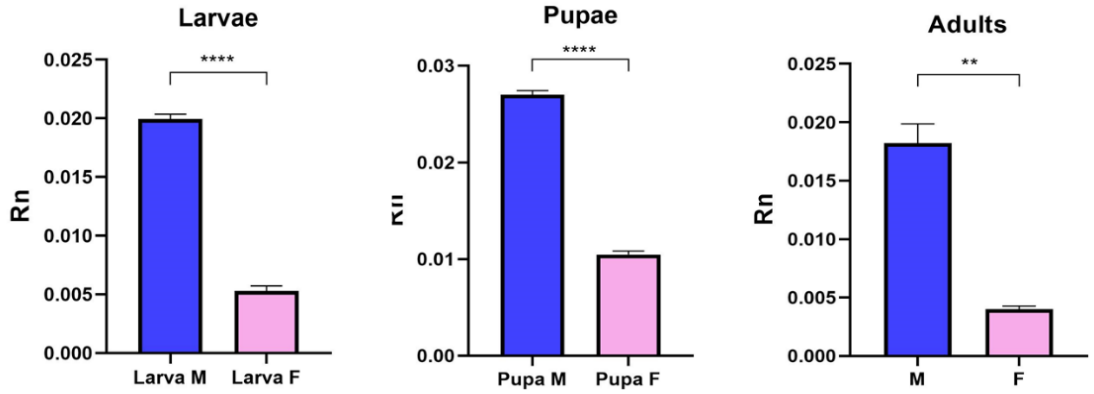


Fig n.3.18– mas expression studied by real time RT-PCR in A. aegypti

*Relative expression pattern of the mas gene studied by real time RT-PCR in different samples of A. aegypti. The expression is reported as Rn. The vertical bars represent the SEM of the biological and technical replicates. t-test, p-Values **** <0.0001; ** <0.0010.*

It is necessary to highlight adult *Aedes* mosquitoes have different lifespans, with males lasting 7 days and females lasting ~3-4 months but it is unclear what is the gene responsible for this phenotype.

The expression of *mas1* is decreased in ageing and oxidatively-stressed *Drosophila* (Zou et al.,2000) as well as in the livers of ageing mice and humans (Cingle et al., 1996; Zhu et al., 2006).

Several lines of evidence indicate that *mas1* is essential during the ageing process (Liu et al., 2009) consequentially, it is an interesting candidate to consider for the regulation of the different *Aedes* mosquito adults' lifespan.

However, more research on this topic is required before the association between *mas* and lifespans is more clearly understood.

RNAi experiments could confirm or not if also in the *Aedes* mosquitoes, down-regulation of *alpha-mannosidase* affects the lifespan, as happens for *C. elegans* and *D. melanogaster*. Following down-regulation of *mas* gene, only in male insects, may be possible to obtain males resistant to stress and with a higher survival rate, making them ideal candidates for SIT programs.

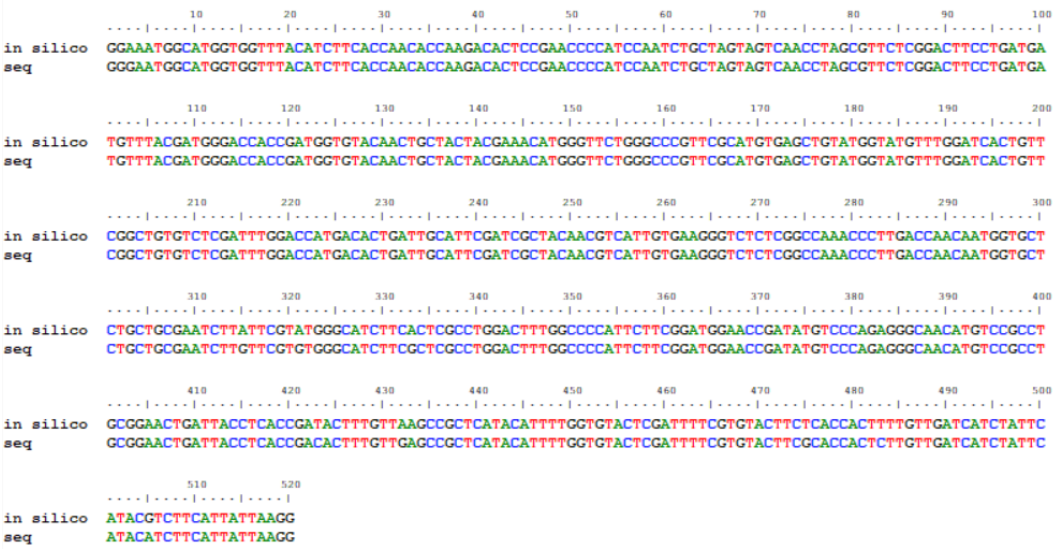
APPENDIX 3.1

Primer sequences

PRIMER NAME	PRIMER SEQUENCES (5'-3')	PURPOSE
rp49+	GACGAAGAAGTTCATCCGCC	cDNA amplifications
rp49-	GTTCTGCTGCGAGCGCAG	cDNA amplifications
nix+	GTTGTTCGTTACAGACTGATG	cDNA amplifications
nix-	CAAAGCTAATGTAAACCATGAC	cDNA amplifications
GPROP3+	CTGTGTTTCATTTCCCTGATC	cDNA amplifications
GPROP3-	AAGAACATGCGCGAACAAGC	cDNA amplifications
GPROP3_real+	GGTTGACGCCCATTGGTATC	qRT-PCR
GPROP3_real+	AGATTGGATGGGGTTCGGAG	qRT-PCR
*rp49+*_real	AGAAGTTCCTGGTCCACAAC	qRT-PCR
*rp49-*_real	GTTCTGCTGCGAGCGCAG	qRT-PCR
*GPROP3*_T7+	taatacgactcactagggCTGTGTTTCATTTCCCTGATC	dsRNA synthesis
*GPROP3*_T7-	taatacgactcactagggAAGAACATGCGCGAACAAGC	dsRNA synthesis
Mas_Aalbo+	GCAGAAGGATCCTAACCGAC	qRT-PCR
Mas_Aalbo-	TGCTTGGTGGGATGTGCCTG	qRT-PCR
Mas_Aaegy+	CCCCGAATGGATGAAAACAAAG	qRT-PCR
Mas_Aaegy-	CCACCCGGGGTCATTATGG	qRT-PCR

