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Aedes albopictus 34k2 SALIVARY PROTEIN AS EPIDEMIOLOGICAL TOOL FOR THE ASSESSMENT OF HUMAN EXPOSURE TO THE TIGER MOSQUITO

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1. INTRODUCTION

Vector-borne diseases, which pose a great threat to the health of societies throughout the world, are caused by viruses, bacteria and parasites transmitted to humans mainly by mosquitoes. The major mosquito-borne diseases account for about 17% of the estimated global burden of communicable diseases and cause more than 700,000 deaths annually. More than 50% of the world's population lives in areas where there is a risk of contracting at least one of the major mosquito-borne diseases. The risk of infection is particularly high in areas, where *Aedes* mosquitoes proliferate thanks to favorable habitat and where there is an important contact with humans. The dynamics and complex nature of mosquito-borne pathogens complicate predictions of the human health impacts of the re-emerging or new diseases. Despite this unpredictability, it is to be expected that new mosquito-borne diseases will appear, and certain existing pathologies will intensify, especially viral diseases transmitted by *Aedes* mosquitoes, closely linked to globalization and climate change. This complexity and unpredictability highlight the pressing need for sustainable epidemiological approaches to evaluate human vector contact and to reduce pathogen transmission in order to drop the burden of disease.

1.1. *Aedes* vectors

From the systematic point of view, mosquito vectors of arboviruses (arthropod-borne viruses) are holometabolous insects belonging to the Diptera Order, Culicidae Family, Culicinae Subfamily, *Aedes* Genus. Subgenus *Stegomyia* comprises most of the medically important *Aedes* species. Of particular importance has been the expansion of *Aedes aegypti* (Linnaeus, 1762) from Africa and *Aedes albopictus* (Skuse, 1894) from Asia, commonly known as tiger mosquito (Figure 1.1)

A B

Figure 1.1. *Aedes albopictus* (A) **and *Aedes aegypti*** (B). Mosquitoes factsheets ECDC.

The adult life span can range from 2 weeks to over a month depending on environmental conditions. Females after copulation have to take a blood meal to complete egg development. On the other hand, males are not blood-sucking and they feed exclusively on sugary juices of vegetable origin. Eggs: after taking a complete blood meal, females produce on an average 100–200 eggs per batch. However, the number of eggs produced is dependent on the size of the blood meal. Eggs are laid on damp surfaces in areas likely to temporarily flood, such as tree holes and man-made containers, and are laid singly, rather than in a mass. Egg to first instar larva transition takes usually a couple of days (Figure 1.2). Larvae: *Aedes* mosquito larvae are often called "wigglers", because they appear to wiggle sporadically in the water upon disturbance. *Aedes* larvae breathe oxygen through a posteriorly located siphon that is held above the water surface, whereas the rest of the body hangs vertically. Larvae are generally found around homes in puddles, pots, cement tanks, tree holes, tires, or within any receptacle retaining water. Duration of larval development is dependent on temperature. The larvae transition through four instars, spending a small duration in the first three instars, and up to 3 days in the fourth instar. Males generally pupate earlier because they develop faster than females. Pupae: after the fourth instar, *Aedes* larvae enter the pupal stage. Pupae, also called "tumblers," do not feed and take around 3–4 days to develop. Adults emerge by ingesting air to expand the abdomen, which helps splitting the pupal case, with the head emerging first. Adult: adult *Aedes* mosquitoes are distinguished from other types of mosquitoes by their narrow and typically black body, unique patterns of light and dark scales on the abdomen and thorax, and alternating light and dark bands on the legs. Females are further distinguished by the shape of the abdomen, which usually comes to a point at its tip, and by their maxillary palps (sensory structures associated with the mouthparts), which are shorter than the proboscis. On the contrary males are generally smaller than female mosquitoes and have more flagella or fine hairs on their antennae (it appears noticeably bushy to the naked eye). *Aedes* mosquitoes characteristically hold their bodies low and parallel to the ground with the proboscis angled downward when landed. *Aedes* mosquitoes feed during the day, with peak activity at dawn and dusk. They tend to feed aggressively on humans and on more than one person. Although many species appear to prefer to feed and breed outdoors, others live in close association with humans and lay their eggs indoors.

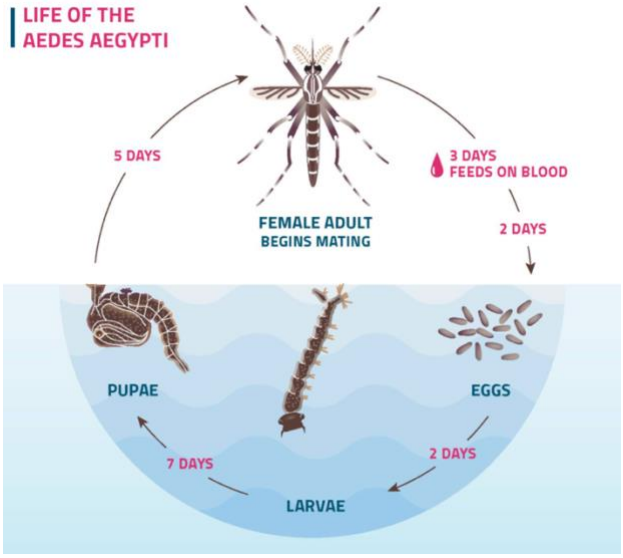


Figure 1.2. *Ae. aegypti* life cycle. CDC, Division of Vector- Borne Diseases, 2020.

Multiple *Aedes* species play a role in disease transmission to humans and, in many instances any of several different species can transmit the same pathogen (see 1.4.4. for additional details). *Ae. aegypti* and *Ae. albopictus* are the most important and widespread vectors of a wide variety of arboviruses belonging mainly to three main families: the *Togaviridae* comprising the genus *Alphavirus* (chikungunya virus), the *Flaviviridae* with the genus *Flavivirus* (Yellow fever virus, dengue 1–4 viruses, Zika fever) and the *Bunyaviridae* including the genera *Bunyavirus* and *Phlebovirus*.

1.2. *Aedes* mosquito-borne diseases

During the last decades, the burden of re-emerging infectious diseases has increased to represent a substantial threat to global health, security, and economy growth. About 75% of re-emerging infectious diseases are zoonotic diseases (Jones *et al.*, 2008). The global re-emergence of vector-borne diseases is helped by international travel, trade, and combination of environmental changes (Kilpatrick and Randolph, 2012). Zoonotic arboviruses transmitted by mosquitoes are the most important re- emerging pathogens because of their geographical spread and their increasing impact on vulnerable human populations. Those belonging to the genus *Aedes* are vectors of the major viral infections including yellow fever, dengue, chikungunya, and Zika fever (Gubler, 2002). Dengue incidence has increased by 30 times over the last 50 years, with about 390 million infections reported annually worldwide (Bhatt *et al.*, 2012). Dengue and chikungunya

outbreaks have resulted in several million cases in the Southwest Indian Ocean region, India, Europe and the Americas (Chikungunya fact sheets, WHO, 2017, Dengue and Severe dengue fact sheets, WHO, 2020). Zika virus (ZIKAV) disease emerged in 87 countries and its infection during pregnancy can cause microcephaly in newborns and is becoming a major threat due to its long-term sanitary and economic impacts, especially in Latin America (Global vector control response, WHO, 2017). Even the yellow fever virus (YFV), for which a safe and effective vaccine is available since decades, and whose transmission has been in decline for several years, is currently endemic in 47 countries in Africa and Central/South America, and a modelling study estimated a disease burden of at least 85,000 cases and 30,000 deaths in 2013 (Yellow fever fact sheets, WHO, 2018).

Besides being vector of these mayor epidemic arboviruses, *Ae. albopictus* has been experimentally shown to be a competent vector of at least 22 other arboviruses including, Rift Valley fever virus, Japanese encephalitis virus, West Nile virus and Sindbis virus (all of which are relevant to Europe), Potosi virus, Cache Valley virus, La Crosse virus, Eastern equine encephalitis virus, Mayaro virus, Ross River virus, Western equine encephalitis virus, Venezuelan equine encephalitis virus, Oropouche virus, Jamestown Canyon virus, San Angelo virus and Trivittatus viruses (Medlock *et al.*, 2015, Schaffner *et al.*, 2013). *Ae. aegypti* is suggested to be a potential vector of Venezuelan Equine Encephalitis virus (Larsen and Ashley 1971) and a competent vector of West Nile virus. Actually, West Nile virus has also been isolated from this mosquito species in the field (Turell *et al.*, 2005).

1.3. Strategies and targets to fight vector borne diseases

During the past three decades, the geographic range of *Aedes* mosquito vectors has dramatically increased and some arboviruses have become endemic in areas where they previously were not creating major public health problems in tropical, subtropical and temperate regions. The transmission of arboviruses in areas where once were absent depends on factors that influence on the epidemiology of vector borne diseases, which are the arbovirus species, the biological characteristics of the vector, the ecosystem to which it is linked and finally human beings (social organization and degree of immunity). However, with effective vaccination and suitable vector control programs, it is possible to control or even eliminate the human arboviral diseases.

The first vaccine for an arboviral disease, the 17D yellow fever vaccine, was developed in 1937 and has been used extensively and successfully in Africa and it was instrumental in eliminating the urban transmission cycle throughout the Americas. However, as the current yellow fever outbreak in Brazil rages, it provides a reminder that when robust vaccination programs are not sustained and herd immunity wanes below a critical point, Yellow fever virus re-emerges and causes severe human disease and mortality (Wilder-Smith and Monath, 2017). The first chikungunya vaccine development efforts date back to the 1970s, whereas development of Zika vaccines research began only about 2 years ago but has advanced at an unprecedented pace. However, vaccines for both viral diseases face uncertain futures owing to (i) misdiagnosed diseases due to common symptoms with other febrile infections, and (ii) difficulties in developing a robust phase III in a post-epidemic period when diseases only affect sporadically, as exemplified by CHIKV in Asia before 2007 (Weaver *et al.,* 2012). Also, with regard to ZIKV, insufficient knowledge of the mechanisms of ZIKV neurovirulence, amongst other unknowns in the biology of this infection, make the development of a safe vaccine more complex (Britto *et al.,* 2018). A dengue vaccine has recently been licensed but its use is recommended only for individuals with known prior DENV infection (Dengue Vaccine, CDC, 2019), and modelling studies predict achievement of cost-effectiveness only in high-transmission areas of dengue-endemic countries (España *et al.,* 2019). Overall, the best current prospects for controlling most vector-borne diseases rely on diagnosis and treatment of these diseases and reducing contact between the vector and humans. On the first case, one objective is to reduce the mortality and morbidity associated with these vector-transmitted diseases as well as to limit its transmission by reducing the reservoir of infection. To achieve this, efforts are being made to achieve or improve rapid and specific diagnosis, allowing for an early therapy of severe clinical cases. A parallel strategy is to improve the availability of specific drugs and vaccines, specially attention in the case of Zika with pregnant women and their partners, who represent the highest risk for severe disease. On the second case, vector control aims to limit the transmission of arboviruses by reducing or eliminating human contact with the vector. Vector control is a fundamental strategy in the fight against *Aedes* vector borne diseases and, in the past, has been instrumental in the eradication of the disease in determinate areas. A wide range of vector control tools exist, which can be broadly classified into chemical- and non–chemical-based tools. Tools targeting immature vectors can act by killing the immature

stages (Figure.1.3) (e.g., chemical or biological larvicides and predator species) or by removing suitable aquatic habitats (e.g., habitat modification or manipulation).

Figure 1.3. Active control on the larval stage of the vector.

Tools targeting the adult vector function by killing the vectors through indoor-outdoor residual spraying (Figure.1.4) and space spraying. In these cases, the use of drones represents a novel tool in vector control, especially in areas with difficult access (Figure 1.5). Another way to target adult vectors is by reducing vector contact (blood-feeding success) with human and/or animal reservoir hosts, and this may be achieved for example by the use of topical repellents, mosquito nets or insecticide-treated bed nets.
The success of these strategies requires the continuous monitoring of the evolution and spread of resistance mechanisms in target vector populations. In fact, since pyrethroid insecticides resistance appeared, its prevalence and incidence in vector populations increased, year after year, at an alarming rate (Pichler *et al.,* 2018).

Figure 1.4. The spread of residual-acting insecticides represents an example of an active fight against the adult form of the vector.

Figure 1.5. Drones used as a tool for the spread of residual-acting insecticides in the fight against mosquito.

Additional innovative vector control tools as mosquito genetic manipulation or infection by bacteria of the genus *Wolbachia* are presently under development and testing. Gene drive is a method of genetic modification that can be used to spread favorable traits through interbreeding populations of *Aedes* mosquitoes, specifically in *Ae. aegypti* more studies have been carried out (Williams *et al.*, 2020). The technique can be used for population replacement (i.e. the substitution of a mosquito population transmitting a pathogen with a refractory one) or population suppression (reducing the size of the vector population by, e.g., reducing fertility of females or biasing the sex ratio towards males). *Wolbachia* is a genus of bacteria that naturally infect some insects and is able to interfere with species reproduction. Normally, *Wolbachia* is absent in *Aedes* mosquitoes and its introduction into *Aedes* may help reducing mosquito populations and transmission of arboviruses to humans (Ryan *et al.*, 2020).

1.4. Epidemiology

Proper assessment of the arboviral transmission risk level in a given geographical area, requires not only information on the spread of the disease in the population but also on the level of transmission of the virus and on vector density. The different *Aedes* species present in the region, their relative spread and abundance, their feeding preferences, as well as a series of environmental factors that can influence the development and behavior of the vector, such as temperature, humidity, rains and the conformation of the territory must also be examined.

1.4.1. *Aedes aegypti* and *Aedes albopictus* worldwide distribution

Ae. aegypti originated in Africa where its ancestral form was a zoophilic tree hole mosquito named *Ae. aegypti formosus* (Brown *et al.*, 2014). The domestic form *Ae. aegypti* is genetically distinct with discrete geographic niches. *Aedes aegypti* was likely introduced into the New World from Africa with the slave trade, and from there it subsequently spread globally to tropical and sub-tropical regions of the world (Brown *et al.*, 2014). On the other hand, *Ae. albopictus*, originally a zoophilic forest species from Asia, initially spread to islands in the Indian and Pacific Oceans (Delatte *et al.*, 2010). During the 1980s it rapidly expanded its range to Europe, the United States and Brazil (Medlock *et al.*, 2012). Today *Ae. aegypti* and *Ae. albopictus* are present in large part of the world and even in most Asian cities and large parts of the Americas there is a co-presence of both species (Houé *et al.*, 2019) (Figure. 1.6).

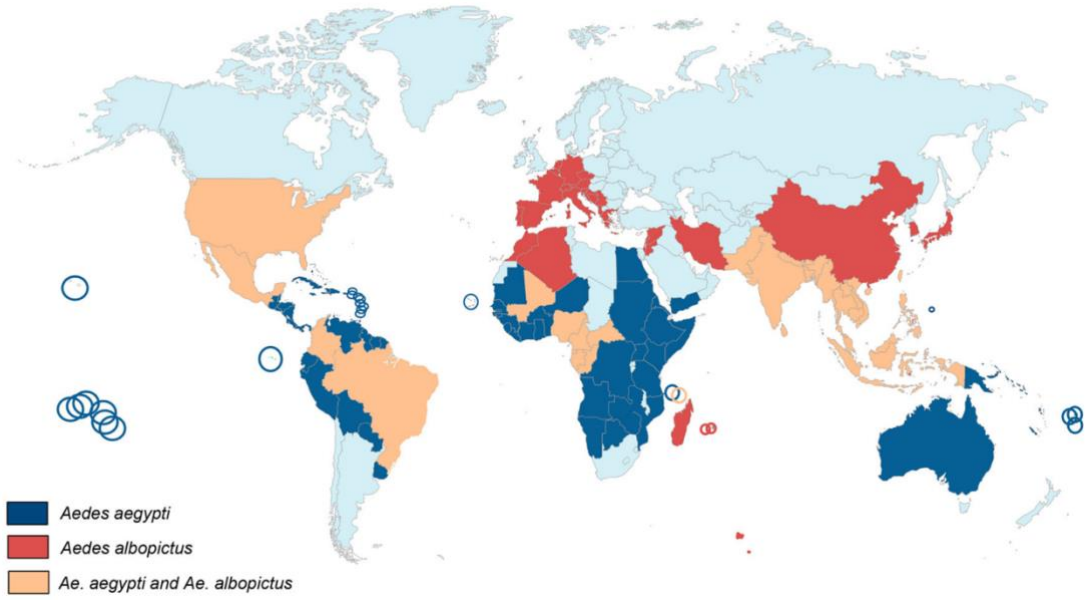


Figure 1.6. World distribution of *Aedes albopictus* and *Aedes aegypti.* (Houé *et al.*, 2019).

1.4.2. Factors of spread

Several different aspects of *Ae. aegypti* and *Ae. albopictus* ecology may have contributed to the present global or regional distributions of these vectors. The success of the invasion of *Ae. albopictus* is due to a number of factors including: its ecological plasticity, strong competitive aptitude, globalization, increase of trade and travel, lack of surveillance and lack of efficient control (Paupy *et al.,* 2009). The success of *Ae. aegypti* has largely been due to globalization. It thrives in densely populated areas which lack reliable water supplies, waste management and sanitation (Honorio *et al.,* 2009). It has been suggested that *Ae. aegypti* evolved its domestic behavior in West Africa. Historically, *Ae. aegypti* moved from continent to continent via ships and its widespread colonization and distribution in the tropics led to highly efficient inter-human transmission of arboviruses (Weaver and Reisen, 2010).

Furthermore, diverse environmental factors may play relevant roles in the spread of mosquitoes by affecting vector behavior, distribution and abundance. Climate changes (especially in temperature and humidity) may alter the dynamics of *Aedes* mosquitoes by modifying vectors behavior, biting rate, vectorial capacity, survival and distribution (including seasonal diapause that allows for the establishment of invasive species in temperate regions), abundance (hatching rates and adult mortality), and seasonal range. The ability to lay diapausing eggs was certainly crucial for *Ae. albopictus* expansion: eggs laid during late summer or early autumn, when daylight hours are reducing, enter facultative diapause. In this conditions hatching is suppressed which allows for overwintering in temperate regions, and this was extremely important for *Ae. albopictus* establishment in more northern latitudes in Asia, North America and Europe. Diapausing eggs of *Ae. albopictus* have been shown to be able to survive a cold spell of -10 ^{o}C (Medlock *et al.,* 2006). Also, *Ae. albopictus* adult populations in Italy are showing signs of cold acclimation, remaining active throughout winter (Romi *et al.,* 2006). *Ae. aegypti*, unlike *Ae. albopictus*, is not able to undergo winter diapause and this certainly limits its ability to exploit more northerly temperate regions. However, it may establish in regions European areas with humid subtropical climate, as in parts of Mediterranean and Black Sea countries (Kotsakiozi *et al.,* 2018). Precipitation is also likely to affect mosquito distribution and, usually, its abundance is usually positively associated to precipitation (Almeida *et al.*, 2007, Bhatt *et al.*, 2013).

Regarding climatic factors, establishing specific thresholds for different *Aedes* mosquito vector species is very useful in order to model and predict the present and future

seasonal and geographic distribution of those involved in human diseases. Climate change predictions suggest that *Ae. albopictus* may continue to be very successful invasive species, spreading beyond its current geographical boundaries (Gould and Higgs, 2009). On the other hand, even though establishment of *Ae. aegypti* in temperate regions is restricted by the high egg mortality due to the frost during winter season (Gould and Higgs, 2009), there is no reason why it should not become widely established again in the Mediterranean basin. This could change in the future with global climate change resulting in more northern and southern expansion.

1.4.3. Biting habits and host preferences

The tiger mosquito, *Ae. albopictus* is an opportunistic feeder (Turell *et al.*, 2005) and its hosts include humans, domestic and wild animals, reptiles, birds and amphibians. However, some preference for a human blood meal has also been reported (Eritja *et al.*, 2005). *Ae. albopictus* is currently considered a serious biting nuisance for humans. Adult females bite aggressively, usually during the day and preferably outdoors, although endophilic and indoor-biting behaviors have been described (Delatte *et al.*, 2010). In European countries *Ae. albopictus* blood-fed females were mainly found indoors, indicating that local mosquito populations could spend time resting indoors after a blood meal (Valerio *et al.*, 2010). *Ae. albopictus* females were also found indoors within containers as flower vases, empty paint cans and sinks (Dieng *et al.*, 2010). A laboratory study found that *Ae. albopictus* could survive for long periods indoors by obtaining sugars from lucky bamboo and other ornamental plants (Qualls *et al.*, 2013). *Aedes aegypti* prefers mammalian hosts and will preferentially feed on humans, even in the presence of alternative hosts (Scott and Takken, 2012). They may also feed multiple times during the same gonotrophic cycle (feeding, egg-producing cycle), which has implications for disease transmission. *Ae. aegypti* also prefers human habitations as they provide resting and host-seeking possibilities and, as a result, will readily enter buildings. Their activity is both diurnal and crepuscular (Turell *et al.*, 2005).

1.4.4. Other invasive *Aedes* species

The *Aedes* genus, which is divided into several subgenera, comprises over 900 species including about 23 known vectors of diseases. Even though *Ae. albopictus* and *Ae. aegypti* are by far the main vectors of human arboviral diseases, additional *Aedes* species play a critical role on the spill-over phenomena in arbovirus transmission. In Africa, *Aedes africanus* is considered the main sylvatic vector of yellow fever virus (Haddow *et al.*, 1948) and can also act as a bridge vector to humans, together with *Aedes furcifer*, and species of the *Aedes simpsoni* complex. Sylvatic dengue viruses in Africa are transmitted among non-human primates by *Ae. furcifer* and *Ae. luteocephalus*, and usually cross over to humans through biting by *Ae. fur*cifer (Hanley *et al.*, 2013). In French Polynesia, as well as in other Pacific islands and territories, the Polynesian tiger mosquito *Aedes polynesiensis*, is the main vector of lymphatic filariasis and a secondary vector of dengue. It has also been involved in Ross River virus transmission in Tahiti (Mitchell and Gubler, 1987, Aubry *et al.*, 2013,). Besides French Polynesia, it is found in abundance in Fiji, Wallis and Futuna, Tuvalu, Kiribati, Tokelau, Samoa, American Samoa, Cook Islands, and Pitcairn. In Asia, *Aedes koreic*us has been proven to be a vector for Japanese encephalitis virus in parts of Russia (Miles, 1964) and may be able to transmit *Brugia malayi* to humans (Korean Center for Disease Control, 2007). *Aedes triseriatus* is known to be the primary vector of La Crosse virus, which has caused serious disease in humans in North America (Borucki *et al.*, 2002) and has been suggested as a possible bridge vector for West Nile virus (Mosquito species producing WNV positives by year, CDC, 2009), with field-collected adult mosquitoes testing positive for the virus.