APPENDIX 3.2

Nucleotide sequence alignment of *GPROP3 in silico* assembled and *GPROP3* sequencing



CHAPTER 4

Citizen-science strategy for *A. albopictus* control

INTRODUCTION

Citizen-science is scientific research conducted, in whole or in part, by amateur (or non-professional) scientists (Gura., 2013). *Citizen-science* is sometimes described as "public participation in scientific research," participatory monitoring, and participatory action research whose outcomes are often advancements in scientific research by improving the scientific communities capacity, as well as increasing the public's understanding of science (Tulloch et al., 2013; Doyle et al., 2019). Citizen participation in scientific projects has become a factor in promoting scientific curiosity and knowledge while providing unprecedented engagement between professional scientists and the general public (Doyle et al., 2019).

To support mosquito monitoring performed by experts through conventional trapping, in some EU countries community-based surveillance activities have been launched. These programs are based on the public participation through active monitoring, as for the “Mückenatlas” (mosquito atlas) in Germany (www.mueckenatlas.de) and the Mosquito Recording Scheme (MRS) in the UK (www.brc.ac.uk), or through smartphone-based mosquito data collection applications (the so called “passive” monitoring), as for the “Muggenradar” (mosquito radar) in the Netherlands (www.muggenradar.nl), “Hunting the tiger” (mosquito Alert) in Spain (www.mosquitoalert.com), the iMoustique® in France, the MosquitoWEB in Portugal (www.mosquitoweb.pt), and the ZanzaMapp in Italy (http://www.zanzamapp.it/).

Procida island: a promising Mediterranean site for the assessment of innovative and community-based integrated pest management methods

Procida (Fig. 4.1) is a small island off the Phlegraean archipelago, situated in the Naples gulf, not far from Ischia and the mainland. It is a flat volcanic island (average 27 m above sea level) with a 16 km-long rough coastline and with a total surface area of only 4.1 km^2, including the uninhabited tiny satellite island of Vivara (0.4 km^2). Except for Vivara, a natural reserve, Procida’s territory is largely urban and accessible. Most of Procida’s private properties include a garden with ornamental flowers, vegetable cultivations and/or orchards with citrus plants and family-type farming of chickens and rabbits. Despite its small surface, Procida has a very high and urban-like population density with 27.83 inhabitants/ha (ISTAT 31/12/2020).

According to residents’ perception, *A. albopictus* arrived on the island around the year 2000, most probably introduced by tourists and/or maritime transport of goods. Official data about Asian tiger

mosquito presence are available only for the satellite island of Vivara, where *A. albopictus* was found for the first time in 2002 (D'Antonio and Zeccolella., 2007).

Several novels and films have been filmed on the island, giving it a world-renowned image that may facilitate wide media coverage in response to positive results for population suppression tests, thereby raising funds for future larger population control tests.

In 2015 the STOPTIGRE project of the Department of Biology of the University of Naples Federico II has been launched. The project has been designed to contribute to the development of an innovative, sustainable and effective approach to the monitoring and control of the Asian tiger mosquito and it is part of a memorandum of understanding between the Department of Biology of the University of Naples Federico II and the Municipality of Procida. The project was designed to encourage, from its very early stages, the direct participation of citizens and the administration of the island and to involve them in *Citizen-science* actions aimed at studying the spatial distribution and the trend over time of the local tiger mosquito population.