In Europe, West Nile fever is mainly transmitted by widespread mosquitoes of the *Culex* genus, although species from other genera such as *Coquillettidiae* and *Aedes (Ochlerotatus) caspius* could be also involved in transmission. In Japan and Korea, its normal native range, *Ae. japonicus* is not considered an important disease vector. There is a concern however that this species may become a pest problem or be involved in the transmission of North American arboviruses such as West Nile virus (Sardelis, 2001).

1.4.5. *Aedes* vector competence

Vector competence refers to the inherent capacity by a vector to become infected and ultimately transmit a given pathogen. For mosquito-borne viruses, this requires: (i) infection of the epithelial cells of the mosquito midgut following blood meal acquisition and digestion; (ii) efficient replication of the pathogen in the gut; (iii) traversing of the basal lamina of the midgut to enter the hemocoel; (iv) infection of/replication in the salivary glands, and sufficient accumulation of infectious particles in saliva for transmission to competent hosts (Monteiro *et al.*, 2019) (Figure. 1.7).

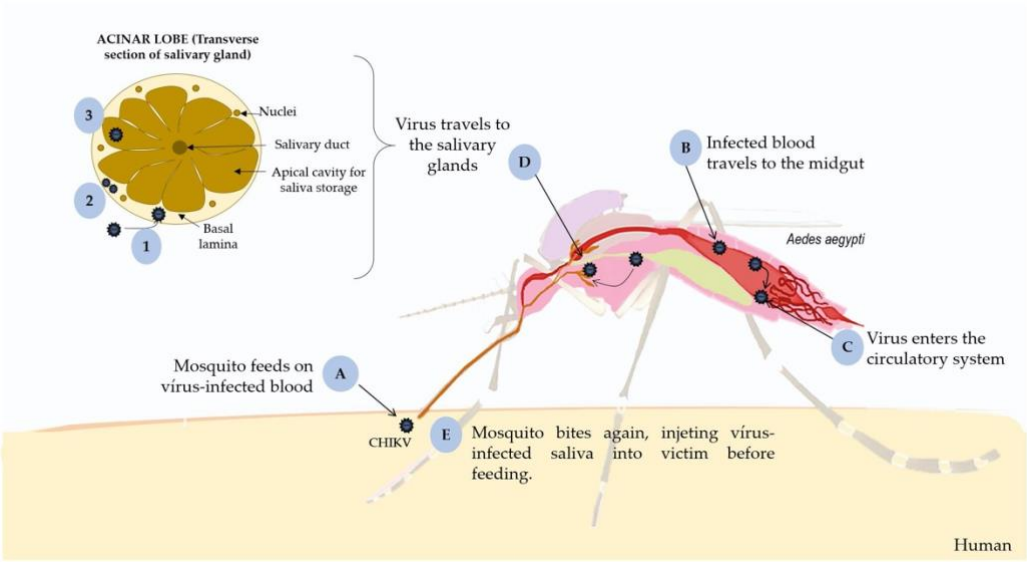


Figure 1.7. Replication of CHIKV in mosquito. (Monteiro *et al.*, 2019).

Vector competence, which is a component of the vectorial capacity, is determined by both genetic (depending on mosquito species/population, virus genotype/strain and their interactions) and non-genetic factors as environmental components. *Ae. albopictus* and *Ae. aegypti* are highly susceptible to different CHIKV strains. The virus escaped from a sylvatic cycle to cause urban outbreaks in South East Asia and Africa since the 1960s, with *Ae. aegypti* as the main vector (Powers and Logue, 2007). However, in 2005, *Ae. albopictus* was the primary vector of a large CHIKV outbreak on the Réunion Island, where *Ae. aegypti* was present as remote populations (Delatte *et al.*, 2008). On this island, CHIKV acquired a mutation in the glycoprotein E1 (E1-A226V) (Schuffenecker *et al.*, 2006) that increased its infectivity to *Ae. albopictus* but no to *Ae. aegypti*, causing numerous outbreaks in Europe.

Even though *Ae. albopictus* is considered a secondary vector for DENV, its susceptibility to DENV infection compared to *Ae. aegypti* remains questionable (Alto *et al.*, 2008). *Aedes albopictus* generally shows a higher midgut susceptibility to DENV infection but a lower rate of virus dissemination compared to *Ae. aegypti* (Lambrechts *et al.*, 2010). Also, experimental studies showed that European *Ae. albopictus* populations from Europe are susceptible to Zika virus infection, allowing viral replication and dissemination up to salivary glands. However, the short persistence of the virus in the mosquito saliva revealed a lower vector competence of *Ae. albopictus* as compared to *Aedes aegypti* (Di Luca *et al.*, 2016).

1.4.6. Entomological measurements

Evaluation of human-vector contact is essential to assess the risk of transmission of mosquito-borne diseases and to guide planning and implementation of vector control by public health authorities. For mosquitoes this evaluation is currently obtained by entomological methods as ovitraps, larval/pupal indices, adult traps or human landing catches (HLC) (Figure 1.8), which provide estimates of adult and/or immature mosquito densities in a given area (Guidelines for the Surveillance of Invasive Mosquitoes in Europe, ECDC, 2012). However, entomological measurements have some limitations and drawbacks. First, they only provide an indirect estimation of human exposure to vectors at community level and cannot be used to gauge the heterogeneity of individual exposure. They are not accurate to assess individual attractiveness to mosquitoes or other environmental and socioeconomic factors which could induce important variations in individual exposure to vector bites. Second, they can be expensive, labor-intensive and/or difficult to carry out in some epidemiological settings (e.g., logistic constraints or low vector densities) or may raise ethical issues (e.g., for HLC). In order to improve vector control and to predict the risk of arboviruses transmission, complementary methods and indicators are urgently needed to evaluate the real human exposure to *Aedes* bites.

Figure 1.8. Human landing catches (HLC). (Colucci and Müller, 2018).

1.4.7. Predicted models based on serological indices

Considering the limits given by entomological methods, recently there has been developed other additional methods for assessing the risk of exposure to *Aedes* mosquito bite. Numerous prediction models have been created from serological surveys data and climate models enriched with information regarding rainfall, temperature and relative humidity. Climate factors are certainly important factors not only for the survival and reproduction of the insect vector but also for the arboviral development within the vector. However, although predicted model based only on climate factors fit with empirical data, they are based on elements that may vary. Therefore, serological data is essential to assess proper predicted models due to its direct measurements of infection rate.

This system of prediction models, where serological data are applied to know the infection rate, have been studied in malaria. Over the years, serological studies for antibody responses to anti-plasmodium antigens have been used to predict transmission intensity (Dewasurenda *et al.*, 2017), to describe differences in *P. falciparum* and *P. vivax* endemicity in a low-transmission setting (Asthon *et al.*, 2015) and to follow its reduction after the application of control measures (McCord and Anttila-Hughes, 2017). Serological analyzes have also been used to identify hotspot boundaries (Stresman *et al.*, 2017).

Regarding to the development of arboviral prediction models, the studies are not so advanced. However, these types of studies have been published with two of the best-known re-emerging arboviruses with an important impact on public health, Dengue fever and Yellow fever. On the one hand, most dengue infections are subclinical and therefore

undetected, coupled with the trouble on serological diagnosis due to cross-reactivity in immunoassays with Zika virus (Langerak *et al.,* 2019) and Yellow fever (Souza *et al.*, 2019). This observational problem has wide ranging implications as it hampers our ability to estimate the underlying level of infection in the community, to characterize individual risk factors for infection and severity. Recently, through Bayesian models a framework that simultaneously characterizes antibody titers to each of DENV1, DENV2, DENV3, and DENV4 serotypes and identifies subclinical dengue infections has been developed from detailed cohort data (Salje *et al.,* 2018). The usefulness of these models in the active surveillance of arboviruses is very high since they allow to detect variability in the kinetics of antibodies between individuals and infections, and even more importantly, undetected infections (subclinical infections during active surveillance or unknown symptom status outside the surveillance windows) and the serotype of the infections.

On the other hand, since the discovery and administration of the yellow fever vaccine, the rate of this disease has dropped dramatically. However, after a period of low vaccination coverage, yellow fever has resurged (Yellow fever fact sheets, WHO, 2018). Since 2006 there has been substantial funding for large preventive mass vaccination campaigns in the most affected countries to curb the rising burden of disease and control future outbreaks. With estimation methods developed on the basis of serological data from the more recent episodes of yellow fever, spatial estimates of transmission intensity can be combined with vaccination coverage levels to evaluate the impact of past or proposed vaccination campaigns, thereby helping to allocate resources efficiently for yellow fever control (Graske *et al.,* 2014). This method has been used by the Global Alliance for Vaccines and Immunization (GAVI Alliance) to estimate the potential impact of future vaccination campaigns (GAVI Alliance, 2013).

Recently, a multinomial logistic model to analyze co-infection between arbovirus and malaria including serological data has been published (Loum *et al.,* 2019). Due to the co-circulation of malaria parasites and arbovirus (dengue, chikungunya, Zika, yellow fever and Rift Valley fever) in same regions, concurrent infections were observed and posed a challenge for medical diagnosis. Misdiagnosis of arbovirus co-infections as malaria infections may increase the spread of arbovirus diseases in areas where fast diagnostic assays are not available. This multinomial logistic model includes serological data about the presence or absence of malaria parasites in blood and the detection of specific anti-arbovirus IgM, proposing an appropriate statistical methodology that can assist in the elaboration of the differential diagnosis of febrile cases for arboviruses.

However, even if such tools have been developed and have gained importance in the last decade, most of them were not used in the public health context because of their complexity and the extensive need for input data. Make investment on prediction models, estimate vector control efficacy, and plan mitigation strategies, are important for public health policymakers, vaccine developers and vector control specialists.

1.5. Mosquito-host interaction

1.5.1. Salivary glands and saliva

Salivary glands of mosquito vectors represent an extremely important organ in arboviral transmission. Each mosquito has a pair of glands located in the ventral portion of the thorax. *Aedes* salivary glands are sexually dimorphic, which can be ascribed to the fact that mosquito females, in addition to feed sugary liquids of vegetable origin, also get blood meals. This fact is essential for reproduction as it is used to produce eggs. Female glands are about five times larger than the male ones and differ not only morphologically but also in the biochemical properties of the surface molecules as well as in salivary secretions (Ribeiro *et al.,* 2016). Each gland is made up of three lobes, two lateral and one median (Figure 1.9), with the lateral ones distinguishable in a proximal and a distal portion. Gene expression studies conducted on *Ae. aegypti* and *Anopheles gambiae* have shown that the genes involved in the intake of the blood meal are mainly expressed in the distal-lateral portion; instead the proximal-lateral portion expresses genes involved in the digestion of sugars or in other more general glandular functions (Arcà *et al.,* 1999). The median lobe is connected to the two lateral lobes by a short region, similar to a bottle neck, and its role in the transport of fluids has been hypothesized. Each of the three lobes is composed of a single layer of epithelial cells that line a central duct; the three ducts of each gland fuse together converging into a single salivary canal that opens at the level of the hypopharynx.

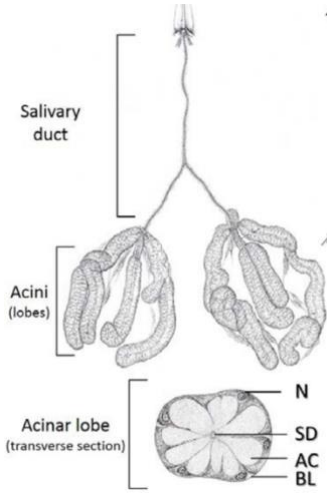


Figure 1.9. Structure of *Ae. albopictus* salivary glands. Schematic representation of salivary glands in *Aedinae* mosquito. N: nuclei. SD: salivary duct. AC: apical cavity for saliva storage. BL: basal lamina. (Vega- Rúa *et al.*, 2015).

During the blood meal, the mosquito injects saliva into its host. Salivary secretions facilitate the intake of the blood meal thanks to the presence of anti-haemostatic, anti-inflammatory and immunomodulatory factors. The study of the saliva of bloodsucking arthropods allowed to identify factors capable of counteracting all three components of the vertebrate haemostatic response: coagulation, platelet aggregation and vasoconstriction. Although only a limited number of the more than 15,000 species of bloodsucking arthropods have been studied, a profound diversity of their salivary protein repertories emerged. This confirms the idea that adaptation to hematophagy has originated independently several times in and within different insect orders, thus representing a good example of convergent evolution (Ribeiro, 1995; Ribeiro and Arcà, 2009).

Extremely different mechanisms are used by different bloodsucking arthropods to achieve vasodilation. For example, the Hemipteran *Cimex lectularius* and *Rhodnius prolixus* use nitrophorins: these are proteins capable of transporting and releasing nitric oxide (NO) at the injection site and subsequently binding histamine, an important modulator of the inflammatory response (Valenzuela and Ribeiro, 1998; Champagne *et al.*, 1995). Anophelines employ an enzyme with peroxidase activity capable of destroying biogenic amines such as serotonin and norepinephrine, thus inhibiting vasoconstriction (Ribeiro and Nussenzveig, 1993). Finally, *Aedes aegypti*, on the other hand, uses two

small peptides, sialokinins I and II, decapeptides capable of stimulating the release of NO by endothelial cells.

A similar wide variety was found for anticoagulants. The substances mainly synthesized by the Hemiptera *R. prolixus* and *Eutriatoma maculatus* target factor VIII and thrombin, respectively. The black fly *Simulum vittatum* (Diptera) synthesizes inhibitors of factor Xa, factor VII and an antithrombin. Since the trigger times for coagulation are higher than the average duration of a blood meal, it is assumed that these substances play an important role in preventing the formation of clots that could obstruct the mouth apparatus. Mosquitoes of the *Aedes* genus target Factor Xa of the clotting cascade while those belonging to the *Anopheles* genus the thrombin.

On the other hand, antiplatelet drugs show less heterogeneity in the target and most bloodsucking arthropods employ molecules with apyrase activity. This enzyme is responsible for the conversion of ATP and ADP into AMP and inorganic phosphate; this way the apyrase inhibits the ADP-dependent recruitment of platelets and their aggregation. Molecular analysis revealed the existence of 3 classes of apyrases with different evolutionary origins. In *Aedes* and *Anopheles* the apyrase activity is performed by a member of the 5'-nucleotidase family (Champagne *et al.,* 1995; Lombardo *et al.*, 2000). In Hemiptera (*C. lectularius* and *R. prolixus*) and in the san fly *Phlebotomus papatasi* this activity is carried out by a different family of proteins strictly dependent on calcium (Valenzuela *et al.,* 2001). The third class of apyrase belongs to the CD39 family and was originally isolated in the flea *Xenopsylla cheopis*. Apyrase CD39 family appears as the candidate for the salivary nucleotide hydrolyzing activity in *X. cheopis* (Andersen *et al.,* 2007).

1.5.2. Application of salivary gland studies

In the last years, the study of the salivary glands of vectors of diseases such as dengue, Zika, malaria or leishmaniasis has attracted considerable interest for molecular entomologists. This is due to the need for a more adequate understanding of their role in the transmission of the pathogen and of the biochemical-pharmacological properties of saliva. However, there are also important implications linked to these studies, such as the possibility of developing vaccine components (Manning *et al.,* 2018) and it use as biomarker to evaluate human exposure to vector of diseases (Schwartz *et al.,* 1990)

1.5.2.1. Saliva-based vaccines

Numerous indications suggest that the saliva of vector insects plays an important role in the transmission of parasites and arbovirus. By targeting the vector-pathogen-host interface, vector saliva-based vaccines might bypass in vivo pathogen-specific immunological phenomena because they act very early at the site of the vector bite in the skin, pre-empting or complementing host antiviral immune responses (Reed *et al.,* 2016). Preclinical models of *Leishmania* infection in rhesus macaques offer a proof-of-concept vaccine strategy using sand fly salivary components (Oliveira *et al.,* 2015). As a result, saliva-based prophylaxis against leishmaniasis protected macaques from subsequent infections and contributed to parasite killing in the dermis and primed specific immunity to leishmaniasis. In addition, it has been demonstrated that in mouse models of malaria repeated host exposure to vector saliva shifts the immune response from a Th2 to a Th1 response, correlated with reduced development of disease (Donovan *et al.,* 2007). This suggests that if a vertebrate host is immunized with synthetic immunodominant peptides derived from mosquito saliva, subsequent exposure to the same mosquito saliva may allow immunomodulation of the host response.

One example on arboviral disease is represented in a murine model of Zika virus. Hastings and colleagues found that NeSt1 protein activates neutrophils *ex vivo*, and the blocking NeSt1 through passive mice immunization by *Ae. aegypti* saliva showed significantly lower viral titers, indicating that NeSt1 contributes to early viral replication during ZIKV infection by mosquito bite. (Hastings *et al.,* 2019). So, these results opening up the possibility of using a vaccination strategy against this protein for protection from ZIKV either alone or in conjunction with a traditional vaccine like those that are in various stages of development. Novel and creative approaches are needed as vector-borne pathogens continue to emerge. Acceleration of vector saliva-based vaccine candidates into clinical trials is one of the next critical steps in expanding to fight against new epidemics.

1.5.2.2. Saliva as a biomarker of vector bite exposure

A further interesting approach is based on the idea that exposure could be directly assessed by measuring human-vector contact as reflected by the human antibody response to mosquito salivary proteins. The female mosquito saliva contains biologically active molecules to favor feeding and some of these are highly immunogenic. To validate the proof of concept 'anti-saliva response = biomarker of vector bite exposure', it was necessary to find new methods to reach this goal. This approach represents a great benefit for testing the validity of the *Aedes* salivary biomarker since it allows the taking into consideration of specific conditions related to the epidemiology of *Aedes* mosquito-borne diseases. In this regard, human antibody responses to the saliva of a number of vectors, including *Triatoma* (Chagas disease) (Nascimento *et al.,* 2001), *Phlebotomus* (Leishmaniasis) (Rohousova *et al.,* 2005) and *Ixodes* tick (Borrelia burgdorferi) (Schwartz *et al.,* 1990), have already been properly identified as promising biomarkers for vector exposure. Further, human antibody responses to the saliva of *Glossina* (the vector of Human African Trypanosomiasis) have been shown to have high diagnostic value (Poinsignon *et al.,* 2008b). For mosquitoes, human antibody responses to whole saliva have been correlated to human exposure to *Anopheles gambiae* (Remoue *et al.,* 2006), *Anopheles dirus* (Waitayakul *et al.,* 2006) and *Anopheles darlingi* (Andrade *et al.,* 2009), vectors of *Plasmodium* parasite. Besides, it has been shown that the IgG responses to whole *An. gambiae* saliva could be a useful biomarker for evaluating the efficacy of malaria vector control (Drame *et al.,* 2010).

Several studies were undertaken to evaluate the relevance of using saliva as biomarker to determine the risk of exposure to *Aedes* bites, thus to the viruses that they transmit. Studies on human antibody responses to *Aedes* saliva demonstrated that IgM and IgG responses to *Ae. aegypti* saliva could be used to estimate exposure in transiently exposed populations (Orlandi- Padrines *et al.,* 2007). Furthermore, IgE and IgG responses to *Ae. aegypti* salivary gland extracts (SGEs) showed variations during the rainy season of high exposure to *Ae. aegypti*, compared to dry season in Senegal (Remoue *et al.,* 2007). Other study, made in south-eastern France, showed a positive association between the average levels of IgG responses against *Ae. caspius* saliva and spatial *Ae. caspius* densities (Fonatine *et al.,* 2011). Concerning *Ae. albopictus*, a rapid decrease of anti-*Ae. albopictus* SGE IgG levels was also reported after the implementation of control measures in an urban city in Reunion Island (Doucoure *et al.,* 2014). The relationship between anti-SGE antibody responses and arbovirus infections was also investigated by Doucoure and

collaborators. In this study, performed in Bolivia, a positive correlation was observed between detected IgG levels and the risk of being infected by Dengue virus, due to the fact that dengue outbreaks are regularly reported in this area (Doucoure *et al.*, 2012b).These results demonstrated that human antibody responses to *Aedes* SGEs can be a valuable biomarker for evaluating the level of human exposure to *Aedes* bites, the risk of arbovirus infection or transmission, and the effectiveness of vector control strategies.

The use of saliva as a marker of exposure is extremely useful but is limited by some important factors. First of all, the collection of saliva or salivary gland extracts is tedious and time consuming and saliva batches are not reproducible in terms of protein quantities and antigenicity. Accordingly, it will be difficult or even impossible to have an adequate production of mosquito saliva needed for large scale epidemiological studies. The standardization of immunological assay using whole saliva appeared difficult and time consuming. Therefore, *Aedes* mosquito saliva, like that of other mosquito species and other bloodsucking arthropods, is a complex mixture consisting of different proteins with different degrees of immunogenicity. It is likely that some cross-reactivity with salivary proteins of other mosquitoes (or other bloodsucking arthropods) exist. This potential cross-reactivity limit significantly the significance of a given measurement, complicating its interpretation. This cross-reactivity has been demonstrated by Doucoure and colleagues in La Reunion island. In an area of chikungunya transmission, it was shown that the level of IgG against *Ae. albopictus* SGE can be used to identify individuals who have been exposed to the bites of this important vector. Cross-reactivity was observed with *Ae. aegypti* SGE suggesting that an *Aedes* species-specific salivary antigen needs to be identified (Doucoure *et al.*, 2012a). Furthermore, the evaluation of vector control effectiveness or the risk of arbovirus infection based on the immunogenicity of SGE could be under or overestimated due to possible cross-reactivity between common epitopes.

From this point of view, a big leap in quality is represented by the identification of individual immunogenic *Aedes* salivary proteins to be used, in place of saliva in its entirety, for the measurement of IgG levels. In this regard, the analysis of the salivary transcriptome of *Ae. albopictus* and other mosquitoes (Arcà *et al.*, 1999, Valenzuela *et al.*, 2003, Ribeiro *et al.*, 2007; Arcà *et al.*, 2007, Arcà and Ribeiro 2018) allowed to identify a series of salivary proteins *Aedes* genus-specific, that are absent in the other genera and other bloodsucking insects (Table 1.1 Arcà *et al.*, 2007 and Ribeiro *et al.*, 2007).

Table 1.1. List of putative secreted proteins identified in the salivary glands of *Ae. albopictus.*

Protein or protein family[1]	Comment	Expression pattern[1,2]	Other aedini[3]	Culicine[3]
D7 (6)	D7 family of salivary proteins	sg (4), sg/m (2)	+	+
Serpins (3)	Serpins	sg (2), enr (1)	+	+
Mucins (4)	Salivary mucins	sg (3), sg/m (1)	+	+
Enzymes (5)	Salivary enzymes	sg (1), enr (1), sg/m (3)	+	+
Immunity related peptides (3)	Immunity related peptides	sg/m (3)	+	+
Angiopoietin/ fibrinogen-related proteins (2)	Angiopoietin/ fibrinogen-related proteins	sg (2)	+	+
Lectins (3)	lectins	sg (2), enr (1)	+	+
Antigen 5 family (4)	Antigen 5 family	sg (2), enr (1), sg/m (1)	+	+
TIL domain-	TIL domain-containing peptides	sg/m (1)	+	+
30 kDa (3)	30 kDa allergen	sg (3)	+	+
56 kDa	56.5 kDa protein	sg/m	+	+
62 kDa (2)	62 kDa family	sg (2)	+	+
41.9 kDa	41 kDa protein	sg/m	+	+
34 kDa (2)	34 kDa family	sg (1), enr (1)	+	+
27 kDa	28 kDa protein	sg/m	+	+
23.4 kDa	23.4 kDa protein	enr	+	+
WPCWW 8.9 kDa	W rich 8.9 kDa peptide	sg/m	+	+
7.6 kDa family (2)	7.6 kDa family	sg (2)	+	+
Basic 3.8 kDa	3.8 kDa peptide	enr	+	+
13 kDa (3)	13 kDa family	enr (3)	+	+

1. The number in parentheses refers to the number of genes present in the same family.

2. The expression pattern is determined by RT-PCR (Arcà *et al.*, 2007): sg, present exclusively in the salivary glands of adult females; enr, enriched in the salivary glands of adult females; sg / m, salivary glands of adult females and adult males.

3.+ or -, presence or absence of homologous sequences in the salivary transcriptomes of other mosquito species determined by blastp analysis against the NCBI database.

Within the family *Culicidae,* groups of anopheline- and culicine-specific salivary proteins have already been identified and validated as good biomarkers of exposure and a clear proof of concept has been provided for the gSG6 salivary protein from *Anopheles gambiae*. In fact, the gSG6 protein has already been validated as marker of human exposure to bites of the main African malaria vectors (*An. gambiae, Anopheles arabiensis* and *Anopheles funestus*) and it can determine spatial and seasonal variation of mosquito density (Rizzo *et al.,* 2011a, Rizzo *et al.,* 2011b, Rizzo *et al.,* 2014a, Rizzo *et al.,* 2014b). In addition, it was demonstrated that gSG6 antigen may be used as a marker of exposure to bites of Asian malaria vectors in the Pacific (Idris *et al.,* 2017).

The recombinant protein strategy based on the identification of genus-specific salivary proteins have been used also for the identification of specific biomarkers of *Aedes* exposure. However, the production of recombinant proteins is not an easy task and it is also challenging to produce protein with a high degree of purity and with total reproducibility between production batches to ensure a correct assessment of the anti-saliva immune response. In addition, recombinant proteins may carry more than one epitope which could increase the risk of immune cross-reactivity, impairing the specificity of candidate biomarkers. To address these limitations linked to the laborious procedures of expression, purification and renaturation of recombinant proteins and to reduce the risk of immune cross-reactivity, a design specific peptide approach based on bioinformatics analysis of the sialotranscriptomic data was developed. The peptide design approach was used for the identification of the well validated gSG6-P1 salivary peptide from the *An. gambiae* gSG6 salivary protein as a biomarker of human exposure to *Anopheles* bites (Poinsignon *et al.,* 2008a, Poinsignon *et al.,* 2009, Drame *et al.,* 2015, Ya-Umphan *et al.,* 2017). Using a similar approach for the gSG6-P1 peptide, the N-terminal extremity peptide (Nterm-34kDa peptide) of the 34kDa salivary protein was validated as specific biomarker to *Ae. aegypti* bites to detect the heterogeneity, and evolution in human exposure to *Ae. aegypti* bites, and thus to evaluate the risk of arbovirus transmission (Elanga Ndille *et al.,* 2012, Elanga Ndille *et al.,* 2014). Moreover, it has been demonstrated that Nterm-34kDa peptide can detect the short-time variations of human exposure to *Aedes* mosquito bite after vector control implementation (Elanga Ndille *et al.,* 2016).

1.5.3. *Aedes* 34kDa salivary protein family

Proteins of the 34kDa family are only found in culicine mosquitoes where they are specially expressed or enriched in adult female salivary glands. The 34kDa family of salivary proteins was originally identified in *Ae. aegypti* and found to be composed three members (Ribeiro *et al.,* 2007), two of which were named 34k1 and 34k2kDa salivary proteins. Orthologs of these two *Ae. aegypti* proteins, with a similar expression profile, were later found in *Ae. albopictus*. The 34k1 and 34k2 *Ae. albopictus* putative proteins share 33% amino acid identity (32% in *Ae. aegypti*). 34k1 orthologues from *Ae. albopictus* (al34k1) and *Ae. aegypti* (ae34k1) share 65% amino acid residues while 34k2 orthologues (al34k2 and ae34k2) show a 62% amino acid sequence identity (Table 1.2). Members of the 34kDa family appear to be present also in *Culex* species (Ribeiro *et al.*, 2018), however they are only distantly related to the *Aedes* proteins (23% to 28% identity).

Table 1.2. Percentage of similarity in the amino acid sequence of the 34k2 salivary proteins from *Ae. albopictus* and *Ae. aegypti.*

	al34k1	**al34k2**	**ae34k1**	**ae34k2**
al34k1		33% (57%)	65% (80%)	33% (58%)
al34k2	33% (57%)		36% (60%)	62% (80%)
ae34k1	65% (80%)	36% (60%)		32% (60%)
ae34k2	33% (58%)	62% (80%)	32% (60%)	

In parentheses percentage of similarity of the nucleotide sequence between the homologues and orthologues of the 34kDa salivary proteins family.

The physiological function of 34kDa proteins in mosquito saliva is hitherto unknown. However, the *Ae. aegypti* 34k1 protein strongly enhanced DENV replication in human keratinocytes, likely as a consequence of its strong suppressive effects on the IRF signaling pathway, resulting in the abrogation of type I IFN production. These results suggest that 34k1 salivary protein from *Ae. aegypti* could play a major role in DENV infection in human keratinocytes by suppressing antiviral immune response in the earliest stages of infection (Surasombatpattana *et al.*, 2014). In agreement with these observations it was later found that 34k1 gene silencing in the mosquito by RNAi reduced DENV2 replication in the salivary glands (Sri-In *et al.*, 2019).

2. AIM OF THESIS AND EXPERIMENTAL PLAN

As previously mentioned, during the blood feeding the mosquito injects into the host a cocktail of salivary proteins whose diverse biochemical and pharmacological activities help the mosquito to effectively blood feed. At the same time, some of these salivary proteins induce in the host a specific immune response. This response to mosquito saliva can be exploited to assess host exposure to mosquito vectors of arboviruses such as dengue, chikungunya, Zika or Yellow fever and, eventually, to evaluate the risk of transmission of these diseases. However, saliva is a complex mixture and the use of IgG responses to whole saliva as a serological marker can lead to bias in the estimates due to potential cross-reactions with antigens of other bloodsucking arthropods. An interesting aspect, which is emerging from the comparative analysis of the salivary transcriptomes of mosquitoes belonging to the genera *Aedes, Anopheles, Culex* and other bloodsucking insects, is the existence of *Aedes*-specific salivary proteins. These proteins, if immunogenic, could represent valid serological markers of exposure to aedine bites and therefore be extremely useful in the immuno-epidemiological analysis of arboviruses.

The tiger mosquito *Ae. albopictus* has been responsible of quite a few cases of arboviral transmission in Europe and of relatively large chikungunya outbreaks in Italy, pointing out the need of an improved surveillance and control of this mosquito. My PhD project is part of a study aimed at identifying *Ae. albopictus* salivary proteins to be used as epidemiological markers of human exposure to bites of the tiger mosquito. In particular, I mainly focused my attention on host IgG responses to the 34k2 salivary protein from *Ae. albopictus* (al34k2) a small culicine-specific protein. I also tested the immunogenicity of the *Ae. aegypti* orthologue ae34k2 and evaluated the potential cross-reactivity between host antibody responses to these two orthologous proteins. The importance of these comparisons lies in the fact that these two species are close relative and they represent the main vectors of re-emergent arboviruses. Consequently, it is of fundamental importance to understand the cross-reactivity of the antibody responses to the al34k2 and ae34k2 in order to be able to develop effective protocols for exposure to bites of these *Aedes* mosquito species.

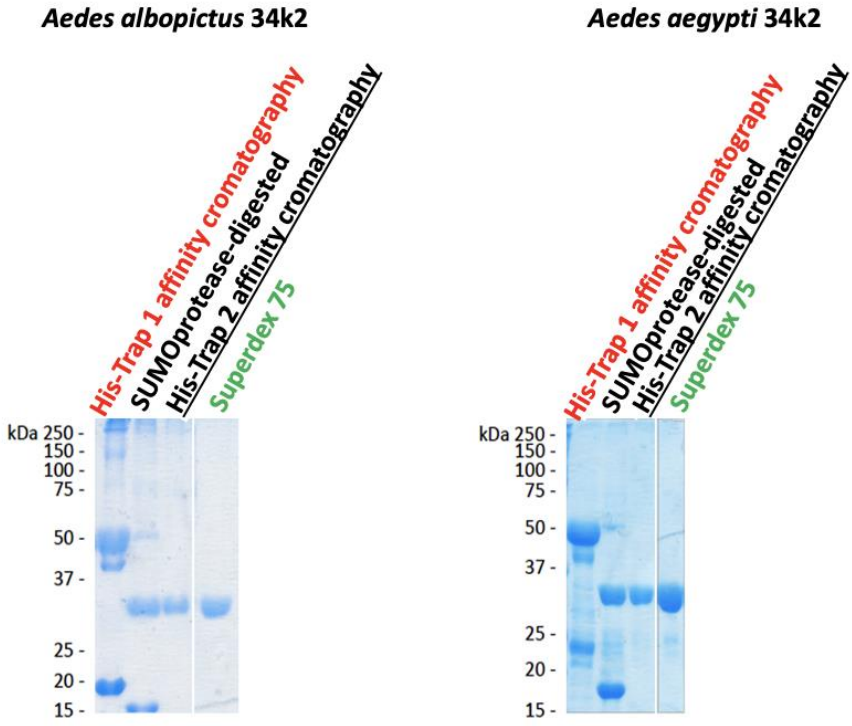
In order to obtain information on the suitability of these biomarkers I first used a murine model and then validated in human the promising indications obtained in mice. One of the advantages of using a murine model is that it allows for a controlled regimen

of exposure to mosquito bites, which is obviously not possible with humans. The exposure of mice to bites of different mosquito species was expected to provide precious information not only on antigen immunogenicity, but also on species-specificity and on the kinetics of antibody responses. I also used a single human hyperimmune serum both as a control and to get some preliminary but valuable indication on the antigenicity to humans of the recombinant 34k2 salivary protein from *Ae. albopictus*. I then moved to validation in human and measured the anti al34k2 humoral response in relatively large groups of healthy individuals naturally subjected to *Ae. albopictus* bites from two locations with different mosquito densities in Northeast Italy (Padova and Belluno) and in two different seasons (high and low tiger mosquito density). Finally, to validate the species-specificity and the suitability of the 34k2 antigens in epidemiological settings where arboviral transmission is endemic, I measured the IgG responses to the al34k2 and the ae34k2 salivary antigens in cohorts of individuals from the Réunion Island (only exposed to *Ae. albopictus*) and from Bolivia (only ever been exposed to *Ae. aegypti*).

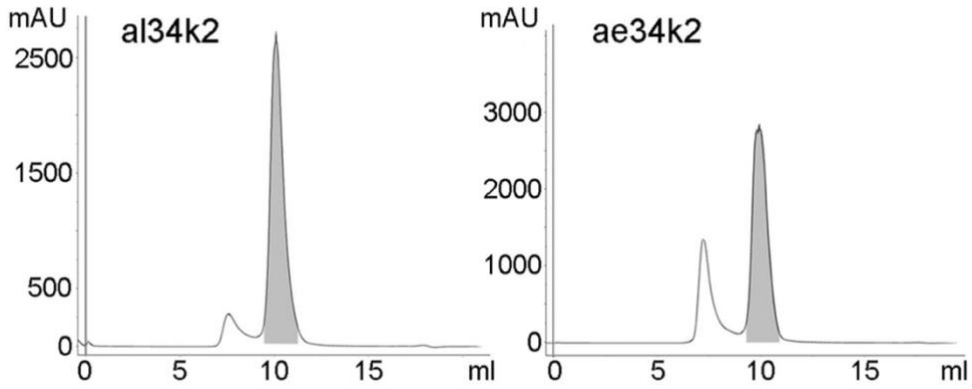
3. MATERIALS AND METHODS

3.1. Expression and purification of the 34k2 recombinant proteins

The *Ae. albopictus* (mRNA AY826118, protein AAV90690) and *Ae. aegypti* (mRNA AF466595, protein AAL76018) 34k2 salivary proteins were expressed in recombinant form and purified by our collaborators Dr. Paolo Gabrieli and Prof. Federico Forneris at University of Pavia (Dept. of Biology and Biotechnology). We will refer to these two salivary proteins as al34k2 (*Ae. albopictus*) and ae34k2 (*Ae. aegypti*). The procedure for expression and purification of these proteins has already been reported in detail in Buezo Montero *et al.*, 2019 and will be only shortly described here. The regions encoding the mature 34k2 proteins was obtained either by Reverse Transcription-Polymerase Chain Reaction (RT-PCR), in the case of al34k2, or directly by gene synthesis for ae34k2. Coding regions were directionally subcloned into a vector suitable for the expression of recombinant proteins fused at their N-terminus to an 8xHis-tag and SUMO protein in order to help purification and increase solubility, respectively (Kuo *et al.*, 2014). Recombinant proteins were expressed in *E. coli* and purified by affinity chromatography on a HisTrap excel column (GE Healthcare) using an Äkta system (GE Healthcare). After elution, the His-SUMO tag was removed by a His-SUMO protease and the 34k2 proteins purified through a second passage on the HisTrap column followed by gel filtration onto a Superdex 75 10/300 GL column. Protein concentration was evaluated determining the absorbance at 280 nm and assuming, according to the Expasy ProtParam tool (Gasteiger *et al.*, 2005), extinction coefficients of 0.82 and 0.84 for the *Ae. albopictus* and the *Ae. aegypti* protein, respectively. Figure 3.1 shows the purity of the two proteins after the different steps of purification.



(A)



(B)

Figure 3.1. Purification of the *Ae. albopictus* and *Ae. aegypti* 34k2 recombinant proteins. (A) SDS-PAGE analysis of protein fractions obtained during the different steps of purification of the recombinant *Ae. albopictus* al34k2 (left) and *Ae. aegypti* ae34k2 (right) proteins. HT1, after the first His-Trap affinity cromatography; SUMO, after SUMO protease digestion; HT2, after the second His-Trap affinity cromatography; S75, after Superdex 75. Gels stained with Coomassie Brilliant Blue R-250. (B) Size exclusion chromatograms (Superdex-75 10/ 300 GL, GE Healthcare) showing the peaks (shaded) corresponding to the pure *Ae. albopictus* and *Ae. aegypti* 34k2 salivary proteins.

3.2. Peptide design

Peptides were designed on the *Ae. albopictus* salivary proteins previously identified as restricted to culicine mosquitoes (Ribeiro *et al.*, 2010, Arcà *et al.*, 2007, Ribeiro *et al.*, 2007, Ribeiro *et al.*, 2010) and exhibiting limited amino acid identity (<50%) to *Culex* species. Potentially immunogenic peptides were selected using four different bioinformatic tools for the prediction of B-cell epitopes and immunogenic regions: BepiPred (Jespersen *et al.*, 2007), ABCpred (Saha and Raghasa, 2006), Bcepred (Saha and Raghasa, 2004) and Epitopia (Rubinsteins *et al.*, 2009a, Rubinstein *et al.*, 2009b). Five peptides 21–23 amino acids in length were designed on three *Ae. albopictus* salivary proteins and chemically synthesized by Biomatik Corporation (Canada): alb34k1-P1 (HPLPEEATSDAAIKCTLSEED), representing the N-terminus of the 34k1 protein (AAV90689); alb34k2-P2 (TVSEEDLTTIRNAIQKASRASLD) and alb34k2-P3 (ALKFYPKTGNKEANEADIRGRQF), designed in the N- and C-terminal regions of the 34k2 salivary protein (AAV90690); alb62k1-P4 (LTHIEKPIYTEEAESETSDSDE) and alb62k1-P5 (YGLSGMRSGGIPDNHAEWKLNA) designed in the N- and C-terminal regions of the 62k1 protein (AAV90683).

3.3. Mosquito colonies and salivary gland extracts preparation

Aedes albopictus (originally collected in Rome, Italy), and *An. coluzzii* (originally collected in Cameroon) were reared in the insectary of Sapienza University of Rome and Istituto Superiore di Sanità under standard conditions (27±1˚C, 70 ±10% relative humidity, 14:10 hours light: dark photoperiod) and colony maintenance achieved by feeding on guinea pigs. *Aedes aegypti* (originally collected in Reynosa, Mexico) was provided by our collaborators at Istituto Superiore di Sanità in Rome (Drs. Francesco Severini and Marco Di Luca) and the colony kept in their insectary under the same standard condition and maintained by membrane feeding using rabbit blood. Adult female mosquitoes 3–8 days post-emergence (dpe), and never fed on blood before, were used for all the experiments. Mosquitoes were starved for at least 6–8 hours before exposure to mice.

Salivary glands were dissected in Phosphate Buffered Saline (PBS), transferred into a tube containing 20 µl of PBS and frozen at -80˚C in batches of 20–40 salivary glands. Salivary gland extracts (SGE) from *Ae. albopictus* (alSGE), *Ae. aegypti* (aeSGE)

and *An. coluzzii* (coSGE) were prepared by three cycles of freezing and thawing followed by centrifugation at 16,000 x g at 4˚C. For each set of experiments the supernatants were collected and the different batches were pooled in order to generate a homogeneous SGE stock for each mosquito species to be used for all the ELISA assays. Protein concentration were measured by the Bradford method (Bio-Rad, 5000002) using the Take3 microvolume plate in a BioTek microplate reader (BioTek Synergy HT). The different SGE stocks were aliquoted and stored at -20˚C until use.

3.4. Mice exposure and sera collection

Female BALB/c mice, aged 6–8 weeks were obtained from Charles River Laboratories and kept in the animal facility of our collaborators (Dr. Marta Ponzi and Sig. Leonardo Picci) at Istituto Superiore di Sanità in Rome according to approved Institutional Animal Care and Use Committee protocols. Cohorts, composed of 4 naïve mice each, were anesthetized and exposed to bites of either *Ae. albopictus* or *Ae. aegypti* or *An. coluzzii*. Briefly, the abdomen of each mice was exposed for ~20 minutes to one of four paper cups covered with a mesh net and containing 33–47 adult female mosquitoes (*Ae. albopictus*, *Ae. aegypti* or *An. coluzzii*) per mice. Blood feeding efficiency was unexpectedly higher for *Ae. aegypti* (80.6%) than for *Ae. albopictus* (49.6%) and *An. coluzzii* (63.4%), with an average of 28, 21 and 23 fed mosquitoes/mouse/exposure, respectively (Table 1). All mice were exposed on the same day every 2 weeks for 6 weeks (total 4 times), an exposure regimen similar to those previously employed for immunization to anopheline mosquito saliva (Donovan *et al.*, 2007, Kebaier *et al.*, 2010). An additional group of mice not exposed to any mosquito was also included in the experimental plan as a further negative control. Small blood aliquots (~50–100 µl) were collected from the tail vein for serum preparation at different time points: one week before the 1st exposure (B, baseline), one week after the 2nd exposure (M, midterm), one week after the 4th/last exposure (T, top) and then 1, 2 and 3 months after the end of the exposure regimen (+30, +60 and +90, respectively). Finally, 5 months after the last exposure (+150) mice were sacrificed and larger blood volumes (> 600 µl) collected by cardiac puncture. After blood clotting sera were separated by centrifugation at 10,000 g for 15 minutes and the different aliquots stored at -20˚C.

Table 1. Mean number of mosquitoes and percentage feeding.

	mosquito n (range)	fed n (range)	fed % (range)
Ae. aegypti	35 (33.0-37.5)	28 (25.5-30.3)	80.4 (74.0-87.0)
Ae. albopictus	44 (41.5-47.0)	21 (17.8-24.0)	47.2 (39.4-54.4)
An. coluzzii	36 (33.0-39.7)	23 (19.2-25.0)	63.4 (58.3-70.8)

Number of total mosquitoes and fed mosquitoes, percentages and ranges are expressed as mean per mouse.

3.5. Human hyperimmune serum

An hyperimmune serum was obtained in February 2013 from a volunteer who had been regularly feeding, for his own purposes (colony maintenance) and independently from this study, an *Ae. albopictus* colony fortnightly in the previous 4 months. Thirty-nine months later, in May 2016, a second serum aliquot was obtained from the same donor who had not been feeding *Ae. albopictus* or other *Aedes* spp colonies for at least twenty-four months and had eventually only natural exposure to *Aedes* mosquitoes. Written informed consent for participation to this study was provided from the volunteer.

3.6. Study areas, human sera and entomological data

3.6.1. Padova and Belluno

A first study on human antibody response to mosquito salivary antigens was carried out in the Veneto region, Northeast Italy, in the cities of Padova and Belluno (Figure 3.2). Padova (45°24’23”N, 11°52’40”E) is located in a plain area (27 meters a.s.l.), has a relatively high population density (2.287 inhabitants/km²) and counts roughly 213,000 inhabitants. Belluno (46°08′27″N, 12°12′56″E) is situated in a valley at 389 meters a.s.l. and is surrounded by Bellunesi Prealps and Dolomites; total population is of approximately 36,000 inhabitants with a population density lower than Padova (243 inhabitants/km²). *Aedes albopictus* is widely spread almost all-over Northeast Italy and Padova is one of the first cities in Europe colonized by this species. After its first finding in 1991 (Dalla Pozza and Majori, 1992) the tiger mosquito got very well established in the area and quickly became an important pest due to its aggressive behavior and daytime biting activity. Afterwards, the tiger mosquito progressively expanded its distribution to the entire Veneto region. Currently, it is by far the most abundant *Aedes* species in the urban areas of Italy, and these two cities were selected as sites with high (Padova) and

low to moderate (Belluno) exposure to bites of *Ae. albopictus*. This assumption was mainly based on entomological data from the two areas in the years preceding this study (Montarsi *et al.,* 2015; Montarsi *et al.,* unpublished observations) and on history of colonization. In fact, even though *Ae. albopictus* is well established in both municipalities, the two sites markedly differ for the timing of colonization. Padova should be considered of "ancient" colonization: the tiger mosquito was first reported at the beginning of nineties (Dalla Pozza and Majori, 1992) and therefore, at the time of this study, it was established in the city since at least 25 years. On the contrary, Belluno is of "recent" colonization: *Ae. albopictus* reached the area approximately 20 years later, in 2012 (Gobbi *et al.,* 2014), and for this reason at the time of our study was established in Belluno since approximately 5 years. Notably, another exotic mosquito species, *Aedes koreicus*, was found shortly earlier in the Belluno area (Capelli *et al.,* 2011); however, according to entomological surveys performed in the period 2014-2015, the most abundant mosquito species in the Belluno city was *Ae. albopictus* (57%), followed by *Culex pipiens* (32.1%) and *Ae. koreicus* (9.2%), with other *Aedes* species only occasionally found and accounting globally for less than 0.8% of the collected mosquitoes (Baldacchino *et al.*, 2017). It should be also mentioned that another mosquito of Asian origin, *Aedes japonicus japonicus*, was found in 2018 in the far Northeast area of the Belluno province, towards the borders with Austria (Montarsi *et al.* 2019); however, there is no indication of the presence of *Ae. j. japonicus* in Belluno during the study period.