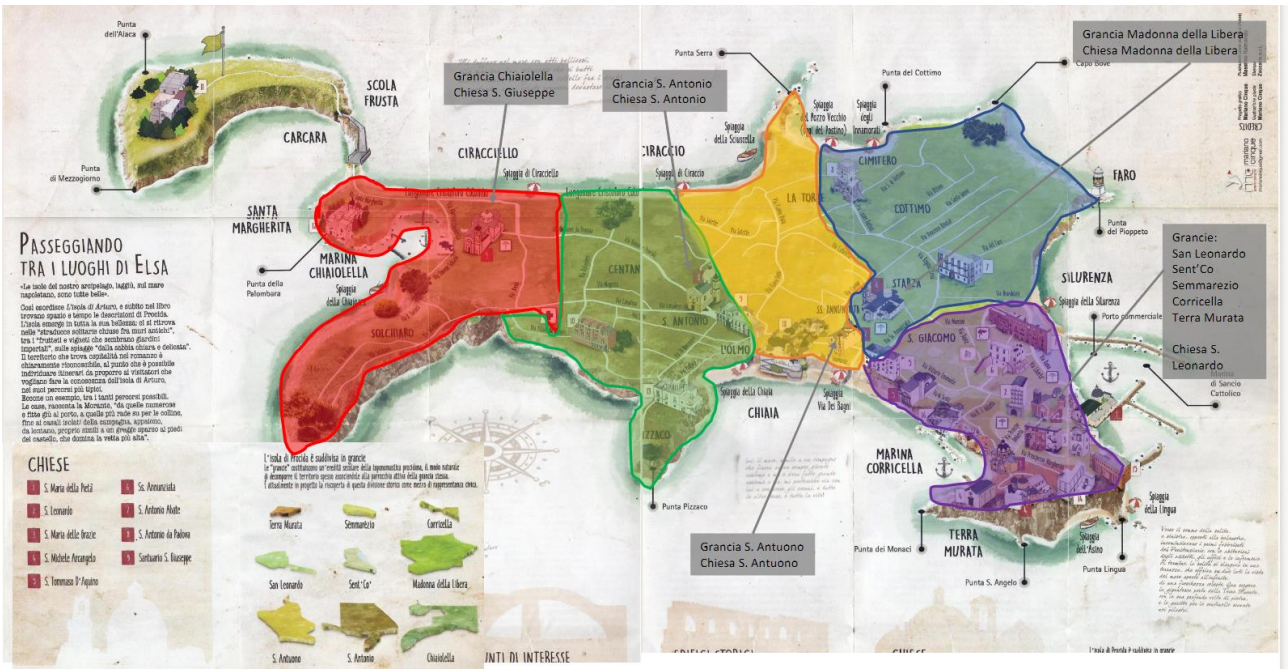


Fig n.4.1 – Procida island map

An illustration of Procida island. The island is divided into five coloured zones, identified by the presence of different Churches.

MATERIALS AND METHODS

Study area

Procida has unique and intriguing characteristics for field testing mosquito IVMs, including SIT: i) very small size; ii) completely urbanized and accessible territory; iii) high human population density; iv) year-round presence of *A. albopictus*.

The island is organized in hundreds of small residential buildings surrounded by private green areas. This landscape is associated with an extensive presence of anthropic water sources, representing ideal breeding sites for *A. albopictus* larvae.

Thanks to very beneficial host and climatic conditions, with an average annual temperature of 16.2 °C and average annual precipitation of 797 mm (http://bit.ly/ecdata_procida; accessed: 07th May 2018), and very abundant availability of water containers in private gardens, *A. albopictus* spread quickly over the entire island, reaching high population densities in some areas and becoming a serious nuisance in the last years.

Monitoring strategies

The monitoring for collecting *A. albopictu*s eggs, were done used "*ovitraps*" (Figure 4.2): cylindrical black plastic jars 15 x 12 cm in size. Ovitraps were filled with 500 mL of tap water and heavy-weight seed germination paper strips (*Anchor Paper Co. USA*) were used as oviposition substrate. Ovitraps were placed on the ground, in shaded areas near vegetation.

Fig n.4.2– Ovitrap placed in the vegetation

An ovitrap is a mosquito trap. It is a black, cylindrical container filled with water that appears to be an ideal location for a female Aedes mosquito to lay eggs. The female lays her eggs on germination paper strips, later removed.

The ovitraps surveillance system is an alternative for long-term vector monitoring to provide insight into population dynamics and the spatiotemporal distribution of mosquito vectors for improving prevention and control programs.

The adults monitoring were performed by using *BG-Sentinel 2*^{TM} trap (BGS2) (Ritchie et al. 2008; Meeraus et al., 2008). The BGS2's primary structure is a collapsible white plastic cylindrical container with an open top covered and equipped with white gauze (Kroeckel et al., 2006). A black cylinder suspended in the center of the trap opening holds captured mosquitos in an attached catch bag. A 12V fan located beneath the catch bag creates suction, sucking mosquitos within a few centimeters of the trap opening into the catch bag of the trap located above the fan (Figure n. 4.3).

Fig n.4.3 – BG-sentinel 2 ^{TM}
The BG2 uses a combination of visual cues, olfactory signals (excluding CO2), and convection currents (similar to those generated by humans) to attract mosquitoes (Kroeckel et al., 2006). The olfactory attractants used with the trap are delivered by the BG-Lure®.

The female adult monitoring were performed by using the **G**ravid ***A****edes* **T**rap (BG-GAT) (Figure n.4.4), attracts female *Aedes* mosquitoes with water and oviposition cues. Mosquitoes trying to find an oviposition site enter the transparent chamber via the black funnel on top of the trap and in the transparent chamber they are exposed to a sticky surface. The transparent chamber makes it difficult for the mosquitoes to escape, and the black mesh net provides a barrier between mosquitoes and the water. Dead mosquitoes are easily collected from the sticky surface.

Fig n.4.4 – Gravid Aedes trap BG-GAT2 (Biogents., AG)

Females of the Asian tiger mosquito are attracted to water for oviposition. Mosquitoes enter the BG-GAT, pass through the black funnel and find themselves in the transparent chamber. Once inside the room, the mosquitoes will try to escape through the transparent windows, but the adhesive sheet will catch them since they are exposed to it. The grate forms a barrier between mosquitoes and water in the lower chamber. The adhesive sheet in the transparent chamber will kill mosquitoes.

RESULTS

Temporal and spatial analysis of *A. albopictus* on Procida island and Vivara natural reserve

The monitoring program of *A. albopictus* held in July 2019 consisted of distributing BG-sentinel2 (tool for the capture of adult individuals) and ovitraps (tool for eggs collection) throughout the perimeter of the Vivara nature reserve, collecting data about the density and distribution of the mosquito population.

Fig n.4.5 – Monitoring of A. albopictus on Vivara natural reserve

In order to analyze the mosquito spatial distribution in the reserve area, we monitored the oviposition rate by 31 ovitraps in 2019. All ovitraps were confirmed positive for *A. albopictus* eggs with 213 eggs/trap/week. In addition, we performed adult collections by 4 BG-sentinel traps baited with BG-lure, collecting 19 males and 27 females. Moreover, the study revealed that *A. albopictus* occurs uniformly and widely throughout the Procida and Vivara island (Figure n. 4.6).