Human sera collection in Padova and Belluno was possible thanks to the collaboration of Coordinamento Regionale Attività Trasfusionale (CRAT, Padova, Italy: Dr. Antonio Breda), of the Department of Immunohematology and Transfusion Medicine (San Martino Hospital, Belluno, Italy; Dr. Alessio Borean and Dr. Stefano Capelli) and of the Department of Transfusion Medicine (Padova University Hospital, Italy; Dr. Massimo La Raja and Dr. Giustina De Silvestro). The study was approved by the Ethics Committee of Sapienza University (306/17 RIF.CE: 4479, April 10th, 2017). All volunteers participating to the study provided written informed consent on the use of their sera to measure antibody responses to mosquito salivary antigens. Sera were collected among adult healthy volunteers who referred for routine blood donation to the immune transfusion centers of Padova and Belluno. A first collection of sera took place in 2017 from May 2nd to May 12th in Padova (PD1, n=130) and from May 4th to June 1st in Belluno (BL1, n=130). According to previous data on mosquito seasonality in the areas (Baldacchino *et al.* 2017), these sera can be considered as representative of individuals

who were not significantly exposed to *Ae. albopictus* bites in the previous 4 to 5 months. I will refer to these collections as PD1 and BL1 or, more generically, as before (summer) = at the end of the low-density mosquito period. A second collection was done, always in 2017, after the summer period of high mosquito density: from September 11th to November 22nd in Padova (PD2, n=132) and from September 14th to November 21st in Belluno (BL2, n=131). These sera can be considered as representative of individuals who were significantly exposed to *Ae. albopictus* bites. In the text I will refer to this second round of collections as PD2 and BL2 or, more generically, as after (summer) = after the high-density mosquito period. A subset of individuals from Padova (n=69) and Belluno (n=97) could be enrolled in both surveys. Volunteers participating to the study were also invited to fill a short questionnaire finalized to gather information on (i) cutaneous reaction to mosquito bites (from 0=absent to 5=very intense) as well as, with specific reference to the six-months preceding the donation, on (ii) travels outside Italy and country visited, (iii) perception of intensity of mosquito bites (from 0=not bitten to 5=very many bites) and (iv) timing of mosquito bites (during day, at night, day and night).

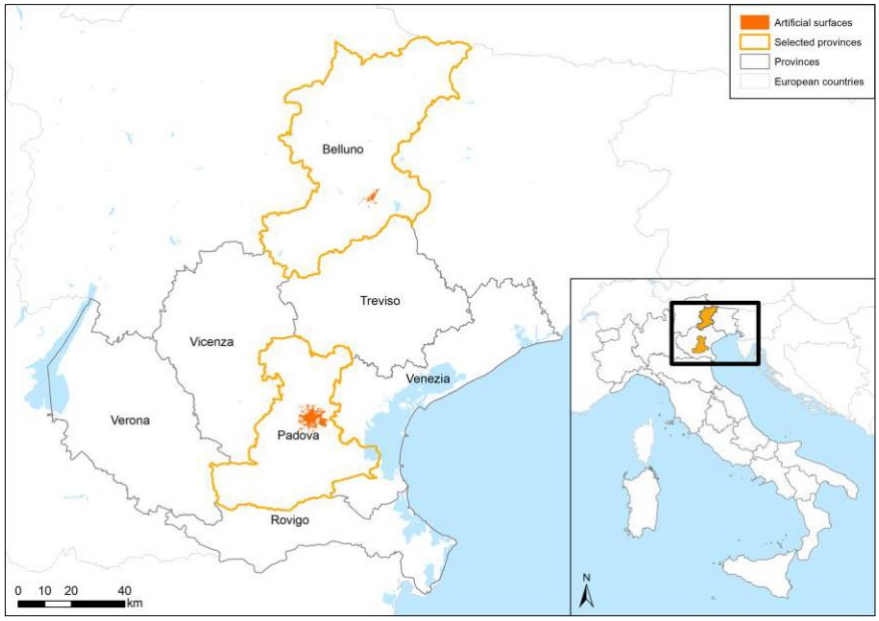


Figure 3.2. Map of the study sites. Map of the Veneto region: the boundaries of the Belluno and Padova provinces are shown in yellow and the cities of Padova and Belluno by the orange spots (artificial surfaces). The inset shows the location of Veneto in Northeast Italy and the Belluno and Padova provinces (yellow areas).

To evaluate the occurrence and population density of *Ae. albopictus* the two selected sites, Padova and Belluno, were monitored from end of May to July 2017 (low-density) and from end of August to beginning of October 2017 (high-density). The surveys were performed using ovitraps (oviposition standard traps), which is the most used kind of trap for monitoring *Aedes* mosquito species (Velo *et al.*, 2016, Manica *et al.*, 2017). Ovitraps consist of black cylindrical vessels (9.0×11.0 cm) with an overflow hole (at 7.0 cm from the bottom) containing ~300 mL of standing water. A wooden stick (Masonite strip, 10.0×2.5 cm) was used as a substrate for oviposition. A larvicide (Bacillus turingensis var. israelensis, BTI) was added into the ovitraps to avoid larval development. Selection of sites where to set the ovitraps was made by dividing the urban areas into hypothetical squares of 4 km2 and positioning three traps inside each square. According to these criteria, ovitraps were placed in geo-referenced sites and checked biweekly, with a total of 20 ovitraps in Belluno and 40 in Padova (Figure 3.3). The mean number of eggs per positive ovitraps and the proportion of positive ovitraps (number of traps with eggs over total number of ovitraps) were calculated to estimate the seasonal mosquito density. Entomological monitoring was possible thanks to our collaborators at Istituto Zooprofilatico Sperimentale delle Venezie (Padova, Italy; Dr. Gioia Capelli and Dr. Fabrizio Montarsi).

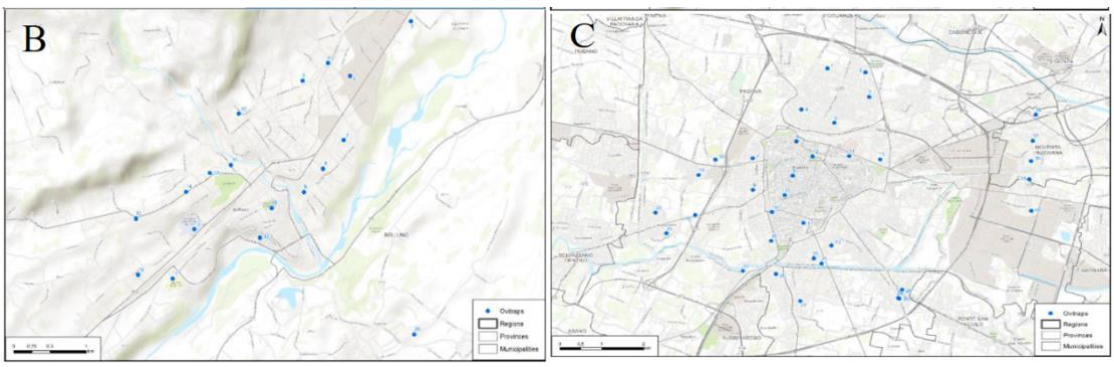


Figure 3.3. Map of the study sites and ovitraps position (b) Map of Belluno and ovitraps position (blue dots). **(c)** Map of Padova and ovitraps position (blue dots).

3.6.2. The Réunion Island and Bolivia

A second study on humans was performed in collaboration with the group of Dr. Franck Remoue [MIVEGEC unit, Institut de Recherche pour de Développement (IRD) Montpellier, France] who made available sera from previous studies on human exposure to *Ae. albopictus* and *Ae. aegypti* carried out in the Réunion Island (RE) and Bolivia (BO), respectively (Doucoure *et al.,* 2012a, Doucoure *et al.,* 2012b). In the south of Réunion Island (Le Tampon), *Ae. albopictus* is abundantly present (up to 1200 meters a.s.l.) and has been responsible of an important Chikungunya outbreak during the 2006 (Delatte *et al.*, 2008). Noteworthy, in this area human population is exposed to *Ae. albopictus* but no to *Ae. aegypti*. Blood samples were collected in Le tampon during May–June 2009 during the seasonal peak of *Ae. albopictus* exposure, from adults of between 18-30 years of age (n= 108). The other survey, including subjects exposed only to *Ae. aegypti* but no to *Ae. albopictus,* was conducted, as part of a large multidisciplinary study, in an urban area in the city of Santa Cruz de la Sierra, Bolivia. *Aedes aegypti* is found in this area and was responsible of several dengue outbreaks in the previous years. During the study period, a large dengue epidemic (DENV-2 and DENV-3) occurred in 2007 in Santa Cruz. Households were selected by cluster survey. Sera were collected in April–May 2007 from 1,049 individuals 3–94 years of age. Informed consent was obtained from all adult participants and from the parents or legal guardians of minor subjects. To make sure not to introduce any bias, a subset of these Bolivian samples Bolivia (n= 105), pair-matched for age with the Réunion Island subjects (Doucoure *et al.*, 2012b) but otherwise randomly selected were used for our study. A small set of individuals (n= 18) from a region in the North of France free of either *Ae. albopictus* or *Ae. aegypti* were used as an unexposed control group.

Entomological measurements in Bolivia were made every day for five weeks (April 23–May 30, 2007) in the morning (8:00 AM–noon) and afternoon (2:00 PM–6:00 PM). A total of 896 prospection units (households) were visited. In each prospection unit, all sites containing *Ae. aegypti* larvae and pupae were identified and characterized. All aquatic stages (L1–L4 larvae stage and pupae) were collected and counted. According to the count of larvae and pupae, two entomologic parameters (exposure 1 and exposure 2) were defined to assess the level of exposure to *Ae. aegypti*. In brief, the exposure 1 parameter provides a measurement of the risk of immediate exposure to adult *Ae. aegypti*. This parameter, which was based on older immature stages, was used as a proxy of the adult mosquito density over the next few days. The exposure 2 parameter provides a

measurement of the number of long-lasting breeding sites. No specific entomological measurements were performed in the Réunion Island during the samples collection.

3.7. ELISA

The ELISA assay (Enzyme Linked ImmunoSorbent Assay), which allows to detect antigen-antibody complexes, was used to verify the presence of specific IgG directed against mosquito SGEs, recombinant salivary proteins or peptides. ELISA assays were essentially performed as previously described for the *An. gambiae* gSG6 protein (Rizzo *et al.,* 2011). Coating was performed in 96-well plates (Nunc, Multiwell immune plate Maxisorp, M9410) in 50 µl of diluted antigen in coating buffer (15mM Na2CO3, 35mM NaHCO3, 3mM NaN3, pH 9.6) for 3 hours at room temperature. After washings [always 4 washings: the first with PBS-T (1x PBS, 0.05% Tween 20) and the others with distilled water], wells were blocked 3 hours at room temperature (RT) in 150 µl of 1% w/v skimmed dry milk in PBST, washed again and then incubated overnight at 4°C with 50 µl of serum diluted in blocking buffer. The different sera dilution used in the different experiments are summarized in Table 2. Serum samples were analyzed in duplicate with the antigen and once without antigen (coating buffer only). After washings, plates were incubated (3 hours, RT) with 100 µl of polyclonal rabbit anti-human IgG/Horseradish Peroxidase (HRP) antibody (Dako P0214). After washing, the colorimetric development was carried out (15 minutes, 25°C in the dark) with 100 µl of o-phenylenediamine dihydrochloride (OPD, Sigma P8287). The reaction was terminated adding 25 µl of 2M H2SO4 and the optical density at 492 nm (OD_{492}) was determined using a Biotek Synergy HT microplate reader equipped with the GenExpert 5 1.09 software. Antigen concentrations, sera dilutions, type and dilutions of secondary antibodies used for the different mouse and human experiment are summarized in Table 2 and Table 3, respectively.

Table 2. Antigens, dilutions and secondary antibody used for experiments with mice sera.

Ig	antigen (Ag)	[Ag]	sera dilution	secondary Ab
IgG	alSGE	8.6 μg/ml	1:50	anti-mouse IgG/HRP Pierce 31430 (1:10000)
	aeSGE	11 μg/ml	1:50	
	coSGE	10.6 μg/ml	1:50	
	al34k2	5 μg/ml	1:50	
	ae34k2	5 μg/ml	1:50	
	peptides	20 μg/ml	1:20	

HRP (Horse peroxidase enzyme)

Table 3. Antigens, dilutions and secondary antibody used for experiments with human sera.

Ig	antigen (Ag)	[Ag]	sera dilution	secondary Ab
IgG	alSGE	5.6 μg/ml	PD-BL 1:50	anti-human IgG HRP Dako P0214 1:5000
	al34k2	5 μg/ml	PD-BL 1:50	
			RE-BO 1:25	
	ae34k2	5 μg/ml	PD-BL 1:50	
			RE-BO 1:25	
IgG1	al34k2	5 μg/ml	PD1-PD2 1:20	anti-human IgG1 Binding Site AP006 1:1000
IgG4	al34k2	5 μg/ml	PD1-PD2 1:20	anti-human IgG4 Binding Site AP009 1:1000

PD-BL (Padova and Belluno surveys), PD1- PD2 (Padova surveys before and after summer period), RE-BO (Réunion Island and Bolivia surveys).

3.8. Statistical analysis

All samples were analyzed in duplicate with the antigen and once with no antigen. The no-antigen well was used for background subtraction and results were expressed as ΔOD values, which were calculated according to the formula ΔOD = ODX−ODN, where ODX represents the mean of the duplicate with the antigen and ODN the value in the well without antigen. Samples whose duplicates showed a coefficient of variation (CV) >20% were re-assayed or not included in the analysis. To control for intra- and inter-assay variation, in the experiments involving the use of human sera, IgG levels were determined including in each plate negative controls as well as a standard curve made by 2-fold dilution series (1:25- 1:1600) of a human hyperimmune serum for Padova and Belluno surveys and (1:3- 1:6561) of high responders from Padova for the Réunion Island and Bolivia surveys. OD values were normalized using titration curves and the Excel software (Microsoft) with a three variable sigmoid model and the Solver add-in application as previously described by Corran and collaborators, 2008. IgG1 and IgG4 OD levels were converted to concentrations (ng/ml) including on each plate standard curves set up as follows. As capturing factors goat anti-human IgG (5µg/ml, Jackson ImmunoResearch 109005098) or mouse anti-human IgG4 (2µg/ml, BD Pharmingen 555881) were used for coating in the same conditions described above. After washing, blocking and washing again, wells were incubated overnight at 4◦C with two-fold dilution series from 1µg/ml to 0.0078µg/ml of purified native human IgG1 (Bio-Rad PHP010) or IgG4 (ABD Serotec 5254–3004) in 50µl of blocking reagent. Incubation with anti-human IgG1/HRP or IgG4/HRP and colorimetric detection were performed as described above.

Datasets were tested for normality and lognormality by different tests (Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk, Kolmogorov-Smirnov). No dataset passed any normality test and only some datasets passed lognormality tests. For these reasons the statistical analysis was performed using non-parametric tests. Multiple comparisons were performed by the Kruskal-Wallis test. Mann-Whitney U test was used to compare IgG levels between two independent groups. The Wilcoxon matched-pairs test was used for comparison of two paired groups. Graph preparation and statistical analyses were performed using the Prism 8.0 GraphPad Software (San Diego, CA).

4. RESULTS

4.1. Selection of candidate *Ae. albopictus* salivary proteins and peptide design

As a first approach toward the identification of candidate salivary antigens for the development of immunoassays to evaluate host exposure to *Ae. albopictus* we decided to try the design of peptides, which could be tested using sera from the immunized mice. Therefore, a group of *Ae. albopictus* salivary proteins were selected (i) on the basis of culicine-specificity, i.e. their absence in the saliva of anophelines or other blood feeding arthropods (Ribeiro *et al*., 2010, Arcà *et al*., 2007, Ribeiro *et al*., 2007), (ii) according to their limited identity (< 50%) to homologs from *Culex* species and (iii) taking also into account previous indications of immunogenicity, if available (Doucoure *et al*., 2013). Considering only peptides whose antigenicity was predicted by multiple tools (Materials and Methods section), five candidates from three different *Ae. albopictus* salivary proteins were selected: alb34k1-P1, alb34k2-P2, alb34k2-P3, alb62k1-P4 and alb62k1-P5. The peptide alb34k1-P1 is designed on the N-terminus of the *Ae. albopictus* 34k1 protein, in a position corresponding to the Nterm-34kDa salivary peptide designed by Elanga Ndille and collaborators on the *Ae. aegypti* ortholog. These two peptides appear significantly divergent, with 11/19 identical amino acids and the alb34k1-P1 exhibiting a three aminoacidic insertion, as shown in the alignment in Figure 4.1. N-term34kDa was proposed as biomarker for evaluation of human exposure to *Ae. aegypti* (Elanga Ndille *et al*., 2012). IgG responses to these 5 peptides were analyzed by ELISA in mice immunized to *Ae. albopictus* or *Ae. aegypti* saliva. However, even using low sera dilutions (1:20) and high peptide concentrations (20 μg/ml), and also mixing together the five peptides, no response was observed in any mice. IgG responses to the peptides were also analyzed in the same conditions using a human serum from a donor hyperimmune to *Ae. albopictus* saliva but no IgG recognizing the peptides could be revealed. Considering these discouraging results the following experiments were concentrated on the recombinant 34k2 proteins from *Ae. albopictus* and *Ae. aegypti.*

Figure 4.1. Alignment of alb34k1-P1 and Nterm34kDa salivary peptides. The sequence of the two peptides, corresponding to the N-terminal regions of the *Ae. albopictus* and *Ae. aegypti* 34k1 salivary proteins, are shown. Conserved sites (*) as well as conservative (:) and semiconservative (.) replacements are indicated.

4.2. Recombinant 34k2 salivary proteins from *Ae. albopictus* and *Ae. aegypti*

As a second parallel approach, the expression in recombinant form of a few candidate salivary proteins from *Ae. albopictus*, including the 62k1 and 62k2 proteins, was attempted. Specifically, conditions for expression and purification of the *Ae. albopictus* 34k2 salivary protein, for which previous indication of immunogenicity were available (Doucoure *et al*., 2013), were optimized. Following the first indication of immunogenicity to mice and humans of the *Ae. albopictus* 34k2, the orthologue from *Ae. aegypti* was also expressed and purifies in recombinant from (Materials and Methods section).

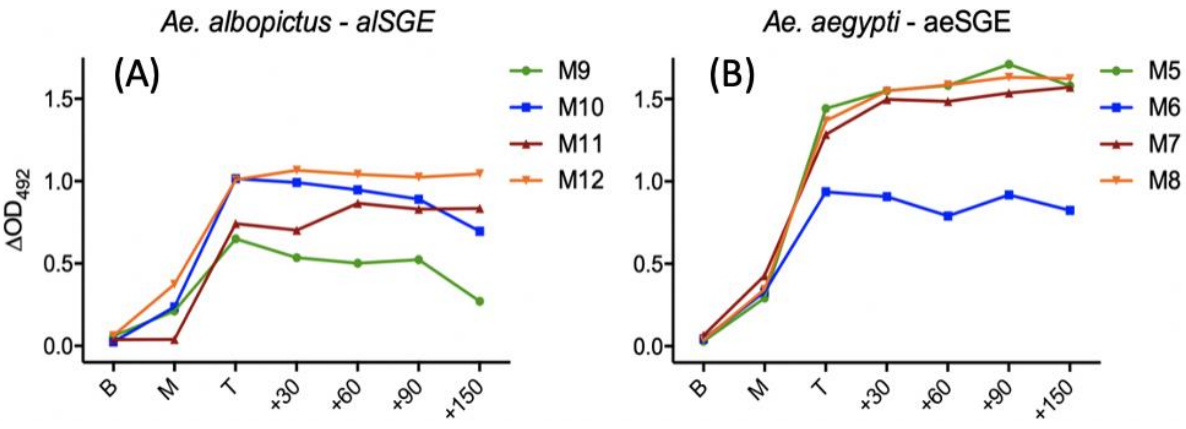
The 34kDa family of salivary proteins was originally found in *Ae aegypti*, where it is composed by at least three members, two of which, named 34k1 (ABF18170) and 34k2 (AL76018), are abundant in saliva and enriched or specifically expressed in adult female salivary glands (Ribeiro *et al*., 2007). Two family members, orthologs of the *Ae. aegypti* 34k1 and 34k2 and with a similar expression profile, were found in *Ae. albopictus* (Arcà *et al*., 2007). Orthologs between the two *Aedes* species share 65% (34k1) and 62% (34k2) amino acid identity, whereas paralogs exhibit 32–33% identity. Members of the 34kDa family appear to be present also in *Culex* species (Ribeiro *et al*., 2004, Ribeiro *et al*., 2018), however they are only distantly related to the *Aedes* proteins (23% to 28% identity).

4.3. Mice IgG responses to Salivary Gland Extracts and 34k2 salivary proteins

4.3.1. IgG responses to Salivary Gland Extracts

Mice immunization was verified measuring by ELISA the IgG responses to salivary gland extracts (SGE) of the corresponding mosquito species. All mice exposed to *Ae. albopictus* developed an antibody response to alSGE, with anti-saliva IgG levels increasing after the second exposure, reaching a peak one week after the fourth/last exposure and remaining essentially unchanged up to 3–5 months post-exposure (Figure 4.2A). A similar pattern was found in mice exposed to *Ae. aegypti*, even though IgG levels against aeSGE appeared higher in most mice (Figure 4.2B). On the contrary, antibody response to coSGE of mice exposed to *An. coluzzii* was markedly lower in intensity, with two mice not showing any IgG antibody response against coSGE (Figure 4.2C). Overall, independently from inter-individual and inter-species quantitative differences, these observations indicate that the exposure regimen was effective both for *Ae. aegypti* and *Ae. albopictus*, with all mice developing anti-SGE IgG responses; instead, surprising, immunization was not successful in mice exposed to *An. coluzzii*.

The salivary proteins of *Ae. albopictus* and *Ae. aegypti* were estimated to share, on average, ~70% amino acid identity (Arcà *et al*., 2007); therefore, we wondered if mice exposed to *Ae. albopictus* could recognize aeSGE and vice versa. Not surprisingly, IgG raised by exposure to saliva of one species could recognize SGE from the other species (Figure 4.3), indicating a certain degree of cross-reactivity due to the common and relatively conserved repertoire of salivary proteins (Arcà *et al*., 2007, Ribeiro *et al*., 2007, Arcà and Ribeiro, 2018).



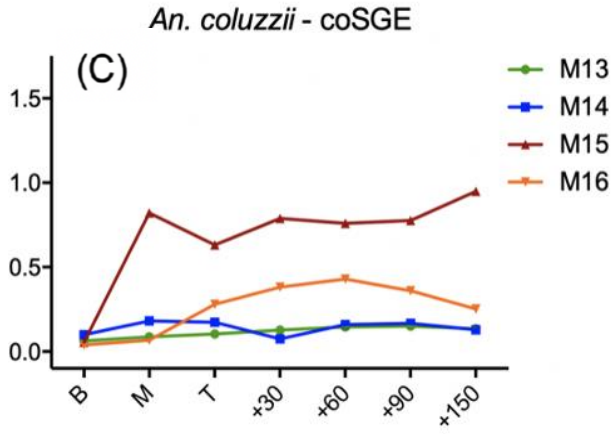
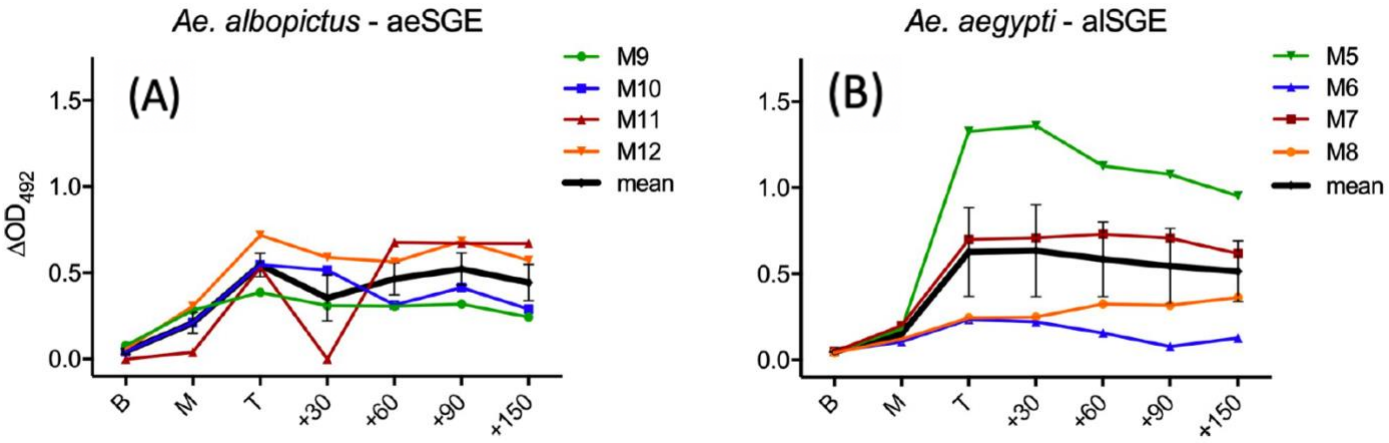


Figure 4.2. **Anti-SGE IgG responses of mice exposed to bites of *Ae. albopictus, Ae. aegypti* or *An. coluzzii***. IgG responses of *Ae. albopictus*-exposed mice to SGE from *Ae. albopictus* (alSGE) is shown in panel A. IgG responses of *Ae. aegypti*-exposed mice to SGE from *Ae. aegypti* (aeSGE) is reported in panel B. IgG responses of *An. coluzzii*-exposed mice to SGE from *An. coluzzii* (coSGE) and is shown in panel C. IgG levels are expressed as ΔOD values at 492 nm. The response of the individual mice is in color as reported in the legends. The different time points are as follows: B = baseline, one week before exposure; M= midterm, one week after the second exposure; T = top, one week after the fourth and last exposure; +30/+60/+90/+150, 30/60/90/150 days post-exposure.



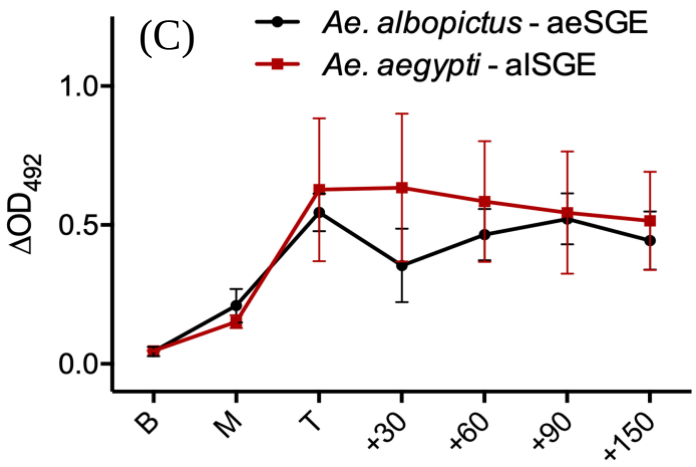
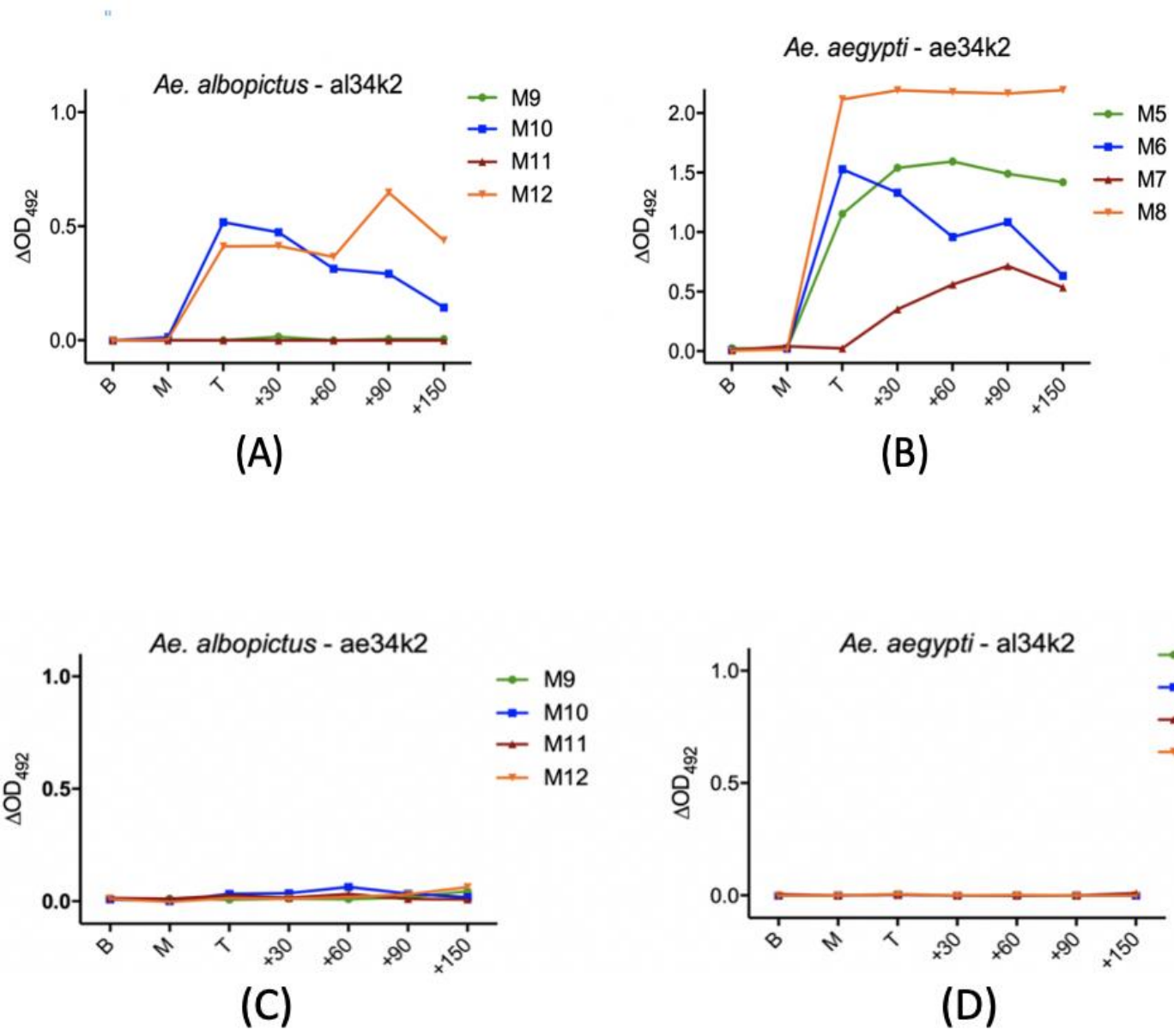


Figure 4.3. Cross-reactivity in mice exposed to bites of *Ae. albopictus* or *Ae. aegypti*. IgG responses of *Ae. albopictus*-exposed mice to SGE from *Ae. aegypti* is shown in panel A. IgG responses of *Ae. aegypti*-exposed mice to alSGE is reported in panel B. Thick black lines represent mean ΔOD values, bars denote standard errors. To facilitate the comparison, mean ΔOD values (thick lines) shown in A and B are shown in black and red, respectively, in panel C. Time points and colour legend as in Figure 4.2.

4.3.2. IgG responses to recombinant 34k2 salivary proteins

IgG antibody levels against the al34k2 and ae34k2 were measured by ELISA in mice exposed to bites of *Ae. albopictus, Ae. aegypti* or *An. coluzzii*, respectively. Two out of four *Ae. albopictus*-exposed mice (M10 and M12) showed IgG responses to al34k2. In both mice the response attained a peak one week after the last exposure and was stable up to 2 months after the end of the exposure regimen. The response then decreased gradually in M10 and, instead, persisted or even had some increase in M12. No anti-al34k2 IgG responses were detectable in the other two mice (M9 and M11) at any time point (Figure 4.4A). As far as the *Ae. aegypti*-exposed mice are concerned, all mice exhibited IgG responses to ae34k2, although at a different degree and with slightly different kinetics (Figure 4.4B). The response reached a peak one week to one month after the last exposure and then stayed unchanged in M5 and M8, continued to slightly increase in M7 and showed a trend to decrease in M6. These results indicate that, despite some inter-individual variability, both al34k2 and ae34k2 are immunogenic to mice. The higher IgG levels and the responses of all *Ae. aegypti*-exposed mice may be due to the more effective immunization to saliva achieved in these mice (likely because of the higher number of bites/mouse/exposure) as also indicated by the IgG responses to SGE (Figure 4.2).

Interestingly, considering the relatively high conservation of the 34k2 proteins in the two *Aedes* species, no immune cross-reaction was observed. Indeed, IgG antibodies directed against al34k2 could not recognize the *Ae. aegypti* protein and, vice versa, anti-ae34k2 IgG did not recognize the *Ae. albopictus* protein (Figure 4.4C-D). These observations suggest that the 34k2 proteins from *Ae. aegypti* and *Ae. albopictus* may represent interesting species-specific markers to evaluate host exposure to these two *Aedes* species. Furthermore, the four mice exposed to *An. coluzzii* did not show any IgG antibody response against al34k2 and ae34k2 (Figure 4.4E-F), nonetheless this is an expected result because 34kDa family of proteins are culicine-specific, i.e. are absent in anopheline mosquitoes.



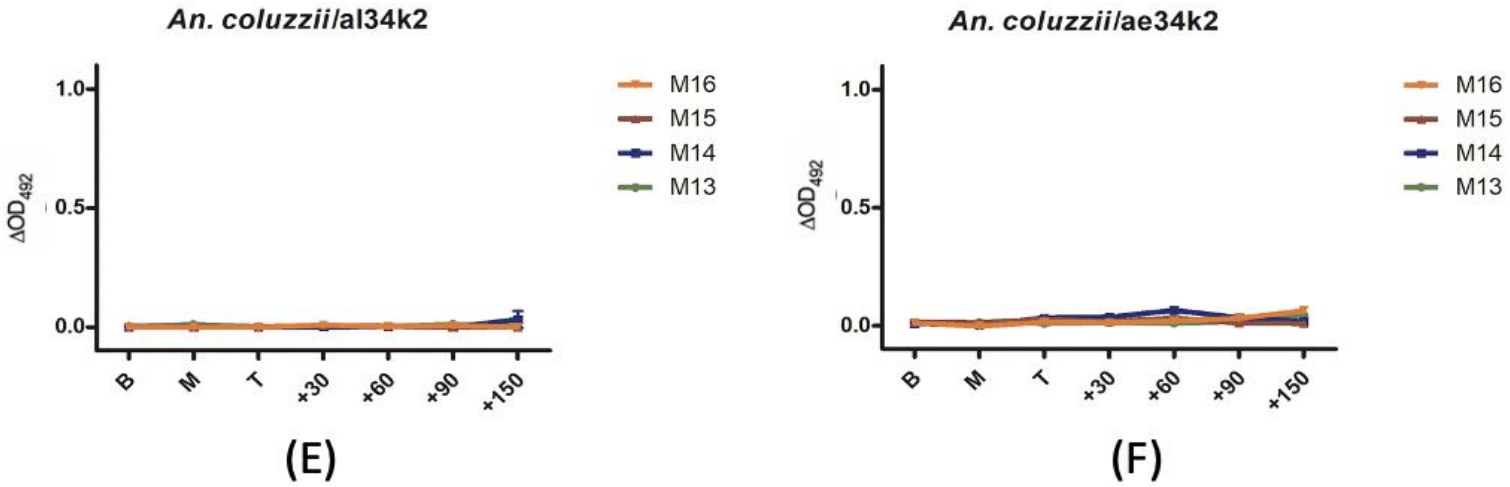


Figure 4.4. **IgG responses to al34k2 and ae34k2 of *Ae. albopictus*- *Ae. aegypti*-and *An. coluzzii* -exposed mice**. Anti-al34k2 (A) and anti-ae34k2 (C) IgG levels in *Ae. albopictus*-exposed mice. IgG responses of *Ae. aegypti*-exposed mice to ae34k2 and al34k2 are shown in (B) and (D), respectively. Anti-al34k2 (E) and anti-ae34k2 (F) IgG levels in *An. coluzzi*-exposed mice. Time points as in Figure 4.3.

4.4. IgG responses to the SGE and 34k2 salivary proteins in a human hyperimmune serum

The availability of a single human serum hyperimmune to *Ae. albopictus* saliva offered the opportunity to collect some preliminary indication on the immunogenicity to humans of al34k2, and eventually on the immune cross-reactivity to ae34k2. The human serum was obtained from a donor at two different time points: (i) in February 2013 (T1), after feeding fortnightly for a period of approximately four months an *Ae. albopictus* colony, and (ii) in May 2016 (T2), after the volunteer had not been feeding *Ae. albopictus* nor other *Aedes spp* colonies for at least two years (and had, likely, only natural exposure to *Aedes* mosquitoes). An intense IgG response against both alSGE and aeSGE was detectable at T1, confirming the hyperimmunization of the donor against *Ae. albopictus* saliva and indicating a wide IgG cross-reactivity to SGE from *Ae. aegypti*. On the contrary, the IgG response to al34k2 appeared considerably higher as compared to the response to ae34k2. At the time point T2 the IgG response to both alSGE and aeSGE persisted, even though at a slightly lower level. Instead, the specific IgG response to al34k2 had a remarkable decrease and also levels of anti-ae34k2 IgG dropped significantly. Chiefly, despite the obvious intrinsic limitations due to the availability of a single human serum and by the hyperimmune status, these observations suggest that al34k2 is immunogenic to humans and that, as observed in mice, there may be a limited cross-reactivity to the two orthologous 34k2 proteins (Figure 4.5).

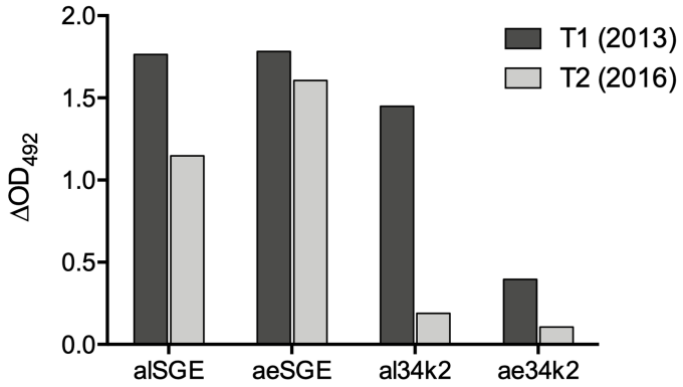


Figure 4.5. IgG responses to SGE and 34k2 recombinant proteins from *Ae. albopictus* and *Ae. aegypti* of a human donor hyperimmune to *Ae. albopictus* saliva. Levels of IgG directed against alSGE, aeSGE, al34k2 and ae34k2 were determined in the serum of a human volunteer obtained at two different time points: (i) T1 (2013), shortly after regularly feeding an *Ae. albopictus* colony approximately every two weeks for 4 months; (ii) T2 (2016), after the donor had not been feeding *Ae. albopictus* nor other *Aedes* spp colonies for at least two years

4.5. Entomological monitoring in Padova and Belluno

Oviposition traps were placed in Padova and Belluno in the time frame between the two sera collections in order to supply an estimation on the relative population dynamics of *Ae. albopictus*. Both the mean number of eggs per ovitrap and the percentage of positive ovitraps indicated that mosquitoes started appearing around the last week of May in Padova (19.9 eggs/ovitrap, 34.4 % positive ovitraps) and shortly later, around the first week of June, in Belluno (36.3 eggs /ovitrap, 30.0 % positive ovitraps). The number of eggs per ovitrap progressively increased during the summer period reaching a peak the last week of August in Padova (281.4 eggs/ovitrap, 94.6 % positive ovitraps) and first week of September in Belluno (342.1 eggs/ovitrap, 88.9 % positive ovitraps) and decreasing subsequently (Figure 4.6). Regardless of the original hypothesis of Padova being an area of higher *Ae. albopictus* density than Belluno, ovitraps data did not show a clear difference between the two study sites. On the contrary, the temporal dynamic fully supports the expectations that (i) individuals whose sera were collected before summer were not significantly exposed to *Ae. albopictus* bites for at least 4 to 5 months and (ii) individuals surveyed after summer were naturally exposed to the tiger mosquito during the warm months, from June to September.

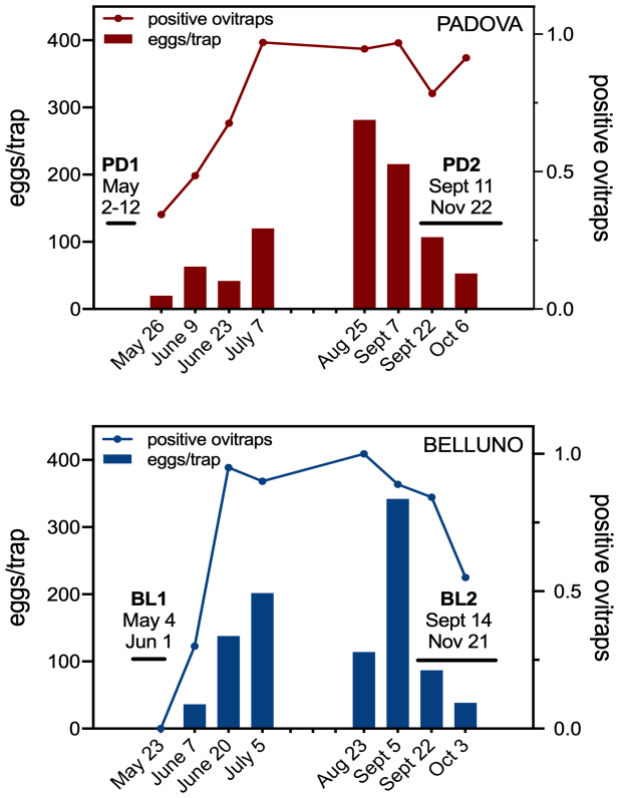


Figure 4.6. Entomological monitoring by ovitraps in the study areas. Bars show the average number of eggs per positive ovitraps (eggs/trap, left Y axis). Lines represent the proportion of positive ovitraps, i.e. the number of ovitraps with eggs over the total number of ovitraps (positive ovitraps, right Y axis). The time intervals for the two sera collections in each study area are reported.

4.6. IgG response to alSGE and 34k2 salivary proteins of *Ae. albopictus* and *Ae. aegypti* in human donors from Padova and Belluno

The main characteristics of the studied population and the individual perception of mosquito bites have been described in detail in the method section and are summarized in Table 1.

Table 1. Features of the studied population and individual perception of mosquito bites.

			PD1	PD2	BL1	BL2	Total
date survey (2017)	start end		May 2 May 12	Sept 11 Nov 22	May 4 June 1	Sept 14 Nov 21	
sampled individuals			130	132	130	131	
age range (years)			18-67	19-66	19-65	19-65	
median age			45.5	47.0	44.0	44.0	
mean age ± 95% CI			43.8 ±	45.1 ±	43.9 ±	44.8 ±	
females (F)			39	34	12	16	
males (M)			91	98	118	115	
paired samples			PD, n=69		BL, n=97		
travel abroad in the preceding 6 months	country with *Ae. albopictus*		19	30	14	29	96
	country with no *Ae. albopictus*		7	4	7	6	20
	not specified		1	4	-	-	5
	no travel		103	94	109	96	402
		total	130	132	130	131	523
cutaneous reaction	low (0-1)		73	77	61	92	303
	mid (2-3)		49	49	18	25	141
	high (4-5)		8	3	4	3	18
		total	130	129	83	120	462
number of bites (0-5)	low (0-1)		117	53	124	64	358
	mid (2-3)		13	49	6	59	127
	high (4-5)		0	28	0	8	36
		total	130	130	130	131	521
timing of bites	day		11	61	12	38	122
	night		7	22	7	43	79
	day & night		6	46	7	36	95
		total	24	129	26	117	296

Collected sera were used to measure IgG responses to *Ae. albopictus* salivary gland protein extracts (alSGE) and to the recombinant *Ae. albopictus* and *Ae. aegypti* 34k2 salivary proteins, al34k2 and ae34k2 respectively. Considering that male volunteers were largely predominant, and to make sure not to introduce any bias, a preliminary comparison of the IgG responses to SGE in males versus females was performed. No significant difference was found in two sexes in the four different surveys (Figure 4.7). and similar results were obtained comparing anti-al34k2 IgG responses in the two sexes (not shown).