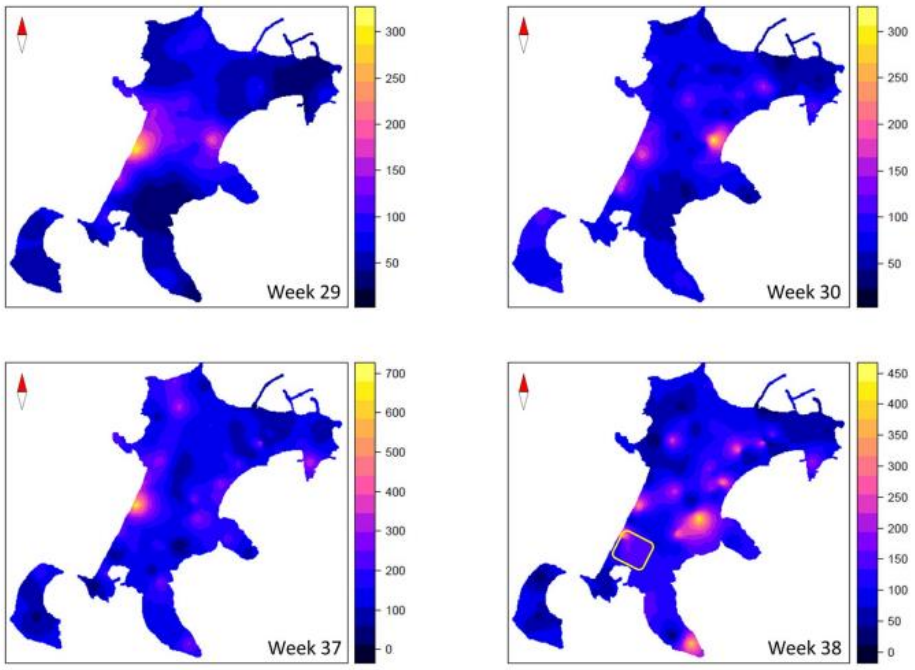


Fig n.4.6 – Eggs density on Procida and Vivara island (Source: PLoS Negl Trop Dis)
*Four weeks of **o**vitraps monitoring reveals uniform distribution of eggs through the islands. The colour gradient corresponds to the variation range of the estimated egg numbers.*

The data collected by ovitraps between the first monitoring program in September 2015 and the second one in July 2019, allowed us to characterize the temporal and spatial dynamics of the distribution of *A. albopictus* on the island and provide the basis for developing predictive models useful for the optimization of interventions.

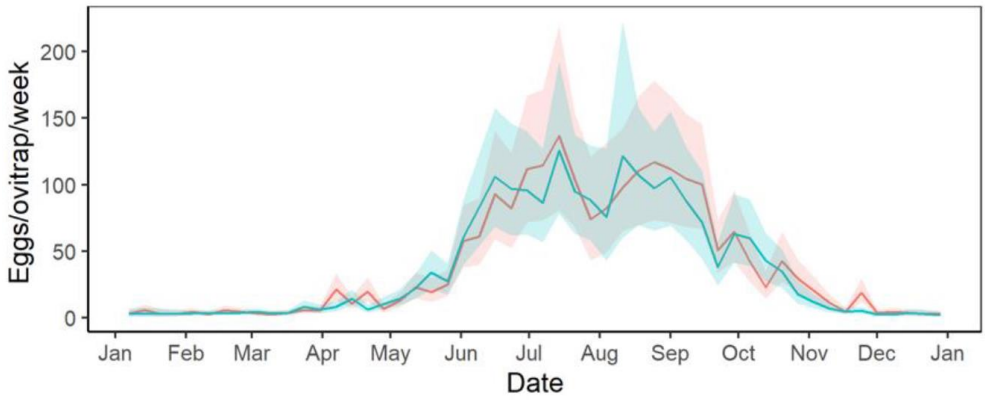


Fig n.4.7 – Monthly distribution of A. albopictus eggs

Eventually, the study revealed that *A. albopictus* begins to be present on the island in April and declines between October and early November, providing crucial information to establish the optimal period to implement the control strategy through SIT (Caputo et al., 2021).

Community-engagement assessment

A new control and monitoring program, called "*Stop Tiger*" started in 2022, in order to collect data about the temporal and spatial dynamics of the mosquito population during the reproductive season of the insect.

We involved ~500 residents as citizen scientists or collaborators in the program, residing in the five different parts of the island.

The monitory activities consisted of the distribution of 500 Gravid Traps (BG-GAT) in the garden of the volunteers, who take responsibility for the traps.
The map of the distributed Gravid Traps is illustrated in the following figure.

Fig n.4.8 – Gravid Traps distribution on Procida island
The map shows the 500 Gravid Traps placed in the island's accessible territory

From April 2022 to September 2022, two times a month, the volunteers have to remove the sticky surface and take a picture of the captured mosquitoes using the application ZZZAPP (https://play.google.com/store/search?q=zzzapp&c=apps&gl=IT).