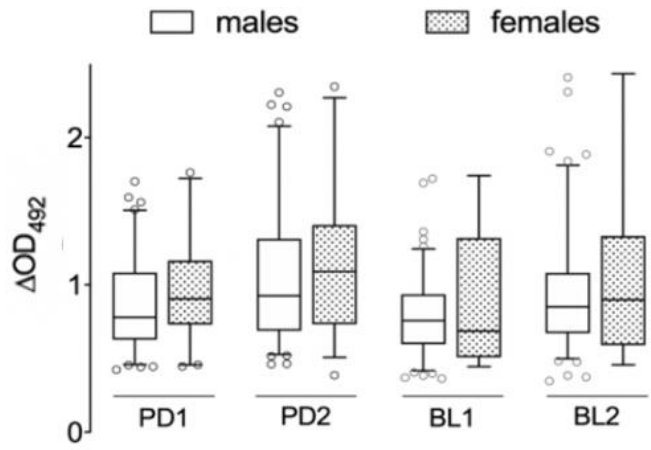


Figure 4.7. IgG responses to *Ae. albopictus* SGE according to sex. Anti-SGE IgG levels in males and females in the four different surveys. The four different surveys (PD1, PD2, BL1 and BL2) are indicated at the bottom. Boxes display median OD values, 25th and 75th percentiles; whiskers represent 5th and 95th percentiles and dots the outliers. Number of individuals for each survey according to Table1. In all cases pairwise comparisons within each survey showed no significant difference (Mann-Whitney U test, p value >0.05).

Moreover, in the six months preceding the surveys a variable proportion of individuals (16.1 to 28.8%) had travelled to countries where *Ae. albopictus* was either present or absent. No significant variation of anti-SGE IgG levels was found by pairwise comparisons between individuals who did not travel and those who: (i) travelled, (ii) travelled to countries where *Ae. albopictus* was present or (iii) travelled to countries where *Ae. albopictus* was absent (Figure 4.8).

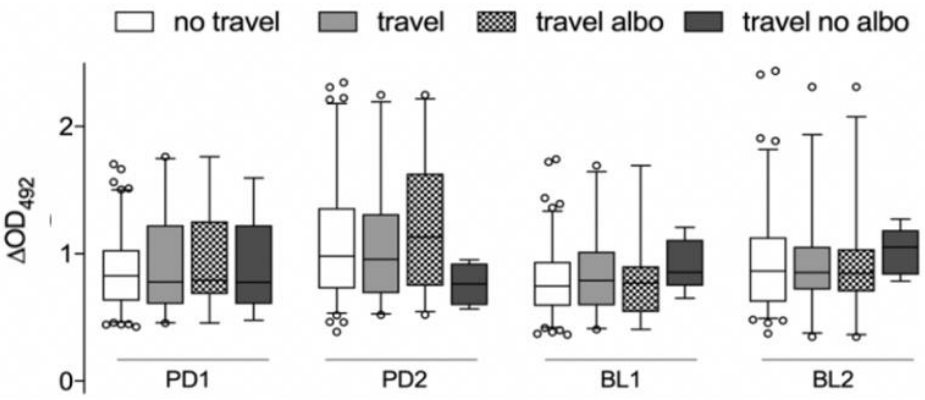


Figure 4.8. IgG responses to *Ae. albopictus* SGE according to travelling. Anti-SGE IgG levels in individuals who, in the six months before the survey, did not travel abroad (no travel), travelled abroad (travel), travelled to countries where *Ae. albopictus* was present (travel albo) or absent (travel no albo). IgG levels are expressed as OD values. Surveys and statistical test as figure 4.7.

According to these observations, the analyses described below were performed including all the samples collected in the different surveys (Buezo Montero *et al.,* 2020).

4.6.1. IgG responses to *Ae. albopictus* Salivary Gland Extracts

IgG antibody responses against mosquito saliva or salivary gland extracts have been previously shown to reliably reflect the intensity of human exposure to bites of either *Anopheles* or *Aedes* species (Remoue *et al.,* 2006, Orlandi-Pradines *et al.,* 2007, Fontaine *et al.,* 2011, Doucoure *et al.,* 2012b, Doucoure *et al.,* 2014). Hence, firstly IgG responses to *Ae. albopictus* SGE were analyzed in sera collected before and after summer in the two study areas. Anti-SGE IgG responses were significantly higher in sera collected after the summer period of high mosquito density both in Padova (PD2) and Belluno (BL2) as compared to those collected before summer in the same areas (Padova, p<0,0001; Belluno, p=0.0009) (Figure 4.9). Moreover, IgG antibody levels against SGE were higher in Padova than Belluno both before (p=0.0341) and after (p=0.0070) the high-density mosquito seasons. These observations, in contrast to ovitraps data, seems to confirm the original assumption of Padova being an area of higher exposure to *Ae. albopictus* than Belluno.

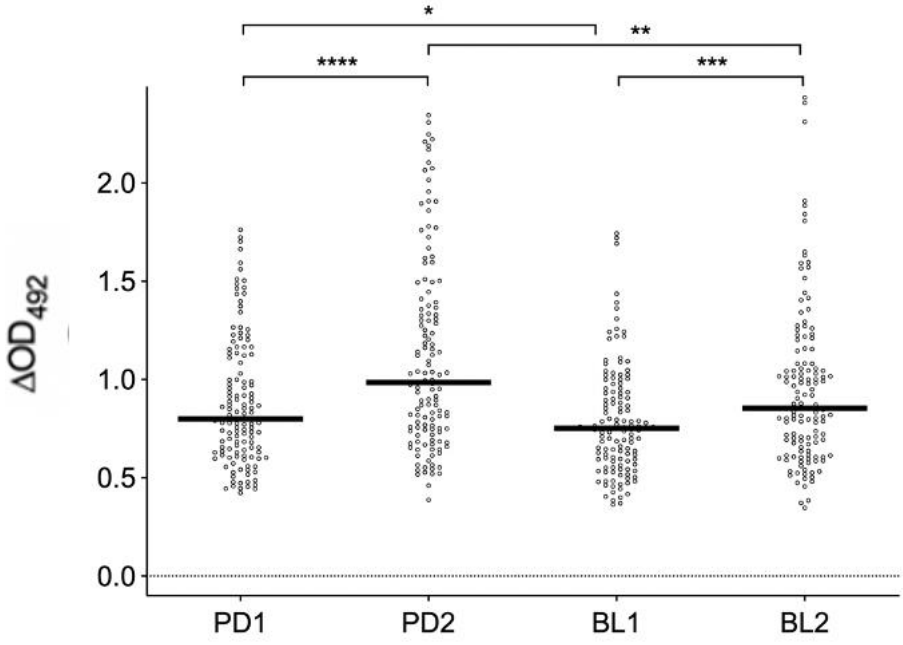
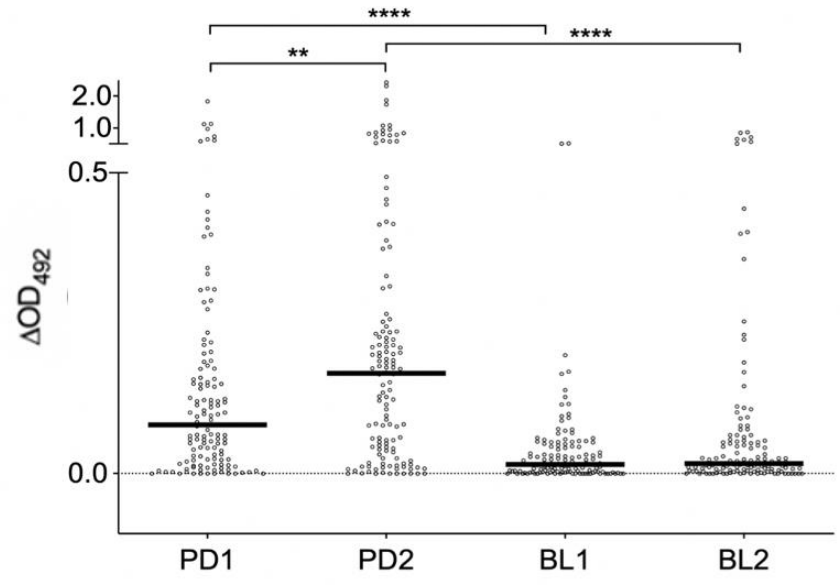


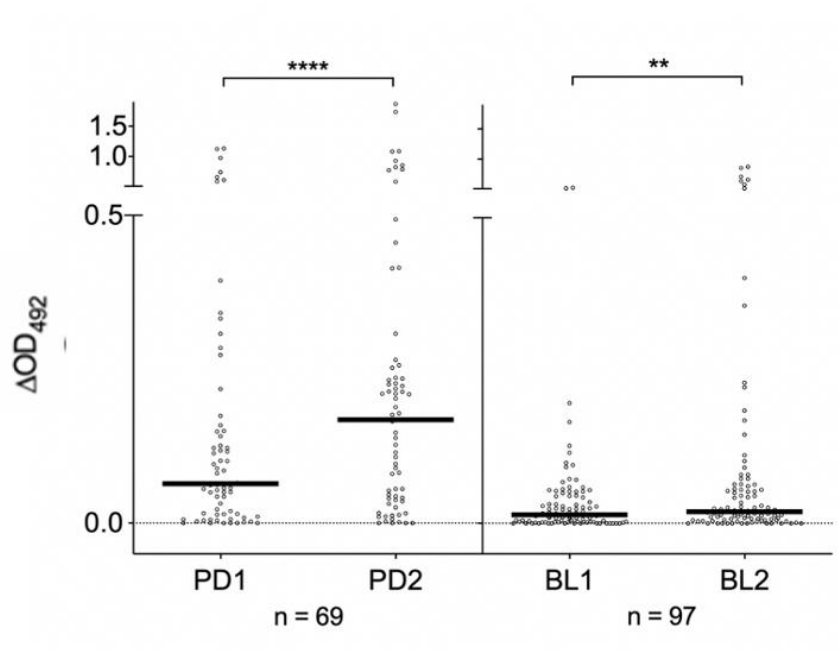
Figure 4.9. IgG responses to *Ae. albopictus* salivary gland protein extracts. Anti-SGE IgG levels in participants to the four different surveys (PD1, PD2, BL1 and BL2) as indicated at the bottom. IgG levels are expressed as ΔOD values. Number of individuals for each survey according to Table 1. Dots mark the individual values and horizontal bars represent the medians. Significant difference in the pairwise comparisons (Mann-Whitney U test) is also reported: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

4.6.2. IgG responses to *Ae. albopictus* and *Ae. aegypti* 34k2 salivary proteins

IgG responses to SGE and al34k2 showed a weak, but clearly positive correlation (Spearman r=0.43, 95% CI 0.36-0.50, n=523, p<0.0001). When the different surveys were compared, a seasonal variation of IgG levels was found in Padova (p=0.0043) and anti-al34k2 IgG responses were higher in Padova than in Belluno both before (p<0.0001) and shortly after the summer season (p<0.0001). Comparison of anti-al34k2 IgG antibody levels between the two sets of sera collected in Belluno failed to show a significant seasonal variation (Figure 4.10A). However, when only paired samples from the two localities were analyzed (i.e. those individuals whose sera were collected both in the first and the second survey), a significant seasonal increase was found not only in Padova (n= 69, p<0.0001) but also in Belluno (n=97, p=0.0032) (Figure 4.10B). Overall, despite the relatively weak correlation, anti-al34k2 IgG responses exhibited a pattern of variation fully consistent with the anti-SGE IgG responses. Therefore, these observations convincingly suggest that IgG responses to al34k2 may be suitable to assess spatial and temporal variation of human exposure to bites of the tiger mosquito *Ae. albopictus*.



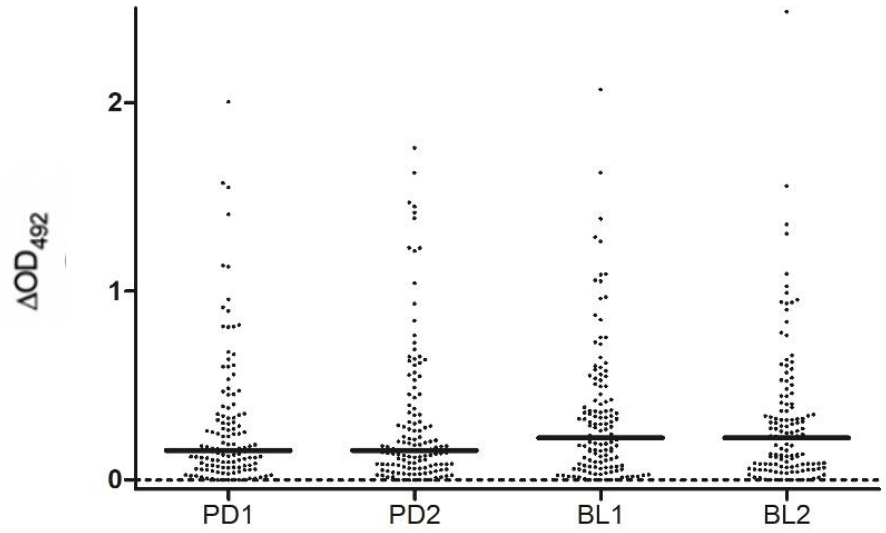
(A)



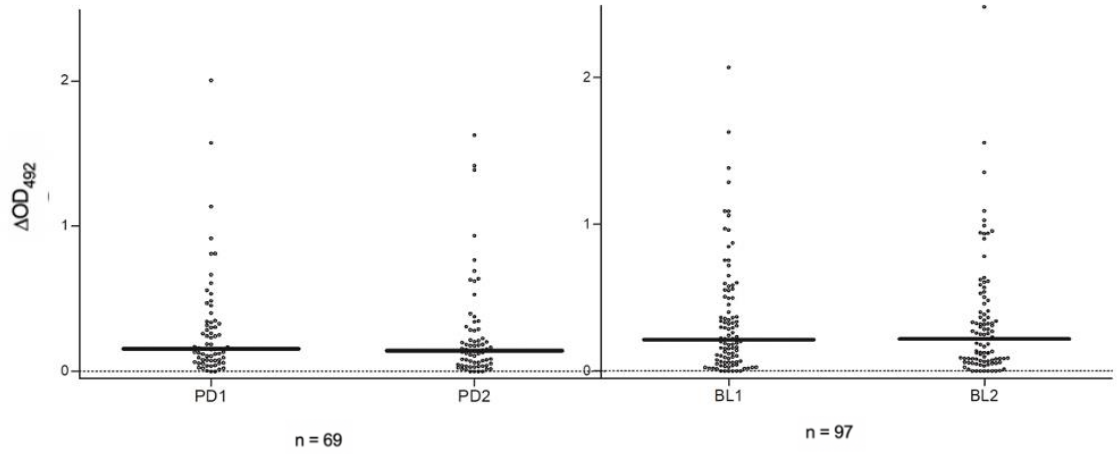
(B)

Figure 4.10. IgG responses to the *Ae. albopictus* salivary gland protein al34k2. (**A**) Anti-al34k2 IgG levels in all participants to the four different surveys. IgG levels, number of individuals, dots, bars and p values as in Figure 4.9. (**B**) IgG responses in paired samples from Padova (left panel) and Belluno (right panel). The number of individuals is indicated at the bottom. Dots and bars as in (A). Significant difference in the pairwise comparisons (Wilcoxon matched-pairs test): **p<0.01; ***p<0.001, ****p<0.0001.

On the other hand, IgG responses to ae34k2 did not show difference between the two sets of sera collected in Padova and Belluno. Even when only paired samples from the two localities were analyzed, no significant seasonal variations were found either in Padova or in Belluno (Figure 4.11). These observations indicate the unspecific nature of the anti-ae34k2 IgG response observed in these areas where *Ae. aegypti* and, again, seem to confirm the absence or low level of cross-reactivity with the anti-al34k2 IgG responses.



(A)



(B)

Figure 4.11. IgG responses to the *Ae. aegypti* salivary gland protein ae34k2. (**A**) Anti-ae34k2 IgG levels in all participants to the four different surveys. IgG levels, number of individuals, dots, bars and p values as in Figure 4.10. (**B**) IgG responses in paired samples from Padova (left panel) and Belluno (right panel). The number of individuals is indicated at the bottom. Dots and bars as in (A).

4.6.3. IgG1 and IgG4 responses to the *Ae. albopictus* 34k2 salivary protein

Preceding studies showed that the *An. gambiae* gSG6 and cE5 salivary proteins induce in naturally exposed individuals differential antibody responses, with the gSG6 antigen evoking high levels of IgG4 antibodies and cE5, on the contrary, triggering an IgG1-dominated response (Rizzo *et al.*, 2014a, Rizzo *et al.*, 2014b). To get insights into IgG subclass-specificity of antibody responses to the al34k2 protein we determined IgG1 and IgG4 antibody titers in the sera collected in Padova before (PD1) and after (PD2) the high-density mosquito season. As expected, a positive correlation was found between anti-al34k2 IgG and IgG1 levels (Spearman r=0.64, 95% CI 0.56-0.70, n=262 p<0.0001), and similar results were obtained for IgG and IgG4 levels (Spearman r=0.68, 95% CI 0.61-0.74, n=262, p<0.0001) levels. Median IgG1 titers appeared to be over ten-fold higher than corresponding IgG4 titers in both surveys (Figure 4.12A; p<0.0001). A highly significant increase of both anti-al34k2 IgG1 and IgG4 levels was observed in PD2 by pairwise comparisons between paired samples (Figure 4.12B; n=69, p<0.0001). Instead, only IgG4 levels showed a weakly significant increase after summer when all samples were considered (Figure 4.12A; p=0.0326). These observations clearly indicate the large predominance of IgG1 antibodies in the anti-al34k2 IgG responses.

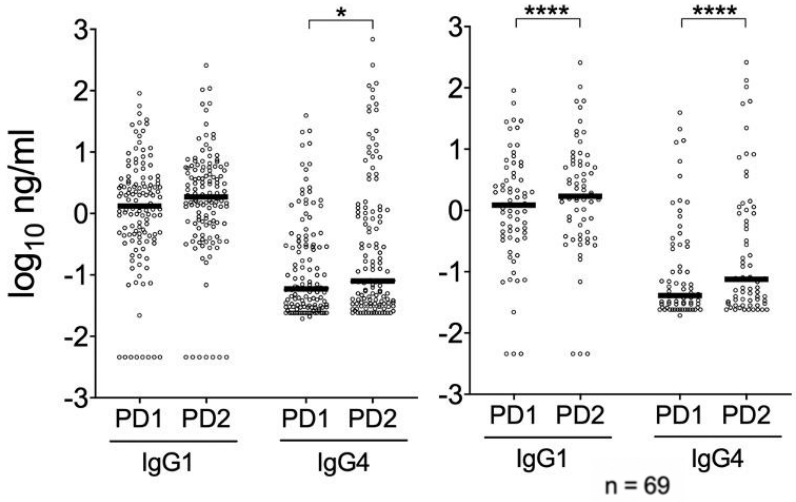


Figure 4.12. IgG1and IgG4 responses to the *Ae. albopictus* salivary gland protein al34k2 in Padova. (**A**) Individual anti-al34k2 IgG1 and IgG4 levels in participants to the PD1 (n=128) and PD2 (n=128) surveys. IgG1 and IgG4 levels are expressed in ng/ml. Dots, bars and p values as in Figure 4.9. (**B**) IgG1 and IgG4 responses against al34k2 in paired samples from Padova (n=69). IgG1 and IgG4 levels, dots and bars as above. Pairwise comparisons by the Wilcoxon matched-pairs test: *p<0.05, ****p<0.0001.

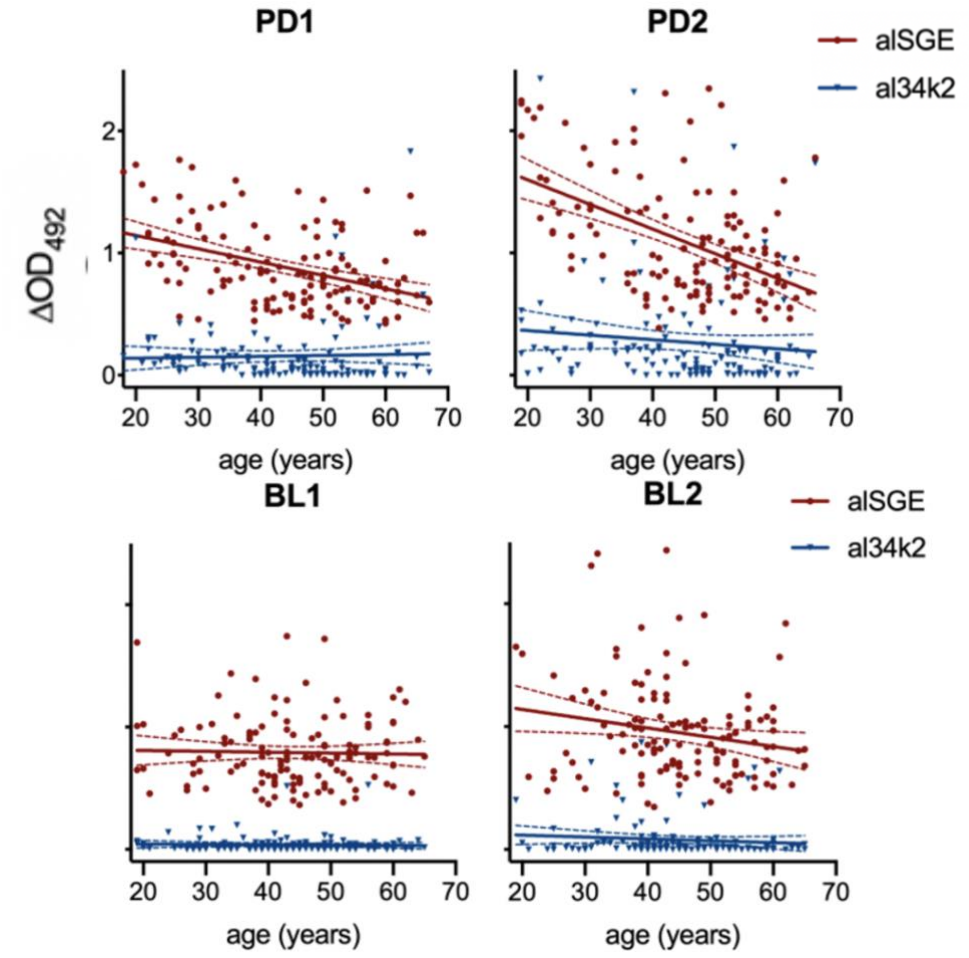
4.6.4. IgG responses to *Ae. albopictus* SGE and al34k2 according to age

Individual IgG responses to *Ae. albopictus* SGE and to al34k2 were also analyzed according to age. Overall, a negative correlation was found between age and IgG responses to *Ae. albopictus* SGE or al34k2. Spearman's rank correlation analysis indicated a clear trend of antibody responses to decrease with age for both Padova surveys, especially when considering the anti-SGE IgG responses; on the contrary, in Belluno a weakly significant negative correlation was only found for the anti-al34k2 IgG responses in the BL2 survey (Table 2).