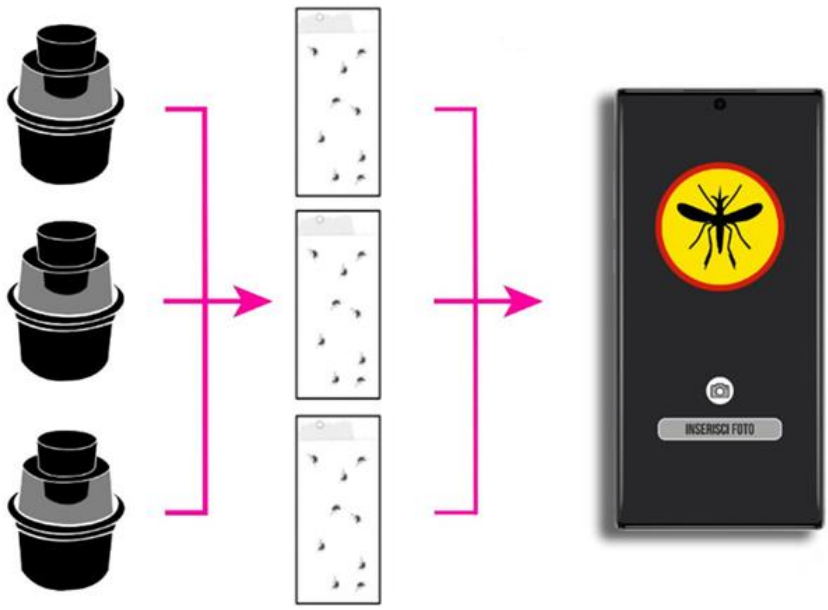


Fig n.4.9 – Schematic representation of mosquito signaling system
Mosquitos collected using sticky papers were photographed using the ZZZAPP. The images are directly transmitted to the server, which counts mosquitoes.

It has been possible to contact such a high number of volunteers in the monitoring project thanks to the collaboration with the "*Accademia di Belle Arti di Napoli*", which has created a project for innovative community engagement called "*Non io ma Noi*". Through this project, the scientific message has been spread using an innovative artistic approach.

The STOPTIGRE project and the *Non io ma noi* project converged into the project called "*Open Science*" which is part of the official program of the cultural activities of *Procida Italian Capital of Culture 2022* (Figure n. 4.10).

Fig n.4.10 – Community-engagement assessment

The first preliminary results show that citizens are actively involved, based on the number of photos uploaded through the ZZZAPP, e-mail and private chats (Figure n. 4.11).

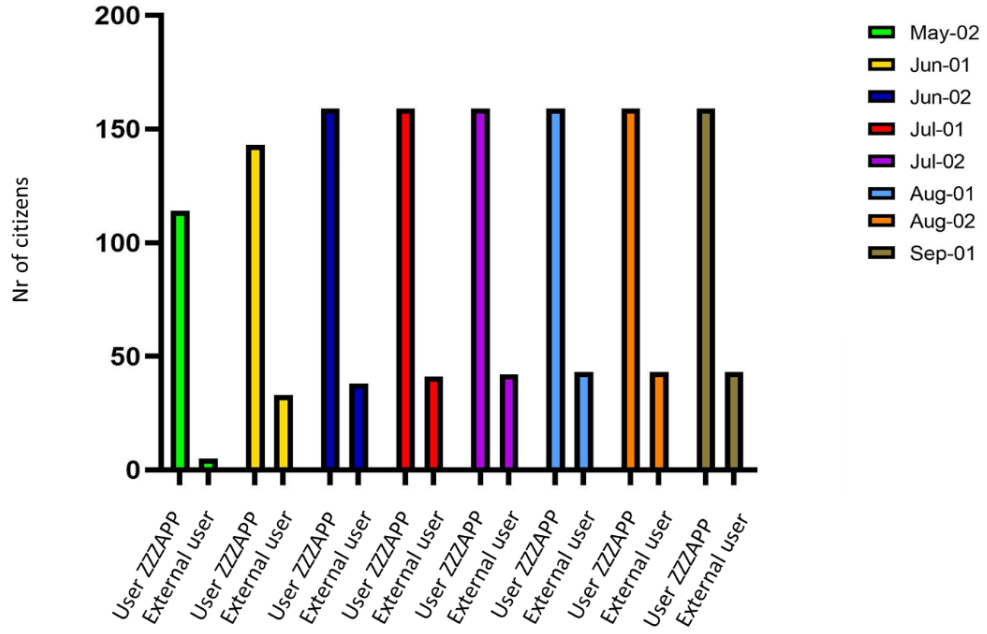


Fig n.4.11 – Number of citizens actively participating in the monitoring

It is estimated that during the first two weeks of the monitoring activities, the traps collected about 2950 mosquitoes, and two weeks later the number rapidly increased, to about 6230 (Figure n. 4.12).

The number of female mosquitoes captured from Gravid traps increased week after week, with about 17536 mosquitoes captured in July. The number of mosquitoes captured began to decline in the first two weeks of August, consistent with population dynamics data collected in previous years using the ovitraps system. Subsequently, mosquitoes collected in September increased (nearly 14829 mosquitoes), indicating a second peak in population density (Figure 4.7).

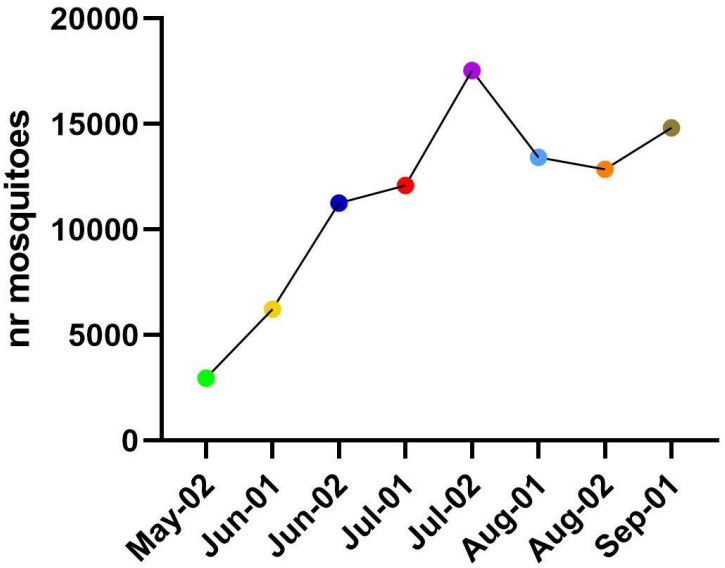


Fig n.4.12 – Mosquito population dynamics estimated by Gravid Traps captures

Whitin the next few months, it will be possible to estimate the total number of adult females caught during this reproductive season and determine also the distribution in different parts of the island.

In addition, in May 2022, residents were asked to fill out questionnaires to collect information about how the population perceived the tiger mosquito problem. Furthermore, through the questions, it was also possible to evaluate their level of knowledge regarding the potential spread of diseases that occurs through the insect. Our mission included educating them on the strategies that can be used to protect themselves, such as eliminating stagnant water. The results of the questionnaire were compared with those of a similar questionnaire distributed to a sample of the Procida population in September 2015 and July 2019, to evaluate the effectiveness of the environmental education programs conducted on the island in recent years.

The results of the questionnaires made it possible to identify a good increase of people who know about the sanitary issue related to *A. albopictus* with 84 % against 77 % in 2015. The key finding is that 54 % affirm to remove the standing water against 11 % in 2015 and 2019, indicating that scientific activity enhances community responsibility. Moreover, it is possible to estimate more significant interest in the mosquito control program and a higher enthusiasm to support or participate in monitoring and control actions on the island with 97 % against 45,5 % recorded in 2019 and only 29% in 2015.

Questionnaire question	Responses 2015 (%)		Responses 2019 (%)		Responses 2022 (%)	
	YES	NO	YES	NO	YES	NO
Do you know that the Asian tiger mosquito can transmit viral diseases to humans?	77	23	73	27	84	16
Do you use protective measures against mosquitoes?	85	15	91	9	95	5
Do you use electric diffusers?	58	42	37	63	28	72
Do you use mosquito nets?	54	46	71	29	77	23
Do you use insect repellents?	45	55	47	53	62	38
Do you use larvicides?	3	97	2	98	7	93
Do you remove standing water?	11	89	11	89	54	46
Would you welcome a regional/municipal mosquito control programme?	88	12	96	4	98	2
Would you agree to the installation in your property of traps for the capture and monitoring of mosquitoes?	44	56	78	22	100	0
Would you agree to contribute personally to the financing of a mosquito control project?	33	67	54	46	67	33
Are you interested in participating, as a volunteer, to a mosquito monitoring and control programme in Procida?	25	75	33	67	97	3

Fig n.4.11 – Results of public surveys
Questionnaire results conducted among Procida Island residents in 2015 (N = 200); 2019 (N = 191) and 2022 (N=303).

Ovitraps monitoring during the *Citizen-science* activities

The *citizen-science* activities have aimed at the control of the mosquito population dimension using active participation of citizens. During the monitoring activities (April-September 2022), 40 ovitraps were installed in two selected zones of Procida, called "Control zone" and "Test zone" (Figure n. 4.12). Specifically, the "Test zone" was the area where mosquito control activities were conducted strategically and the volunteer citizen involved in the *Citizen-science* activities were more numerous than in the control zone.

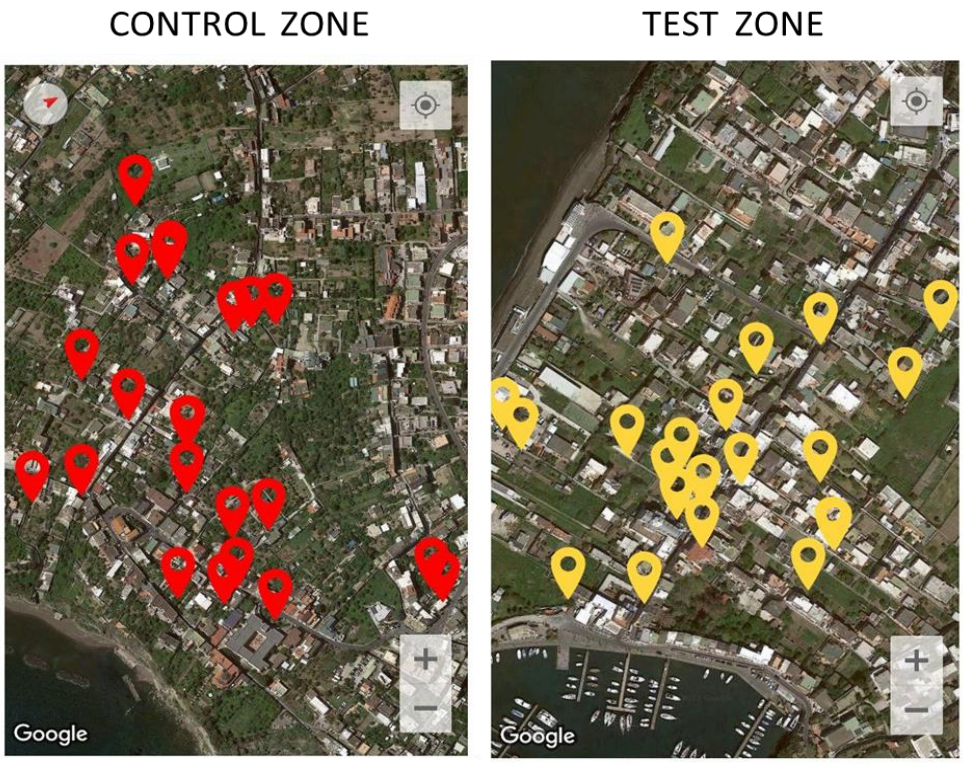


Fig n.4.12 – Ovitraps distribution on Procida
Two Procida's areas were selected: control and test zone. Starting from April 2022, 20 ovitraps were placed in each of the two zones, in order to collect A. albopictus eggs.