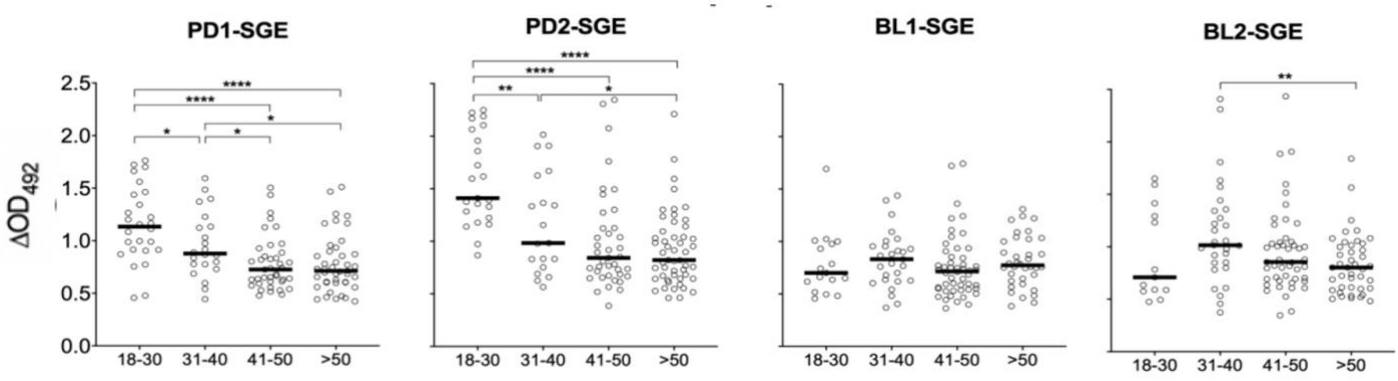
Table 2. Correlation between age and IgG levels.

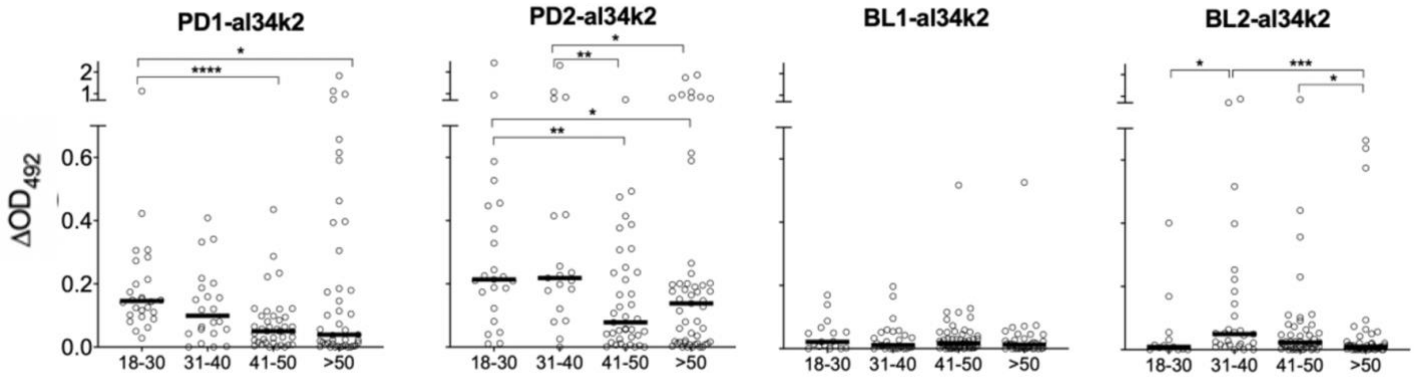
	Spearman r	95% CI	n	p value
PD1-SGE	-0.3861	-0.53 to -0.22	130	<0.0001
PD2-SGE	-0.4570	-0.59 to -0.31	132	<0.0001
BL1-SGE	0.0007	-0.18 to 0.18	130	ns
BL2-SGE	-0.1648	-0.33 to 0.01	131	ns
PD1-al34k2	-0.2548	-0.42 to -0.09	130	0.0030
PD2- al34k2	-0.2435	-0.40 to -0.07	132	0.0049
BL1- al34k2	-0.0961	-0.27 to 0.08	130	ns
BL2- al34k2	-0.2149	-0.38 to -0.04	131	0.0137

This general tendency was confirmed when participants to the surveys were divided in four different age groups (18-30, 31-40, 41-50 and >50 years old). Pairwise comparisons indicated a clear and significant decrease with age of the anti-SGE and anti-al34k2 IgG responses in Padova; again, this was not the case for Belluno where some decrease was only observed in the over 50 years old category in the BL2 survey (Figure 4.13B). To make sure that age distribution did not represent a source of bias we compared the age of the different cohorts of individuals (PD1, PD2, PD paired, BL1, BL2, BL paired) by the Kruskal-Wallis or Mann-Whitney tests and found no significant difference.


PD1
PD2
alSGE
al34k2
ΔOD492
age (years)
BL1
BL2

(A)


PD1-SGE
PD2-SGE
BL1-SGE
BL2-SGE
ΔOD492
18-30
31-40
41-50
>50



(B)

Figure 4.13. Anti-SGE and anti-al34k2 IgG antibody responses according to age. (A) The scatter plots show the IgG responses to SGE (red) and al34k2 (blue) as function of age in participants to the four different surveys. Best-fit lines (solid lines) and confidence interval bands (dashed lines) are shown. Results of correlation analysis are reported in Table 2. **(B)** Anti-SGE and anti-al34k2 IgG responses in the four age-groups and different surveys are shown in the upper and lower panel, respectively. IgG levels are expressed as OD values. Dots mark the individual values and horizontal bars represent the medians. Number of individuals in the different age groups as follows: PD1 (18-30, n=26; 31-40, n=22; 41-50, n=39; >50, n=43), PD2 (18-30, n=23; 31-40, n=19; 41-50, n=39; >50, n=51), BL1 (18-30, n=17; 31-40, n=27; 41-50, n=50; >50, n=36), BL2 (18-30, n=13; 31-40, n=29; 41-50, n=47; >50, n=42). Significant difference in the pairwise comparisons (Mann-Whitney U test) is reported: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

We also calculated the frequencies of the four age groups in the different cohorts (not shown) and compared them by the Chi-square test without finding any difference (chi-square 16.37; df 15; p=0.358). Finally, we also compared IgG levels before and after the mosquito season in the two sites by age groups, using both paired and unpaired samples. Median anti-SGE and anti-al34k2 IgG levels were higher after the summer season in almost all the pairwise comparisons (30/32), even though statistical significance was only reached in ~40% of cases (56% for SGE and 25% for al34k2) (Figure 4.14), likely because of the relatively small sample size. Overall, these observations suggest that age should be taken into consideration in similar studies but also indicate that it does not appear to be a relevant source of bias in the investigation.

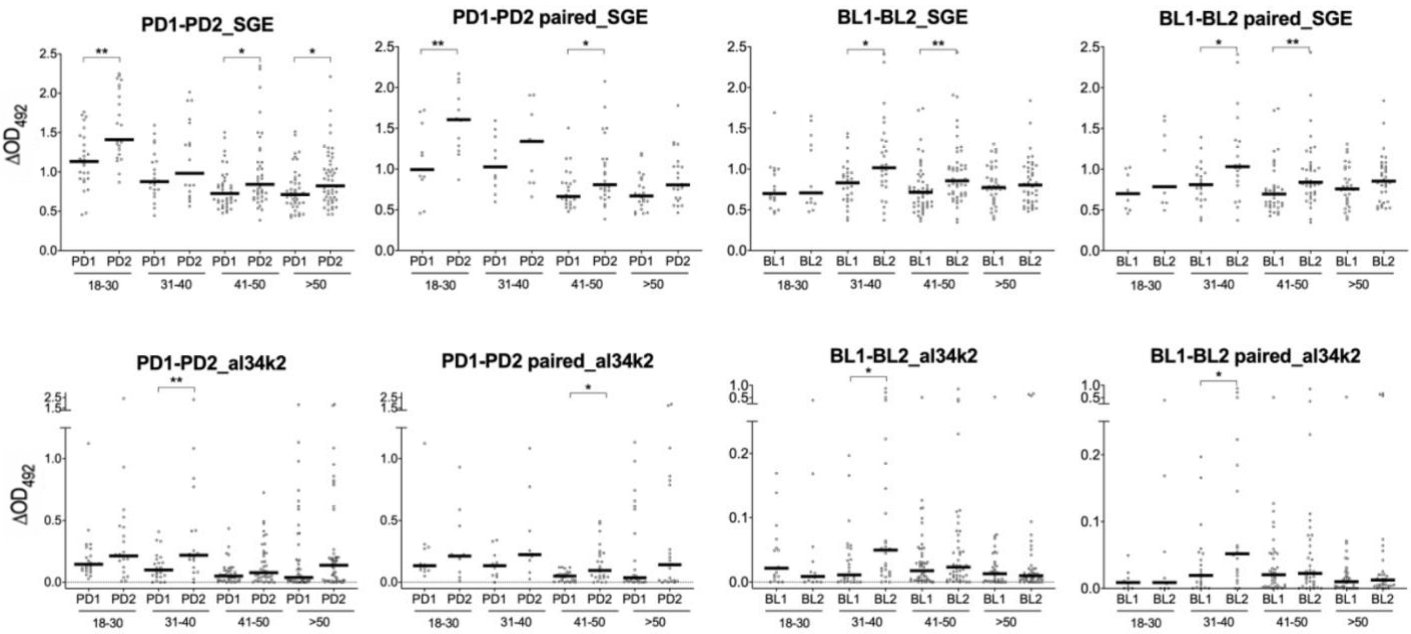
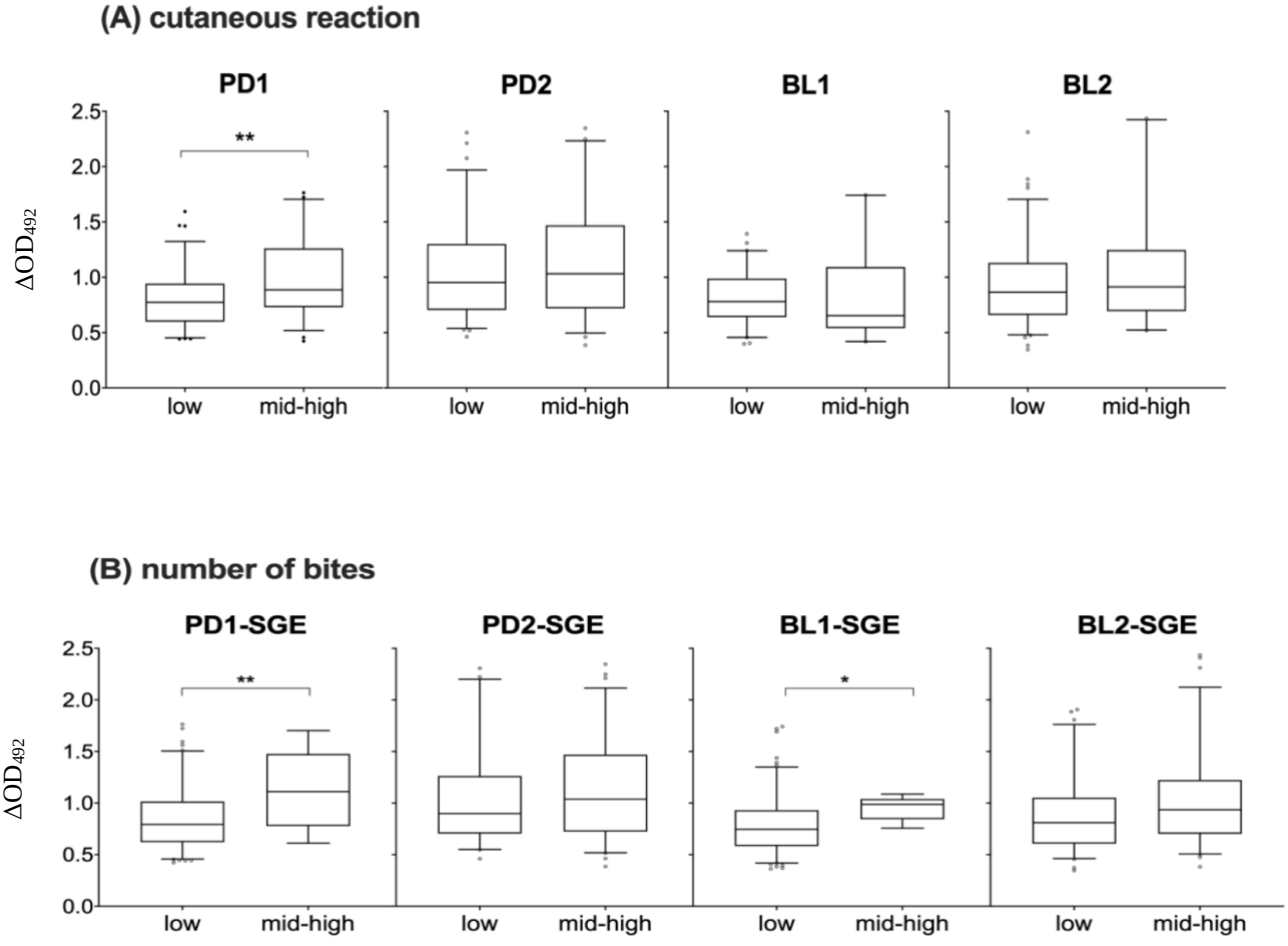


Figure 4.14. Distribution of participants to the different surveys by age groups and comparison of the IgG antibody responses before and after the summer season according to age. Comparison of anti-SGE and anti-al34k2 IgG responses before and after the high-density mosquito season in the four age groups and different surveys as indicated. IgG levels are expressed as ΔOD values. Dots mark the individual values and horizontal bars represent the medians. Number of individuals per age group in the unpaired. Number of individuals per age group in the paired samples as follows: PD paired (18-30, n=12; 31-40, n=10; 41-50, n=25; >50, n=22), BL paired (18-30, n=9; 31-40, n=20; 41-50, n=36; >50, n=32). Significant difference in the pairwise comparisons (Mann-Whitney U test) is reported: *p<0.05; **p<0.01.

4.6.5. Anti-saliva IgG responses and individual perception of exposure to mosquito bites

Participants to the surveys, along with the informed consent, were asked to fill a short questionnaire on their individual perception of cutaneous reaction to mosquito bites, intensity/number of bites and timing of occurrence (Table 1). Despite the intrinsic limitations of this subjective self-assessment, the possible correlation between anti-SGE and anti-al34k2 IgG antibody levels was verified. Overall, individuals reporting mid to high (2-5) cutaneous reactions showed higher anti-SGE IgG levels as compared to those with absent or low (0-1) reactions. This was supported by the observation that median ΔOD values, as well as 25th and 75th percentiles, were in most cases (PD1, PD2 and BL2) higher for the mid-to-high category, although the difference reached statistical significance only for the PD1 survey (p =0.0036) (Figure 4.15A). IgG responses to both *Ae. albopictus* SGE and to al34k2 were also compared in individuals reporting a low number of bites (score 0-1) versus those accounting for mid to high number of bites (score

2-5). For both antigens, the 25th and 75th percentiles and median ΔOD values were, in the very large majority of cases, higher in the mid to high category; however, statistical significance was only found when considering the IgG response to SGE in the PD1 (p=0.0075) and BL1 surveys (p=0,0320) (Figure 4.15B). No general common trend and/or significant difference was recognizable when IgG responses were compared in individuals reporting mainly day- versus night-time bites, only exception being the BL2 survey where anti-al34k2 IgG levels were slightly higher (p=0.0280) in individuals accusing a larger number of bites during daytime.



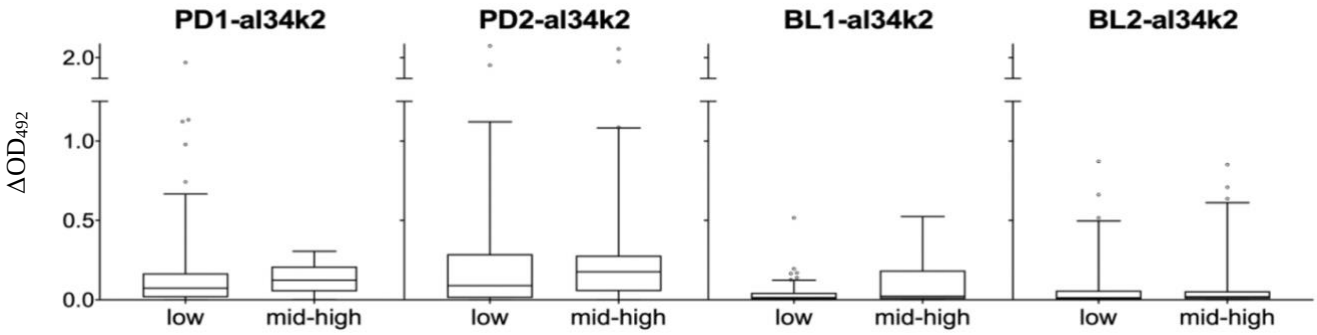


Figure 4.15. IgG responses to salivary antigens and individual perception of mosquito bites. (**A**) IgG responses to *Ae. albopictus* SGE according to intensity of cutaneous reactions to mosquito bites. Boxplots of values among individuals in the PD1, PD2, BL1 and BL2 surveys. Boxes display median ΔOD values, 25th and 75th percentiles; whiskers represent 5th and 95th percentiles, dots the outliers. Low=0-1, absent to low intensity reaction (PD1, n=73; PD2, n=77; BL1, n=61; BL2, n=92). Mid-high=2-5, moderate to intense reaction (PD1, n=57; PD2, n=52; BL1, n=22; BL2, n=28). Pairwise comparisons by Mann-Whitney U test. (**B**) IgG responses to *Ae. albopictus* SGE and to al34k2 in the four surveys according to the subjective perception of intensity of mosquito bites: low=0-1, low number of bites, (PD1, n=117; PD2, n=53; BL1, n=124; BL2, n=64); mid-high=2-5, moderate to very high number of bites, (PD1, n=13; PD2, n=77; BL1, n=6; BL2, n=67). Boxplots and pairwise comparisons as above.

4.7. IgG responses to 34k2 salivary proteins of *Ae. albopictus* and *Ae. aegypti* in human donors from the Réunion Island and Bolivia

To provide further validation and get additional insights into specificity and suitability of IgG responses to the *Ae. albopictus* and *Ae. aegypti* 34k2 salivary proteins as markers of human exposure to *Aedes* mosquitoes, we also measured the specific IgG responses to these two proteins in individuals: (i) from the Réunion Island, (ii) from Bolivia and (iii) from North of France. These are a subset of sera used in previous studies to measure IgG responses to SGE of *Ae. albopictus* and *Ae. aegypti* (Doucoure *et al.*, 2012a and Doucoure *et al.*, 2012b) made available from our collaborator Dr. Franck Remoue (IRD, Montpellier, France). In the Réunion Island, the omnipresence of *Ae. albopictus* is present in all urban areas (and up to 1200 m altitude) and throughout the year on the coast, underlined the need to extend health education, surveillance and mosquito control actions to *Ae. albopictus* vector. Tiger mosquito is considered as the main vector of dengue (2004) and chikungunya (2005-2007) epidemics on the island. Individuals from this cohort were exposed to *Ae. albopictus* but no to *Ae. aegypti*. Subjects from Bolivia on the contrary, were only exposed to *Ae. aegypti* but no to *Ae.*

albopictus. Blood samples were collected in an urban setting in Bolivia where *Ae. aegypti* is the only vector of dengue and dengue outbreaks are reported regularly. The use of these samples from endemic areas are useful to evaluate the specific IgG response to 34k2 salivary proteins in individuals from different epidemiological settings where arboviruses are endemic and maintained by either *Ae. albopictus* or *Ae. aegypti*. In addition, they are appropriate to answer some questions left open by the previous analyses as the potential cross-reactivity of al34k2 and to evaluate the suitability of the specific IgG response to ae34k2 as a marker of exposure to *Ae. aegypti*. A small number of sera collected in North of France in a region free of either *Ae. albopictus* or *Ae. aegypti*, were used as a negative unexposed control and allowed for the determination of cut-off values for seropositivity. The main characteristics of the studied population are summarized in Table 3.

Table 3. Features of the studied population.

	The Réunion Island	**Bolivia**	**North of France**
***Aedes* specie present**	*Ae. albopictus*	*Ae. aegypti*	Not exposed
Date survey	May-June 2009	April-May 2007	2009
Sampled individual	108	105	18
Age range (years)	18-30	17-78	-
Median age	-	30	-
Mean age	24	35,3	-
Females (F)	-	79	-
Males (M)	-	36	-

IgG responses to 34k2 salivary proteins from *Ae. albopictus* and *Ae. aegypti* in individuals from the Réunion Island and North of France are shown in Figure 4.16. Anti-al34k2 IgG responses were significantly higher in subjects from the Réunion Island than in the unexposed cohort from North of France ($p<0.0001$). Noticeably, the antibody response observed in the unexposed controls was very low, confirming the low

background and cross- reactivity and the good specificity and immunogenicity of anti-al34k2 IgG responses in individuals from endemic areas. Comparison of anti-al34k2 and anti-ae34k2 IgG antibody levels in the Réunion Island cohort showed a significantly higher response to the *Ae. albopictus* 34k2 salivary protein ($p<0.0001$). Remarkably, anti-ae34k2 IgG responses were (i) significantly higher that anti-al34k2 antibody responses in unexposed controls ($p=0.0003$) and (ii) not significantly different between the cohorts collected in the Réunion Island and North of France. Overall, these results reveal that IgG responses to al34k2 may be a suitable marker to assess human exposure to *Ae. albopictus* in endemic areas where arboviral diseases are endemic and maintained by the tiger mosquito. Furthermore, the high anti-ae34k2 IgG levels observed in control individuals from North of France (not exposed either *Ae. albopictus* or *Ae. aegypti*), as well as the comparable levels observed in subjects from the Réunion (not exposed to *Ae. aegypti)*, appeared in agreement with the observations previously obtained in Padova and Belluno and suggest that IgG responses to ae34k2 suffer of a relatively high background (at least in individuals not exposed to *Ae. aegypti)*, possibly because of cross-reactivity with some other unknown antigen.