The ovitraps were monitored every two weeks to collect the eggs and analyze the evolution of the population dynamic during the control and suppressor activities.

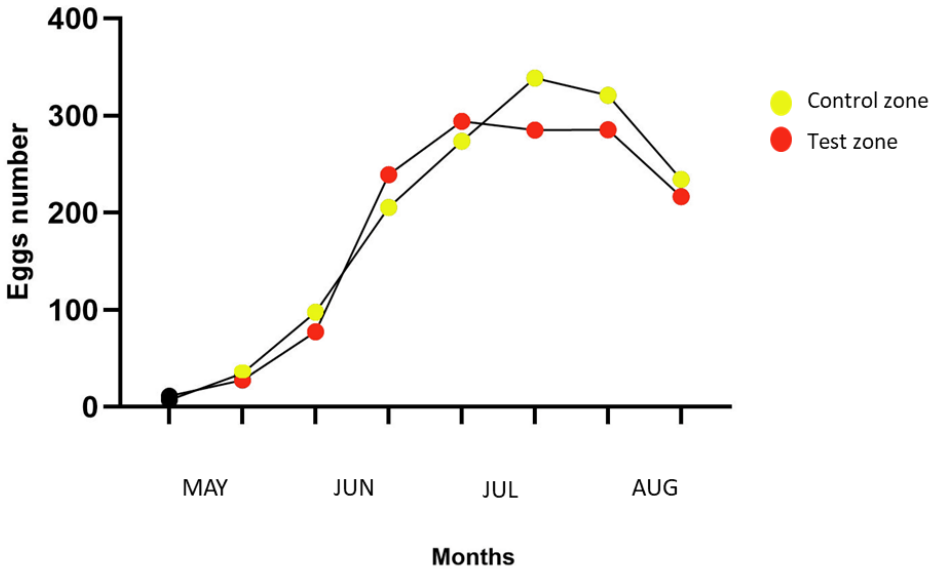


Fig n.4.13 – Eggs collected by Ovitraps
Estimates of population dynamics generated by collected eggs agree with estimates generated by Gravid traps

The number of eggs in the control and test zone follows the same trend: the eggs increased in June and the pick density is recorded in July, followed by a decrease.

It is possible to affirm that the number of eggs collected in the test zone is lower than the control, proving a diminution of mosquitoes in this area thanks to the success of the collaboration activities performed with the citizenship.

CHAPTER 5

Conclusions

My research aimed to study and characterize peculiar genes of the *A. albopictus* mosquito in order to find innovative biotechnological strategies for vector control. Here, I briefly summarize the results and suggest directions for future studies on this intriguing topic.

Mosquitoes are ideal subjects for exploring the biological basis of sexual dimorphism since they display innate sexually dimorphic behaviors. For example, only male mosquitoes initiate mating and only females consume blood, essential to the development of their eggs and transmit disease (Bowen, 1991; Galun et al., 1963; Jove´ et al., 2020; Klowden., 1995). In addition, males of *Aedes* and *Anopheles* have large bushy antennae whereas females of the same species have more slender antennae (Wheelwright et al., 2020). Apart from having an olfactory function, antennae also mediate sound detection in mosquitoes (Warren et al., 2010; Lapshin., 2012; Windmill et al., 2018; Su et al., 2018).

Despite the *A. albopictus* sex determination has been studied over the years, the knowledge of this pathway and the development of effective strategies for fighting the vector are in process. Consequentially, my research is composed and focused on the analysis of the genes involved in the sex determination pathway of *A. albopictus* in order to better understand the sex regulation in this insect.

In this work, in *A. albopictus* male antennae were found a high and significant expression of the *fruitless* gene assuming specific roles in male behavior regulation.

To confirm the male behavior function of the gene *fru* a functional analysis was performed by transiently silencing *fru* in early embryos of *A. albopictus*. The results from embryonic silencing confirmed that early *fru* activity is required for the differentiation of male-specific sexual behavior. Our results are consistent with the idea suggested by Basrur et al. (2020) that the neural circuits that promote female attraction to human scent or warm blood are latent in males and suppressed by the expression of *fru* either during development or during adulthood. *Fru* might function in the antenna to modulate the detection of sexual stimuli in male mosquitoes, perhaps by turning the functional or anatomical properties of olfactory sensory neurons.

These results reveal that *fru*, a gene that controls sex-specific mating behaviors in other insects, also controls sex-specific feeding behavior in *Aedes* mosquitoes.

Identifying the genes that regulate blood-feeding behavior in adult females could lead to new vector control targets (Tomchaney et al., 2014).

Furthermore, the study showed expression analysis of the *Sex-lethal* gene revealed a result never identified before in mosquitoes in which *Sxl* has a different expression in the sexes and specific tissues, such as the male abdomen and female head. By analyzing the data presented on these two genes, it was possible to explore mechanisms not yet understood in the *A. albopictus* mosquito. Future functional studies are essential to clarify the implication of the *Sxl* gene in regulating sexual development. The next step of the research is the down-regulation of *Sxl* to observe differences or anomalies in male development.

Understanding the molecular basis of sex determination can help to improve existing or develop novel genetic strategies. In this section, I present a new possible model to explain the sex determination pathway of *A. albopictus*.