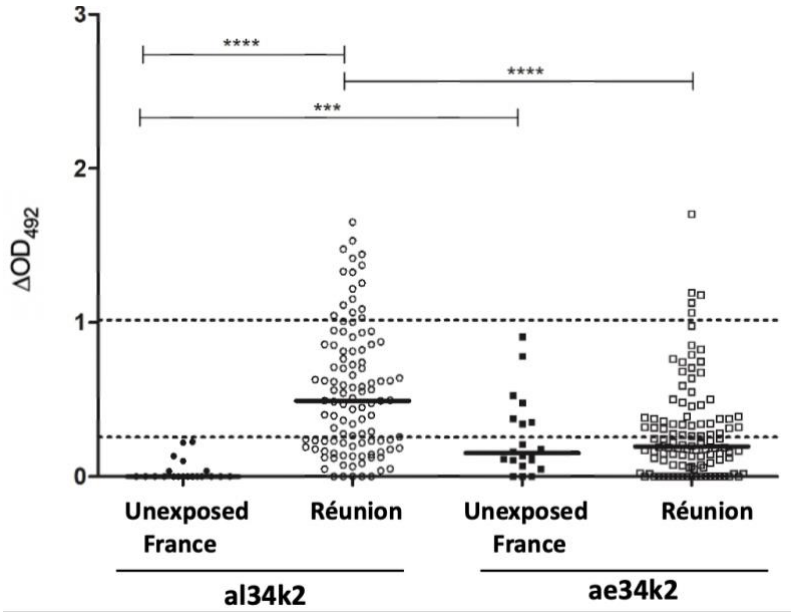


Figure 4.16. Antibody responses to the *Ae. albopictus* and *Ae. aegypti* 34k2 salivary proteins in individuals from the Réunion Island and in unexposed controls. The figure presents the individual IgG responses (ΔOD) against the 34k orthologous proteins in French unexposed controls (n = 18) and in individuals from the Réunion Island (n = 108). Filled and empty circles and squares represents the individual samples. Horizontal bars indicate the median values. The dotted lines correspond to the positivity thresholds calculated from the cohort of unexposed French individuals, not exposed to these *Aedes* species (0.257 for al34k2 and 1,017 for ae34k2). The non-parametric Wilcoxon matched-pairs and Mann-Whitney tests were used to compare paired and unpaired groups, respectively: ****$p< 0.0001$.

Anti-al34k2 and anti-ae34k2 IgG responses observed in individuals from the Réunion Island exhibited a pattern of variation consistent with the IgG responses to SGE from *Ae. albopictus* and *Ae. aegypti* measured by our collaborators at IRD (Doucoure *et al*., 2012a). In fact, anti-alSGE IgG responses were significantly higher in sera of subjects from the Réunion Island than in sera of unexposed individuals from North of France ($p<0,0001$) (Figure 4.17). Moreover, IgG antibody levels against alSGE were higher than anti-aeSGE IgG responses in individuals from the Réunion Island ($p<0,0001$). These results fully agree and corroborate those obtained on the same sub-cohort from the Réunion Island with al34k2 and ae34k2 salivary proteins.

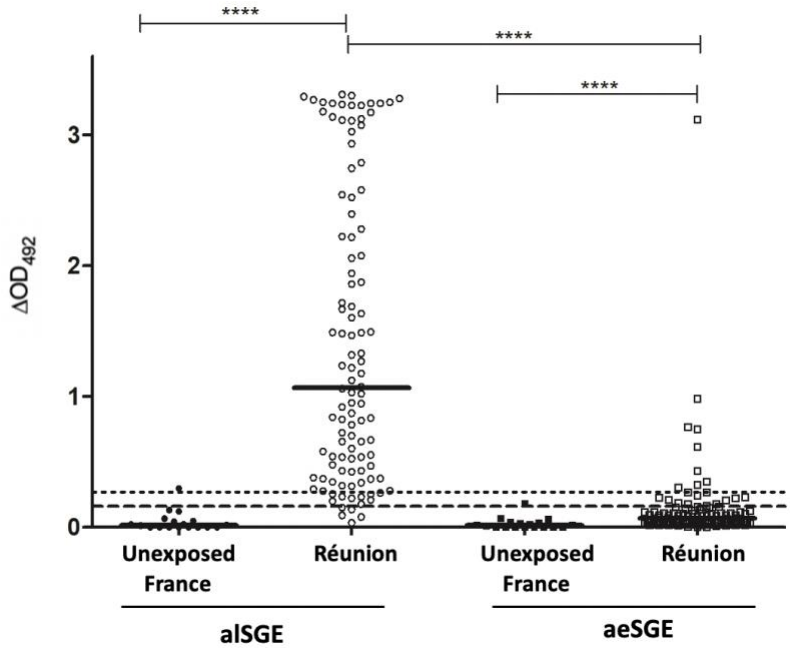


Figure 4.17. Antibody responses to *Ae. albopictus* and *Ae. aegypti* 34k2 salivary gland protein extracts in individuals from the Réunion Island and in unexposed controls. The figure reports the individual IgG responses (ΔOD) against alSGE and aeSGE in French unexposed controls (n = 18) and in individuals from the Réunion Island (n = 108) individuals. Filled and empty circles and squares represents the individual samples and the horizontal bars indicate the median values. The dotted lines correspond to the positivity thresholds calculated from the cohort of unexposed French residents, not exposed to these *Aedes* species (0.269 for alSGE and 0.160 for aeSGE). The non-parametric Wilcoxon matched-pairs and Mann-Whitney tests were used to compare paired and unpaired groups, respectively: **** $p< 0.0001$.

The al34k2 cross-reactivity and the ae34k2 suitability as specific marker of exposure were further evaluated in individuals only exposed to *Ae. aegypti* from Bolivia and compared to the same unexposed cohort from North of France (Figure 4.18). IgG responses to ae34k2 in individuals from Bolivia were (i) significantly higher than anti-al34k2 IgG levels in the same individuals ($p<0,0001$) but (ii) no significantly higher than in unexposed controls suggesting, once again, that IgG responses to ae34k2 appear to be not suitable as marker to assess human exposure to *Ae. aegypti* due to the background and low specificity of the ae34k2 IgG response. Interestingly, IgG responses to al34k2 were higher in the Bolivian samples than in the unexposed cohort from North of France ($p=0.0011$). This result may indicate that the IgG response to al34k2 may detect exposure to *Ae. aegypti*, although the sensitivity may be low as indicate by the observation that the median anti-al34k2 IgG level is below the cut-off.

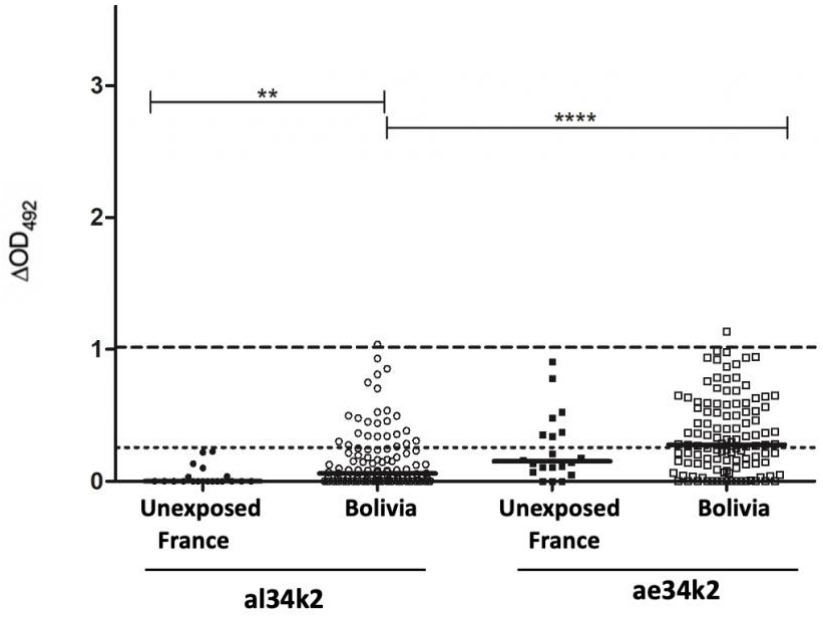


Figure 4.18. Antibody responses to the *Ae. albopictus* and *Ae. aegypti* 34k2 salivary protein in individuals from Bolivia individuals and in unexposed controls. The figure presents the individual IgG responses (ΔOD) against the ae34k2 orthologous proteins in French unexposed controls (n = 18) and in individuals form Bolivia (n = 115). Filled and empty circles and squares represent the individual sample. Horizontal bars indicate the median values. The dotted lines correspond to the positivity thresholds calculated from the cohort of unexposed French residents, not exposed to these *Aedes* species (0.257 for al34k2 and 1.017 for ae34k2). The non-parametric Wilcoxon matched-pairs and Mann-Whitney tests were used to compare paired and unpaired groups, respectively: ** $p< 0.01$; **** $p< 0.0001$.

The differences observed looking at IgG antibody levels are even more evident if we consider the seroprevalences among individuals from the Réunion Island and Bolivia (Figure 4.19). The frequency of responders to al34k2 in the Réunion Island is significantly higher (0.66) than in Bolivia (0.22; p <0.0001; Chi-square test). The observation that 22% of the sampled individuals from Bolivia, who are not exposed to *Ae. albopictus*, respond to al34k2 suggest some low degree of cross-reactivity with the response to ae34k2 confirming the idea that al34k2 may detect exposure to *Ae. aegypti*. This interpretation is also supported by the very low background observed in the French individuals not exposed either to *Ae. albopictus* or *Ae. aegypti*. On the contrary, the frequency of responders to ae34k2 was not different between Réunion Island (0,04) and Bolivia (0,008), similarly to what previously observed analyzing IgG levels.

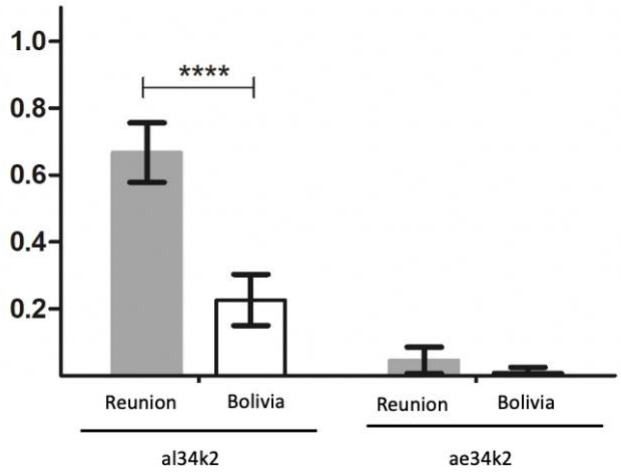


Figure 4.19. Seroprevalence to al34k2 and ae34k2 in individuals form the Réunion Island and Bolivia. Whiskers: 95% CI, the P-values were calculated using the Chi-square test (**** p< 0,0001). The Réunion Island (n=108), Bolivia (n = 115).

5. DISCUSSION

Monitoring and control of mosquito vectors of the *Aedes* genus is of crucial importance considering both their worldwide progressive spreading and the increasing occurrence of arboviral diseases of public health relevance such as dengue, Zika, chikungunya and Yellow fever (Gubler, 2002). Historically, vector control has always played a major role in the control of mosquito-borne diseases, and this holds especially true when affective vaccines and/or drugs are not available. Since pathogens are transmitted by mosquitoes during blood feeding, the evaluation of human-vector contact is of major importance for evaluating the risk of disease transmission and for guiding implementation of vector control strategies by public health authorities. Currently, human-vector contact is indirectly assessed through methodologies based on entomological measurements as ovitraps, larval/pupal indices, adult traps or human landing catches (HLC) (Guidelines for the Surveillance of Invasive Mosquitoes in Europe, ECDC, 2012). However, these methods are not very sensitive in situations of low or not present *Ae. albopictus* densities and require more skills and resources that are not always available. However, in the last 10-15 years, with the increasing understanding of the complexity of blood feeding arthropod saliva (Ribeiro and Arcà, 2009; Ribeiro *et al.*, 2010; Arcà and Ribeiro 2018) the possibility of using host antibody response to mosquito salivary proteins has emerged as an innovative and useful complimentary tool (Schwartz *et al.,* 1990). While the employment of mosquito saliva has several limitations and drawbacks, the use of genus-specific salivary proteins appears very promising, and a soundproof of concept have been provided for anopheline malaria mosquitoes (Rizzo *et al.,* 2011a). Furthermore, a methodology of this type has the advantage of allowing an assessment of real human-vector contract, that is the direct exposure to *Ae. albopictus* bites.

As far as *Aedes* mosquitoes are concerned, some promising indications have been provided using the Nterm-34kDa peptide, which is designed on the culicine-specific 34k1 salivary protein from *Ae. aegypti* (Elanga Ndille *et al.,* 2012). Studies in Benin, Cotê d'Ivoire and Laos (Elanga Ndille *et al.,* 2012; Elanga Ndille *et al.,* 2014; Yobo *et al.*, 2018) suggested that the Nterm-34kDa peptide may allow to detect variation in human exposure to *Ae. aegypti* bites. Moreover, even though 34k1 salivary proteins from *Ae. aegypti* and *Ae. albopictus* are relatively divergent in the N-terminal region, the IgG response to the Nterm-34kDa peptide has been employed to assess vector control

implementation in an urban area at the Réunion Island, where individuals were exposed to *Ae. albopictus* (Elanga Ndille *et al.*, 2016). As a consequence, IgG antibody response to the Nterm-34kDa salivary peptide has been proposed as a relevant short-term indicator to evaluate the efficacy of vector control interventions against *Aedes* mosquito species.

The main objective of the present study is the development of human exposure markers to *Aedes* mosquito bites and specially to the tiger mosquito *Ae. albopictus*. From this point of view, it is appropriate consider how the use of synthetic peptides, though presenting some relevant advantages, it also has some limitations. In fact, the use of peptides allows to avoid laborious procedures of expression, purification and renaturation of recombinant proteins, however not always crowned from success, and can guarantee less variability from preparation of the antigen. Also, synthetic peptides often have one limited sensitivity, due to loss of epitopes conformational of native proteins, and require the use of more concentrated serums, which can be a problem in some epidemiological conditions. On the contrary, recombinant proteins, carrying the conformational epitopes typical of the native forms, may provide higher sensitivity but, their expression/purification can be difficult, time consuming and less reproducible. Besides, it should be notice that, apart from the Nterm-34kda peptide, the availability of extra markers of exposure to the tiger mosquito may result useful for several reasons. First, the Nterm-34kDa (Sagna *et al.*, 2018) peptide is designed on the *Ae. aegypti* 34k1 salivary protein and the appropriate peptide from the *Ae. albopictus* ortholog is considerably divergent (12/19 identical residues with 3 amino acids gap). This may entail a relatively low sensitivity, which might be a limiting factor in settings of low mosquito density, when also classical entomological approaches become less trustworthy. Second, human immune responses to mosquito salivary antigens display significant individual variability, as shown before for the *An. gambiae* gSG6 and cE5 (Rizzo *et al.*, 2014a). Accordingly, various antigens may be very helpful bringing a more comprehensive view and eventually increasing the sensitivity and/or specificity of the immunoassays.

The results reported in this thesis demonstrate for the first time, that the al34k2 recombinant salivary protein has been shown to be a reliable marker of exposure to bites of the arboviral vector *Ae. albopictus* in different epidemiological areas. These results open up new scenarios for the development of risk assessment protocols for exposure and verification of control interventions and indicate the possibility of using the anti-al34k2 antibody response as a reliable method able to complement effectively classical entomological measures. Several experimental observations support al34k2 as a strong

candidate marker of human exposure to bites of the tiger mosquito *Ae. albopictus*. First, in the surveys from both Padova and Belluno (Northeast Italy), human humoral response to al34k2 was suitably related to **seasonality**. In fact, we observed an increase of antibody titers shortly after the summer exposure and a decline after the winter period of non-exposure to *Ae. albopictus*: these results highlighted the **short-term duration of the anti-al34k2 IgG responses**, even though this was more evident in Padova than in Belluno. Padova was originally selected as an area at higher *Ae. albopictus* density than Belluno. This assumption was supported both by history of colonization and by previous entomological monitoring (Montarsi *et al.,* 2015); however, ovitraps data did not show the expected difference between the study areas. Despite this, we found that both anti-SGE and anti-al34k2 IgG responses were higher in Padova than Belluno both before and after summer, a result that matched perfectly with the original expectation of Padova being a higher density area for *Ae. albopictus*. Considering that (i) IgG responses to mosquito saliva have been previously shown, in different settings, to be a reliable marker of host exposure to mosquito bites (Remoue *et al.,* 2006; Orlandi-Pradines *et al.,* 2007; Fontaine *et al.,* 2011; Doucoure *et al.,* 2012a, 2014) and that, (ii) differently from entomological measures, they provide a direct evidence of human-vector contact, we believe that IgG responses to the al34k2 salivary protein are also a reliable marker also to detect **spatial variation** of human exposure to *Ae. albopictus*. These properties are basic and fundamentally important for the effectiveness of a serological marker.

Our cohorts from Padova and Belluno were composed form adult healthy volunteers (blood donors). We do not know how individuals naturally exposed to *Ae. albopictus* bites respond to the al34k2 salivary antigen before adulthood (children and teenagers). However, we wondered of there was any variation with age of the antibody titers to SGE and al34k2. We found an **age dependence** trend that decrease progressively with age in sera collected in Padova but only hardly detectable in those from Belluno. Previous studies showed that, while IgG responses to mosquito saliva (a cocktail of ~100–150 proteins) decrease with age, the situation with individual salivary proteins is antigen dependent. For example, a decrease in the antibody response with age was previously reported for the *An. gambiae* gSG6 (Poinsignon *et al.,* 2009; Rizzo *et al.,* 2011, 2014b; Montiel *et al.,* 2020) and for *Anopheles albimanus* salivary peptides (Londono-Renteria *et al.,* 2020); opposite to that noted for the *An. gambiae* cE5 (Rizzo *et al.,* 2014a) and the *Ae. aegypti* D7s4 (Ribeiro *et al.,* 2007; Londono-Renteria *et al.,* 2018). We found that in Padova anti-al34k2 antibody responses were higher in younger adults (18-30 years

old) and decreased progressively with age; this was not the case for Belluno, where some decrease was only observed in the over 50 years old category. It is known that human cutaneous reactions and immune responses to mosquito saliva are known to vary over time according to intensity and persistence of exposure to salivary antigens, and that natural desensitization to salivary antigens may eventually occur (Mellanby, 1946; Feingold *et al.,* 1968; Peng and Simons, 1998, 2004; Doucoure *et al.,* 2012b; Cardenas *et al.,* 2019; Montiel *et al.,* 2020). This may give a reason for the different trends we observed in Padova, an area colonized since more than 25 years, and in Belluno, where individuals were exposed to *Ae. albopictus* bites for no more than 5 years.

Human IgG responses to mosquito saliva are mainly characterized by antibodies of the IgG1 and IgG4 subclasses and very low IgG2 and IgG3 concentrations (Reunala *et al.,* 1994; Brummer-Korvenkontio *et al.,* 1994). High levels of antigen-specific IgG4 antibodies may be related to allergenic properties of insect salivary proteins, and eventually associated with immune tolerance (Peng and Simons, 2004). We determined anti-al34k2 IgG1 and IgG4 levels in individuals from the PD1 and PD2 surveys and, in both cases, median IgG1 titers were at least 10-fold higher than corresponding IgG4 levels. An analogous finding was previously reported for the *An. gambiae* salivary protein cE5 in naturally exposed individuals from a malaria hyperendemic area of Burkina Faso. However, the same individuals carried high levels of anti-gSG6 IgG4 antibodies. This contrasting responses to the cE5 and gSG6 proteins have been explained as a possible indicator of Th1-type and Th2-type polarized immune responses, respectively (Bretscher, 2014; Rizzo *et al.,* 2014a, b). Our findings suggest that the *Ae. albopictus* al34k2 protein may be of limited allergenicity and leads in naturally exposed individuals an IgG1-dominated antibody response that may be indicative of a Th1-type polarization.

The culicine-specific 34kDa family is absent in anopheline mosquitoes and other blood sucking arthropods. Within *Aedes* species the 34k2 salivary proteins from *Ae. albopictus* and *Ae. aegypti* have a 62% amino acid sequence identity, leaving an open question about the possible degree of cross-reactivity. We observed absence of cross-reactivity in our murine model (Buezo Montero *et al.,* 2019): in fact, sera of mice immunized to *Ae. aegypti* saliva did not carry IgG antibodies recognizing the al34k2 proteins, and vice versa. A limited level of cross- reactivity was found in a single individual hyperimmune to *Ae. albopictus* saliva, however, this result should be interpreted with caution because of both, the use of a single human serum and the hyperimmune status. No variation of IgG responses to ae34k2 were found in Padova and

Belluno, indicating its unsuitability for the evaluation of human exposure to the tiger mosquito. Nevertheless, detectable levels of anti-ae34k2 IgG were found in Padova and Belluno. Other *Aedes* mosquitoes (*Aedes koreicus, Aedes japonicus japonicus*) are present in these two localities (Capelli *et al.,* 2011 and Montarsi *et al.,* 2019) and, in principle, a possible cross-reaction between the IgG responses to ae34k2 and to 34k2 proteins from these species cannot be excluded *a priori*. However, the absence of spatial and/or temporal variation, and the low abundance of these species in comparison to *Ae. albopictus*, suggest a low specificity of the IgG responses to the ae34k2 salivary protein, at least in areas where *Ae. aegypti* is not present. To get some further insights we analyzed the IgG responses to al34k2 and ae34k2 in a cohort of individuals from Bolivia, only ever exposed to *Ae. aegypti,* and in a group of individuals from the Réunion Island, only ever exposed to *Ae. albopictus*. A group of French individuals not exposed to either *Ae. albopictus* and *Ae. aegypti* was used as negative control. This analysis provided clear evidence that IgG responses to al34k2 may represent a suitable **marker of human exposure to *Ae. albopictus* also in areas with arboviral transmission.** Moreover, IgG responses to al34k2 may, at least in part, also capture exposure to *Ae. aegypti* suggesting a possible use in areas where both vectors are present. On the contrary, IgG responses to ae34k2 appear unsuitable as marker of human exposure to *Ae. aegypti* due to the relatively high background, as indicated by the high anti-ae34k2 IgG levels measured among unexposed controls.

CONCLUDING REMARKS

In conclusion, results achieved in this thesis provide reliable indications that IgG antibody responses to the *Ae. albopictus* 34k2 salivary protein can be exploited to detect spatial and temporal variations of human exposure to the tiger mosquito and, perhaps, may be useful in areas where both *Ae. albopictus* and *Ae. aegypti* are present. Further targeted studies will be needed to verify whether anti-al34k2 IgG responses may be also employed for the evaluation of efficacy of vector control interventions, including the innovative *Wolbachia*-based mosquito suppression strategies currently under evaluation (Williams *et al.,* 2020). The availability of a serological tool providing direct indication of human-vector contact may certainly be helpful for control strategies targeting vectors of major arboviruses such as dengue, chikungunya or Zika. Such complementary tool,

which can be employed for epidemiological studies and possibly for the evaluation of transmission risk, may be especially useful when implementation of classical entomological methods is challenging (low vector density, logistic constraints, limited resources, etc.) or when the simultaneous determination of exposure to vector and to specific circulating pathogen(s) by serological measurements may be needed. Despite the need of further testing in different epidemiological settings, I believe that the research work reported in this thesis provides the basis for a novel methodology, based on the use of recombinant salivary antigens, for the assessment of human exposure to the tiger mosquito *Ae. albopictus*, a mosquito that is impressively expanding its geographic distribution and in recent years caused important arboviral outbreaks in both tropical and temperate regions.

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