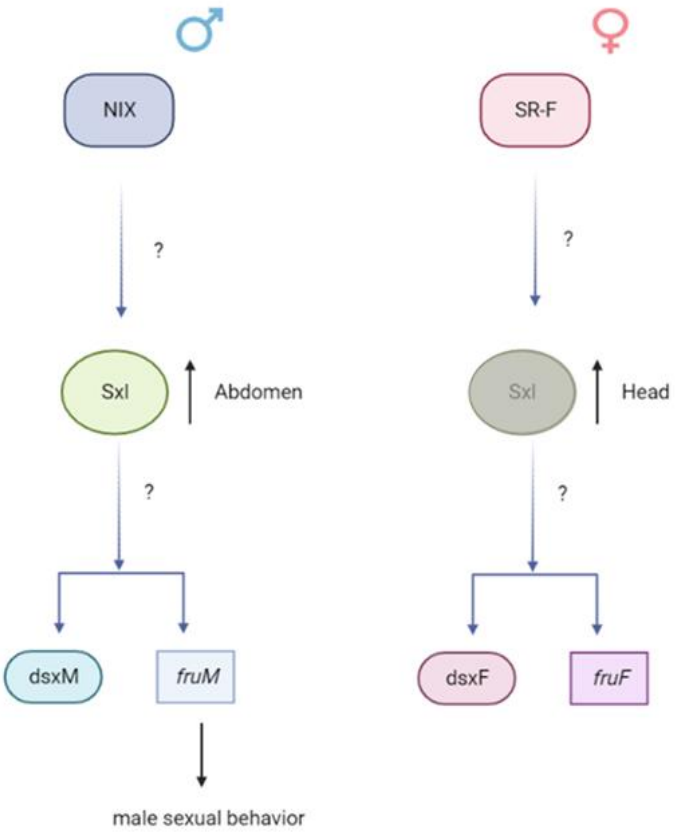


Fig n. 5.1 – New model for sex determination cascade in A. albopictus
The downstream genes of the pathway, fru and dsx are sex-specific spliced in A. albopictus, as it occurs in the other insects. Fru regulates sexual behavior in male mosquitoes. Sxl has a different expression in the sexes and specific tissues but it is unknown its role in the regulation of fru and/or dsx.

The *Sterile Insect Technique* (SIT) is most effective when the sterile release populations consist solely of males and novel genetic methods that use sex-linked markers may improve high throughput sex sorting accuracy and efficiency. Besides, the behavioral study of the periodicity of

courtship and mating is of fundamental importance for a full understanding of the reproductive dynamics of the species. In order to be able to apply the SIT optimally the key to reading is certainly a more in-depth study of the reproductive and behavioral genetics of the insect that helps to develop biotechnological systems for the containment of the invasive population.

Investigating genes not only involved in the sex determination cascade can be helpful for identifying genes with particular patterns of expression that provide opportunities to create innovative strategies for separating the sexes or improving the quality of life of sterilized males. Especially, my researches found that *A. albopictus* larvae run away from a source of light stimulus to a shadow area. To establish the gene responsible for this phenotype, the long-weight rhodopsin *GPROP3* is a potential candidate for its specific expression at the larval stage. The function of *GPROP3* was studied by transiently silencing the gene in early embryos of *A. albopictus* using double-stranded RNA. The results from embryonic silencing confirm that early activity of the rhodopsin *GPROP3* is required to generate a specific larval mechanism for the response to a light stimulus. Precisely, the *GPROP3* down-regulation causes a change in larvae response to light which are able to stay in a lighted zone.
As a result, the genetic manipulation of the gene *GPROP3* would allow the development of a larval separation system based on larvae's perception of light. Producing by genetics automated strategy large number of males who act to light differently from females may help to improve the critical separation of the sexes during the SIT technique. The purpose will be the generation of an *A. albopictus* strain in which the *GPROP3* gene could be down-regulated only in female insects, in order to obtain female larvae able to stay at the light and automatically collect a large number of male larvae who run away from the light stimulus.

Another important goal of a SIT program is the quality and longevity of sterile males. *Aedes* mosquito adults have different lifespans, with males lasting 7 days and females lasting ~3-4 months but it is unclear what is the gene responsible for this phenotype.
The study of the *alpha-mannosidase, a* gene that affects the maturation rate of proteins influencing longevity, reveals a male-specific expression throughout the *A. albopictus* and *A. aegypti* mosquitoes life, making it an intriguing gene to study. Additional studies are needed to determine whether the *alpha-mannosidase* gene influences longevity in the *Aedes* mosquito. The next step will be the down-regulation of this gene to evaluate the difference in longevity, consequentially creating long-lived males to use in the extremely efficient SIT program.

Procida island has unique and intriguing features facilitating the field testing of mosquito integrated vector management (IVM) approaches and control methods, including the SIT. The citizens of Procida contributed actively as volunteers in monitoring the seasonal and spatial distribution of *A. albopictus* since 2015.

Our results highlight an overall uniform and massive presence of the *A. albopictus* mosquito (Caputo et al., 2021). The ecological characteristics of the island, the high human and *A. albopictus* densities, the positive attitude of the resident population in being active parts in innovative mosquito control projects, and the acquired knowledge on *A. albopictus* spatial and temporal distribution provide the ground for evidence-based planning of the interventions and for the assessment of their effectiveness.

The control and monitoring program "*Stop Tigre*" started in April 2022 aims to analyze the population dynamic of mosquitoes on the Procida island using the C*itizen-science* approach.

500 Gravid Traps were distributed in all the island territory in order to collect female mosquitoes until the end of their reproductive season, in September 2022. The data sent to us monthly from the voluntaries made us confirm the good citizen participation and engagement in the monitoring project. At the end of September, we will be able to collect crucial information about the density and the distribution of the *A. albopictus* on the island, necessary to develop an optimal sterile male release strategy for the island.

These results indicate the efficiency of the innovative *Citizen-science* strategy proposed, involving synergy between arts and science, allow us to concretize the future male-sterile release in collaboration with the citizens.

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